Biochem. J. (1962) **84**, 240

Some Observations on the Preparation and Properties of Dihydronicotinamide–Adenine Dinucleotide

BY K. DALZIEL*
Department of Biochemistry, University of Sheffield, Sheffield 10

(*Received* 28 *December* 1961)

In neutral and acid solution, the maximum rate of oxidation of commercial preparations of dihydronicotinamide–adenine dinucleotide by acetaldehyde or butyraldehyde, catalysed by liver alcohol dehydrogenase (alcohol–NAD oxidoreductase, EC 1.1.1.1), varies markedly with the purity of the preparations (Dalziel, 1961*a*, *b*). This phenomenon has important implications for kinetic studies of this enzyme, and perhaps of others also (Dalziel, 1962*a*). The purity and properties of coenzyme prepared by enzymic reduction of nicotinamide–adenine dinucleotide, and isolated first as the barium salt and then as the sodium salt, are compared here with those of a number of commercial samples, and with data recorded in the literature. Brief kinetic studies of the inhibition of liver alcohol dehydrogenase by adenosine diphosphate ribose are also reported.

EXPERIMENTAL

Enzymes. Crystalline alcohol dehydrogenase was prepared from horse liver as described by Dalziel (1961*a*). Yeast alcohol dehydrogenase was obtained from British Drug Houses Ltd. and Worthington Biochemical Corp.

Adenosine diphosphate ribose. The sodium salt monohydrate, mol.wt. 599, from Pabst Laboratories was assayed as 97% by weight from extinction measurements at 260 mμ and a millimolar extinction coefficient of 15·4 (Burton, 1959).

Nicotinamide–adenine dinucleotide. A preparation from Sigma Chemical Co. contained 98% NAD tetrahydrate by enzymic assay according to the method of Dalziel (1961*a*).

Dihydronicotinamide–adenine dinucleotide. The barium salt was prepared by reduction of NAD with ethanol and yeast alcohol dehydrogenase as described by Rafter & Colowick (1957). The yield from 200 mg. of NAD was 230 mg. of pale-yellow powder, dried *in vacuo* over $CaCl_2$. Liver alcohol dehydrogenase has also been used instead of the yeast enzyme, but is less convenient: the turnover number is smaller and because of substrate inhibition the ethanol is best added in successive portions. The barium salt also contains considerable alcohol-dehydrogenase activity in this case; it is removed during conversion into the sodium salt, whose properties are similar to those of the yeast enzyme.

To 200 mg. of barium salt dissolved in 6 ml. of water was added 0·6 ml. of M-Na_2SO_4; the mixture was centrifuged at room temperature, and 14 ml. of ethanol was added to the supernatant solution. After 1 hr. at 0°, the small precipitate was removed by centrifuging and discarded. The remaining steps were carried out at −10°. A mixture of 28 ml. of ethanol and 42 ml. of ether was added; the flocculent precipitate was separated by centrifuging after 1 hr., washed twice with ethanol–ether (1:1, v/v) and twice with ether, and dried *in vacuo* over $CaCl_2$ for 1 hr. at room temperature. The white solid was then powdered and put back in the desiccator for 24 hr. at −15°. The yield was 170 mg.

Samples of the disodium salt of $NADH_2$ were also purchased from Sigma Chemical Co. (β-$DPNH_2$), Pabst Laboratories and Mann Research Laboratories, and were kept desiccated at −15°.

To assay $NADH_2$ 7–10 mg. was quickly weighed and dissolved in 10 ml. of water. To a 1 cm. optical cell containing 3 ml. of phosphate buffer, pH 7, was added 0·2 ml. of $NADH_2$ solution, and the extinction measured at 260 mμ (E_{260}) and 340 mμ (E_{340}). After oxidation by the addition of 0·03 ml. of 0·5M-acetaldehyde and 0·01 ml. of 10 μM-liver alcohol dehydrogenase, the final stable value of E_{340} (residual E_{340}) was recorded. The relatively slow oxidation of the less pure commercial samples was striking. The concentration of active coenzyme was calculated from (initial E_{340}) − (residual E_{340}) and a millimolar extinction coefficient of 6·22 (Horecker & Kornberg, 1948). The purity of the solid samples was calculated as percent of $NADH_2$ (disodium salt) tetrahydrate.

