The use of pH-gradient ion-exchange chromatography to separate sheep liver cytoplasmic aldehyde dehydrogenase from mitochondrial enzyme contamination, and observations on the interaction between the pure cytoplasmic enzyme and disulfiram

F. Mark DICKINSON, Graham J. HART and Trevor M. KITSON*
Department of Biochemistry, University of Hull, Hull HU6 7RX, U.K.

(Received 7 May 1981/Accepted 22 July 1981)

1. Sheep liver cytoplasmic aldehyde dehydrogenase can be purified from contamination with the mitochondrial form of the enzyme by pH-gradient ion-exchange chromatography. The method is simple, reproducible and efficient. 2. The purified cytoplasmic enzyme retains about 2% of its original activity in the presence of a large excess of disulfiram. This suggests that the disulfiram-reactive thiol groups are not essential for covalent interaction with the aldehyde substrate during catalysis, as has sometimes been suggested. 3. Between 1.5 and 2.0 molecules of disulfiram per tetrameric enzyme molecule account for the observed loss of activity, suggesting that the enzyme may have only two functional active sites. 4. Experiments show that disulfiram-modified enzyme retains the ability to bind NAD⁺ and NADH.

In the past few years there have been several reports of various properties of the cytoplasmic and mitochondrial aldehyde dehydrogenases from sheep liver (see e.g. Kitson, 1978; Hart & Dickinson, 1978; MacGibbon *et al.*, 1979). Dickinson & Berrieman (1979) showed that their isolation procedure, which was based on that of Crow *et al.* (1974), resulted in cytoplasmic enzyme preparations that were appreciably contaminated with the mitochondrial form. They discussed the importance of obtaining pure preparations of cytoplasmic aldehyde dehydrogenase, and described a procedure for removing mitochondrial aldehyde dehydrogenase contamination by (NH₄)₂SO₄ fractionation. Our experience, involving extensive use of this fractionation procedure, is that the method, though useful on occasions, cannot be applied with uniform success. Accordingly, we sought an alternative technique for separating cytoplasmic and mitochondrial aldehyde dehydrogenases, and the present paper describes how pH-gradient ion-exchange chromatography furnishes a simple, efficient and reproducible answer to the problem.

Having purified the cytoplasmic enzyme, we re-examined the effect on it of disulfiram, since, as

discussed by Dickinson & Berrieman (1979), the presence of mitochondrial aldehyde dehydrogenase contamination can introduce serious errors into the results of such experiments. An unambiguous measure of the extent of inactivation of cytoplasmic aldehyde dehydrogenase by disulfiram is quite important in understanding the properties of this enzyme, as discussed below. [Disulfiram, or tetraethylthioperoxydicarbonic diamide, is a drug used in the treatment of chronic alcoholics; see Kitson (1977).]

Disulfiram

Experimental

Materials

NAD⁺ (grades I and II) and NADH (grade I) were purchased from Boehringer Corp. (London), London W.5, U.K. Disulfiram was obtained from Sigma (London) Chemical Co., London S.W.6, U.K. All other chemicals were analytical-reagent grade whenever available, purchased from Fisons Chemicals, Loughborough, Leics., U.K., or BDH Chemicals, Poole, Dorset, U.K. Solutions of acetal-

* Permanent address: Department of Chemistry, Biochemistry and Biophysics, Massey University, Palmerston North, New Zealand.

dehyde were made up daily from 1M stock solutions (kept frozen), which were prepared from freshly distilled acetaldehyde. Sheep livers were obtained from the local slaughterhouse, and enzyme isolation was started as soon as possible after the death of the animal.

Methods

All dialysis tubing was boiled in 10mM-EDTA, pH 7.0, and washed well with water before use. All solutions were prepared in glass-distilled water.

Protein concentrations. In the early stages of the enzyme-purification procedure, protein concentrations were determined spectrophotometrically by assuming $A_{1\text{cm}}^{1\%} = 10$ at 280nm. For purified cytoplasmic aldehyde dehydrogenase solutions, a specific absorption coefficient at 280nm of $A_{1\text{cm}}^{1\%} = 11.3$ was used, determined from dry-weight measurements.

