The effects of Mg^{2+} on certain steps in the mechanisms of the dehydrogenase and esterase reactions catalysed by sheep liver aldehyde dehydrogenase

Support for the view that dehydrogenase and esterase activities occur at the same site on the enzyme

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Stopped-flow experiments in spectrophotometric and fluorescence modes reveal different aspects of the aldehyde dehydrogenase mechanism. Spectrophotometric experiments show a rapid burst of NADH production whose course is not affected by Mg^{2+} . The slower burst seen in the fluorescence mode is markedly accelerated by Mg^{2+} . It is argued that the fluorescence burst accompanies acyl-enzyme hydrolysis and, therefore, that Mg^{2+} increases the rate of this process. Experiments on the hydrolysis of *p*-nitrophenyl propionate indicate that acyl-enzyme hydrolysis is indeed accelerated by Mg^{2+} and a combination of Mg^{2+} and NADH. V_{max} values for *p*-nitrophenyl propionate hydrolysis in the presence of NADH and NADH and Mg^{2+} agree closely with the specific rates of acyl hydrolysis from the E·NADH·acyl and E·NADH·acyl· Mg^{2+} complexes seen in the dehydrogenase reaction with propionaldehyde. These observations support the view that esterase and dehydrogenase activities occur at the same site on the enzyme. Other evidence is presented to support this conclusion.

INTRODUCTION

The findings of Takahashi & Weiner (1980) and Takahashi *et al.* (1980) that incubation of the horse liver mitochondrial aldehyde dehydrogenase with Mg^{2+} led to dissociation of the enzyme into dimers and to a doubling of the active-site concentration led in turn to similar studies with the sheep liver cytosolic enzyme (Dickinson & Hart, 1982; Bennett *et al.*, 1983). These investigations showed that Mg^{2+} and other bivalent-metal ions are not activators, but strong inhibitors, of the cytosolic enzyme, and cause neither dissociation nor a significant increase in the number of coenzyme-binding sites. The inhibition of the dehydrogenase activity is due to the formation of an E·NADH· Mg^{2+} complex that is considerably more stable than the normal rate-limiting E·NADH complex. The decreased rate of dissociation of NADH from this complex largely determines the inhibited rate.

The esterase activity of the cytosolic enzyme, which is, of course, normally studied in the absence of added coenzyme, is not inhibited by Mg^{2+} , but slightly activated (Dickinson & Hart, 1982). This observation suggests a potential for Mg^{2+} to affect steps in the dehydrogenase mechanism other than those concerned with coenzyme binding or release. The effect of Mg^{2+} on one such step forms the main subject matter of the present paper.

EXPERIMENTAL

Materials

NADH (grade I) and NAD⁺ (grade II) were from Boehringer Corp., London W.5, U.K. *p*-Nitrophenyl propionate was from Sigma Chemical Co. Ltd., Poole, Dorset, U.K. Other chemicals were analytical-reagent grade, where available, obtained from Fisons Chemicals,

Loughborough, Leics., U.K. Propionaldehyde was redistilled before use.

Cytosolic aldehyde dehydrogenase was prepared as described by Dickinson *et al.* (1981), and was assayed by the method of Hart & Dickinson (1977). Protein concentrations were calculated by using an $A_{280,1cm}^{1\%}$ value of 11.3 (Hart & Dickinson, 1983). Enzyme solutions were normally prepared for use by dialysis against 50 mM sodium phosphate buffer, pH 7.0, containing 0.3 mM-EDTA and 100 μ M-dithiothreitol.

Methods

Buffer solutions for conventional assays contained 100 μ M-EDTA and 10 μ M-dithiothreitol. For experiments involving the use of disulfiram (tetraethylthioperoxycarbonyl diamide) the dithiothreitol was omitted.

The relative molecular mass of the enzyme is taken to be 212000 (MacGibbon *et al.*, 1979).

