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The Purification of Nicotinamide Adenine Dinucleotide and the Kinetic Effects of Nucleotide Impurities

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Large variations of the maximal rate of aldehyde reduction by liver alcohol dehydrogenase and reduced nicotinamide adenine dinucleotide (NADH) showed that commercial preparations of this coenzyme contain variable amounts of an inhibitor (1). The observed kinetic effects were reproduced quantitatively by a known competitive inhibitor, adenosine diphosphate ribose, added in constant molar ratio to the coenzyme, and were consistent with theoretical treatment of this unfamiliar situation (2, 3). It was evident from the theory that in the reverse reaction, small amounts of inactive nucleotide impurity in the oxidized coenzyme, NAD^+, might cause large errors in the estimation of the Michaelis constant for the substrate alcohol, in neutral and acid solution. Such errors would explain the deviations of initial rate data for liver alcohol dehydrogenase from the requirements of a compulsory order mechanism, first reported some years ago (4, 5) and recently confirmed by more detailed and precise measurements (6). The detection and separation of such an inhibitor from several commercial preparations of NAD^+ of high purity and the general kinetic effects of competing impurities in coenzymes, especially in relation to tests of mechanism, are described in this paper. Kinetic evidence of the mechanism of liver alcohol dehydrogenase will be reconsidered in the light of these findings in a later paper.[1] Preliminary accounts of aspects of this work have been published (7, 8).

EXPERIMENTAL PROCEDURE

Five commercial NAD^+ preparations were used. The active coenzyme content was estimated from the increase of light absorption at 340 mμ accompanying complete reduction by alcohol dehydrogenase and excess ethanol at pH 10, and the molar absorbancy of NADH, 6.22×10^6 cm² per mole† (9). Three samples from Sigma Chemical Company (designated 98 to 99% β-DPN, corrected for 2.5 or 5 moles of H_2O) contained 89 to 90% NAD^+ (mol. wt. 663) by weight, uncorrected. A preparation from C. F. Boehringer und Soehne contained 85%, and one from Pabst Laboratories 76%, NAD^+.

The quinine salt of NAD^+ was prepared and recrystallized as described by Wallenfels and Christian (10). ADP-ribose was obtained from Pabst Laboratories.

The preparation and assay of liver alcohol dehydrogenase, and measurements of initial rates with a recording fluorometer, were carried out with the techniques and apparatus described previously (6, 11).

Diethylaminoethyl cellulose (Whatman) was washed with 0.5 N NaOH + 0.5 M NaCl, then with 0.2 M phosphate buffer, pH 6.0, and finally with 5×10^{-3} M phosphate, pH 6.0, until the conductivity of the buffer remained unchanged. Columns 50 cm in length and 2.5 cm in diameter were packed by gravity and gave flow rates of about 250 ml per hour. Chromatography of NAD^+ was carried out at +2°. The coenzyme (0.1 to 0.45 g) was applied to the column in 5 to 10 ml of the dilute buffer and was washed in with a further 20 ml. A concentration gradient of the same buffer was then applied by running 0.2 M phosphate, pH 6.0, into a 500-ml reservoir of 5×10^{-3} M buffer at the flow rate of the column. The quinine salt of NAD^+, dissolved in 30 ml of dilute buffer with heating, was chromatographed in the same way, except that the quinine was first eluted with the dilute buffer.

RESULTS

Kinetic Effects of Competing Impurities in Coenzymes—The steady state initial rate equation for a compulsory order mechanism, with or without rate-limiting ternary complexes, in which the coenzyme S_1 forms a binary complex with the enzyme, but the substrate S_2 does not (*cf.* (4), Type II) may be written

$$E/v_0 = \phi_0 + \phi_1/S_1 + \phi_2/S_2 + \phi_{12}/S_1S_2 \quad (1)$$

where E represents the total enzyme concentration, S_1 and S_2 represent the initial concentrations of coenzyme and substrate, respectively, and v_0 is the initial rate. The coefficients in the equation may be determined experimentally as the slopes and intercepts of double reciprocal plots (4).

