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The Separation of Sheep Liver Cytoplasmic and Mitochondrial Aldehyde Dehydrogenases by Isoelectric Focusing, and Observations on the Purity of Preparations of the Cytoplasmic Enzyme, and their Sensitivity towards Inhibition by Disulfiram

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Preparations of sheep liver cytoplasmic aldehyde dehydrogenase obtained by published methods were found by analytical isoelectric focusing in the pH range 5–8 to contain 5–10% by weight of the mitochondrial aldehyde dehydrogenase. Under the conditions used the pI of the cytoplasmic enzyme is 6.2 and that of the mitochondrial enzyme 6.6. The mitochondrial enzyme can be removed from the preparation by selective precipitation of the cytoplasmic enzyme with $(NH_4)_2SO_4$. Kinetic experiments and inhibition experiments with disulfiram show that the properties of the two sheep liver enzymes are so different that the presence of 10% mitochondrial enzyme in preparations of the cytoplasmic enzyme can introduce serious errors into results. Our results suggest that the presence of 10μM-disulfiram in assays may completely inactivate the pure cytoplasmic enzyme. This result is in contrast with a previous report [Kitson (1978) *Biochem. J.* **175**, 83–90].

Aldehyde dehydrogenases (EC 1.2.1.3) from mammalian liver have become the subjects of increased interest and study now that stable and apparently homogeneous preparations can be readily obtained. The findings that, for example, the aldehyde dehydrogenases of sheep liver are of mitochondrial and cytoplasmic origin (Crow *et al.*, 1974) and that despite similar molecular weight and subunit structure these enzymes have rather different catalytic properties (Kitson, 1975; Hart & Dickinson, 1977) are important observations. It is obviously necessary to study the properties of the two enzymes separately and to be sure that preparations of one are essentially free of the other. In our recent work we have found that sheep liver mitochondrial and cytoplasmic enzymes can be readily separated and identified by analytical isoelectric focusing. It has also become apparent by use of this technique that preparations of the cytoplasmic enzyme made by established methods (Crow *et al.*, 1974) may contain significant amounts of the mitochondrial enzyme. The present paper describes these experiments and shows how this contamination can lead to erroneous results, at least in so far as steady-state kinetic studies and inhibition by disulfiram (tetraethylthioperoxydicarbonic diamide) are concerned.

Materials and Methods

NAD^+ was chromatographically purified before use by the method of Dalziel & Dickinson (1966). Sheep liver cytoplasmic aldehyde dehydrogenase was prepared essentially by the method of Crow *et al.* (1974) and the mitochondrial enzyme was prepared by the method of Hart & Dickinson (1977) and was kindly given by Mr. G. J. Hart of this Department. Both enzymes were stored at 0–4°C in 50mM-sodium phosphate buffer, pH 7.0, containing 0.1mM-dithiothreitol and 0.3mM-EDTA. The enzymes were assayed fluorimetrically at pH 8.0 and 25°C by the method of Hart & Dickinson (1977). In the assay purified cytoplasmic enzyme had a specific activity of 0.13–0.16unit/mg (1unit = 1μmol/min) and the mitochondrial enzyme 0.20unit/mg.

Isoelectric focusing was performed in 4% polyacrylamide gels (5mm×55mm) containing 0.7% pH 5–8 Ampholines (LKB Producter, Bromma, Sweden). The cathode compartment contained 0.3M-NH_3 +0.05M-NH_4Cl and the anode compartment 0.3M-acetic acid and 0.05M-sodium acetate. The gels were pre-run at 0–4°C (1.5mA/gel) for 15–30min, then the enzyme samples (100–250μg) were applied in 0.1ml of 40% (w/v) sucrose containing 1% pH 5–8 Ampholine and this was then overlaid with 0.1ml of 10% sucrose containing 0.6% pH 7–10 Ampholine. Focusing (1.5mA/gel) was performed for 5h at 0–4°C, by which time a stable pH-gradient was established. Recovered gels were stained for protein and enzyme activity. The protein stain contained: Coomassie Brilliant Blue G, 100mg; trichloroacetic acid, 10g; sulphosalicyclic acid, 3g; methanol, 25ml, made up to 100ml in water. The activity stain contained: NAD^+, 20mg; Nitro Blue Tetrazolium, 5mg; acetaldehyde, 0.02mmol; and