Substrate and coenzyme concentrations

Propionaldehyde concentrations were determined enzymically at pH 7.0 with aldehyde dehydrogenase and NAD⁺. NADH was assayed enzymically with yeast alcohol dehydrogenase by the methods of Dalziel (1962, 1963). For all these methods $\epsilon = 6.22 \times 10^3 \text{ M}^{-1} \cdot \text{cm}^{-1}$ for NADH at 340 nm (Horecker & Kornberg, 1948). *p*-Nitrophenyl propionate was made up in acetone. For experiments with this substrate the acetone concentration in assays was < 1% and was held constant within each experiment. A value of $\epsilon = 18300 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 400 nm was used for the *p*-nitrophenoxide ion (Kézdy & Bender, 1962) and a pK_a of 7.1 for *p*-nitrophenol (Behme & Cordes, 1967) was taken.

Fig. 1. Fluorescence titration of aldehyde dehydrogenase with NADH in the presence and absence of Mg^{2+}

The mixtures contained 50 mM-Hepes buffer, pH 7.0, and were maintained at 25 °C. $\circ$, no enzyme; $\triangle$, 4 μM -enzyme; $\bullet$, 4 μM -enzyme + 1 M- MgCl_2 . Small quench corrections have been applied to the upper trace for the four highest NADH concentrations. The corrections allow for the small deviation from linearity seen in the trace with no enzyme present.

Stopped-flow experiments

The apparatus used was that described by Hart & Dickinson (1982a). The performance of the apparatus was tested as described earlier (Dickinson, 1985). The effective cell path-length for the spectrophotometric experiments was 2.2 cm.

RESULTS AND DISCUSSION

Fig. 1 shows the results of fluorescence titrations of 4 μM cytosolic aldehyde dehydrogenase with NADH at pH 7.0 in the presence and absence of 1 M- MgCl_2 . The presence of the MgCl_2 clearly enhances the fluorescence through the formation of an $\text{E} \cdot \text{NADH} \cdot \text{Mg}^{2+}$ complex (Dickinson & Hart, 1982; Bennett *et al.*, 1983) and the binding of NADH is considerably strengthened. The intersection of the two straight-line portions of the upper trace gives a direct estimate of the concentration of NADH binding sites (4.4 μM binding sites, 1.1 binding sites/enzyme tetramer). This determination provides further confirmation that Mg^{2+} does not significantly increase the number of NADH binding sites on the enzyme. The extrapolation procedure used in Fig. 1 cannot be adopted for titrations in the absence of Mg^{2+} . Although it is not obvious from Fig. 1, the plot in this case is too curved to allow a valid estimate to be made. Bennett *et al.* (1983) reported about 1 binding site/tetramer and a dissociation constant of 0.2 μM at pH 7.6 in the presence of 1.6 mM- MgCl_2 . With this concentration of MgCl_2 the plot of fluorescence versus NADH concentration was a smooth curve and the results quoted were obtained from subsequent analysis. Our estimate of the dissociation constant of NADH from Fig. 1 was about 0.1 μM . This was obtained from the points on the curve that fall below the straight-line sections. Because the

proportions of free to bound enzyme and NADH are small, the estimate is likely to be rather inaccurate.

The finding of one coenzyme binding site/tetramer is a result that requires explanation, but this is not yet forthcoming. There is no doubt, however, that the value recorded above is a reliable estimate. Detailed studies using a variety of alternative methods with various preparations of enzyme have consistently given values of about one binding site/tetramer (Hart & Dickinson, 1983). The detailed amino-acid sequence data now available for the human and horse cytosolic aldehyde dehydrogenases do not provide any explanation of this difficulty. They show that the preparations are essentially homogenous and that the enzymes are tetramers composed of chemically identical subunits (von Bahr-Lindstrom *et al.*, 1984; Hempel *et al.*, 1984). The fact that the human cytosolic enzyme is 85% inhibited by reaction with 1 mol/mol of iodoacetamide is broadly similar to our finding (Dickinson *et al.*, 1981) that reaction with 1.5 mol/mol of disulfiram causes almost complete loss of enzyme activity. The purified sheep enzyme appears to be a highly purified and undegraded preparation (Hart & Dickinson, 1983). It seems that the low coenzyme-binding capacity is not an artefact. The enzyme protein presumably fulfils other, as yet unobserved, functions.