If an inhibitor is present which competes with S_1 and forms an inactive complex EI, with dissociation constant K_i, the initial rate equation is

$$E/v_0 = \phi_0 + \phi_1 (1 + I/K_i)/S_1 + \phi_2/S_2 + \phi_{12} (1 + I/K_i)/S_1S_2 \quad (2)$$

If the inhibitor is present in constant molar ratio to the coenzyme, $r = I/S_1$, as in the case of an impurity in the latter, then this equation becomes

$$E/v_0 = \phi_0(1 + K_{m_1}r/K_i) + \phi_1/S_1 + \phi_2 (1 + K_1r/K_i)/S_2 + \phi_{12}/S_1S_2 \quad (3)$$

K_{m_1} is the Michaelis constant for the coenzyme, defined as the concentration of S_1 which, with an infinitely large concentration of S_2, gives half of the maximal rate; and K_1 is the dissociation constant of the coenzyme complex ES_1. Equation 1 may also be written in the form $1/v_0 = 1/V + K_{m_1}/VS_1 + K_{m_2}/VS_2 + K_1K_{m_2}/VS_1S_2$, since (4) the two sets of parameters are related as follows: $V = E/\phi_0$, $K_{m_1} = \phi_1/\phi_0$, $K_{m_2} = \phi_2/\phi_0$, and $K_1 = \phi_{12}/\phi_2$.

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[1] To be published.

† Editor's note: 6.22×10^6 cm² per mole = 6.22×10^3 M⁻¹ cm⁻¹. The latter units are preferred by the *Journal*.

Equation 3 is of the same form as Equation 1; if a competing impurity is present in the coenzyme, experimental determination of the kinetic coefficients, in the usual way, will give the correct values for ϕ_1 and ϕ_{12}, but the apparent values of ϕ_0 and ϕ_2 will be greater than the true values, by the two inhibition factors within the brackets in Equation 3. The relative values of these two factors depend upon the relative values of K_{m_1} and K_1.

These conclusions were tested by two series of kinetic experiments with liver alcohol dehydrogenase and ADP-ribose at pH 6.0. In the first, the inhibition constant, K_i, was estimated in the usual manner from initial rate measurements with a constant ADP-ribose concentration and several concentrations of coenzyme and substrate, with Equation 2. In the second, the effects of an inhibiting impurity in the coenzyme were simulated by similar measurements in which the molar ratio of ADP-ribose to coenzyme was kept constant. Results of both kinds of experiment are shown in Fig. 1 as secondary plots of the reciprocal specific rates with infinitely large ethanol concentrations (*i.e.* partial maximal rates estimated in the usual way as intercepts of primary plots with reciprocal ethanol concentrations as variable) against reciprocal NAD^+ concentration. With a fixed ADP-ribose concentration of 21 μM, conventional competition with NAD^+ is observed, and the value obtained for K_i, 6.2 μM, agrees reasonably well with the value of 8.5 μM previously reported from similar measurements on the reverse reaction (2). With a fixed molar ratio of ADP-ribose to NAD^+ of 1.3, the intercept (ϕ_0) is almost doubled, whereas the slope (ϕ_1) is unchanged, in qualitative agreement with Equation 3 and with similar experiments on the reverse reaction (2).

The alternative plots of the partial maximal rates with infinitely large coenzyme concentration against the reciprocal of the substrate concentration, for the reaction in both directions (Figs. 2 and 3), show the very different effects produced on the forward and reverse reaction by a fixed molar ratio of inhibitor to coenzyme. With NAD^+ and ethanol, the intercept is again doubled, of course (although the scale of Fig. 2 is not suited to show this), but the slope (ϕ_2) is increased by a much larger factor. In contrast, with NADH and acetaldehyde in Fig. 3, the slope is not significantly affected by a molar ratio of ADP-ribose to NADH which causes a 3-fold increase in the intercept.

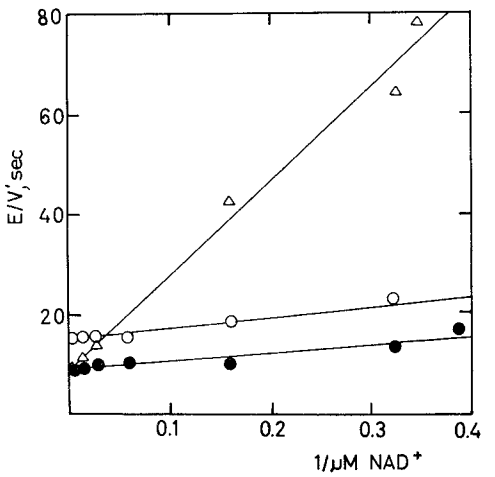

FIG. 1. Inhibition of liver alcohol dehydrogenase by ADP-ribose at pH 6.0 and 23.5°. Plots of the reciprocals of the partial maximal rates with infinitely large ethanol concentrations and unit enzyme concentrations, E/V', against the reciprocals of the NAD^+ concentration. Values for E/V' were obtained as intercepts of plots of E/v_0 against reciprocals of ethanol concentrations. ●, No inhibitor; △, 21 μM ADP-ribose; ○, molar ratio of ADP-ribose to NAD^+ (1.3).