Enzyme assay. This was performed fluorimetrically as described by Hart & Dickinson (1977).

Preparation of cytoplasmic aldehyde dehydrogenase. The preparation of this enzyme was based on the procedure of Crow *et al.* (1974). Since differences in isolation procedures may result in significant differences in the purity of the product, our method is described here in full.

All operations were performed at 0–4°C. Portions (250g) from 1.3–1.5kg of chopped liver were homogenized for 60s in an MSE Ato-Mix blender with 800ml of 5mM-sodium phosphate buffer, pH 7.4, containing 0.25M-sucrose, 0.3mM-EDTA and 0.1% (v/v) 2-mercaptoethanol. (Unless otherwise stated, all subsequent buffer solutions contained the same concentrations of EDTA and 2-mercaptoethanol.) The homogenates were combined, then centrifuged at 500g for 5min, and the supernatant was then re-centrifuged at 20000g for 15min to sediment the mitochondria. The pH of the supernatant was lowered to 5.3 [by the dropwise addition of aq. 50% (v/v) acetic acid] and centrifugation at 20000g for 15min was repeated. The pH of the supernatant was quickly re-adjusted to 7.4 (with 4M-NH₃). Protamine sulphate solution (10mg/ml) was added to the supernatant to the extent of 2mg of protamine sulphate/100mg of protein, and the mixture was centrifuged at 20000g for 15min. (NH₄)₂SO₄ (240g/litre of extract) was added to the supernatant, and the precipitate formed after 20min was removed by centrifugation (20000g for 15min). More (NH₄)₂SO₄ (180g/litre of extract) was then added, and after 20min the precipitate (which contained the aldehyde dehydrogenase activity) was collected by centrifugation at 20000g for 15min, dissolved in 25mM-sodium phosphate buffer, pH 7.4, and dialysed overnight against three 4-litre changes of the same buffer. [During the addition of (NH₄)₂SO₄ the pH of the solution was kept at approx. 7.0 by the addition of 4M-NH₃.]

After dialysis, insoluble material was removed by centrifugation, and the protein solution was applied to a column (7.5cm × 50cm) of DEAE-cellulose (Whatman DE-23), previously equilibrated with 25mM-phosphate buffer, pH 7.4. The enzyme was eluted from the column with the same buffer, 50–100ml fractions being collected. (NH₄)₂SO₄ (400g/litre of solution) was added to the pooled aldehyde dehydrogenase-containing fractions, and after 20min the precipitate was collected by centrifugation (20000g for 15min). The precipitate was dissolved in the minimum amount of 5mM-phosphate buffer, pH 7.4, and dialysed against four 4-litre changes of the same buffer.

After dialysis, insoluble material was removed by centrifugation, and the protein solution was applied to a column (5cm × 50cm) of DEAE-cellulose (Whatman DE-23), previously equilibrated with 5mM-phosphate buffer, pH 7.4. The column was washed with this buffer until the A_{280} of the effluent in 1cm-path-length cells had become negligible. Aldehyde dehydrogenase activity was then eluted with a gradient made by running 1 litre of 70mM-phosphate buffer, pH 7.4, into 1 litre of 5mM-phosphate buffer, pH 7.4, fractions of volume approx. 15ml being collected. The protein in the aldehyde dehydrogenase-containing fractions was precipitated by dialysis overnight against 50mM-phosphate buffer, pH 7.4, containing sufficient (NH₄)₂SO₄ to give a final concentration both inside and outside the bag of 400g/litre. The precipitate was dissolved in the minimum volume of 50mM-phosphate buffer, pH 7.4 (containing 15mg of dithiothreitol/litre, in place of 2-mercaptoethanol), and dialysed against two 4-litre changes of the same buffer.