The inhibition of the dehydrogenase reaction by Mg^{2+} has been studied in detail by Dickinson & Hart (1982) and Bennett *et al.* (1983). It appears that the inhibition arises principally from the formation of the $\text{E} \cdot \text{NADH} \cdot \text{Mg}^{2+}$ complex referred to above. This intermediate is not a dead-end complex, but it dissociates NADH much more slowly than the normal $\text{E} \cdot \text{NADH}$ complex. The inhibition is consequently hyperbolic. Mn^{2+} and Ca^{2+} have similar effects to those of Mg^{2+} , and presumably $\text{E} \cdot \text{NADH} \cdot \text{Mn}^{2+}$ and $\text{E} \cdot \text{NADH} \cdot \text{Ca}^{2+}$ complexes are involved (Dickinson & Hart, 1982).

We have now moved on to investigate the effects of Mg^{2+} on transients seen in stopped-flow studies of the dehydrogenase reaction. Fig. 2 shows the spectrophotometric and fluorescence records on mixing enzyme, NAD^+ and propionaldehyde in the presence and absence of 11 mM- MgCl_2 in 50 mM-phosphate buffer, pH 7.0, at 25 °C. The conditions were chosen so that the final NAD^+ (400 μM) and propionaldehyde (50 μM) concentrations saturated the low- K_m activity, but did not enter the region of substrate activation (Hart & Dickinson, 1982a, b; MacGibbon *et al.*, 1977). Under these conditions, in the absence of Mg^{2+} ions, the mechanism of catalysis is expected to conform to a compulsory mechanism with NAD^+ binding before aldehyde and $\text{E} \cdot \text{NADH}$ being the terminal complex, whose rate of breakdown determines the overall rate. It is clear from Fig. 2(a) that Mg^{2+} ions did not alter the amplitude or shape of the transient of the spectrophotometric record. The derived first-order rate constants from the spectrophotometric records of Fig. 2(a) were 9.4 s⁻¹ (no Mg^{2+}) and 11.0 s⁻¹ (11 mM- Mg^{2+}), with amplitudes in the range of 0.9 $\pm$ 0.05 mol of NADH/mol of enzyme assuming $\epsilon = 5500 \text{ M}^{-1} \cdot \text{cm}^{-1}$ at 340 nm for enzyme-bound NADH (Agnew *et al.*, 1981). When the spectrophotometric experiments were repeated in 50 mM-Hepes buffer, pH 7.0, the amplitudes were about the same as before, with the derived rate constants for the transients of 6.5 s⁻¹ (no Mg^{2+}) and 7.4 s⁻¹ (+11 mM- Mg^{2+}). The results just described may seem to be at variance with a previous result (Dickinson & Hart, 1982) in which

Fig. 2. Stopped-flow studies of the aldehyde dehydrogenase reaction in the presence and absence of Mg^{2+}

Solutions of enzyme and NAD^+ were mixed with solutions of NAD^+ and propionaldehyde. The experiments were conducted in 50 mM-sodium phosphate buffer, pH 7.0, at 25 °C. When MgCl_2 was present, the concentration was 11 mM. (a) Apparatus in spectrophotometric mode at 340 nm. Final conditions: enzyme, 5 μM ; NAD^+ , 400 μM ; and propionaldehyde, 50 μM . (b) Apparatus in fluorimetric mode. Final conditions: enzyme, 1 μM ; NAD^+ , 400 μM ; and propionaldehyde, 50 μM . Ten fluorescence units = 0.73 V and 6 μM - NADH gave a signal of 0.8 V. (a) and (b) also show first-order plots of the transients obtained by taking the difference between the observed readings at any time and the reading obtained at the same time by extrapolating back from later times when the steady state was established.