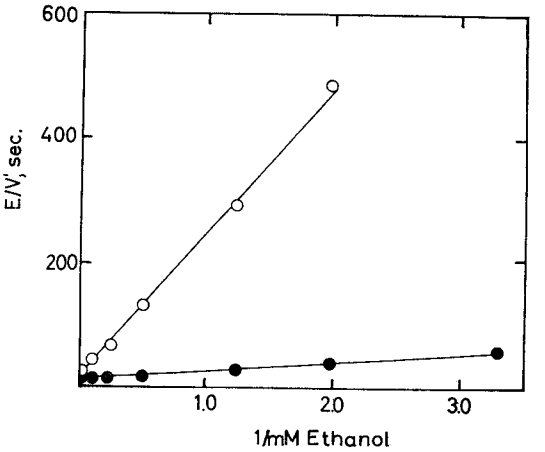

FIG. 2. Inhibition of liver alcohol dehydrogenase by a fixed molar ratio of ADP-ribose to NAD^+ at pH 6.0 and 23.5°. Plots of the reciprocals of the partial maximal rates with infinitely large NAD^+ concentration against the reciprocals of the ethanol concentration. ●, No inhibitor; ○, molar ratio of ADP-ribose to NAD^+ (1.3).

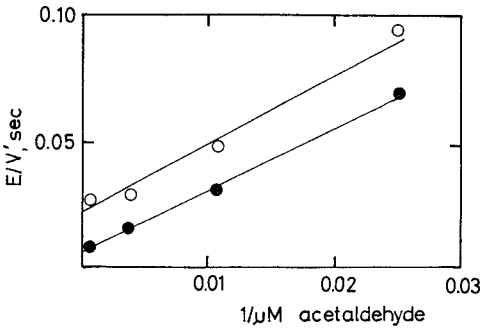

FIG. 3. Inhibition of liver alcohol dehydrogenase by a fixed molar ratio of ADP-ribose to NADH at pH 6.0 and 23.5°. Plots of the reciprocals of the partial maximal rates with infinitely large NADH concentration against the reciprocals of the aldehyde concentration. ●, No inhibitor; ○, molar ratio of ADP-ribose to NADH (1.4).

Quantitative data from these experiments are consistent with Equation 3 and with kinetic values for the Michaelis constants and dissociation constants of the enzyme-coenzyme complexes obtained in earlier work in the absence of added inhibitor. The mean value for K_i is 7 μM. For NADH, K_{m_1} ($=\phi_1/\phi_0$) and K_1 ($=\phi_{12}/\phi_2$) are 14 μM and 0.19 μM, respectively (6). Hence with $r = 1.4$, the inhibition factors in Equation 3 may be calculated: $(1 + K_{m_1}r/K_i) = 3.6$, and $(1 + K_1r/K_i) = 1.03$. The experimental values from the data of Fig. 3 and Equations 1 and 3 are 3.5 and 1.0, respectively. For NAD^+, $K_{m_1} = 4.0$ μM and $K_1 = 88$ μM (6), and with $r = 1.3$, $(1 + K_{m_1}r/K_i) = 1.7$ and $(1 + K_1r/K_i) = 16$. The experimental values from Fig. 2 are 1.7 and 14, respectively.

Each form of the coenzyme will act as an inhibitor of the reverse reaction with $K_i = K_1$ (12). The relative values of K_1 for oxidized and reduced coenzymes just given show that at pH 6.0 quite a high proportion of NAD^+ as an impurity in NADH would have little effect. On the other hand, the kinetic parameter, ϕ_2, for the oxidation of ethanol by NAD^+, and the apparent

