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The removal of cytosolic-type aldehyde dehydrogenase from preparations of sheep liver mitochondrial aldehyde dehydrogenase and the unusual properties of the purified mitochondrial enzyme in assays

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1. The pI ~5.2 isoenzymes of mitochondrial aldehyde dehydrogenase were separated from the other isoenzymes by pH-gradient chromatography on DEAE-Sephacel. The pI ~5.2 material is immunologically identical with cytosolic aldehyde dehydrogenase. It also shows sensitivity to 20 μM-disulfiram and insensitivity to 4 M-urea in assays. These and other criteria seem to establish that the material is identical with the cytosolic enzyme. 2. Mitochondrial enzyme that had been purified to remove pI ~5.2 isoenzymes shows concentration-dependent lag phases in assays. These effects are possibly due to the slow establishment of equilibrium between tetramer and either dimers or monomers, with the dissociated species being intrinsically more active than the tetramer.

In a study of the isoelectric-focusing behaviour of purified preparations of sheep liver mitochondrial aldehyde dehydrogenase, at least seven active isoenzymes were found (Agnew *et al.*, 1981). Two isoenzymes had isoelectric points close to pH 5.2, and the other isoenzymes had pI values in the range 5.48–5.76. The disulfiram-sensitive bands focused at ~pH 5.2 were identified as of cytosolic aldehyde dehydrogenase type, because that enzyme exhibits only those bands on isoelectric focusing (Agnew *et al.*, 1981). No other criteria were applied. It seemed to us that characterization of the pI ~5.2 bands in mitochondrial enzyme preparations could be improved. This would help to decide the likely origin of this material and is one object of the present work.

Fluorimetric assays of mitochondrial aldehyde dehydrogenase have been observed to show concentration-dependent lag phases (Hart & Dickinson, 1978). As it now appears that the enzyme used in that study probably contained cytosolic-type enzyme, it seemed important to re-examine the phenomenon with enzyme which is free of this material. The results of this work form the second subject of this paper.

Materials and methods

Materials

NAD^+ (grade II) and NADH (grade I) were obtained from Boehringer Corp., London W.5, U.K. Disulfiram and *p*-nitrophenyl acetate were obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K. Ampholine polyacrylamide-gel-electrophoresis plates of pH range 3.5–9.5 were supplied by LKB, Bromma, Sweden, and DEAE-Sephacel was supplied by Pharmacia, Uppsala, Sweden. Other chemicals were analytical-reagent grade from Fisons, Loughborough, Leics., U.K. Aldehydes were redistilled before use. Glass-distilled water was used throughout in the preparation of solutions.

Enzyme preparation

Mitochondrial aldehyde dehydrogenase was prepared from fresh sheep livers essentially in the manner described by Hart & Dickinson (1977). The modifications introduced in the present work were that in the pH step the pH was lowered to 5.4 instead of 5.0 and the $(NH_4)_2SO_4$ step after pH-adjustment was omitted. After removal of the mitochondrial debris by centrifugation at pH 5.4, the supernatant was re-adjusted to pH 7.4 with 4 M-NH_3. Ion-exchange chromatography was performed on DEAE-Sepharose instead of DEAE-cellulose. The specific activity of the enzyme produced was about 200 munits/mg when measured at relatively high enzyme concentrations (see below), about the same as in the earlier work (Hart & Dickinson, 1977). The purified enzyme has a specific absorption of $A_{280}^{1cm} = 9.7$, on the basis of dry-weight measurements. M_r of these prepara-

tions has been estimated by ultracentrifugation to be 200000 (Hart & Dickinson, 1977).

Isoelectric focusing

This was carried out on Ampholine polyacrylamide-gel-electrophoresis plates of pH range 3.5–9.5. Electrophoresis took place on a water-cooled base kept at about 4°C at essentially constant power. Focusing took 2–3h and was monitored by using haemoglobin or occasionally ferritin as a marker. Aldehyde dehydrogenase activity was monitored by using an activity stain containing acetaldehyde as described by Dickinson & Berrieman (1979). The pH gradients in gels were measured by cutting a strip of gel, which had not had sample applied to it, in 0.5cm lengths. The pieces of gel were placed in distilled water for several hours and the pH of the liquid was then measured.