50 mM-Tris/HCl buffer was used. In this case a burst of 0.35 mol of NADH /mol of enzyme was observed with no Mg^{2+} present and 0.69 mol of NADH /mol of enzyme with 5 mM- Mg^{2+} . The rate constant for the bursts were 5 s^{-1} (no Mg^{2+}) and 5.5 s^{-1} (+ 5 mM- Mg^{2+}). The change in amplitude observed on adding Mg^{2+} in Tris/HCl buffer is probably due to the fact that Tris reacts with aldehydes to form imines. Thus the effective substrate concentration was much lower in this buffer and was probably not saturating. In the presence of Mg^{2+} the dissociation of NADH is much slower, giving the opportunity for

$\text{E}\cdot\text{NADH}\cdot\text{Mg}^{2+}$ to accumulate to higher steady-state concentrations.

The fluorescence measurements of Fig. 2(b) show an interesting contrast with those of Fig. 2(a). It is clear that the presence of 11 mM- MgCl_2 dramatically increases the amplitude of the fluorescence transient, but also increases its specific rate from 3 s^{-1} (no Mg^{2+}) to 8.5 s^{-1} (+ 11 mM- Mg^{2+}). When the experiments were repeated in 50 mM-Hepes buffer, pH 7.0, similar changes occurred, with the specific rate of the transient increasing from 2.2 s^{-1} (no Mg^{2+}) to 7.5 s^{-1} (+ 11 mM- Mg^{2+}). The increased amplitude of the fluorescence changes in the presence of Mg^{2+} are readily explained by the greater fluorescence of the $\text{E}\cdot\text{NADH}\cdot\text{Mg}^{2+}$ complex relative to that of the $\text{E}\cdot\text{NADH}$ complex (see Fig. 1); the increased rate of the transient process in the presence of Mg^{2+} requires further consideration.

The occurrence of transients on different time scales in the spectrophotometric and fluorescence records has already been reported for this enzyme (Hart & Dickinson, 1982a) under conditions similar to those of Fig. 2. The explanation offered was that the first NADH -containing complex to appear after mixing enzyme with NAD^+ and aldehyde was a non-fluorescent one, presumably $\text{E}\cdot\text{NADH}\cdot\text{acyl}$ and that the fluorescence characteristic of enzyme-bound NADH only developed as the $\text{E}\cdot\text{NADH}\cdot\text{acyl}$ complex underwent further change. The lag phases clearly seen in the early parts of the fluorescence bursts (see the first-order plots) arise, one supposes, because the non-fluorescent complex has to accumulate first. In a recent study using *trans*-cinnamaldehyde as substrate, more evidence has been obtained on this point (Dickinson, 1985). The acyl-enzyme formed in this reaction can be detected spectrophotometrically at about 325 nm and it appears that the hydrolysis of the acyl group from the $\text{E}\cdot\text{NADH}\cdot\text{acyl}$ complex is the process that leads to development of fluorescence in the transients of Fig. 2(b). This is supported by the fact that the fluorescence transient is independent of the *trans*-cinnamaldehyde concentration over the range 6.5–130 μM . The fluorescence 'bursts' of Fig. 2(b) in the absence of MgCl_2 are also independent of the propionaldehyde concentration over the range 50–1000 μM . On the basis of these arguments, then, the effects of Mg^{2+} ions on the fluorescence records in Fig. 2(b) are due to the acyl group being hydrolysed more rapidly from an $\text{E}\cdot\text{NADH}\cdot\text{acyl}\cdot\text{Mg}^{2+}$ complex than from the usual $\text{E}\cdot\text{NADH}\cdot\text{acyl}$ complex. In support of this conclusion may be the fact that the esterase activity of the enzyme towards *p*-nitrophenyl acetate in Tris/HCl buffer, pH 7.0, is slightly activated by Mg^{2+} ions and not drastically inhibited, as is the dehydrogenase reaction (Dickinson & Hart, 1982). The rate-limiting step in the esterase reaction is acyl-enzyme hydrolysis (McGibbon *et al.*, 1978).

