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The Activity of Liver Alcohol Dehydrogenase with Nicotinamide–Adenine Dinucleotide Phosphate as Coenzyme

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1. The separation of nucleotide impurities from commercial NADP preparations by chromatography is described. All the preparations studied contained 0·1–0·2% of NAD. 2. The activity of pure crystalline liver alcohol dehydrogenase with NADP as coenzyme has been confirmed. Initial-rate data are reported for the reaction at pH 6·0 and 7·0 with ethanol and acetaldehyde as substrates. With NADP and $NADPH_2$ of high purity, the maximal specific rates were similar to those obtained with NAD and $NADH_2$, but the Michaelis constants for the former coenzymes were much greater than those for the latter. 3. The oxidation of ethanol by NADP is greatly inhibited by $NADH_2$, and this accounts for low values of certain initial-rate parameters obtained with commercial NADP preparations containing NAD. The kinetics of the inhibition are consistent with competitive inhibition in a compulsory-order mechanism. 4. Initial-rate data with NAD and $NADPH_2$ do not conform to the requirements of the mechanism proposed by Theorell & Chance (1951), in contrast with results previously obtained with NAD and $NADH_2$. The possibility that the deviations are due to competing nucleotide impurity in the oxidized coenzyme cannot be excluded. The data show that the enzyme reacts more slowly with, and has a smaller affinity for, NADP and $NADPH_2$ than NAD and $NADH_2$. 5. Phosphate behaves as a competitive inhibitor towards NADP.

It has been reported that liver alcohol dehydrogenase (alcohol–NAD oxidoreductase, EC 1.1.1.1) catalyses the oxidation of ethanol by NADP at about 1% of the rate obtained with NAD as coenzyme (Pullman, Colowick & Kaplan, 1952). The coenzyme and substrate concentrations used were not stated. With a single finite concentration of each, the low activity with NADP could be due to one or more of several factors: for example, the enzyme may have a smaller affinity for NADP than for NAD; the combination of ethanol with the enzyme–NADP compound may be relatively slow; or the ternary complex may undergo intramolecular reaction, or dissociate into products, relatively slowly. The main objectives of the present work were to confirm the activity of pure crystalline liver alcohol dehydrogenase with NADP and $NADPH_2$ as coenzymes, and to distinguish between these possible explanations for the low activity by initial-rate measurements with several coenzyme and substrate concentrations. Such studies might give further information about the nature of the binding of coenzymes to this enzyme and the mechanism of the overall catalytic reaction.

Detailed kinetic studies with NAD and $NADH_2$ of high purity, and with butanol and butyraldehyde as well as ethanol and acetaldehyde as substrates, have provided fairly strong evidence that neither binary enzyme–substrate complexes nor ternary complexes of enzyme, coenzyme and substrate are kinetically significant in the overall reaction of liver alcohol dehydrogenase (cf. Dalziel, 1962*a*, 1963*a*). The maximum rates and Michaelis constants for the coenzymes were shown to be independent of the nature of the substrate, and dissociation constants for the enzyme–coenzyme compounds calculated from the initial rate parameters agreed with those obtained by direct equilibrium measurements in the absence of substrates (Theorell & McKinley-McKee, 1961). The kinetic data were completely consistent with the limiting case of a compulsory-order mechanism proposed by Theorell & Chance (1951), in which the first step is the combination of enzyme and coenzyme, and the last, which determines the maximum rate, is the dissociation of the compound of enzyme and product coenzyme. Divergencies of previous results from some of the relations required by this mechanism were shown to be caused by small amounts of competitive inhibitors generally present as impurities in commercial NAD and $NADH_2$ preparations (Dalziel, 1962*b*, 1963*b*). It was expected that

similar difficulties would be encountered in work with NADP, especially if the affinity of the enzyme for NADP is relatively small. This has proved to be the case.

MATERIALS AND METHODS

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

Enzymes. Crystalline alcohol dehydrogenase was prepared from horse liver and assayed as described by Dalziel (1961*a*). Yeast alcohol dehydrogenase and glucose 6-phosphate dehydrogenase were purchased from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany.

Substrates. Ethanol and acetaldehyde were redistilled. The acetaldehyde was freshly redistilled on the day of the experiment, because acid formed on storage at $-15°$. By titration, the acid content of freshly redistilled material was 0·5% on a normality basis, and increased to 5% after 2 days and to 20% after several weeks. Spectrophotometric titration of the latter sample with $NADH_2$ and liver alcohol dehydrogenase at pH 7·0 showed the acetaldehyde content to be only 60% by weight. A freshly redistilled sample assayed as 98–100%.

Coenzymes. NADP was purchased from C. F. Boehringer und Soehne G.m.b.H. and Sigma Chemical Co., St Louis, Mo., U.S.A., and was purified by chromatography on DEAE-cellulose (Whatman) at pH 6·0. The technique was essentially as described for the purification of NAD by Dalziel (1963*b*), except that smaller columns (20 cm. × 1·25 cm.) were used. The concentration gradient for development was applied by running 0·1 M-phosphate buffer into a 400 ml. reservoir of 5 mM-phosphate buffer at the flow rate of the column (5 ml./min.). The elution was monitored by transmittance measurements at 254 mμ with a Uvicord absorptiometer and recorder (LKB-Produkter A.B., Stockholm, Sweden), supplemented by extinction measurements at 260 mμ for the estimation of the nucleotide content of particular fractions. NADP was assayed with glucose 6-phosphate dehydrogenase (Horecker & Kornberg, 1957); reduction with ethanol and liver alcohol dehydrogenase was too slow for accuracy and convenience.

$NADPH_2$ (enzymically reduced; C. F. Boehringer und Soehne G.m.b.H.) was readily assayed with acetaldehyde and liver alcohol dehydrogenase, exactly as described for $NADH_2$ by Dalziel (1962*b*), except that ten times as much enzyme (0·05 mg./ml.) was needed for complete oxidation in 15 min. The extinction ratio E_{260}/E_{340} was 2·5 and the residual E_{340} after complete enzymic oxidation was 3% of the initial value. These characteristics are similar to those of the best $NADH_2$ preparations (Dalziel, 1962*b*).