Enzyme assays

Routine assays of the dehydrogenase activity were carried out spectrophotometrically at 340nm in 33mM-sodium phosphate buffer, pH8.0 at 25°C under the conditions described by Hart & Dickinson (1977). The cell housings of the spectrophotometers (Unicam SP.8-100 or SP.1800) were thermostatically controlled, and no significant variation in temperature occurred over the prolonged period of some assays. Assays of the esterase activity were carried out in 25mM-phosphate buffer, pH7.0, with 500μM-*p*-nitrophenyl acetate as substrate. *p*-Nitrophenyl acetate was prepared as a 30mM solution in acetone. The presence of 1.5% (v/v) acetone in assays did not affect the rates. All assays were initiated by adding enzyme, unless otherwise stated. Assays with different enzyme concentrations were performed by withdrawing samples (100μl, 10μl or 1μl) from the same stock solution of enzyme. The micropipettes/microsyringes used for the transfer of enzyme were calibrated spectrophotometrically at 373nm by making dilutions of $K_2Cr_2O_4$ solutions in 0.01M-NaOH.

Preparation of anti-(cytosolic aldehyde dehydrogenase) serum

Over a 3-week period, a rabbit was given three injections containing a total of 2mg of cytosolic aldehyde dehydrogenase previously homogenized with BCG vaccine and Freund's non-ulcerative incomplete adjuvant (Morris). The injections were made subcutaneously at four sites on the back. Starting at the fourth week, 1mg of enzyme per week was injected intravenously into the ear. The rabbit was bled out at the seventh week. Double-diffusion plates were run in 2% agarose in the cold. Pure cytosolic aldehyde dehydrogenase was prepared by the method of Dickinson *et al.* (1981).

Results and discussion

Removal of the cytosolic-type isoenzymes

Isoelectric focusing of purified sheep mitochondrial aldehyde dehydrogenase on wide-pH-range (pH3.5–9.5) polyacrylamide plates revealed a pair of active bands with pI ~5.2 and five further active bands with pI values in the range 5.48–5.76 (Agnew *et al.*, 1981). We have obtained very similar results with our preparations of enzyme. As cytosolic aldehyde dehydrogenase exhibits only a pair of bands with pI ~5.2 on isoelectric focusing under the same conditions (Agnew *et al.*, 1981; Hart & Dickinson, 1983), it seems that the mitochondria may contain a cytosolic-type enzyme. Alternatively, it could be that these bands appear because of contamination of mitochondrial fractions with cytosol. Agnew *et al.* (1981) thought this explanation unlikely, however, because their mitochondrial fractions contained very little of the cytosolic enzyme lactate dehydrogenase. In order to investigate the matter further, we have subjected enzyme preparations to pH-gradient chromatography on DEAE-Sephacel. This procedure has been used effectively to remove impurities of pI ~5.6 from preparations of the cytosolic enzyme (Dickinson *et al.*, 1981). It is clear that the pH gradient achieves an effective separation (Fig. 1), because the early fractions are completely insensitive to the presence of disulfiram in assays, whereas the late fractions are almost totally inhibited. Almost complete inhibition by 20μM-disulfiram in standard assays is a well-known characteristic of the cytosolic form of the enzyme (Dickinson *et al.*, 1981).

Fractions from the pH-gradient column were collected together into four batches, as indicated in Fig. 1, and the enzyme in each batch was concentrated by precipitation with $(NH_4)_2SO_4$, followed by redissolving in a minimum volume of 50mM-phosphate buffer and dialysis against that buffer. When examined, by isoelectric focusing as above, the batch (batch 1) made from fractions emerging first from the pH-gradient column was found to be free of the pI ~5.2 isoenzymes, and the batches from later fractions (batches 2–4) exhibited these isoenzymes in increasing quantities. It is to be expected that materials with lowest pI values will be retained longest by the DEAE-Sephacel column with the pH gradient used here. When the behaviour of the concentrated samples was studied by diffusion against rabbit anti-(cytosolic aldehyde dehydrogenase) serum on Ouchterlony plates, it was clear that the enzyme that had emerged first from the