After dialysis, insoluble material was removed by centrifugation, and the protein mixture was fractionated on a column (5cm × 46cm) of Sephacryl G-200, which was equilibrated and eluted with 50mM-phosphate buffer, pH 7.4, containing dithiothreitol (15mg/litre, final concn. 100μM). The protein in the enzyme-bearing fractions of highest specific activity was precipitated by dialysis against (NH₄)₂SO₄, the precipitate was redissolved, and the solution was dialysed as before. Dickinson & Berrieman (1979) have shown that at this stage of the preparation the cytoplasmic enzyme is uncontaminated by any other protein species apart from various amounts of the corresponding mitochondrial enzyme. This was removed as follows.

Final purification of cytoplasmic aldehyde dehydrogenase by pH-gradient ion-exchange chromatography. Cytoplasmic aldehyde dehydrogenase was dialysed until equilibrated against 10mM-Bistris {2-[bis-(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol} buffer, pH 6.5, containing 0.3mM-EDTA and dithiothreitol (15mg/

litre). The enzyme solution (7–10 ml; 20–25 mg/ml) was then applied to a column (2 cm × 14 cm) of DEAE-Sephacel (Pharmacia Chemicals, Uppsala, Sweden) that had been equilibrated with the above buffer. Elution was performed by using a gradient made by running 400 ml of 10 mM-sodium acetate buffer, pH 4.6, into 400 ml of 10 mM-Bistris buffer, pH 6.5 (both containing EDTA and dithiothreitol). Fractions of volume 8–10 ml were collected. The protein in the cytoplasmic aldehyde dehydrogenase peak was precipitated by dialysis against $(\text{NH}_4)_2\text{SO}_4$, redissolved and dialysed as before. When necessary (e.g. in experiments with disulfiram), the enzyme solution was dialysed to remove dithiothreitol.

Titration of aldehyde dehydrogenase with disulfiram. This was conducted essentially as described by Kitson (1978), except that activity was measured fluorimetrically, not spectrophotometrically.

Coenzyme binding. The binding of NADH to aldehyde dehydrogenase was monitored fluorimetrically as described by Hart & Dickinson (1977). Excitation was at 366 nm and a narrow-band-pass interference filter, $\lambda_{\text{max.}} = 430 \text{ nm}$, was placed before the detector. Sequential additions of NADH (in negligibly small volume, 5 or 10 μl) were made to 4 ml of 44 mM-sodium phosphate buffer, pH 7.0. The observed increments in fluorescence after each addition were then subtracted from the corresponding increments in fluorescence obtained with buffer containing native cytoplasmic aldehyde dehydrogenase or disulfiram-modified enzyme (in the presence and absence of NAD^+).

Stopped-flow experiments. These were performed in an apparatus of the type described by Gibson & Milnes (1964). Fluorescence was observed at 90° to the exciting light through Wratten 47B and 2B filters in combination.

Results and discussion

Fig. 1 shows a typical elution profile for pH-gradient ion-exchange chromatography of aldehyde dehydrogenase isolated as described above. The total enzymic activity is resolved into a minor and a major peak corresponding respectively to the mitochondrial and cytoplasmic enzymes. [This particular enzyme sample was quite heavily contaminated with the mitochondrial species (to the extent of approx. 15%), giving a good test of the separatory efficiency of the method.] The average pH values (for seven separate column runs) at which these two peaks were eluted were 5.95 and 5.34. These are the pH values at which the two enzyme species no longer carry sufficient negative charge to bind to the DEAE-cellulose resin and would be expected to be close to (but not below) the isoelectric points. This is

Fig. 1. Elution profile for pH-gradient ion-exchange chromatography of impure cytoplasmic aldehyde dehydrogenase

Cytoplasmic aldehyde dehydrogenase, contaminated with approx. 15% mitochondrial enzyme, was eluted by a pH-gradient from DEAE-Sephacel as described in the Experimental section. ●, Enzyme activity; ○, pH.