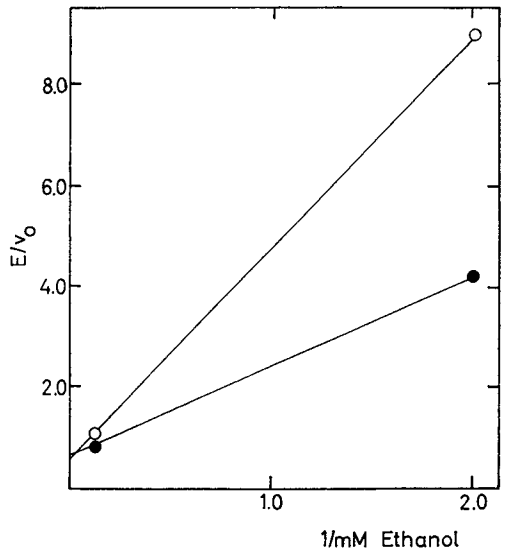

FIG. 4. Inhibition by added NADH of the oxidation of ethanol by NAD^+ and liver alcohol dehydrogenase. Variation of the reciprocal of the initial rate with the reciprocal of the ethanol concentration. The NAD^+ concentration was 5×10^{-4} M in every case. ●, No addition; ○, 1.5×10^{-6} M NADH was added with the reactants.

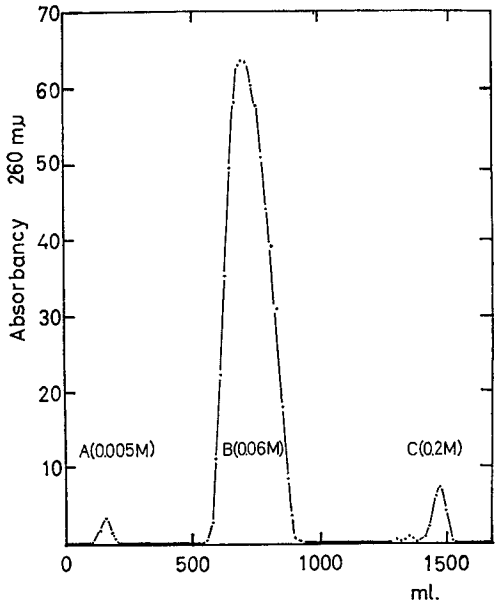

FIG. 5. Elution diagram for the chromatography of 450 mg of NAD^+ (Sigma Chemical Company) on DEAE-cellulose, showing the variation of absorbancy at 260 mμ with volume of eluate. Column dimensions, 50 cm × 2.5 cm. Elution was effected with a concentration gradient of phosphate buffer, pH 6.0.

Michaelis constant for ethanol should be extremely sensitive to contamination of NAD^+ by NADH, or by any nucleotide impurity which resembles NADH rather than NAD^+ in its affinity for the enzyme. This is illustrated experimentally in Fig. 4 by the effect of only 3 moles of NADH per 1000 moles of NAD^+ on initial rates of reaction of NAD^+ and ethanol. In accordance with Equation 3, the apparent Michaelis constant for ethanol is increased considerably, whereas the maximal rate is not affected significantly. Errors of this kind and magnitude would completely explain the deviations between initial rate data for liver alcohol dehydrogenase and the requirements of a compulsory order mechanism (6).

Separation of a Competitive Inhibitor from NAD^+ Preparations—Solutions of commercial preparations of NAD^+ have a measurable fluorescence and also a small absorbancy at 340 mμ. The intensity of the fluorescence varies considerably from preparation to preparation, and in some cases amounts to as much as

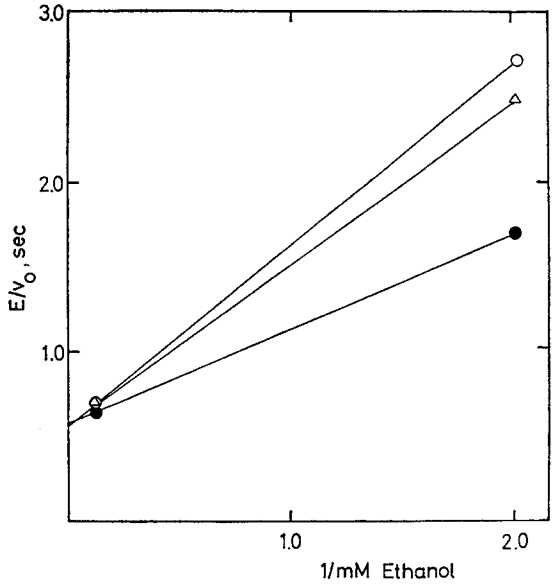

FIG. 6. Plots according to Lineweaver and Burk (13), showing initial rates of oxidation of ethanol by liver alcohol dehydrogenase with the same concentration, 2.5×10^{-4} M, of three NAD^+ preparations. ○, Sigma 1; △, Sigma 2; ●, Sigma 1 after purification by chromatography on DEAE-cellulose, pH 6.0, 23.5°.