Initial-rate measurements

Measurements of the initial rates of oxidation of $NADH_2$ by acetaldehyde and liver alcohol dehydrogenase at pH 6·0 and 23·5° were made with a recording fluorimeter (Dalziel, 1962*b*). The standard reactant concentrations were 20 μM-$NADH_2$ and 1·3 mM-acetaldehyde in phosphate buffer, I 0·1, which gives rates, v_0, close to maximal. EDTA was not added, since control experiments showed that it did not affect the results.

Chromatography

Paper chromatography by the descending method with acetone–water (3:1) as solvent gave a good separation of NAD, R_F 0·05, from $NADH_2$, R_F 0·11, and adenosine diphosphate ribose, R_F 0·12. The last-named compound was detected by $AgNO_3$ spray. Quicker and more complete separation of adenosine diphosphate ribose and $NADH_2$ was obtained by paper electrophoresis with pyridine–acetic acid buffer, pH 6·4.

* Sorby Research Fellow of the Royal Society.

Table 1. *Purity, extinction ratios and relative initial rates of oxidation, v_0, by acetaldehyde and liver alcohol dehydrogenase at* pH 6·0, *of dihydronicotinamide–adenine dinucleotide preparations*

Experimental details are given in the text.

Preparation	Purity (% $NADH_2$)	$\frac{E_{260}}{E_{340}}$	Residual* $\frac{E_{340}}{E_{340}}$	v_0 (relative)
1 (Laboratory)	89	2·4	0·020	100
2 (Laboratory)	91	2·4	0·020	100
2*a* (Laboratory)	86	2·55	0·025	84
3 (Laboratory)	81	2·85	0·080	85
4 (Sigma)	97	2·4	0·030	82
5 (Sigma)	88	2·55	0·025	77
6 (Sigma)	94	2·55	0·040	74
7 (Sigma)	87	2·6	0·055	63
8 (Sigma)	86	2·65	0·050	58
9 (Sigma)	82	2·85	0·080	37
10 (Pabst)	80	3·0	0·055	39
11 (Mann)	42	5·0	0·200	8

* After complete oxidation by acetaldehyde and liver alcohol dehydrogenase.

RESULTS

Assay values, extinction ratios and initial rates of oxidation for several $NADH_2$ (disodium salt) samples are shown in Table 1. Preparations 1 and 2 were freshly made as described with yeast alcohol dehydrogenase; their properties were unchanged after storage for 6 weeks at −15°. After a similar period at room temperature, *in vacuo* over anhydrous calcium chloride and in the dark, the purity and rate of oxidation had decreased slightly (preparation 2*a*). Preparation 3 was also made as described, with liver alcohol dehydrogenase, except that after reduction of the NAD the reaction mixture was heated at 90–100° for 10 min. to inactivate the enzyme. Before heating, assay showed the residual E_{340}, after enzymic oxidation, to be 1·5 % of the initial E_{340}; after heating it was 8 %, and remained at this high level in the isolated barium and sodium salts. The commercial preparations 4, 5, 8, 9 and 10 were tested within a few days of delivery, the remaining preparations after storage at −15° for various periods. Preparation 11 was in the form of large orange crystals, and contained 7 % of NAD. Of the other samples, the purest were white powders, the less pure pale-yellow powders, and all contained < 2 % of NAD. In control experiments the addition of NAD in concentration equal to that of $NADH_2$ (20 μM) decreased the initial rate of oxidation by only 10 %.

$NADH_2$ turns yellow on exposure to a moist atmosphere. When concentrated solutions of white preparations (about 300 mg./ml. in water or 0·4 M-disodium hydrogen phosphate) were dried in air or over anhydrous calcium chloride at room temperature, large yellow crystals were obtained. Compared with the original material, this product showed little loss of active coenzyme and only a slightly greater E_{260}/E_{340} ratio, but v_0 was less than half the original value. Similar changes occurred when such concentrated solutions were allowed to stand at room temperature without being dried, whereas dilute solutions (1 mg./ml.) were stable. The results of an experiment in which 20 mg. of preparation 5 was dissolved in 0·06 ml. of 0·4 M-disodium hydrogen phosphate in a stoppered tube and kept in the dark are shown in Table 2. At intervals, 5·1 μl. samples were diluted in 2 ml. of water, and assays and initial rate measurements were made. The absolute extinction values increased by 10 % in 22 hr. owing to evaporation. The increase of the ratio E_{260}/E_{340}, together with the increase of residual E_{340} (Table 2), indicate that in 22 hr. not more than about 8 % of the active coenzyme had been destroyed, but v_0 had fallen to half the initial value. Apart from the small changes just mentioned, the absorption spectrum and fluorescence emission and excitation spectra of the product were identical with those of the original material.