Fig. 2. Titration of purified cytoplasmic aldehyde dehydrogenase with disulfiram

Enzyme activity was measured at pH 7.0 as described in the Experimental section. Two examples are shown, obtained with enzyme from two separate pH-gradient purifications. The enzyme concentration was calculated for the tetrameric enzyme species based on mol.wt. 212 000 (MacGibbon *et al.*, 1979) and $A_{\text{cm}}^{1\%} = 11.3$ at 280 nm.

in fact the case. The mitochondrial and cytoplasmic aldehyde dehydrogenases of sheep liver have isoelectric points of 5.5–5.8 and 5.2 respectively, as determined in isoelectric-focusing experiments by L. F. Blackwell & K. E. Crow (personal communication). This result has been confirmed by us in isoelectric-focusing experiments in polyacrylamide gels with 2% Ampholine, but otherwise as described by Dickinson & Berrieman (1979). In the earlier work in this laboratory isoelectric points of 6.6 and

6.2 were obtained for mitochondrial and cytoplasmic enzymes. These higher values could have resulted from destabilization of the pH gradient, a phenomenon known to occur in this type of experiment (Chrumbach *et al.*, 1973). In the present work we have attempted to counter the effect by using higher Ampholine concentrations and by incorporating a haemoglobin marker. The current was switched off after the haemoglobin had focused into a sharp band and just as the band began to move at the onset of pH drift. This drift was first seen about 2 h after the start of the experiment. Whatever the cause of the discrepancy over pI values, it is plain that important points from the earlier work are confirmed. Thus the isoelectric point for the cytoplasmic enzyme is some 0.4 pH unit more acid than that of the mitochondrial enzyme, and therefore contamination of cytoplasmic enzyme preparations by the mitochondrial enzyme may be readily detected in isoelectric-focusing experiments.

The identity of the enzyme in the two peaks of Fig. 1 was confirmed by examining the effect of disulfiram on the enzymic activity. A sample of the minor peak retained 91% (average of six column runs) of its original activity when assayed in the presence of $10\mu\text{M}$ -disulfiram, as would be expected for the mitochondrial enzyme (Kitson, 1975; Dickinson & Berrieman, 1979). The effect of disulfiram on the cytoplasmic peak is discussed below.

We have found that the extent of mitochondrial enzyme contamination (as estimated from the effect of $10\mu\text{M}$ -disulfiram; Dickinson & Berrieman, 1979) of ostensibly cytoplasmic aldehyde dehydrogenase samples varies from one preparation to the next; the extent is usually 5–10%, but it has been as much as 17%. For reliable work a method of removing this contamination is obviously essential. The pH-gradient chromatography method reported here is straightforward in operation, gives good separation of the two enzyme forms and gives excellent recovery of enzyme activity (approx. 100%). It is a valuable additional step to the basic isolation procedure for cytoplasmic aldehyde dehydrogenase, and should displace the less-reliable $(\text{NH}_4)_2\text{SO}_4$ fractionation procedure of Dickinson & Berrieman (1979). In our experience, this latter technique works well only with cytoplasmic aldehyde dehydrogenase preparations that are relatively pure to start with, i.e. containing about 5% of mitochondrial enzyme; samples heavily contaminated with the mitochondrial enzyme (i.e. 10–17%) give unsatisfactory results with the $(\text{NH}_4)_2\text{SO}_4$ fractionation method because contamination by the mitochondrial enzyme is not completely removed.

Having used the pH-gradient method to complete the purification of cytoplasmic aldehyde dehydrogenase, an obvious step was for us to re-examine the inactivatory effect of disulfiram (as studied by

Kitson, 1978), since the presence of mitochondrial enzyme will lead to erroneous conclusions in such studies (Dickinson & Berrieman, 1979). The reaction is thought to be a disulphide-interchange reaction between the reagent and an important thiol group on the enzyme. Typical results of titrating the enzyme with small amounts of disulfiram are shown in Fig. 2. Previously (Kitson, 1978) it appeared that such titration profiles were curved; however, it is now clear that over most of the titration range there is, within experimental error, a linear relationship between amount of disulfiram added and loss of enzymic activity, but that at high disulfiram concentration there is a limiting small residual activity.