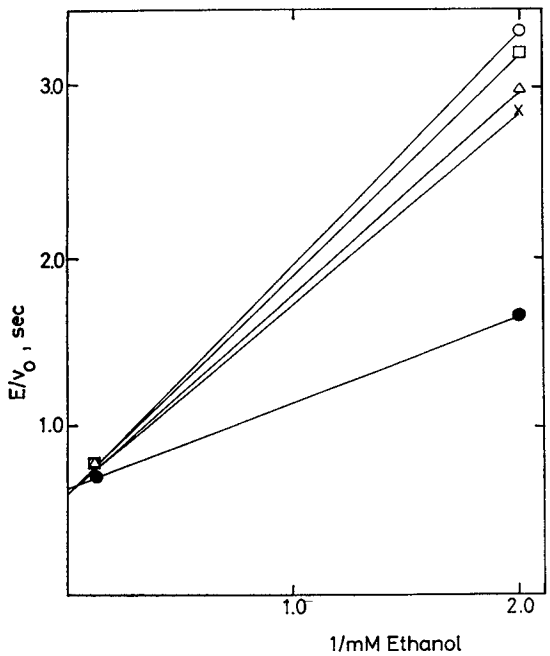

FIG. 7. Initial rates of oxidation of ethanol with liver alcohol dehydrogenase at pH 6.0 and 23.5°, plotted according to Lineweaver and Burk (13), with the same concentrations, 5×10^{-4} M, of four NAD^+ preparations. ○, Sigma 3; □, Boehringer; △, Pabst; ●, Pabst after chromatographic purification. The effect of restoring the main impurity to the latter is also shown (×).

1% of that due to an equivalent concentration of NADH. It is not due to NADH, however, since it is not diminished by an excess of acetaldehyde and liver alcohol dehydrogenase at pH 6.0. The compound responsible has not been identified or isolated, but it is almost entirely removed during chromatographic purification of NAD^+ preparations as described below. It does not inhibit significantly, since no correlation was observed between the intensity of fluorescence of NAD^+ preparations and the rate of oxidation of ethanol in low concentrations.

The separation of two main nucleotide impurities from NAD^+ by chromatography on DEAE-cellulose is shown in the elution diagram of Fig. 5. Similar results were obtained with all five commercial preparations used, and with the quinine salt prepared from two of them. The minor component, *A*, represented 0.5 to 1.5%, and the major impurity, *C*, about 3%, of the total nucleotide absorbancy. Neither impurity was coenzymatically active with liver alcohol dehydrogenase or possessed significant absorbancy at 340 mμ. By combining the most concentrated NAD^+-containing eluates (*B*), about 80% of the total could be recovered as a solution of 3 mg per ml in 0.06 M phosphate.

Figs. 6 and 7 show initial rates of ethanol oxidation at pH 6.0 obtained with NAD^+ purified in this manner and with the original commercial preparations, in the same concentrations. With high ethanol concentration, there are no significant differences, but with low ethanol concentration the eluates give much higher rates than the original materials. In the light of the theory and experiments described earlier, the removal of a competitive inhibitor from the commercial products is indicated. This conclusion is confirmed by the effect on the rate with purified NAD^+ of adding the main impurity, *C* (Fig. 7). This is evidently the principal inhibitor in the original materials. The component *A*, even when added in 5 times its original proportion, did not cause significant inhibition in the presence of the purified coenzyme.

In similar initial rate measurements at pH 7.0, smaller differences between purified and commercial NAD^+ preparations were observed. At pH 9.0 there were no significant differences.

From measurements of the active coenzyme content of the eluates, and from absorbancy measurements at 260 mμ and pH 7.0, the value 17.6 (±0.2) × 10^6 cm^2 per mole was obtained for the molar absorbancy of the purified NAD^+, relative to 6.22 × 10^6 for NADH at 340 mμ, with Sigma and Boehringer coenzyme as the starting material. The eluates of Pabst NAD^+ gave a higher value, indicating the presence of 8% of the inactive α isomer, which is not separated from the β form by chromatography (14). It is interesting, but not unexpected, that the presence of this proportion of α-NAD^+ in the commercial product and in the eluates obtained from it does not cause significant inhibition. This is shown by comparison with the Sigma products, which are free from α isomer, and with purified NAD^+ obtained from them (Figs. 6 and 7).