phenazine methosulphate, 0.2 mg, in 20 ml of sodium phosphate buffer, pH 8.0 ($I = 0.2$). The pH-gradient established in the gels was measured by sectioning specimen gels into 2 mm lengths. The lengths were incubated in 2 ml of degassed distilled water for 2 h and then the pH of the liquid was measured. The measurements indicated that a smooth and reproducible pH-gradient was established. If one 2 mm segment from each end of the gels was ignored the pH in the gels ranged smoothly from 4.7 at the anode end to 8.0 at the cathode end.

Kinetic measurements were performed fluorimetrically by using a filter fluorimeter of the type described by Dalziel (1962). Assays of enzyme activity in the presence of disulfiram were performed essentially by procedure A of Kitson (1978). Assays were performed in 50 mM-phosphate buffer, pH 7.0, with disulfiram incorporated by adding it as 0.1 ml of ethanolic solution. Assay mixtures contained 430 μM-NAD$^+$, 1 mM-acetaldehyde and 0–50 μM-disulfiram in 4 ml of reaction mixture. For these experiments the stock enzyme was first dialysed overnight against 50 mM-phosphate buffer, pH 7.0, containing 0.3 mM-EDTA to remove the dithiothreitol used to stabilize the enzyme in storage.

Results and Discussion

The results of isoelectric focusing of a preparation of cytoplasmic aldehyde dehydrogenase prepared essentially by the method of Crow *et al.* (1974) are shown in Fig. 1, gels (*b*) and (*c*). It is evident that all the protein material is enzymically active, but that there are two species exhibiting about equal activity. One of these corresponds to the main protein band and the other to a much more weakly staining protein band. Experiments with a mixture of purified mitochondrial and cytoplasmic aldehyde dehydrogenases (gel *a*) showed that the mitochondrial enzyme has the same isoelectric point as the weakly staining protein band of gel (*c*) (Fig. 1). It appears therefore that this preparation of the cytoplasmic enzyme was contaminated with significant amounts of mitochondrial enzyme. This is not particularly surprising, the contamination probably arising from rupture of some mitochondria in the early stages of the purification. However, it has not been previously reported for this preparation as far as we know. Indeed, according to MacGibbon *et al.* (1977), quoting Crow (1975), isoelectric focusing of these preparations did not reveal the presence of isoenzymes. Measurement of the pH-gradient in specimen gels run as for Fig. 1 indicated that the isoelectric point of the cytoplasmic enzyme is 6.2 ± 0.1 and for the mitochondrial enzyme is 6.6 ± 0.05. We may note here that the highly purified preparations of mitochondrial aldehyde dehydrogenase prepared as described by Hart & Dickinson (1977) are free of contamination by the cytoplasmic enzyme when examined by the procedure of Fig. 1.