Table 2. *Changes in the properties of dihydronicotinamide–adenine dinucleotide in concentrated solution*

A portion (20 mg.) of preparation 5* was dissolved in 0·06 ml. of 0·4 M-disodium hydrogen phosphate. Samples were withdrawn and diluted at the times stated. Experimental details are given in the text.

Time (hr.)	$\frac{E_{260}}{E_{340}}$	Residual* $\frac{E_{340}}{E_{340}}$	v_0* (relative)
0	2·55	0·025	77
0·2	2·6	0·025	77
3·5	2·6	0·035	66
22	2·7	0·040	38

* See Table 1.

Inhibition by adenosine diphosphate ribose

The presence in the $NADH_2$ preparations of various amounts of adenosine diphosphate ribose could account qualitatively for their differences in

properties, insofar as this compound is a rather potent inhibitor of liver alcohol dehydrogenase at pH 6 but not at pH 10, and not with yeast alcohol dehydrogenase (Dalziel, 1961*b*). Small amounts of a compound with the same mobility as adenosine diphosphate ribose were detected by paper chromatography and electrophoresis in several samples of $NADH_2$, especially in preparation 11, and in a concentrated solution after 22 hr. (Table 2). Qualitative comparison with artificial mixtures showed, however, that the amounts were much too small to account for the apparent inhibitions.

The kinetics of inhibition of liver alcohol dehydrogenase by adenosine diphosphate ribose were studied at pH 6·0, both in the conventional manner with a constant concentration of inhibitor and several concentrations of $NADH_2$ and acetaldehyde, and with a constant molar ratio of adenosine diphosphate ribose to $NADH_2$, to simulate the presence of an inhibitor in the coenzyme preparation. The results of both kinds of experiment are shown (Figs. 1A and 1B) as secondary plots of reciprocal specific rates with infinitely large coenzyme (or substrate) concentration, estimated in the usual way as intercepts of primary plots according to Lineweaver & Burk (1934), against the reciprocal substrate (or coenzyme) concentration (cf. Dalziel, 1957). The results with a fixed concentration of 2·8 μM-adenosine diphosphate ribose indicate conventional competitive behaviour towards $NADH_2$: the maximum rate, and the Michaelis constant for acetaldehyde (Fig. 1B), are unchanged, but the slope in Fig. 1A, and therefore the Michaelis constant for $NADH_2$, are increased by a factor of 1·33. The apparent inhibitor constant, K_i, is therefore 2·8/0·33, i.e. 8·5, μM.

The results of the second kind of experiment, in which adenosine diphosphate ribose was present in a constant molar ratio to $NADH_2$ of 1·4, show that the maximum rate is decreased by a factor of 3·5: the slopes of the plots are not affected significantly, and the Michaelis constants of both substrate and coenzyme are therefore depressed by the same factor.

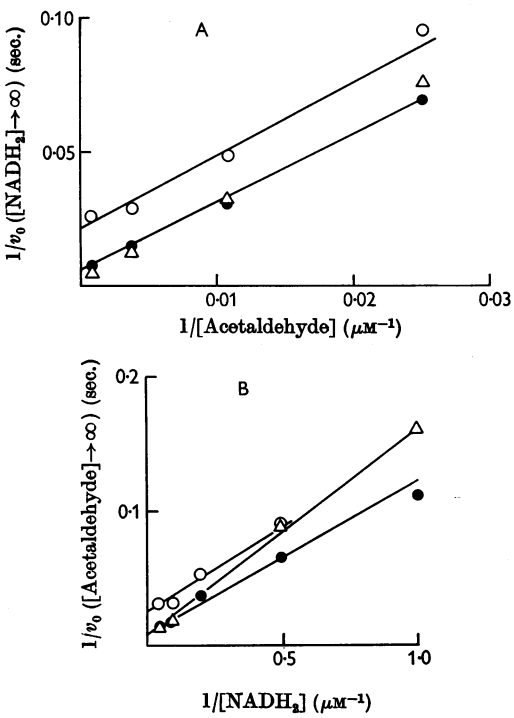

Fig. 1. Inhibition of liver alcohol dehydrogenase by adenosine diphosphate ribose at pH 6·0, and 23·5°. A, Plots of reciprocals of the specific rates ([$NADH_2$]→∞) against reciprocals of the acetaldehyde concentrations; B, plots of reciprocals of the specific rates ([acetaldehyde]→∞) against reciprocals of [$NADH_2$]. ●, No inhibitor; △, 2·8 μM-adenosine diphosphate ribose; ○, molar ratio of adenosine diphosphate ribose to $NADH_2$, 1·4.