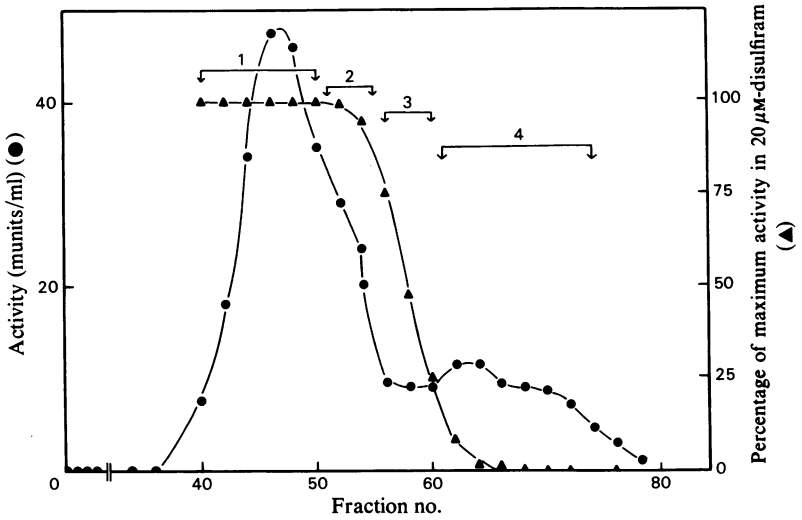

Fig. 1. *Fractionation of mitochondrial aldehyde dehydrogenase on DEAE-Sephacel*

Enzyme (70mg) was applied to a column (4.5cm × 2cm diam.) equilibrated with 5mM-Bistris {2-[bis-(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol} buffer, pH6.5. The column was developed with a gradient formed from 280ml of 5mM-Bistris buffer, pH6.5, and 280ml of 5mM-sodium acetate buffer, pH4.6. Buffers contained 0.3mM-EDTA and 100μM-dithiothreitol. The experiment was conducted essentially as described by Dickinson *et al.* (1981). Assays were conducted as described by Hart & Dickinson (1977) in the absence (●) and presence (▲) of 20μM-disulfiram. Eluted fractions (5ml) were collected together as indicated into batches 1–4. The enzyme in the batches was precipitated with $(NH_4)_2SO_4$, redissolved and dialysed versus 50mM-sodium phosphate buffer, pH7.0, and used for subsequent experiments.

pH-gradient column (batch 1) and was free of the pI~5.2 isoenzymes did not form precipitin lines. Later fractions (batches 2–4), which contained the pI~5.2 isoenzyme and which were sensitive to disulfiram, reacted increasingly strongly with the antiserum, though batch 2 showed only a very weak reaction. These results appear in Fig. 2. The late fractions (batch 4) reacted to antiserum identically with purified cytosolic enzyme taken at the same concentration.

Further tests established the similarity of the pI~5.2 isoenzymes to the cytosolic enzyme. The latter is insensitive to the inclusion of 4M-urea in assays (S. Allanson & F. M. Dickinson, unpublished work). The early fractions emerging from the pH column were sensitive to 4M-urea (75% inhibition), whereas the later fractions were little affected (5% inhibition). It has been observed (Dickinson & Berrieman, 1979) that the cytoplasmic enzyme exhibits a much lower activity than the mitochondrial enzyme in the Nitro Blue Tetrazolium-based activity stain used in developing electrophoresis slabs. In the present instance we found that the fractions emerging late from the pH-column had a 4-fold lower activity in the activity stain than did the early fractions. The same amount of dehydrogenase activity was taken in each case.

The experiments described show that the pI~5.2 isoenzymes found in preparations of the mitochondrial enzyme have many of the properties of authentic cytoplasmic aldehyde dehydrogenase. It is possible that these isoenzymes originate in the mitochondria, but it seems more likely that they arise because of contamination of the mitochondria fraction with some cytosol. Certainly, very thoroughly washed mitochondria yielded activity which was insensitive to disulfiram. For routine enzyme preparations, mitochondria are washed only once, because they are fragile and we wish to retain as much enzyme as possible. The cytosol, however, contains 3–4-fold more aldehyde dehydrogenase activity than the mitochondrial compartment, and contamination of the mitochondria with 5% of the cytoplasmic fraction would lead to a disproportionate contribution to the activity of the mitochondrial fraction. Subsequent fractionation procedures may favour a relative increase in the concentration of the cytosolic enzyme. In $(NH_4)_2SO_4$ precipitations, for example, the cytosolic enzyme precipitates much earlier (see below) and would be definitely favoured in steps where precipitation is used to concentrate enzyme from column eluates and other dilute solutions.