DISCUSSION

Chromatography on DEAE-cellulose (15) is shown to provide a rapid and sensitive method for the detection and separation of nucleotide impurities present in currently available commercial preparations of NAD^+. These impurities, amounting to about 3 to 4% of the total nucleotide, are not removed by recrystallization as the quinine salt. The cellulose derivative permits a simpler procedure and milder conditions than were employed in earlier ion exchange methods (10, 16, 17). A concentrated solution of NAD^+ in pH 6.0 phosphate buffer can be obtained in 2 hours, and is stable at 0° for at least 4 days, as judged by enzymatic assay as NADH and by initial rate measurements with liver alcohol dehydrogenase and low concentrations of ethanol (5×10^{-4} M) at pH 6.0.

The purified coenzyme was not isolated in the solid form. Wallenfels and Christian (10) found that pure NAD^+ prepared from the quinine salt by ion exchange chromatography lost 10% active coenzyme content during dry storage of the solid at 0°. These workers did not report the molar absorbancy at 260 mμ. The value of 17.6 × 10^6 cm^2 per mole found in the present work is slightly smaller than the generally accepted value of 18.0 × 10^6 cm^2 per mole (17). For a commercial preparation freed from the α isomer by ion exchange chromatography after enzymatic reduction, 17.8 × 10^6 cm^2 per mole has been reported (18).

Until recently, little attention had been paid to the effects of inhibitors which might be present as impurities in coenzymes or substrates. It is obvious that even a small proportion of competitive inhibitor which has a much greater affinity than the coenzyme for the enzyme would cause large errors. However, the precise nature of the errors, and the possibility that they may be large even if the inhibitor has a much smaller affinity for the enzyme than the coenzyme, are perhaps less obvious. It has been shown by steady state analysis that the effects of an inhibitor which competes with, and is present in, the coenzyme are confined to two of the four kinetic coefficients in the reciprocal form of the initial rate equation, namely, ϕ_0 and ϕ_2. The apparent values for the maximal rate $1/\phi_0$, and the Michaelis constant for the coenzyme, ϕ_1/ϕ_0, will always be smaller than the true values by the same factor, $K_i/(K_i + K_{m_1}r)$. The apparent Michaelis constant for the substrate, $\phi_2(1 + K_1r/K_i)/\phi_0 (1 + K_{m_1}r/K_i) = \phi_2 (K_i + K_1r)/\phi_0 (K_i + K_{m_1}r)$, may be greater or smaller than the true value, ϕ_2/ϕ_0, according to the relative values of the dissociation constant, K_1, and the Michaelis constant, K_{m_1}, for the coenzyme.

These conclusions are supported by experiments with liver alcohol dehydrogenase and the competitive inhibitor, adenosine diphosphate ribose. This enzyme provides examples in its reversible reaction at pH 6.0 of two extreme cases.

Case 1—If $K_{m_1} \gg K_1$, a small proportion of inhibitor, even though it binds to the enzyme no more firmly than the coenzyme (*i.e.* $K_i \geq K_1$), may nevertheless give an apparent value for ϕ_0 much greater than the true value, whereas ϕ_2 may not be significantly in error. The maximal rate and the Michaelis constants for both coenzyme and substrate will then be depressed in the same proportion.

This is the case for the reduction of acetaldehyde by NADH at pH 6.0, and from this and other evidence it has been concluded (2) that large variations of the maximal rate, without variations of ϕ_1, ϕ_2, and ϕ_{12}, are due to variable small amounts of a competing nucleotide in different coenzyme preparations. Further, the fact that such variations were not observed in alkaline solution is consistent with the observed decrease of K_{m_1} with increase of pH (2).

Case 2—If $K_{m_1} \ll K_1$, a small proportion of a competitive inhibitor will be effective only if it has a relatively large affinity for the enzyme, and its effect will be greatest on ϕ_2. The maximal rate and Michaelis constant for the coenzyme may not be affected significantly, but the apparent Michaelis constant for the *substrate* will be greater than the true value. The effect of the inhibitor would then be detectable only in measurements with low substrate concentrations.