DISCUSSION

$NADH_2$, prepared as the barium salt by the method of Rafter & Colowick (1957) and subsequently isolated as the sodium salt, gives maximum rates of aldehyde reduction (catalysed by liver alcohol dehydrogenase at pH 6) which are higher than those obtained with any of the commercial samples tested. Although the purity by weight of the laboratory preparations is less than that of the best commercial samples, the extinction ratio E_{260}/E_{340} and the proportion of coenzymically inactive material absorbing at 340 mμ are smaller and it is likely, therefore, that the main impurities are inorganic salts. Rafter & Colowick (1957) state that the purity of their product is equal to that of the NAD used; they do not record extinction ratios, but quote values of 16 and 6·25 for the millimolar extinction coefficients of $NADH_2$ at 260 and 340 mμ respectively, which give a ratio of 2·56. The present value of 2·4, obtained for both the barium salt and the sodium salt, may be compared with 2·72 (Drabkin, 1945) and 2·72–2·88 (Lehninger, 1952) for preparations isolated after chemical reduction of NAP. Siegel, Montgomery & Bock (1959) estimated the ratio to be 2·32 by enzymic oxidation of NAD, but did not isolate the $NADH_2$. The observation of Lehninger (1952) that the extinction ratio increased during dry storage at room temperature has been confirmed. It is possible that the purest commercial samples, which are little inferior to the laboratory preparations, suffered some decomposition during transport.

In previous enzymic methods of preparation of $NADH_2$ the enzyme was inactivated by heat

(Bonnichsen, 1950; Pullman, Colowick & Kaplan, 1952). This causes significant destruction of $NADH_2$ with increases of both the E_{260}/E_{340} ratio and the proportion of inactive material absorbing at 340 mμ. The rate of oxidation of this product by liver alcohol dehydrogenase was only slightly slower than that of the best preparations, in contrast with commercial preparations with comparable extinction ratios.

Both for the commercial samples and for material kept in concentrated solution there is a reasonably good correlation between the extinction ratio E_{260}/E_{340}, the proportion of inactive material absorbing at 340 mμ, and the rate of oxidation by liver alcohol dehydrogenase. The most obvious explanation is the presence of an inhibiting breakdown product of $NADH_2$. It is surprising, however, that preparations which assay as 80–82 % of active $NADH_2$ and have a nominal purity of 84–88 %, based on E_{340}, should show an apparent inhibition of more than 60 %; and, equally, that changes of absorption characteristics in concentrated solution which correspond to a loss of only about 8 % of the coenzyme should be accompanied by a decrease of 50 % in the maximum rate. This last observation underlines the importance of drying $NADH_2$ preparations quickly with organic solvents (Theorell & Nygaard, 1955; Rafter & Colowick, 1957); isolation by drying aqueous solutions (Bonnichsen, 1950) is clearly undesirable.

Attempts to isolate an inhibitor from $NADH_2$ have been unsuccessful, and it is difficult to characterize the type of inhibition in the usual manner. The tentative explanation of the experimental findings, that they are due to a breakdown product of $NADH_2$ which competes with the coenzyme, is supported by kinetic studies with adenosine diphosphate ribose. This hydrolysis product of $NADH_2$ was shown to behave as a competitive inhibitor. When present in constant molar ratio to the coenzyme, it depresses both the maximum rate and the Michaelis constants of substrate and coenzyme: that is, in the initial rate equation (Dalziel, 1957):

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

where e is the enzyme concentration, the kinetic coefficient ϕ_0 is increased and ϕ_1 and ϕ_2 are unchanged. Dalziel (1962*b*) has shown that samples of $NADH_2$ which give values for ϕ_0 differing by a factor of 3 (cf. Table 1) also give the same values for ϕ_1 and ϕ_2, consistent with the presence of a competitive inhibitor. It cannot be identified with adenosine diphosphate ribose, since an equimolar ratio of the latter to $NADH_2$ is needed to depress the maximum rate to the same extent as that observed with $NADH_2$ samples containing 80 % by weight of active coenzyme. An intermediate in the formation of adenosine diphosphate ribose from $NADH_2$ seems a likely possibility.