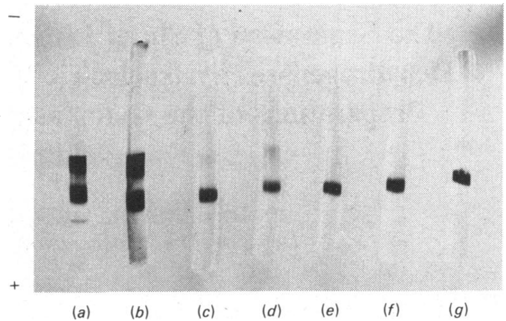

Fig. 1. *Isoelectric focusing of various preparations of sheep liver cytoplasmic aldehyde dehydrogenase*
Enzyme (150 μg of protein) was applied to each gel and subjected to isoelectric focusing at 0–4°C in the pH-range 5–8 as described in the text. Gel (*a*) shows the results of protein staining on a mixture of purified mitochondrial and cytoplasmic enzymes. Gels (*b*) and (*c*) show the results of activity and protein staining on a preparation of enzyme made by the procedure of Crow *et al.* (1974). Gels (*d*), (*e*) and (*f*) show the results of protein staining of the first and second $(NH_4)_2SO_4$ supernatants and the redissolved second $(NH_4)_2SO_4$ precipitate after fractionation of the enzyme preparation by the procedure described in the text. Gel (*g*) shows the activity stain corresponding to the protein stain of gel (*f*).

One interesting finding to emerge from Fig. 1 is that the mitochondrial enzyme is much more active in the activity stain used than is the cytoplasmic enzyme. By following the increase in A_{550} in the activity stain after initiating the reaction by addition of either highly purified cytoplasmic (see below) or mitochondrial aldehyde dehydrogenase, we estimated that the mitochondrial enzyme is 11.5 times more active in the activity stain than cytoplasmic enzyme of an equivalent specific activity determined in the standard assay system. This finding explains the intense activity stain given by the weakly staining protein band in Fig. 1, gel (*c*). Scanning of gel (*c*) (Fig. 1) indicates that the preparation contained 5–10% by weight of mitochondrial enzyme.

The results with analytical isoelectric focusing indicate that the mitochondrial enzyme could be removed from preparations of the cytoplasmic enzyme by isoelectric focusing on a preparative scale. Such a procedure is, however, expensive and rather time-consuming, and we sought a more convenient method. Instead we have managed to achieve the purification of the enzyme by fractionation with $(NH_4)_2SO_4$. The basis of the method is that the cytoplasmic enzyme may be less soluble and therefore preferentially precipitated at its isoelectric

point. The fractionation is carried out at 0°C in 50mM-phosphate buffer, pH6.3, containing 0.3M-EDTA and 0.1mM-dithiothreitol with an initial protein concentration of 10mg/ml. Solid $(NH_4)_2SO_4$ is added slowly until 80–90% of the protein is precipitated: this occurs at about 45% saturation (320g/litre). The precipitate is removed by centrifugation and redissolved in 50mM-phosphate buffer, pH6.3, containing 0.3mM-EDTA and 0.1mM-dithiothreitol. Then the protein concentration is re-adjusted to 10mg/ml by dilution with buffer, and the precipitation of 80–90% of the protein by addition of solid $(NH_4)_2SO_4$ is repeated. Finally the precipitate is redissolved in 50mM-phosphate buffer, pH7.0, containing 0.3M-EDTA and 0.1mM-dithiothreitol, dialysed against the same buffer and then stored at 0–4°C.

The progress of the fractionation procedure is readily monitored by isoelectric focusing, and representative results are shown in Fig. 1, gels (*d*)–(*g*). It is clear that the first $(NH_4)_2SO_4$ supernatant is enriched with respect to the mitochondrial enzyme (gel *d*) and that the final precipitate is free of mitochondrial enzyme (gels *f* and *g*). A crude indication of the success of the procedure is obtained in the standard fluorimetric assays of enzyme activity. As has been reported (Hart & Dickinson, 1977), the assays of the mitochondrial enzymes are characterized by lag phases in reaction progress curves. Progress curves for the cytoplasmic enzyme under the same conditions are, however, linear. Assays of the fractions obtained in the $(NH_4)_2SO_4$ precipitations show the expected characteristics. That is, the activity in the first $(NH_4)_2SO_4$ supernatant shows a distinct lag phase, whereas those in the precipitates show linear progress curves.