The pH-gradient column achieves an effective

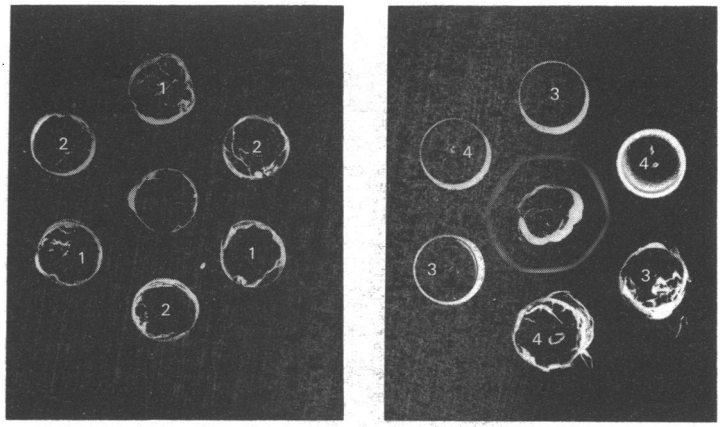

Fig. 2. *Enzyme–antibody reactions on double-diffusion plates*
Anti-(cytosolic aldehyde dehydrogenase) serum was placed in the centre wells, and mitochondrial aldehyde dehydrogenase fractions (batches 1–4) were placed in the outer wells as indicated. The aldehyde dehydrogenase fractions were all taken at 1.25mg/ml. The cytosolic aldehyde dehydrogenase used for antibody formation was purified as described by Dickinson *et al.* (1981).

separation of the pI~5.2 isoenzymes from those with higher pI. It is likely that the procedure achieves at least a partial separation of the other isoenzymes. It might indeed prove possible to separate these other isoenzymes sufficiently to tackle the question of their structural relationship to one another. To the present we have not pursued this question. We have confined our efforts to obtaining as great amounts of the mitochondrial enzyme as possible which are quite free of the cytoplasmic-type enzyme. To this end, fractions totally insensitive to 20 μM-disulfiram have been collected together (i.e. batch 1 in Fig. 1), and the concentrated enzyme has been used for subsequent experiments. It is noted in passing here that an effective, though incomplete, removal of the cytosolic-type enzyme can be achieved by $(NH_4)_2SO_4$ precipitation. A sample of mitochondrial enzyme (25mg/ml in 50mM-phosphate buffer, pH7.0) was fractionated with solid $(NH_4)_2SO_4$, and the fractions precipitating at 40–50, 50–60 and 60–70% saturation were collected. The 40–50%-satd. fraction was 85% inhibited by disulfiram, whereas the 60–70%-satd. fraction was hardly inhibited at all (<5%). The 60–70%-satd. fraction was not completely free of the disulfiram-sensitive component, because a reaction was observed on diffusion against anti-(cytoplasmic aldehyde dehydrogenase) serum.

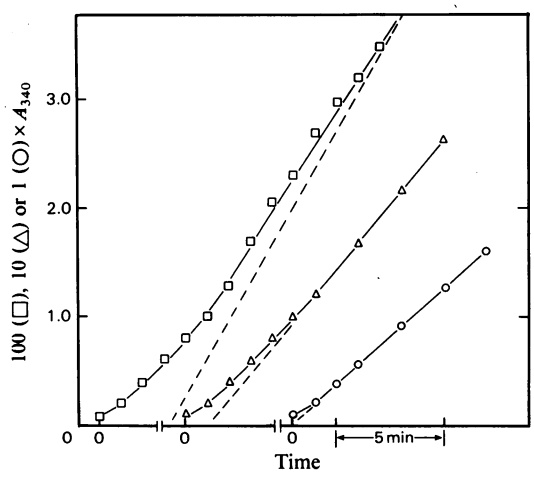

Fig. 3. *Spectrophotometric assays of mitochondrial aldehyde dehydrogenase at pH8.0, 25°C*
Assays were conducted with various enzyme concentrations, 0.9 μM (○), 0.09 μM (△) and 0.009 μM (□), as described by Hart & Dickinson (1977). Assays were initiated by adding pre-warmed samples (1, 10, 100 μl) of stock enzyme (5.5mg/ml; batch 1, Fig. 1) to the pre-warmed assays. Calibrated micro-syringes were used for enzyme transfer.

Assays of the purified mitochondrial enzyme

Routine spectrophotometric assays of mitochondrial enzyme from which the cytosolic-type enzyme has been removed exhibit rather unusual features, as shown in Fig. 3. The assays show lags in NADH production, and the length of the lag is greater as the enzyme concentration is decreased. Further, the steady-state rate achieved is greater at the lower enzyme concentrations, so that the measured specific activity of the enzyme

preparation depends on the enzyme concentration taken (Fig. 4). There is about a 2–3-fold change in specific activity over the accessible range of enzyme concentrations. It is to be particularly noted, however, that the true initial rate (though necessarily subject to significant errors because of acceleration of the progress curves) appears to be directly proportional to the enzyme concentration.