TABLE I

Ratios of Michaelis constants and dissociation constants for enzyme-coenzyme complexes, and ratios of kinetic coefficients, calculated from published initial rate data

Enzyme	pH	K_{m_1}'/K_1 (NADH)	K_{m_1}'/K_1 (NAD)	K_1'/K_1	$\phi_1\phi_2/\phi_{12}\phi_0'$	$\phi_1'\phi_2'/\phi_{12}'\phi_0$
Lactate dehydrogenase (beef heart) (21)	6.15	3.3	0.7	67	0.25	9.4
	6.98	6.3	0.4	66	0.86	2.9
Yeast alcohol dehydrogenase (22)	6.0	6.0	0.76	40	0.61	7.6
	7.15	6.0	0.19	37	0.67	1.6
Malic dehydrogenase (23)	8.0	3.4	0.29	140	1.0	1.0
Ribitol dehydrogenase (24)	8.0	0.87	0.12	26	0.28	0.36
Lactate dehydrogenase (rabbit muscle) (25)	6.8	1.28	0.4	75	0.26	1.9

This is the situation in the oxidation of ethanol by NAD^+ and liver alcohol dehydrogenase at pH 6.0. A few per cent of a nucleotide impurity common to several commercial coenzyme preparations results in overestimation of ϕ_2, and of the Michaelis constant for ethanol, by a factor of 3 (8). The affinity of the impurity for the enzyme, like that of NADH, must be much greater than that of NAD^+ at this pH value. This is evidently not true of the α isomer, which is not an effective inhibitor in small proportions. The smaller effect at pH 7.0, and the absence of significant inhibition at pH 9.0, are consistent with the marked decrease of the dissociation constant of the enzyme-NAD^+ complex with increase of pH and the smaller dependence of the dissociation constant of the NADH complex, and presumably that of the inhibitor complex, on pH.

The most important general conclusion to be drawn from this work is that for valid kinetic studies the purity of coenzymes must be controlled much more rigorously than has been realized or attempted hitherto. This is especially important in relation to tests of mechanism involving relations between kinetic parameters for forward and reverse reactions (4) and in studies of the variation of kinetic parameters with pH. For a given enzyme, the errors due to an inhibiting impurity will vary with pH according to the relative values of K_i, K_{m_1}, and K_1, and may be large at one pH value and negligible at another; the greatest errors are likely to occur at pH values such that either $K_{m_1} \gg K_1$ or the oxidized and reduced coenzymes have widely different affinities for the enzyme. It has been shown (6), for example, that a pK′ of 6.4 for liver alcohol dehydrogenase adduced from variation of the maximal rate of aldehyde reduction from pH 6.0 to 7.1 (19) was an anomaly caused by inhibitor in the NADH preparation used.

It is likely that published kinetic data for other enzymes have been subject in greater or lesser degree to similar errors. There is evidence that the purity of NADH preparations is fairly critical for lactic dehydrogenase of beef heart (20), whereas yeast alcohol dehydrogenase at pH 6.0 (1) and ribitol dehydrogenase[2] at pH 8.0 are less sensitive in this respect than liver alcohol dehydrogenase, but no detailed studies have yet been made.

[2] The inhibitor present in poor samples of NADH affects φ_0 and φ_2 equally, consistent with the fact that with this enzyme $K_{m_1} \simeq K_1$ for NADH (H. J. Fromm and K. Dalziel, unpublished observations).

It is interesting to consider the probable nature of the kinetic effects of these inhibitors on a number of other dehydrogenases for which sufficiently complete initial rate data are available and have been interpreted on the basis of a compulsory order mechanism. Relative values of Michaelis constants and dissociation constants calculated from published initial rate parameters (21–25) are shown in Table I. Constants for NAD^+ reactions are denoted by primed symbols, and those for NADH reactions, by unprimed symbols. In general, $K_m > K_1$ for NADH, whereas the reverse is true for NAD^+. As with liver alcohol dehydrogenase, therefore, the main effect of the inhibitor in NADH preparations would be on ϕ_0, and that of the competing nucleotide in NAD^+ would be to give too large a value for ϕ_2'. Only experiments with purified coenzymes can show whether these errors are significant, but it is interesting that for all these enzymes the apparent dissociation constant for the NAD^+ complex is considerably greater than that for the NADH complex. This gives grounds for thinking that the competing nucleotide demonstrated in NAD^+ preparations may be an effective inhibitor of these enzymes also. A little consideration also shows that the effects of inhibitors in the coenzymes would be to minimize the three inequalities just discussed.