We have continued this line of investigation by studying the effect of Mg^{2+} ions on the esterase activity of the enzyme using *p*-nitrophenyl propionate as substrate. The K_m for *p*-nitrophenyl propionate was determined to be 13 μM at pH 7.0 and 25 °C in 50 mM-Hepes buffer, pH 7.0. Fig. 3 shows the effects of NADH on the rate of hydrolysis of 300 μM -*p*-nitrophenyl propionate and of MgCl_2 on the rate of the same process in the presence of optimal (150 μM) concentrations of NADH . It is clear that very substantial activation (2-fold) is achieved by NADH , and a further 2-fold activation is achieved by including 400 μM - MgCl_2 . The

Fig. 3. Effect of NADH, and NADH and Mg^{2+} , on the esterase activity

Experiments were conducted at 25 °C in 50 mM-sodium phosphate buffer, pH 7.0. Absorbance measurements were made at 400 nm. The *p*-nitrophenyl propionate concentration was 1 mM in all cases. For the experiments in which MgCl_2 was added (●), 150 μM -NADH was also included in the assays. e is the enzyme concentration in the assays.

Fig. 4. Stopped-flow experiments on the esterase activity and the effects of NADH and NADH and Mg^{2+}

The apparatus was in the spectrophotometric mode at 400 nm. Experiments were conducted at 25 °C in 50 mM-Hepes buffer, pH 7.2, with 1 mM-*p*-nitrophenyl propionate and 5 μM -enzyme after mixing. When included, NADH and MgCl_2 were added to both the enzyme-containing and the substrate-containing syringes. Δ , No further addition; $\circ$, +150 μM -NADH; $\bullet$, +150 μM -NADH and 800 μM - MgCl_2 . The inset shows the first-order plots of the transients.

result with NADH alone parallels the result obtained by MacGibbon *et al.* (1978) with *p*-nitrophenyl acetate as substrate.

Fig. 4 shows the results of spectrophotometric

experiments in the stopped-flow apparatus observing transients in *p*-nitrophenol production on hydrolysis of *p*-nitrophenyl propionate. The experiments were at pH 7.2 and 25 °C in 50 mM-Hepes buffer. With 1 mM-substrate alone a burst of 0.7 mol of *p*-nitrophenol/mol of enzyme occurred with an apparent first-order rate constant of 30 s^{-1} . The 'burst' amplitude is similar to that seen in the dehydrogenase reaction (see above) and contrasts with the much lower 'burst' seen with *p*-nitrophenyl acetate as substrate (Blackwell *et al.*, 1983). In the presence of 150 μM -NADH, the transient amplitude corresponded to 0.5 mol of *p*-nitrophenol/mol of enzyme with a rate constant of 34 s^{-1} . In the presence of 150 μM -NADH and 800 μM - Mg^{2+} the burst was 0.35 mol of *p*-nitrophenol/mol of enzyme and the rate constant 40 s^{-1} . The principal points here are that, under all conditions, deacylation of the acyl-enzyme appears to be the rate-limiting step and that the presence of NADH or NADH and Mg^{2+} together accelerate this process selectively. The acylation reaction appears to be affected to some extent by NADH and NADH and Mg^{2+} , but the increase from 30 s^{-1} to 40 s^{-1} in this process hardly compares with the 4-fold change in the steady-state rate seen on adding NADH and Mg^{2+} . The decrease in the transients recorded above from 0.7 to 0.35 mol of *p*-nitrophenol/mol of enzyme are probably not very significant. As will be seen below (Fig. 5) the presence of NADH and NADH and Mg^{2+} causes substantial increases in the K_m value for *p*-nitrophenyl propionate. In 50 mM-Hepes buffer, pH 7.0, the K_m values were 13 μM (no addition), 240 μM (+150 μM -NADH) and 430 μM (+150 μM -NADH, 400 μM - MgCl_2). Thus 1 mM-*p*-nitrophenyl propionate is no longer saturating with these additional species present.