NAD was purchased from Sigma Chemical Co., and $NADH_2$ was prepared as described by Dalziel (1962*b*).

Initial-rate measurements. Kinetic coefficients in the initial-rate equation

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

were obtained from initial-rate measurements at 23·5° by the usual primary and secondary plots (Dalziel, 1957, 1962*a*). 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 the acetaldehyde–$NADPH_2$ reaction, and ϕ'_0 etc. for those for the ethanol–NADP reaction. These coefficients are functions of rate constants in the mechanism. ϕ_0 is the reciprocal of the maximum rate, and ϕ_1/ϕ_0 and ϕ_2/ϕ_0 are the Michaelis constants for coenzyme and substrate respectively. A recording fluorimeter (Dalziel, 1962*a*) was used for the ethanol–NADP reaction. This method could not be used to estimate ϕ_0 and ϕ_2 for the acetaldehyde–$NADPH_2$ reaction, because of the large Michaelis constant for $NADPH_2$ (ϕ_1/ϕ_0) and the quenching of fluorescence above 40 μM-$NADPH_2$. Progress curves were obtained instead by spectrophotometric measurement at 340 mμ in cells of 2 mm. path length with a Zeiss PMQII spectrophotometer. Estimates of ϕ_1 and ϕ_{12} from initial-rate measurements with low $NADPH_2$ concentrations (2–40 μM) by the fluorimetric method agreed well with those obtained from spectrophotometric measurements with high $NADPH_2$ concentrations.

In the principal experiments, the ranges of concentrations of substrates and coenzymes were: $NADPH_2$, 0·26–1·56 mM; acetaldehyde, 0·1–42 mM; NADP, 0·06–1·2 mM; ethanol, 10–200 mM. The primary plots (Lineweaver & Burk, 1934) and the secondary plots of their slopes and intercepts were linear, within the experimental error, over these concentration ranges. The enzyme concentrations were 0·08–0·16 μM. Reaction mixtures were *I* 0·1 with respect to sodium phosphate buffer unless otherwise stated.

All initial-rate measurements were made in duplicate. Excellent reproducibility of 3% was obtained for the ethanol–NADP reaction because, in spite of the unfavourable equilibrium, progress curves were practically linear: the great sensitivity of the fluorimeter, and the large NADP concentrations, allowed initial-rate measurements from a very small fraction of the total reaction to equilibrium. Progress curves for the acetaldehyde–$NADPH_2$ reaction in the spectrophotometer were not linear, and the reproducibility of initial-rate estimates was only 10%.

RESULTS

Kinetic measurements with commercial NADP. With ethanol concentrations up to 80 mM, progress curves for the reduction of NADP (Sigma) were linear from 15 sec. to at least 3 min. after enzyme addition (Fig. 1). However, extrapolation to zero time showed that a greater increase of fluorescence occurred during the first 15 sec. than could be explained by the subsequent constant rate of $NADPH_2$ formation. With greater ethanol concentration, progress curves were clearly biphasic, the slopes decreasing during the first 30 sec. and then remaining constant (Fig. 1). These results suggested that NADP preparations contained traces of NAD, which is much more rapidly reduced than NADP. With low ethanol concentrations, the reduction of NAD (estimated as about 0·2% of the NADP from the initial increase of fluorescence) evidently reaches equilibrium within a few seconds. With greater ethanol concentrations, the reduction of NAD lasts longer, because it is subject to substrate inhibition with ethanol concentrations

greater than 8mM (Theorell, Nygaard & Bonnichsen, 1955). In the concentration range of these experiments, the rate of NAD reduction decreases, and the rate of NADP reduction increases, with increase of ethanol concentration. The linear parts of the progress curves were assumed to represent the initial rate of NADP reduction, and values for the kinetic coefficients (Table 1) in eqn. (1) were obtained from the secondary plots shown in Fig. 2. The rate of the NADP–ethanol reaction was found to decrease with increase of phosphate concentration. The results in Fig. 3 indicate that the Michaelis constant for NADP increases with phosphate concentration, if it may be assumed that such plots are linear at the higher phosphate concentrations, as they were shown to be at the lower phosphate concentration. Further

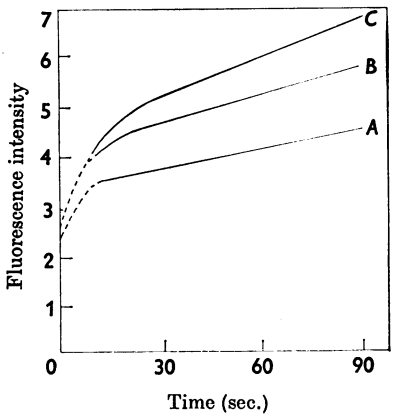
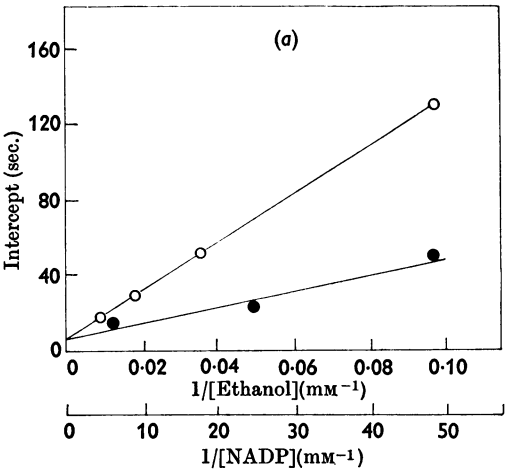


Fig. 1. Progress curves for the reaction of commercial NADP with ethanol at pH 7·0. The NADP concentration was 20 μM in every case. The ethanol concentrations were: *A*, 20mM; *B*, 86mM; *C*, 680mM.