The last two columns of Table I show experimental values for ratios of kinetic coefficients from which conclusions have been drawn regarding the significance of ternary complexes and isomers of binary coenzyme-enzyme complexes.[3] For a simple compulsory order mechanism with one binary complex of each coenzyme, these ratios should be equal to unity if ternary complexes are not formed or are not kinetically significant (the Theorell-Chance mechanism), and less than unity if ternary complexes are rate-limiting (4). For the enzymes considered in Table I, and for liver alcohol dehydrogenase (6), $\phi_1\phi_2/\phi_{12}\phi_0'$ satisfies one or other of these conditions. But for liver and yeast alcohol dehydrogenases (4) and lactic dehydrogenase of beef heart[3] (21), $\phi_1'\phi_2'/\phi_{12}'\phi_0 > 1$ at pH 6 to 7. Mahler, Baker, and Shiner (26) showed by steady state analysis that this could be explained if isomeric coenzyme-enzyme complexes are formed, and recently Bloomfield, Peller, and Alberty[3] restated these conclusions in more detailed and general terms, and emphasized that this relation may provide evidence for (but not against) the existence of isomeric coenzyme complexes, with reference to most of the data in Table I. The present work shows that experimental values of these ratios may be extremely sensitive to the presence of inhibiting impurities in the coenzymes, especially the ratio $\phi_1'\phi_2'/\phi_{12}'\phi_0$. Values greater than unity previously reported for liver alcohol dehydrogenase were due to the effect of the competing nucleotide in NAD^+ on ϕ_2 (8). Studies with purified coenzymes are evidently needed before reliable conclusions can be drawn from these ratios for other enzymes. It is interesting that for lactate dehydrogenase of rabbit muscle, studies of product inhibition indicated that the Theorell-Chance mechanism was valid; for this mechanism, these ratios (which were not considered by the authors) should be equal to unity.

Although attention has been directed here toward NAD^+, similar effects are to be expected from competing impurities in other coenzymes, especially in NADP, and in substrates, for example, inactive optical isomers. The theoretical treatment was based on a compulsory order mechanism with the assumption

[3] V. Bloomfield, L. Peller, and R. A. Alberty, personal communication.

of only one inactive complex, EI, but similar conclusions may be reached if an inactive complex, EIS_2, is taken into account, and also if an alternative pathway or random order mechanism is assumed.

A number of dehydrogenases and oxidases (28–31) appear to conform to a different mechanism, which involves successive oxidation and reduction of the enzyme (or in general, chemical modification by group transfer) by successive reactions with the coenzyme and substrate. The initial rate equation for this mechanism (4, 32) lacks a term in S_1S_2, and consequently the inhibition equation in this case corresponding to Equation 3 is $E/v_0 = \phi_0 (1 + K_{m_1}r/K_i) + \phi_1/S_1 + \phi_2/S_2$. If the last term in S_2 is omitted, this equation also applies to a single substrate reaction,[4] as was shown independently by Tubbs (31).

SUMMARY

1. The kinetic effects of an impurity in a coenzyme which acts as a competitive inhibitor are examined theoretically and by experiments with liver alcohol dehydrogenase and the competitive inhibitor, adenosine diphosphate ribose.

2. A competitive inhibitor has been separated from several commercial preparations of nicotinamide adenine dinucleotide of high purity. It is present in sufficient quantity to cause large errors in the estimation of initial rate parameters for liver alcohol dehydrogenase. Relations between kinetic parameters for forward and reverse reactions, from which conclusions may otherwise be drawn regarding the kinetic significance of ternary complexes and isomeric binary complexes, are thereby altered.

3. It is concluded that for valid kinetic studies of mechanism, and of the effects of pH on rates, the purity requirements for pyridine nucleotides may be very exacting and that published kinetic data for other enzymes, and conclusions drawn from them, may require revision.

4. For nicotinamide adenine dinucleotide purified by ion exchange chromatography, the molar absorbancy at 260 mμ is estimated to be 17.6 ± 0.2 cm² per mole.

Acknowledgment—I am greatly indebted to Mrs. B. Sutton for technical assistance.

[4] If a noncompetitive inhibitor is present as an impurity in the substrate, the initial rate equation takes the same form as that derived by Haldane (33) for substrate inhibition (3).

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