The effects of Mg^{2+} on acyl-enzyme hydrolysis in NADH-containing species occurring in either the esterase or in the dehydrogenase activity appear to be the same, and this in turn suggests that the same group on the enzyme is acylated in turnover. Blackwell *et al.* (1983) reason that this is not so on the basis of inhibition experiments and propose that different sites are involved. The results given below contribute further to this argument.

Fig. 5(a) shows the results of detailed kinetic studies on the hydrolysis of *p*-nitrophenyl propionate at pH 7.0 and at 25 °C with various concentrations of MgCl_2 in the presence of 150 μM -NADH. The activation by MgCl_2 is hyperbolic, as is indicated by Fig. 3. Fig. 5(b) is therefore a secondary plot based on the intercepts of Fig. 5(a), which leads to an estimate of V_{max} for *p*-nitrophenyl propionate hydrolysis in the presence of 150 μM -NADH and saturating MgCl_2 concentrations. In using *p*-nitrophenyl propionate as substrate, the acyl group involved is the same as that in propionaldehyde oxidation. So, if the same enzyme group is involved in the acylation and deacylation reactions, certain comparisons should be possible between data from the dehydrogenase and esterase assays. According to the above argument and the fluorescent transients in Fig. 2(b), the specific rate of hydrolysis of the $\text{E} \cdot \text{NADH} \cdot \text{acyl}$ complex in 50 mM-phosphate buffer, pH 7.0, is 3 s^{-1} and that of the $\text{E} \cdot \text{NADH} \cdot \text{acyl} \cdot \text{Mg}^{2+}$ complex is 8.5 s^{-1} . From Fig. 5(a) the V_{max} of *p*-nitrophenyl propionate hydrolysis in the same buffer is 2.6 s^{-1} in the presence of 150 μM -NADH. The intercept of Fig. 5(b) leads to a V_{max} estimate of 8.6 s^{-1} in the presence of 150 μM -NADH and saturating

Fig. 5. Variation of the specific initial rate of p -nitrophenyl propionate hydrolysis with the p -nitrophenyl propionate and MgCl_2 concentrations

Assays were performed spectrophotometrically at 400 nm in 50 mM-sodium phosphate buffer, pH 7.0, with 150 μM -NADH included. (a) Primary double-reciprocal plots. The reciprocal of the specific initial rate is plotted against the reciprocal of the p -nitrophenyl propionate concentration. The MgCl_2 concentrations were: $\circ$, 0; $\triangle$, 100 μM ; $\square$, 200 μM ; $\bullet$, 400 μM . e is enzyme concentration.

MgCl_2 . These measurements have been repeated fully in 50 mM-Hepes buffer, pH 7.0, at 25 $^\circ\text{C}$. For p -nitrophenyl propionate hydrolysis, V_{max} in the presence of 150 μM -NADH was 1.5 s^{-1} and in the presence of 150 μM -NADH and saturating MgCl_2 was 5.5 s^{-1} . The corresponding values from the fluorescence transients in propionaldehyde oxidation in this buffer were 2.2 s^{-1} and 7.5 s^{-1} . The extrapolations involved in V_{max} determinations lead inevitably to some degree of uncertainty as to the true values. Additional limited experiments with 600 μM -NADH in 50 mM-Hepes buffer have indicated that the 150 μM -NADH used in the experiments may not be fully saturating and that an increase in V_{max} estimates of the order of 25% is required to allow for this. The values given are surely close enough, when the various errors are considered, to permit the preliminary conclusion that the enzyme group involved in acyl transfer in esterase and dehydrogenase activities is the same group.