Theoretical considerations (Dalziel, 1962*a*) lead to the conclusion that the presence of small amounts of a competitive inhibitor in coenzyme (or substrate) preparations may produce large effects on the maximum rate, even though the inhibitor is bound to the enzyme no more firmly than the coenzyme. This is because the magnitude of the inhibition depends on the ratio of the Michaelis constant of the coenzyme to the inhibitor constant, and the former may be much greater than the dissociation constant of the enzyme–coenzyme compound. The experimental results obtained with adenosine diphosphate ribose are consistent with these suggestions. On the simplest hypothesis for the action of a competitive inhibitor present in molar ratio, r, and competing with the coenzyme, S_1, the initial rate equation (1) becomes (Dalziel, 1962*a*):

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

Conventional inhibition studies gave K_i a value of 8·5 μM for adenosine diphosphate ribose. At pH 6·0, the dissociation constant for the compound of liver alcohol dehydrogenase and $NADH_2$, K_1, is 0·21 μM (Theorell & Winer, 1959), and the Michaelis constant for $NADH_2$, $K_m = \phi_1/\phi_0$, is 14 μM (Dalziel, 1962*b*). From equation (2), when adenosine diphosphate ribose is present in constant molar ratio to $NADH_2$, r, of 1·4, the inhibition factor affecting ϕ_0, the reciprocal of the maximum specific rate, should be 3·3. This agrees with the experimental value of 3·5 (Fig. 1). On the other hand, the inhibition factor affecting ϕ_2 is only 1·03, consistent with the experimental finding that ϕ_2 is not significantly affected by the inhibitor (Fig. 1A). Equation (2) was deduced from the simplest assumption that the inhibitor forms only a binary inactive complex with the enzyme, EI, but it can readily be shown that the same general conclusions may be reached if it is assumed that an inactive complex EIS_2 is also formed, although the inhibition factors are then more complex.

Such effects emphasize the importance of distinguishing between Michaelis constants and dissociation constants, especially in coenzyme–substrate reactions. With liver alcohol dehydrogenase, the difference between these constants for $NADH_2$ decreases with increase of pH (cf. Theorell & Winer, 1959; Dalziel, 1962*b*; Theorell & McKinley McKee, 1961), and if, as seems likely, K_i and K_1 are affected in a similar manner by pH, the inhibition effect on the maximum rate would decrease also.

This has been found both with adenosine diphosphate ribose and with different $NADH_2$ preparations (Dalziel, 1961*b*).

SUMMARY

1. $NADH_2$ 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_{260}/E_{340} and 2% for the proportion of inactive material absorbing at 340 mμ 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 $NADH_2$. 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.

I am grateful to Mrs B. Sutton for technical assistance in this work.

REFERENCES

Bonnichsen, R. K. (1950). *Acta chem. scand.* **4**, 714.
Burton, K. (1959). In *Data for Biochemical Research*, p. 74. Ed. by Dawson, R. M. C., Elliott, D. C., Elliott, W. H. & Jones, K. M. Oxford: University Press.
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*). *Nature, Lond.* (in the Press).
Dalziel, K. (1962*b*). *Biochem. J.* **84**, 244.
Drabkin, D. L. (1945). *J. biol. Chem.* **157**, 563.
Horecker, B. L. & Kornberg, A. (1948). *J. biol. Chem.* **175**, 385.
Lehninger, A. L. (1952). *Biochem. Prep.* **2**, 92.
Lineweaver, H. & Burk, D. A. (1934). *J. Amer. chem. Soc.* **56**, 658.
Pullman, M. E., Colowick, S. P. & Kaplan, N. O. (1952). *J. biol. Chem.* **194**, 593.
Rafter, G. W. & Colowick, S. P. (1957). In *Methods in Enzymology*, vol. 3, p. 887. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Siegel, J. M., Montgomery, G. A. & Bock, R. M. (1959). *Arch. Biochem. Biophys.* **82**, 289.
Theorell, H. & McKinley McKee, J. S. (1961). *Nature, Lond.*, **192**, 47.
Theorell, H. & Nygaard, A. P. (1955). *Acta chem. scand.* **9**, 1300.
Theorell, H. & Winer, A. D. (1959). *Arch. Biochem. Biophys.* **83**, 291.

Biochem. J. (1962) **84**, 244

Kinetic Studies of Liver Alcohol Dehydrogenase

BY K. DALZIEL*
Department of Biochemistry, University of Sheffield, Sheffield 10

(*Received* 28 *December* 1961)

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.