In the earlier work (Dickinson & Berrieman, 1979) it was suggested that the low residual activity observed in the presence of $10\mu\text{M}$ -disulfiram was due to contamination by traces of mitochondrial enzyme. This seemed reasonable at the time, because the method for removing the mitochondrial enzyme, which depended on the preferential precipitation of the cytoplasmic enzyme by $(\text{NH}_4)_2\text{SO}_4$, might not have been completely effective. With the pH-gradient method now introduced, the separation of the two enzymes is efficient, and it is clear that the residual activity in the presence of $10\mu\text{M}$ -disulfiram is not likely to be due to contamination with mitochondrial enzyme. This conclusion is reinforced by the finding that the residual activity was still exhibited by a sample of purified cytoplasmic enzyme that had been subjected to a second pH-gradient chromatography run. It is further supported by the results of isoelectric-focusing experiments. In the activity stain that we use, the mitochondrial enzyme is some 11-fold more active than the cytoplasmic enzyme (Dickinson & Berrieman, 1979). The fact that no mitochondrial enzyme was detected on gels loaded with our highly purified enzyme suggests that the amount of mitochondrial enzyme that could be present is significantly less than 0.5%.

It seems important to note here that there is difficulty in assaying accurately the very low residual activity of aldehyde dehydrogenase in the presence of $10\mu\text{M}$ -disulfiram. A sensitive fluorescence assay is required, but, because of the presence of a light-sensitive fluorescent impurity in commercial NAD^+ , pronounced lag phases are observed when assaying low aldehyde dehydrogenase activities. In some cases the true activity may not be expressed for as much as 5–8 min after initiation of the reaction. The problem is, however, very much diminished if chromatographically purified NAD^+ (Dalziel & Dickinson, 1966) is used.

It does not seem that the inhibition of aldehyde dehydrogenase by disulfiram is similar to the inhibition of glutamate dehydrogenase by pyridoxal 5'-phosphate. In the latter system the 6–7% residual

activity observed after incubation with excess of the reagent is thought to be due to the formation of a freely dissociable non-covalent complex in equilibrium with the covalent Schiff-base complex (Chen & Engel, 1975). The intermediate complex generates fully active enzyme on dilution in assays and explains why some activity is apparently retained when covalent modification leads to a total loss of activity. In the present case the same low residual activity is observed if the incubation mixture containing a 3-fold molar excess of reagent is diluted in assays or when the enzyme is assayed directly in the presence of a large excess (10 or 100 μM) of disulfiram. Furthermore, prolonged dialysis or gel filtration of inhibited enzyme in the absence of 2-mercaptoethanol or dithiothreitol does not yield active enzyme.

The mechanism by which aldehyde dehydrogenase has been postulated to catalyse the oxidation of aldehydes involves nucleophilic attack of an enzymic thiol group on the aldehydic carbonyl group to give a thiohemiacetal; from this, a hydride ion is transferred to NAD^+ in the oxidation-reduction step. The resulting enzymic thioester is subsequently hydrolysed to give the acid product and regenerate the free enzyme (Jakoby, 1963; Li, 1977). One of the main pieces of evidence in support of the existence of such an essential thiol group is the susceptibility of the enzyme to disulfiram and other reagents that react with thiol groups. However, since, as we have reported above, the pure cytoplasmic enzyme (uncontaminated by the mitochondrial species) still retains an appreciable activity in the presence of a large excess of disulfiram, then the groups with which disulfiram reacts cannot be absolutely vital to the catalytic mechanism in the manner proposed (otherwise, of course, modification by disulfiram would lead to total loss of activity). Rather, it appears that reaction with disulfiram affects the enzyme indirectly, such that it can still perform its catalytic function, albeit much less efficiently, and the proposed mechanism of catalysis remains hypothetical. This interpretation of our results does provide the simplest explanation, but it cannot be regarded as conclusively proved. It is possible, for example, that the residual activity represents the full activity of the two unmodified subunits. Alternatively there might be a small amount of a hybrid of the cytoplasmic and mitochondrial enzymes present, although no hybrids have been observed on mixing the two purified enzymes before isoelectric focusing (S. Allanson & F. M. Dickinson, unpublished work). What does seem clear is that the residual activity observed is not due to simple contamination with mitochondrial enzyme.