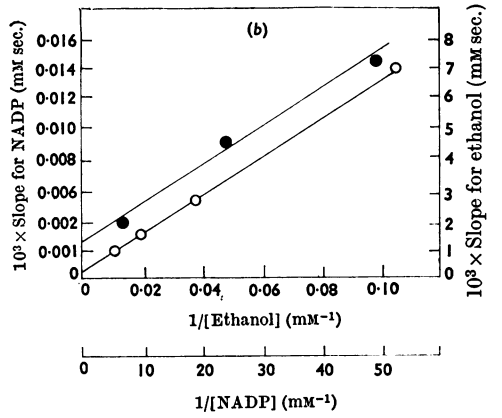


Fig. 2. Secondary plots showing the variation of intercepts (*a*) and slopes (*b*) of primary Lineweaver–Burk plots with the reciprocals of the NADP concentration (○) and of the ethanol concentration (●).

Table 1. *Kinetic coefficients for the NADP–ethanol reaction at* 23·5°

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

$$\frac{e}{v_0} = \phi'_0 + \frac{\phi'}{[S_1]} + \frac{\phi'_2}{[S_2]} + \frac{\phi'_{12}}{[S_1][S_2]}$$

where S_1 is NADP and S_2 is ethanol.

pH	Ionic strength	NADP	ϕ'_0 (sec.)	ϕ'_1 (mM sec.)	ϕ'_2 (mM sec.)	ϕ'_{12} (mM² sec.)
7·0	0·1	Commercial	5·5	2·5	378	128
7·0	0·1	Purified	0·9	1·9	25	105
7·0	0·1–0·15*	Purified	2·0	2·4	310	118
6·0	0·1	Purified	0·3	1·6	45	135
6·0	0·1–0·13*	Purified	2·6	1·7	300	200

* Varied with the NADP concentration (see the text).

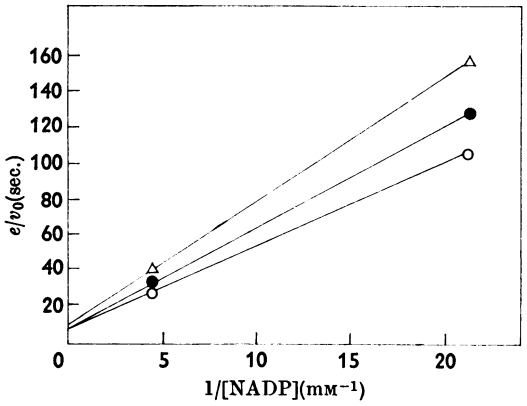


Fig. 3. Initial rates of NADP reduction by ethanol at pH 6 with phosphate buffer concentrations of 0·08 M (○), 0·10 M (●) and 0·14 M (△). The reciprocal of the specific initial rate is plotted against the reciprocal of the NADP concentration. The ethanol concentration was 81 mM in every case.

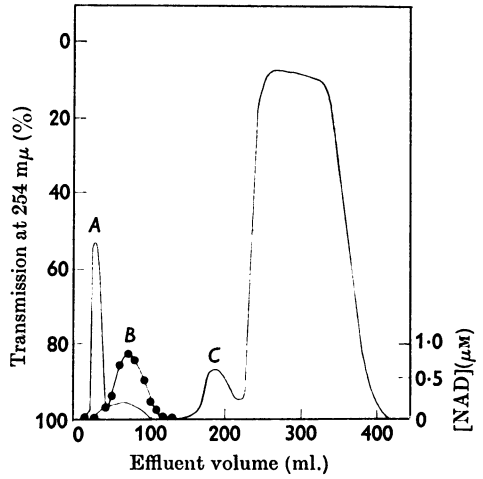


Fig. 4. Elution diagram for the chromatography of 102 mg. of commercial NADP (Boehringer) on DEAE-cellulose, showing variation of transmittance at 254 mμ with volume of eluate. Elution was effected with a concentration gradient of phosphate buffer, pH 6·0.

evidence for this effect of phosphate concentration is given below.

Chromatographic purification of NADP. Preliminary experiments showed that artificial mixtures of NAD, ADP-ribose and NADP could be completely separated by chromatography on DEAE-cellulose with a concentration gradient of phosphate buffer, pH 6·0. Of the two main nucleotide impurities detected in commercial NAD preparations (Dalziel, 1963*b*), the first, which is not retained by the column, was also present in all four NADP preparations studied, to the extent of about 0·5–1·0% (*A* in Fig. 4). The second NAD impurity, probably ADP-ribose and the chief competitive inhibitor of NAD in the liver alcohol-dehydrogenase reaction, was not detected in the NADP preparations. The latter all contained 0·1–0·2% of NAD (*B* in Fig. 4), and 2·4% of another unidentified impurity that was eluted in 0·1 M-phosphate, pH 7·0. Finally, two of the four preparations contained about 0·3% of another impurity that was eluted at pH 6·0 just before the NADP itself (*C* in Fig. 4). The identity of component *B* in Fig. 4 as NAD was established by enzymic assay of successive 10 ml. fractions of the eluate. Samples were brought to pH 10·0 by the addition of glycine buffer, and the formation of $NADH_2$ on the addition of acetaldehyde and either yeast or liver alcohol dehydrogenase was detected by fluorescence. The concentration of NAD in each fraction was estimated from measurements of the increase of fluorescence intensity. The results of these analyses are also shown in Fig. 4. The total content of NAD in the NADP preparation was estimated as 0·14% of the NADP content.