Kitson (1982) has shown that the response of aldehyde dehydrogenase activity and esterase activity at pH 7.0 to the addition of disulfiram is different if the reactions are already proceeding when the inhibitor is added. Enzyme pre-treated with the reagent gave the same inhibition when subsequently assayed for dehydrogenase and esterase activity. The likely explanation for the different behaviour of the two activities towards added disulfiram is that in the dehydrogenase activity the predominant enzyme species is an $\text{E}\cdot\text{NADH}$ complex (i.e. this is the rate-limiting complex), whereas for the esterase activity it is the acyl-enzyme in which the thiol group to be modified by disulfiram is already protected by the acyl group. The $\text{E}\cdot\text{NADH}$ complex would not be expected to protect against disulfiram, for inhibited enzyme binds NADH almost as well as native enzyme (Dickinson *et al.*, 1981). One would predict, however, that the abortive $\text{E}\cdot\text{NADH}\cdot\text{propionaldehyde}$ complex, which is only formed at high (> 10 mM) concentrations of aldehyde (Dickinson, 1985), would protect against disulfiram. The recent experiments of Kitson (1985) seem to show that this prediction is realized. According to Dunn & Buckley (1985), in work on the oxidation of *trans*-4-(*NN*-dimethylamino)cinnamaldehyde, the acyl enzyme is much more stable at low pH. One might expect that, in the oxidation of this and similar compounds, acyl-enzyme hydrolysis could become an important rate-limiting step, rather than NADH dissociation from the terminal $\text{E}\cdot\text{NADH}$ complex. We have examined the effect of disulfiram on *trans*-cinnamaldehyde oxidation at pH 7.0 and pH 5.1 and the results appear in Fig. 6. According to Dunn & Buckley (1985), pH 5.1 would give optimum levels of acyl-enzyme formation with *trans*-4-(*NN*-dimethylamino)cinnamaldehyde and the rate of hydrolysis of the acyl-enzyme would be quite slow. It is apparent from Fig. 6 that working at pH 5.1 confers much protection against disulfiram when comparing with similar experiments at pH 7.0. Also, the protection

(b) Secondary plot obtained from the intercepts of (a). The reciprocal of the difference between the observed maximum velocity with MgCl_2 present and that when no MgCl_2 was present ($1/v - v_0$) is plotted versus the reciprocal of the MgCl_2 concentration. Points are also included from experiments with 300 μM - and 500 μM - MgCl_2 , which were not shown in (a) for the sake of clarity.

Fig. 6. Effect of disulfiram on assays of the enzyme activity

All assays were performed at 25 °C. ●, Oxidation of 150 μM -*trans*-cinnamaldehyde in 50 mM-sodium acetate buffer, pH 5.1. The horizontal arrow marks the addition of 2.5 mM- MgCl_2 . ▲, Oxidation of 150 μM -*trans*-cinnamaldehyde in 50 mM-sodium phosphate buffer, pH 7.0; □, hydrolysis of 300 μM -*p*-nitrophenyl acetate in 50 mM-Hepes buffer, pH 7.0. The vertical arrows mark the addition of 20 μM -disulfiram. The dehydrogenase assays were monitored fluorimetrically with an enzyme concentration of 0.039 μM . The esterase assay was monitored at 400 nm with an enzyme concentration of 0.78 μM .

observed is comparable with that provided by *p*-nitrophenyl propionate at pH 7.0. Fig. 6 also shows the effect of inclusion of 2.5 mM- MgCl_2 on the dehydrogenase activity. It is clear that addition of this reagent accelerates the dehydrogenase activity some 3.5-fold in marked contrast with the 85% inhibition seen at pH 7.0. This finding is consistent with what we have argued above, namely that bound Mg^{2+} accelerates acyl-enzyme hydrolysis and that, at pH 5.1, acyl-enzyme hydrolysis is an important rate-limiting step in the dehydrogenase reaction. It is further noted from Fig. 6 that, in the presence of Mg^{2+} at pH 5.1, the inhibitory effects of disulfiram are more rapidly established than in the absence of Mg^{2+} . The rate immediately observed after addition of disulfiram is 90% of the preceding rate when no Mg^{2+} is present and 10% of the preceding rate when 2.5 mM- MgCl_2 is present. These results are consistent with the view that Mg^{2+} accelerates acyl-enzyme hydrolysis and so lowers the relative concentration of the acyl-enzyme species.