It is interesting, however, that disulfiram does modify the enzyme very rapidly. It has been shown

by fluorescence measurements in a stopped-flow apparatus that, when the enzyme is mixed with NAD^+ and propionaldehyde, a rapid burst of NADH production precedes the establishment of the steady-state rate (MacGibbon *et al.*, 1977b). We have confirmed this by fluorescence measurements on mixing 1 μM enzyme with 40 μM -propionaldehyde and 200 μM - NAD^+ in phosphate buffer, pH 7.0, at 25°C. However, when the enzyme was mixed with the same substrate mixture, but now containing 3 μM -disulfiram, the burst was of the same duration ($t_1 \sim 0.5$ s) as before, but was decreased in amplitude by about 40%, and the fully inhibited rate was established immediately after the burst.

The stoichiometry of the reaction between disulfiram and sheep liver cytoplasmic aldehyde dehydrogenase was previously found to be between 1 and 2 (Kitson, 1978). In the present study, with enzyme samples purified by pH-gradient chromatography, a value of 1.5–2.0 has been obtained (i.e. the number of molecules of disulfiram per tetrameric enzyme molecule necessary to decrease the activity to the small residual value, about 2%). This is obviously the case for the two enzyme samples used in obtaining Fig. 2, and the result was reproduced on numerous other occasions. Even with enzyme preparations of the highest specific activity (approx. 140 nmol of NADH produced/min per mg in the standard assay), and performing the experiment immediately after enzyme isolation to avoid slight losses of activity on storage, the observed stoichiometry did not exceed 2. This suggests that the enzyme, although composed of four subunits (MacGibbon *et al.*, 1979), may have only two functional active sites. The same conclusion was reached by Takahashi & Weiner (1980) with respect to the pI-5 isoenzyme of horse liver. They presented evidence showing that the tetramer has 'half-of-sites' reactivity, but dissociates (under the influence of Mg^{2+}) into dimers with 'all-of-sites' reactivity.

As discussed above, our results suggest that disulfiram does not react with the thiol groups that, it has been proposed, become covalently attached to the aldehyde substrate during the catalytic process. An alternative way in which disulfiram might affect activity is by affecting the binding of enzyme to coenzyme. To test this possibility we performed fluorescence titrations (by the method of Hart & Dickinson, 1977) between the enzyme and NADH under various conditions, the results of which are shown in Fig. 3. It is clear that disulfiram-modified enzyme does bind NADH. (If there were no binding, there would be no enhancement of fluorescence, and the experimental points would follow the horizontal axis.) The modified enzyme augments the fluorescence of NADH less than does the native enzyme; however, the dissociation constants for the native and modified enzymes are very similar, since

Fig. 3. Fluorescence titrations between purified cytoplasmic aldehyde dehydrogenase and NADH under various conditions

The binding of enzyme and NADH was examined by fluorescence titration as described in the Experimental section. The enzyme concentration was in all cases $0.82 \mu\text{M}$. Each addition of NADH represented a concentration of $3.04 \mu\text{M}$. $\circ$, Native enzyme; $\bullet$, native enzyme in the presence of $46 \mu\text{M-NAD}^+$; $\triangle$, disulfiram-modified enzyme; $\blacktriangle$, disulfiram-modified enzyme in the presence of $46 \mu\text{M-NAD}^+$. (Disulfiram-modified enzyme was enzyme that had been treated with a 2-fold excess of disulfiram over the concentration of enzyme tetramers.) All points shown on these curves have had subtracted the corresponding fluorescence observed when NADH was added to buffer in the absence of enzyme.

the titration curves attain saturation at approximately the same concentration of NADH. Furthermore, the presence of NAD^+ affects both NADH-fluorescence titration curves to a similar degree, showing that the disulfiram-modified enzyme still retains its ability to bind NAD^+ . This conclusion, that reaction with disulfiram does not block the binding of NAD^+ , complements the observation that the presence of NAD^+ does not block the enzyme's reaction with disulfiram (T. M. Kitson, unpublished work).