To obtain NADP in the highest possible concentration for initial-rate measurements, the concentration gradient was discontinued when the NADP began to be eluted, and 0·1 M-phosphate, pH 6·0, was applied directly to the column. From 102 mg. of commercial NADP, 84 mg. was recovered as a 1·6 mM solution by combining the most concentrated eluates. In a similar preparation, from 179 mg., 150 mg. was recovered as a 2·4 mM solution. From enzymic assays with glucose 6-phosphate dehydrogenase and extinction measurements at 260 mμ, the molar extinction at this wavelength of the purified NADP was $18{\cdot}0 \times 10^6$ cm.^{2}/mole, relative to $6{\cdot}22 \times 10^6$ cm.^{2}/mole for $NADPH_2$ at 340 mμ (Horecker & Kornberg, 1948). The original commercial material gave a value of $18{\cdot}5 \times 10^6$ cm.^{2}/mole, indicating the removal of about 2·5% of nucleotide impurity by chromatography in this experiment.

Kinetic measurements with purified NADP. Progress curves for the reduction of chromatographically purified NADP by ethanol were linear, and did not show the initial rapid increase of fluorescence observed with commercial preparations. Readdition of NAD fractions separated from the NADP reproduced the biphasic curves, as did the addition of authentic NAD in the proportion of 1·5 moles/1000 moles of NADP. Further, in control experiments with the same concentration of NAD, in the absence of NADP, progress curves for the reduction of NAD corresponded closely to the

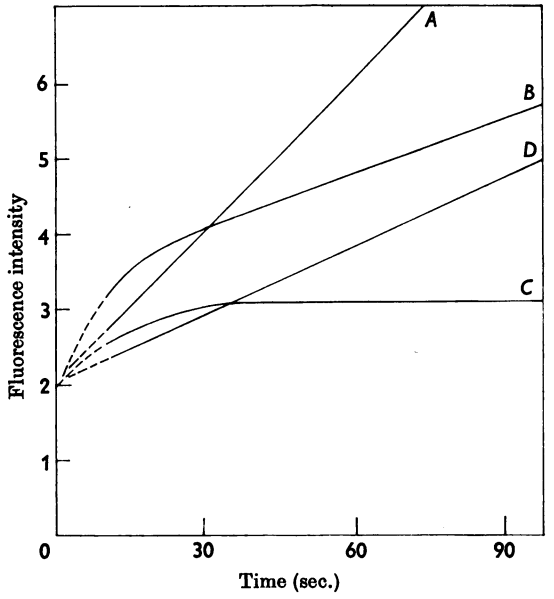


Fig. 5. Progress curves for the reduction of chromatographically purified NADP and NAD by ethanol (800mM) at pH7·0. *A*, 225μM-NADP; *B*, 225μM-NADP+0·32μM-NAD; *C*, 0·32μM-NAD; *D*, 225μM-NADP+0·28μM-$NADH_2$.

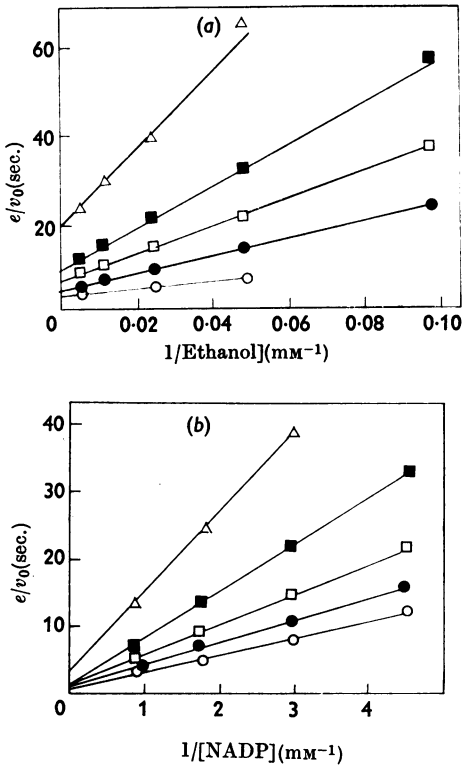


Fig. 6. Primary plots: variation of the reciprocal of the specific initial rate of ethanol oxidation at pH7·0 and 24° with (*a*) the reciprocal of the ethanol concentration, for several constant NADP concentrations (mM): ○, 1·12; ●, 0·56; □, 0·34; ■, 0·23; △, 0·11; and (*b*) the reciprocal of the NADP concentration for several constant ethanol concentrations (mM): ○, 202; ●, 81; □, 40; ■, 20; △, 10.

initial part of the progress curve for the mixture. These results, some of which are shown in Fig. 5, confirm the presence and effects of NAD in commercial NADP preparations. They also show that the rate of NADP reduction in the presence of $NADH_2$, formed by the rapid reduction of the NAD impurity, is considerably smaller than the rate obtained with purified NADP. This is further illustrated in Fig. 5 by the effect of adding 1·2moles of $NADH_2$/1000moles of purified NADP. The results of initial-rate measurements with purified NADP at pH7·0 are shown in Figs. 6 and 7. Values for the kinetic coefficients in eqn. (1) were obtained from two such experiments at pH7·0, and two at pH6·0 (Table 1). It is obvious that the estimates of ϕ_0' and ϕ_2', the intercept and slope respectively of the secondary plots (Fig. 7*a*), are approximate. More accurate values for these parameters could only be obtained from experiments with extremely large NADP concentrations. Nevertheless, it is clear that the values obtained for these parameters with purified NADP are smaller than those obtained with commercial NADP (Table 1).

Since the NADP solution in phosphate buffer forms a significant proportion of the total volume of reaction mixtures, it was necessary to add variable amounts of buffer, according to the volume of NADP solution used, to make the ionic strength accurately 0·1 in all reaction mixtures. The importance of this, and the nature and magnitude of the effect of phosphate concentration on the kinetics, is illustrated by kinetic parameters (Table 1) obtained in experiments in which the contribution of the NADP solution to the phosphate concentration of reaction mixtures was ignored. The ionic strength with respect to phosphate buffer in these experiments therefore varied with the NADP concentration, from 0·104 to 0·125 at pH6·0, and from 0·104 to 0·145 at pH7·0. As a result, ϕ_0' and ϕ_2' are greatly increased, whereas ϕ_1' and ϕ_{12}' are not significantly affected.