Examination of the properties of purified cytoplasmic aldehyde dehydrogenase suggests that at least one recorded property of the enzyme, i.e. the resistance of 10% of the enzyme activity to inhibition by disulfiram (Kitson, 1978), is probably due to contamination by the mitochondrial enzyme. This possibility was discussed in the original report, but was discounted because polyacrylamide-gel electrophoresis at pH8.9 with a protein stain failed to detect a second band, and because Crow (1975) failed to detect the presence of isoenzymes in these preparations by use of an isoelectric-focusing method. We may add that when using polyacrylamide-gel electrophoresis at pH8.3 with 7% gels we also failed to detect the presence of two enzyme species by either protein or activity stain in material later shown by isoelectric focusing to contain mitochondrial enzyme. Electrophoresis at pH8.3 under the same conditions but using purified cytoplasmic and mitochondrial enzymes showed that the proteins have virtually the same mobility and cannot be distinguished under these conditions.

The effects of disulfiram on the activity of purified cytoplasmic and mitochondrial aldehyde dehydrogenase at pH7.0 and 25°C are shown in Fig. 2. Above 1μM-disulfiram less than 1% activity remains in the cytoplasmic enzyme preparation, whereas the mitochondrial enzyme is rather insensitive to the reagent under these conditions. It may be that the trace of residual activity in the cytoplasmic enzyme at high disulfiram concentrations is due to contamination by a small amount of mitochondrial enzyme. The 10% residual activity obtained by Kitson (1978) is most probably due to the presence of larger amounts of mitochondrial enzyme. This degree of contamination is, after all, about that found by us in preparations made by a very similar procedure.

Apart from the disulfiram results, there is another area where the presence of significant amounts of mitochondrial aldehyde dehydrogenase will introduce serious errors. The results of initial-rate experiments with purified mitochondrial and cytoplasmic enzymes at pH7.0 and 15°C in the oxidation of acetaldehyde by 500μM-NAD^+ showed that the kinetic behaviour of the two enzymes is quite different (though for both the reciprocal plots are non-linear and exhibit apparent substrate activation). The enzymes exhibit very similar activities at high acetaldehyde concentrations, but the cytoplasmic enzyme is much less efficient at low acetaldehyde concentrations. Contamination of the cytoplasmic enzyme with approx. 10% mitochondrial enzyme will produce serious errors in measured rates at acetaldehyde concentrations below 10μM. Since for assessing the roles of the mitochondrial and cytoplasmic enzymes in the metabolism of acetaldehyde it may be important to have accurate kinetic information at low aldehyde concentrations, it will be essential to have pure preparations of the cytoplasmic enzyme. The present

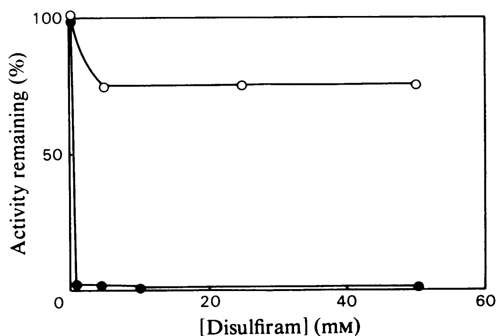

Fig. 2. *Inhibition of sheep liver aldehyde dehydrogenases by disulfiram*

Assays were performed at 25°C and pH7.0 as described in the text by using either 0.032μM-cytoplasmic (●) or 0.016μM-mitochondrial (○) enzyme.

work suggests two criteria either of which may be used to assess whether preparations of cytoplasmic aldehyde dehydrogenase are free of the mitochondrial enzyme. The isoelectric-focusing method in conjunction with the activity stain is peculiarly sensitive to the presence of the mitochondrial enzyme and is convenient for those with the appropriate equipment and chemicals to hand. Most convenient of all, however, is likely to be the method based on the sensitivity of the cytoplasmic enzyme to disulfiram. Straightforward assays at pH 7.0 in the presence or absence of 10 μM-disulfiram will yield a ready indication of the extent of contamination by the mitochondrial enzyme. The residual activity in the presence of disulfiram should not exceed 0.5% of the rate seen in the absence of this reagent, and quite possibly the residual rates of 0.5% observed here are due to the presence of a trace of the mitochondrial enzyme.

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