If the stability of the enzyme-coenzyme complexes is little affected by disulfiram modification, it also seems that the rates of association of the enzyme with coenzyme are little changed. MacGibbon *et al.* (1977a) have shown by stopped-flow fluorescence measurements that the enzyme-NADH association reaction in the presence of excess of

Fig. 4. Association of NADH with aldehyde dehydrogenase

Aldehyde dehydrogenase ($4 \mu\text{M}$) ($\bullet$) or aldehyde dehydrogenase ($4 \mu\text{M}$) pretreated with disulfiram ($8 \mu\text{M}$) ($\circ$) were mixed with NADH ($40 \mu\text{M}$). For details see the Experimental section. The graph shows the logarithm of the difference, ΔF , between the voltage at infinite time and the voltage at each time, plotted against time. The rate constants derived from the lines are 0.81 s^{-1} for native enzyme and 0.96 s^{-1} for enzyme plus disulfiram.

NADH is biphasic, with the slow process being independent of the coenzyme concentration with a first-order rate constant of 0.85 s^{-1} at pH 7.6 at 25°C . We have obtained very similar results at pH 7.0 in experiments where $4 \mu\text{M}$ enzyme was mixed with $40 \mu\text{M-NADH}$. When the experiment was conducted with enzyme pretreated with a 2-fold excess of disulfiram (5% residual activity), a fluorescence trace was obtained of very similar shape but of 65% of the amplitude of that obtained with untreated enzyme. Analyses of both experiments are shown in Fig. 4. MacGibbon *et al.* (1977a) attribute the biphasic nature of these curves to a two-step reaction in which the association of enzyme and NADH is followed by isomerization of the enzyme-coenzyme complex. Evidently neither process is materially affected by disulfiram modification, although the modified group is close enough to the reaction centre to inhibit the enzyme severely and to alter the fluorescence yield of bound NADH.

In conclusion, the results presented here tend to rule out certain ways in which thiol-group modification by disulfiram might cause loss of activity of cytoplasmic aldehyde dehydrogenase. The precise mechanism by which this happens remains to be elucidated.

We are grateful to Ms. Carol Rooks for technical assistance and to Dr. L. F. Blackwell and Dr. K. E. Crow for making their results available before publication.

References

- Chen, S.-S. & Engel, P. C. (1975) *Biochem. J.* **147**, 351–358
- Chrambach, A., Doerr, P., Finleyson, G. R., Miles, L. E. M., Sherins, R. & Rodbard, D. (1973) *Ann. N.Y. Acad. Sci.* **209**, 44–64
- Crow, K. E., Kitson, T. M., MacGibbon, A. K. H. & Batt, R. D. (1974) *Biochim. Biophys. Acta* **350**, 121–128
- Dalziel, K. & Dickinson, F. M. (1966) *Biochem. Prep.* **11**, 84–88
- Dickinson, F. M. & Berrieman, S. (1979) *Biochem. J.* **179**, 709–712
- Gibson, Q. H. & Milnes, L. (1964) *Biochem. J.* **91**, 161–171
- Hart, G. J. & Dickinson, F. M. (1977) *Biochem. J.* **163**, 261–267
- Hart, G. J. & Dickinson, F. M. (1978) *Biochem. J.* **175**, 899–908
- Jakoby, W. B. (1963) *Enzymes 2nd Ed.* **7**, 203–221
- Kitson, T. M. (1975) *Biochem. J.* **151**, 407–412
- Kitson, T. M. (1977) *J. Stud. Alcohol* **38**, 96–113
- Kitson, T. M. (1978) *Biochem. J.* **175**, 83–90
- Li, T. K. (1977) *Adv. Enzymol.* **45**, 427–483
- MacGibbon, A. K. H., Buckley, P. D. & Blackwell, L. F. (1977a) *Biochem. J.* **165**, 455–462
- MacGibbon, A. K. H., Blackwell, L. F. & Buckley, P. D. (1977b) *Biochem. J.* **167**, 469–477
- MacGibbon, A. K. H., Motion, R. L., Crow, K. E., Buckley, P. D. & Blackwell, L. F. (1979) *Eur. J. Biochem.* **96**, 585–595
- Takahashi, K. & Weiner, H. (1980) *J. Biol. Chem.* **255**, 8206–8209