Kinetic measurements with commercial $NADPH_2$. Kinetic coefficients for the oxidation of $NADPH_2$ by acetaldehyde at pH6·0 and 7·0, given in Table 2, are averages of the results of two experiments at each pH value.

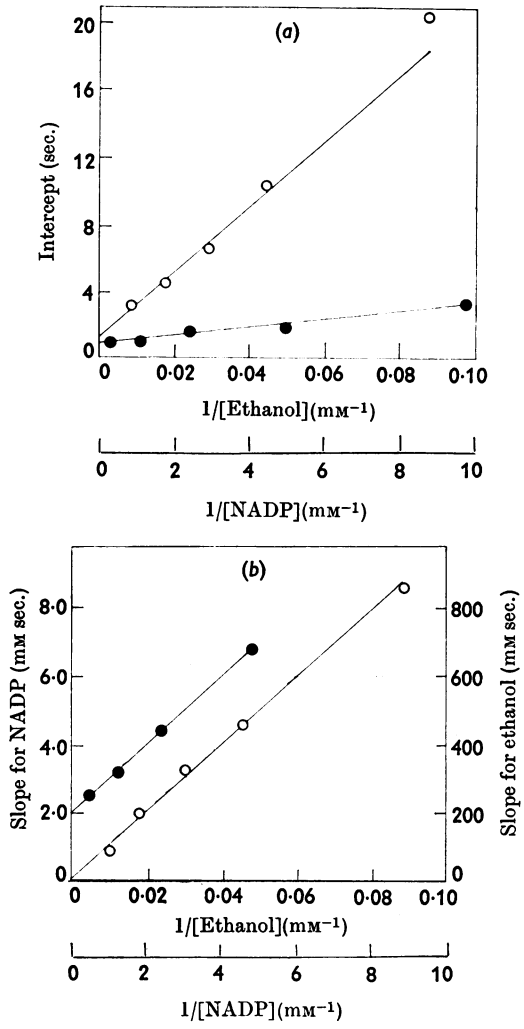


Fig. 7. Secondary plots: variations of the intercepts (*a*) and slopes (*b*) of Lineweaver–Burk plots in Figs. 6(*a*) and 6(*b*) with the reciprocal of the NADP concentration (○) and of the ethanol concentration (●) respectively.

Table 2. *Kinetic coefficients for the $NADPH_2$–acetaldehyde reaction at* 23·5°

The ionic strength was 0·1 with respect to sodium phosphate buffer. The kinetic coefficients are those in the initial-rate equation:

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

pH	ϕ_0 (sec.)	ϕ_1 (mM sec.)	ϕ_2 (mM sec.)	ϕ_{12} (mM^2 sec.)
7·0	0·008	0·030	0·006	0·018
6·0	0·010	0·016	0·005	0·003

Spectrophotometric studies of the reaction of liver alcohol dehydrogenase with $NADPH_2$. Theorell & Bonnichsen (1951) showed that the enzyme reacts directly with $NADH_2$ to form a compound that exhibits an absorption maximum at 325mμ, in place of the maximum at 340mμ exhibited by free $NADH_2$. The dissociation constant of this compound is so small that they were able to show, by spectrophotometric titration at 350mμ, that one molecule of enzyme binds two molecules of $NADH_2$. Attempts to demonstrate a similar reaction with $NADPH_2$ were not successful. In the region 310–360mμ the absorption spectrum of a mixture of 0·14mM-enzyme and 0·73mM-$NADPH_2$ in phosphate buffer, pH 7·0, measured in an optical cell of 2mm. path length, was the same as that of $NADPH_2$ alone. If it is assumed that $NADPH_2$ does form a compound, and that it has the same extinction coefficient at 350mμ as the $NADH_2$ compound, this negative result indicates that its dissociation constant must be greater than 1mM.

In the presence of isobutyramide, the affinity of liver alcohol dehydrogenase for $NADH_2$ is greatly increased (Theorell & McKinley-McKee, 1961). By spectrophotometric titration of the enzyme with $NADPH_2$ in the presence of isobutyramide, the formation of a compound with spectral characteristics similar to those of the $NADH_2$ compound was demonstrated. Successive 10μl. volumes of $NADPH_2$ solution (11·2mM) were added to 2·5ml. of enzyme solution (0·205mM) in phosphate buffer, pH 7·0, containing isobutyramide (0·1M), in an

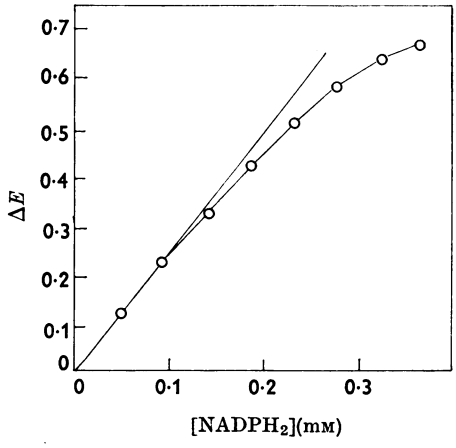


Fig. 8. Titration of 0·20mM-liver alcohol dehydrogenase in phosphate buffer, pH 7·0, containing isobutyramide (0·1M), with $NADPH_2$. The difference of extinction at 350mμ, ΔE, between the enzyme solution and a control solution of isobutyramide and buffer only is plotted against the concentration of $NADPH_2$ added to each.