The kinetic experiments and the disulfiram-protection experiments seem to provide convincing support for the view that the acyl-enzyme species formed in the dehydrogenase and esterase activities of sheep cytosolic aldehyde dehydrogenase involve the same enzyme group (possibly a thiol group). The kinetic results are particularly important. It seems unlikely that the good agreement between the V_{max} values for *p*-nitrophenyl propionate hydrolysis in the presence of NADH and NADH and Mg^{2+} and the rate constants of the fluorescence transients seen in the dehydrogenase reaction with propionaldehyde in the presence and absence of Mg^{2+} is just fortuitous.

Blackwell *et al.* (1983) have argued that different sites are involved in dehydrogenase and esterase activity. They make the point that, if the mechanisms shared a common

pathway, the esterase activity should be inhibited by Mg^{2+} . This, however, is not so. Mg^{2+} inhibits the dehydrogenase because NADH release from the terminal $\text{E} \cdot \text{NADH} \cdot \text{Mg}^{2+}$ complex is relatively slow. It enhances the esterase activity because acyl-enzyme hydrolysis from the $\text{E} \cdot \text{acyl} \cdot \text{Mg}^{2+}$ complex is favoured. The same effect is seen in the dehydrogenase reaction if one looks at fluorescent NADH-containing intermediates in the mechanism by stopped-flow methods (Fig. 2b). This process does not, however, affect the steady-state dehydrogenase rates, because they are principally determined by the slow breakdown of the $\text{E} \cdot \text{NADH} \cdot \text{Mg}^{2+}$ complex.

Blackwell *et al.* (1983) provide evidence from inhibition experiments to support their view that esterase and dehydrogenase activities occur at different sites. This evidence certainly presents difficulties when trying to show that only one site is involved, but it is not clear that these difficulties are insuperable. The esterase activity is inhibited competitively by propionaldehyde. This was shown with *p*-nitrophenyl acetate as substrate by MacGibbon *et al.* (1978) and by ourselves with *p*-nitrophenyl propionate as substrate (G. W. Haywood, & F. M. Dickinson, unpublished work). This evidence could certainly support the one-site theory. More complex experiments described by Blackwell *et al.* (1983) consider the inhibition of the esterase by propionaldehyde in the presence of NAD^+ (i.e. with the dehydrogenase reaction also running). In the new experiments the inhibition by propionaldehyde appears to be competitive, but the secondary plot of slopes is curved downwards towards the abscissa, a feature not apparently compatible with strict competitive behaviour and a one-site model. It is not clear, however, that the latter experiments are open to straightforward interpretation. There will have been at least three enzyme species present capable of catalysing ester hydrolysis. These are, the free enzyme, the $\text{E} \cdot \text{NAD}^+$ complex and the $\text{E} \cdot \text{NADH}$ complex formed in the dehydrogenase reaction. As we have shown above, the free enzyme and $\text{E} \cdot \text{NADH}$ complex have quite different kinetic characteristics in the esterase reaction (V_{max} and K_m markedly increased on adding NADH). The $\text{E} \cdot \text{NAD}^+$ species is likely to be at least slightly different from one or other of the other two. In normal competitive-inhibition experiments one has only one form of active enzyme. The presence of three catalytically different species whose relative concentrations change as the experimental conditions vary makes it difficult to predict what kind of inhibition behaviour one would anticipate. A rather complex pattern would be expected. Nevertheless, the fact that the V_{max} for *p*-nitrophenyl acetate hydrolysis is not inhibited by propionaldehyde even in the presence of its reaction partner, NAD^+ , could surely support our view that the two reactions occur at the same site. The aldehyde thus cannot bind in the presence of saturating concentrations of ester, whether or not NAD^+ is present.

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