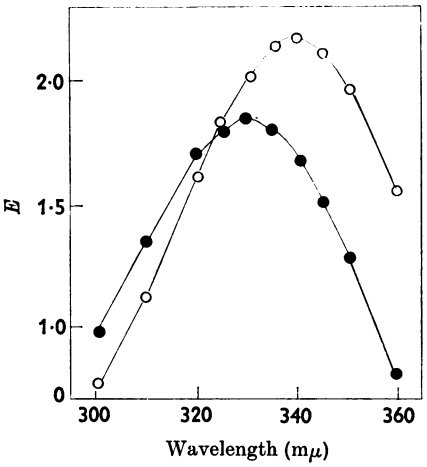


Fig. 9. Absorption spectra of 0·20 mM-liver alcohol dehydrogenase + 0·35 mM-$NADPH_2$ (●) and 0·35 mM-$NADPH_2$ (○), in phosphate buffer, pH 7·0, containing isobutyramide (0·1 M).

optical cell of 1 cm. path length, and also to the same volume of buffer and isobutyramide, without enzyme, in a second cell. After each addition the difference in extinction between the two solutions at 350 mμ was measured and was plotted against the $NADPH_2$ concentration (Fig. 8). The absorption spectra of the two solutions at the end of the titration (corrected for protein absorption) are shown in Fig. 9. The initial slope of $2·5 \times 10^3$ cm.^{2}/mmole in Fig. 8 agrees well with the difference between the extinction coefficients of $NADH_2$ and its compound with liver alcohol dehydrogenase at this wavelength (Theorell & Bonnichsen, 1951; Ehrenberg & Dalziel, 1958). Thus more than one molecule of $NADPH_2$ is bound/molecule of enzyme, but the compound is evidently partly dissociated under these conditions, even in the presence of excess of isobutyramide.

DISCUSSION

The activity of liver alcohol dehydrogenase with NADP and $NADPH_2$ as coenzymes had been confirmed. Kinetic coefficients in the initial-rate equation (1) for the reaction of ethanol with chromatographically purified NADP, and of acetaldehyde with commercial $NADPH_2$ of high purity, are compared in Table 3 with the corresponding parameters for the reactions with NAD and $NADH_2$ under similar conditions (Dalziel, 1963*a*). At pH 6·0 and 7·0, similar maximum turnover numbers ($1/\phi_0$ and $1/\phi'_0$) were obtained with both coenzymes, but the Michaelis constants for NADP and $NADPH_2$ (ϕ'_1/ϕ'_0 and ϕ_1/ϕ_0) were much greater than those for

Table 3. *Comparison of kinetic coefficients at 23·5° for NAD- and NADP-linked reactions of liver alcohol dehydrogenase*

The initial-rate equations are given in Tables 1 and 2. Kinetic coefficients for the reactions with NADP and $NADPH_2$ are from Tables 1 and 2, and those for the reactions with NAD and $NADH_2$ from Dalziel (1963*a*).

		Acetaldehyde reduction					Ethanol oxidation				
pH	Coenzyme	ϕ_0 (sec.)	ϕ_1 (μM sec.)	ϕ_2 (μM sec.)	ϕ_{12} (μM^2 sec.)	ϕ_1/ϕ_0* (μM)	ϕ'_0 (sec.)	ϕ'_1 (μM sec.)	ϕ'_2 (μM sec.)	ϕ'_{12} (μM^2 sec.)	ϕ'_1/ϕ'_0* (μM)
6·0	$NADP(H_2)$	0·010	16	4·5	2800	1600	0·3	1600	45000	135×10^6	5200
	$NAD(H_2)$	0·008	0·115	3·7	1·5	14	0·62	2·5	280	70000	4
7·0	$NADP(H_2)$	0·008	30	6·0	18000	3750	0·9	1900	25000	105×10^6	2100
7·1	$NAD(H_2)$	0·008	0·100	3·3	1·4	13	0·37	1·1	66	7200	3

* Michaelis constant for the coenzyme with infinitely large substrate concentration.

NAD and $NADH_2$. Consequently, the turnover numbers with limiting concentrations of the former coenzymes are relatively small (cf. Pullman *et al.* 1952).

With commercial preparations of NADP, much smaller maximum turnover numbers were obtained (Table 1). This is due to the presence of 0·1–0·2% of NAD, as demonstrated by chromatography and reduction with ethanol and yeast alcohol dehydrogenase (which has no activity with purified NADP). The inhibition is attributed to $NADH_2$, the formation of which can be seen as a rapid initial phase in the progress curves obtained with commercial NADP, and in those obtained with chromatographically purified NADP to which 0·15% of NAD has been added. A similar degree of inhibition of NADP reduction by 0·12% of $NADH_2$ has also been demonstrated.

The pronounced inhibition by such a small proportion of $NADH_2$ is explained by the small dissociation constant of the enzyme–$NADH_2$ compound, together with the large dissociation constant of the enzyme–NADP compound and the large Michaelis constant for NADP. The effect of an inhibitor that competes with, and is present in, a coenzyme can be represented by the following initial-rate equation, derived on the assumption of a compulsory-order mechanism, in which the first step is the combination of enzyme and coenzyme (Dalziel, 1962*c*, 1963*b*):

$$\frac{e}{v_0} = \phi_0'\left(1+\frac{\phi_1' r}{\phi_0' K_i}\right)+\frac{\phi_1'}{[S_1']}+\frac{\phi_2'}{[S_2']}\left(1+\frac{\phi_{12}' r}{\phi_2' K_i}\right)+\frac{\phi_{12}'}{[S_1'][S_2']}$$
$$= \phi_0'\left(1+\frac{K_m' r}{K_i}\right)+\frac{\phi_1'}{[S_1']}+\frac{\phi_2'}{[S_2']}\left(1+\frac{K_1' r}{K_i}\right)+\frac{\phi_{12}'}{[S_1'][S_2']} \qquad (2)$$

In eqn. (2), K_m' is the Michaelis constant for the coenzyme S_1', K_1' is the dissociation constant of ES_1', K_i the dissociation constant of EI, and r the constant molar ratio of inhibitor to coenzyme. If the inhibitor is $NADH_2$, then at pH 7·0 K_i is 0·3 μM (Theorell & McKinley-McKee, 1961). From the initial-rate parameters for the ethanol–NADP reaction obtained with chromatographically purified NADP (Table 1), and assuming 0·1% of $NADH_2$ ($r = 0{\cdot}001$), one may evaluate the inhibition factors in eqn. (2) as $(1+K_m' r/K_i) = 8$ and $(1+K_1' r/K_i) = 14$. The kinetic coefficients obtained with commercial NADP are in satisfactory agreement with these theoretical predictions: the values for ϕ_0' and ϕ_2' were greater by factors 6 and 15 respectively than those obtained with chromatographically purified NADP, whereas ϕ_1' and ϕ_{12}' were not significantly different from the two coenzyme samples (Table 1). Both the apparent Michaelis constants (ϕ_1'/ϕ_0') and the apparent dissociation constants of the enzyme–coenzyme compound (ϕ_{12}'/ϕ_2') obtained with the commercial NADP preparation are therefore smaller than those obtained with the purified material.

The marked effect of phosphate concentration on the rate of the NADP–ethanol reaction also has the kinetic features of competitive inhibition towards the coenzyme. This is shown by the fact that, when additional phosphate was introduced into reaction mixtures with the coenzyme solution, the apparent values for ϕ_0' and ϕ_2' were greatly increased, whereas ϕ_1' and ϕ_{12}' were not altered significantly (Table 1), in accordance with eqn. (2). This equation is simply the usual initial-rate equation for competitive inhibition in a compulsory-order mechanism, for the special case that the inhibitor concentration is not constant, but varies in proportion to the concentration of the coenzyme with which it competes (Dalziel, 1962*c*). Interpretation of the effect of phosphate requires more detailed study, but its magnitude in these experiments underlines the practical importance of maintaining a strictly constant buffer concentration.

For a compulsory-order mechanism, the kinetic coefficient ϕ_1 is equal to the reciprocal of the second-order velocity constant for the combination of enzyme and coenzyme (Dalziel, 1957). The data of Table 3 indicate that these reactions are much slower with NADP(H_2) than with NAD(H_2). These parameters are not affected by the presence of competing impurities in the coenzymes. The coefficients ϕ_2 and ϕ_2' are functions of the rate constants for the combination of the substrates with the enzyme–coenzyme compound, and the subsequent reactions of the ternary complex. It appears from the values of ϕ_2 in Table 1 that for the acetaldehyde reaction these steps are no slower with $NADPH_2$ than with $NADH_2$. The fact that ϕ_2' is greater with NADP than with NAD, on the other hand, might indicate that one or more of these steps in the ethanol reaction is slower with the former coenzyme. It has been seen, however, that competitive inhibitors in the coenzyme can give erroneously large values for ϕ_2'. This point is elaborated below.

For all mechanisms for which an initial-rate equation of the form of eqn. (1) has been derived, the ratio $\phi_{12}[H^+]/\phi_{12}'$ should be equal to the equilibrium constant of the overall reaction of coenzyme and substrate (Dalziel, 1957, 1963*a*). From the kinetic coefficients for the NADP and $NADPH_2$ reactions in Table 3, these ratios are $1{\cdot}7\times10^{-11}$ M at pH 6·0 and $2{\cdot}1\times10^{-11}$ M at pH 7·0. From direct equilibrium studies of the reaction of NAD with ethanol, Bäcklin (1958) obtained $0{\cdot}9\times10^{-11}$ M. The reason for this difference is not clear. There is no good evidence for a significant difference

between the oxidation–reduction potentials of the NAD–$NADH_2$ and NADP–$NADPH_2$ couples (cf. Kaplan, 1960). Values for $\phi_{12}[H^+]/\phi'_{12}$ from initial-rate measurements with NAD and $NADH_2$ were also generally close to 2×10^{-11}M. However, the values of ϕ_{12} were small and difficult to determine accurately with this coenzyme, and the difference from the direct value of $0{\cdot}9 \times 10^{-11}$M was not considered to be significant (Dalziel, 1963*a*). This explanation is less satisfactory for the present data, since ϕ_{12} and ϕ'_{12} are large and reasonably reproducible, but no firm conclusion can be drawn.

The ratios ϕ_{12}/ϕ_2 and ϕ'_{12}/ϕ'_2 should be equal to the dissociation constants of the enzyme–coenzyme compounds (Dalziel, 1957; Frieden, 1957). Such kinetic values for the dissociation constants of the NAD and $NADH_2$ compounds of liver alcohol dehydrogenase (Dalziel, 1963*a*) agree well with direct estimates from equilibrium studies of enzyme and coenzyme in the absence of substrates (Theorell & McKinley-McKee, 1961). Unfortunately, it was not possible to make direct estimates of the dissociation constants of the NADP and $NADPH_2$ compounds. The kinetic values are large compared with those for the NAD and especially the $NADH_2$ compounds (Table 4). The fact that there was no significant change of light absorption in the wavelength region 310–350mμ when the largest practicable concentrations of enzyme and $NADPH_2$ were mixed indicates that the dissociation constant of the compound is greater than 1mM at pH 7·0, and is consistent with the kinetic value. The formation of a compound with spectral characteristics similar to those of the enzyme–$NADH_2$ compound was demonstrated by mixing enzyme and $NADPH_2$ in the presence of isobutyramide, which evidently increases the affinity of the enzyme for $NADPH_2$ as well as for $NADH_2$ (Theorell & McKinley-McKee, 1961). Dissociation was still too large to permit determination of the stoicheiometry, but the results were consistent with the binding of two molecules of $NADPH_2$/molecule of enzyme, as has been shown for $NADH_2$ (Theorell & Bonnichsen, 1951; Ehrenberg & Dalziel, 1958) and certain analogues (Li, Ulmer & Valee, 1962).

Table 4. *Dissociation constants* (μM) *of coenzyme compounds of liver alcohol dehydrogenase*

The values for NADP and $NADPH_2$ are calculated from kinetic coefficients in Table 3 as ϕ'_{12}/ϕ'_2 and ϕ_{12}/ϕ_2 respectively. The values for NAD and $NADH_2$ are from direct equilibrium studies by Theorell & McKinley-McKee (1961).

pH	NADP	$NADPH_2$	NAD	$NADH_2$
6·0	3000	620	266	0·23
7·0	4200	3000	160	0·31

Theoretical considerations have indicated that values of the ratios $\phi_1\phi_2/\phi_{12}\phi'_0$ and $\phi'_1\phi'_2/\phi'_{12}\phi_0$ may provide evidence of the occurrence of isomeric enzyme–coenzyme compounds in a compulsory-order mechanism, and of the kinetic significance of ternary complexes. For the simplest mechanism, suggested by Theorell & Chance (1951), in which it is assumed that ternary complexes are not rate-limiting, these ratios should be equal to unity (Dalziel, 1957). This is true for liver alcohol dehydrogenase with NAD and $NADH_2$ provided that coenzyme preparations of high purity are used (Dalziel, 1963*a*). If ternary complexes were rate-limiting, these ratios would be less than unity. From the present results with NADP and $NADPH_2$ (Table 3), the ratios at pH 6·0 and 7·0 are $\phi_1\phi_2/\phi_{12}\phi'_0 = 0{\cdot}09$ and 0·01, and $\phi'_1\phi'_2/\phi'_{12}\phi_0 = 53$ and 57. It has been shown theoretically that such results are consistent with the presence of more than one form of compound of enzyme and oxidized coenzyme in a compulsory-order mechanism (Mahler, Baker & Shiner, 1962), and this explanation has been suggested for similar, although much less marked, deviations of these ratios from unity that have been noted for several other dehydrogenases (Bloomfield, Peller & Alberty, 1962).

Though this explanation of the present data cannot be dismissed, we have not been able to reconcile it in any simple way with the complete conformity of initial-rate data for the NAD reaction to the requirements of the Theorell–Chance mechanism. It has been pointed out previously that the kinetic effects of a competing impurity in the coenzyme are indistinguishable from those of isomeric compounds of enzyme and coenzyme, and therefore kinetic evidence for the existence of the latter is equivocal. The present experiments are peculiar in that the coenzyme under investigation is very weakly bound by the enzyme, whereas closely related compounds such as $NADH_2$ and ADP-ribose, and perhaps others, are very firmly bound. It therefore seems possible that the chromatographically purified NADP still contains traces of nucleotide impurity that result in erroneously large values for ϕ'_0 and ϕ'_2. In these circumstances it is thought that no conclusions can be drawn about the role of isomeric enzyme–coenzyme compounds in the reaction, nor does it seem likely that decisive experimental evidence could be obtained readily. Estimates of dissociation constants, maximal turnover numbers and Michaelis constants may be accepted as minimum values.

The results of these experiments indicate that the main reason, and perhaps the only one, for the relatively low activity of liver dehydrogenase with finite concentrations of NADP and $NADPH_2$ is that it combines only slowly with, and has small affinity for, these coenzymes. It has been suggested that

since ADP-ribose is firmly bound by liver alcohol dehydrogenase, whereas ADP is not, the terminal ribose residue of the former compound may be important for firm binding (Dalziel, 1961*b*; Yonetani, 1963). The effect of the 2′-phosphate of $NADPH_2$ on the coenzyme binding further suggests that both ribose residues are important for the remarkably firm binding of $NADH_2$.

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REFERENCES

Bäcklin, K. (1958). *Acta chem. scand.* **12**, 1279.
Bloomfield, V., Peller, L. & Alberty, R. A. (1962). *J. Amer. chem. Soc.* **84**, 4367.
Dalziel, K. (1957). *Acta chem. scand.* **11**, 1706.
Dalziel, K. (1961*a*). *Biochem. J.* **80**, 440.
Dalziel, K. (1961*b*). *Nature, Lond.*, **191**, 1098.
Dalziel, K. (1962*a*). *Biochem. J.* **84**, 244.
Dalziel, K. (1962*b*). *Biochem. J.* **84**, 240.
Dalziel, K. (1962*c*). *Nature, Lond.*, **195**, 384.
Dalziel, K. (1963*a*). *J. biol. Chem.* **238**, 2850.
Dalziel, K. (1963*b*). *J. biol. Chem.* **238**, 1538.
Ehrenberg, A. & Dalziel, K. (1958). *Acta chem. scand.* **12**, 465.
Frieden, C. (1957). *J. Amer. chem. Soc.* **79**, 1894.
Horecker, B. L. & Kornberg, A. (1948). *J. biol. Chem.* **175**, 385.
Horecker, B. L. & Kornberg, A. (1957). In *Methods in Enzymology*, vol. 3, p. 879. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Kaplan, N. O. (1960). In *The Enzymes*, vol. 3, part B, p. 150. Ed. by Boyer, P. D., Lardy, H. & Myrbäck, K. New York: Academic Press Inc.
Li, T. K., Ulmer, D. D. & Vallee, B. L. (1962). *Biochemistry*, **1**, 115.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Mahler, H. R., Baker, R. H., jun. & Shiner, V. J., jun. (1962). *Biochemistry*, **1**, 47.
Pullman, M. E., Colowick, S. P. & Kaplan, N. O. (1952). *J. biol. Chem.* **194**, 593.
Theorell, H. & Bonnichsen, R. (1951). *Acta chem. scand.* **5**, 1105.
Theorell, H. & Chance, B. (1951). *Acta chem. scand.* **5**, 1127.
Theorell, H. & McKinley-McKee, J. S. (1961). *Acta chem. scand.* **15**, 1811.
Theorell, H., Nygaard, A. P. & Bonnichsen, R. (1955). *Acta chem. scand.* **9**, 1148.
Yonetani, T. (1963). *Biochem. Z.* **338**, 316.