The cytosolic enzyme does not show unusual assay characteristics. The two enzymes have similar specific activities, but the former exhibits linear progress curves and a constant specific activity in assays with enzyme concentrations in the range 3–1540nM. This has been shown with the same equipment and solutions as used for Fig. 3. It may be noted that the fractions eluted from the pH-gradient column as described above (Fig. 1) exhibited this same diversity of behaviour in assays. Thus the early fractions exhibited marked lag phases at low concentrations of enzyme, whereas the late fractions containing the cytosolic-type enzyme did not.

Concentration-dependent lags in the assay of the mitochondrial aldehyde dehydrogenase were first noted by Hart & Dickinson (1978), using fluorimetric techniques to follow NADH production. The variation of specific activity with concentration was, however, not noted by these workers. Fluorimetric assays suffer from the disadvantage that fluorescence is not proportional to concentration over an extended range because of inner-filter effects and resultant quenching. Rates were apparently measured too early in the progress curve (after about 5min with 3nM enzyme, whereas Fig. 3 suggests that the true steady-state rate is not established at such low concentrations for 10–15min), so that the increase in specific activity at the low enzyme concentrations was not seen. One interesting finding by Hart & Dickinson (1978) was that preincubation of enzyme in assays with NAD^+ before addition of aldehyde did dramatically shorten the lag phase. We have confirmed this. In one case, the lag was decreased from 7min to 30s by the preincubation with 1mM-NAD^+. The steady-state rates achieved after the lag phase were the same in each case. In a separate experiment an assay was conducted with 0.9μM enzyme and, when the lag was complete, a sample was removed from the assay and used to initiate another assay. The enzyme concentration in the second assay was 0.009μM. A lag phase was observed just as if the first assay had not been conducted. This clearly demonstrates that turnover itself is not sufficient to promote the change of the enzyme to a higher activity, but that the activation process is concentration-dependent.

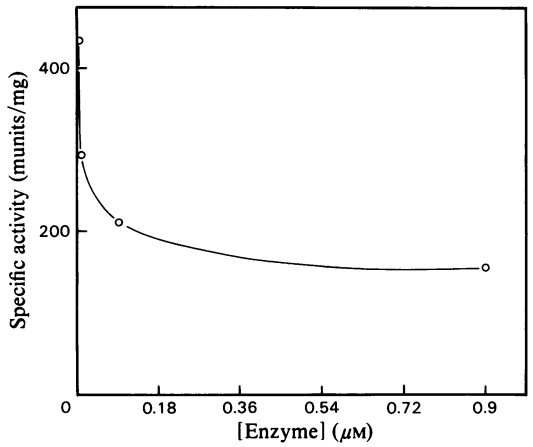

Fig. 4. *Variation of the specific activity of mitochondrial aldehyde dehydrogenase assays with enzyme concentration*
The steady-state rates are taken from the data presented in Fig. 3, but with addition of data from an assay conducted with an enzyme concentration of 0.0009μM. Specific activities are computed from the observed rates in the usual way.

We have analysed the curves in Fig. 3 by plotting log ΔA_{340} versus time in the manner of Dalziel *et al.* (1978). ΔA_{340} is obtained as the difference between the observed A_{340} at time t and that at the same time obtained by extrapolation from the portion of the reaction profile where the steady state is established. Linear plots were obtained on analysing the results of Fig. 3, but with other results we have observed a slight curvature, the slope increasing by about 40% over the period of the lag. Analysis of results like these in Fig. 3 is open to considerable error. The values of ΔA_{340} are small, deciding where the true steady-state rate is established is subjective and the extrapolations to obtain ΔA_{340} are long. If the slopes of the plots are taken as an estimate of the rate of the transient process, then the apparent first order rate constant for the lag increased from 0.27min^{-1} with 0.009μM enzyme, to 0.37min^{-1} with 0.09μM enzyme, to 1.07min^{-1} with 0.9μM enzyme. With 0.0009μM enzyme it appeared that the lag was about the same as for 0.009μM enzyme. The process shows only limited dependence on the enzyme concentration.

The esterase activity of the enzyme (Hart & Dickinson, 1978) shows the same characteristics as the dehydrogenase assays. Assays at pH7.0 with 500μM-*p*-nitrophenyl acetate as substrate showed concentration-dependent lags, with the specific activity determined from the steady-state rate increasing 1.2-fold over a 10-fold (0.09–0.9μM) range of enzyme concentration. Analysis of the data by the method of Dalziel *et al.* (1978) yielded slightly curved plots. Experiments with the esterase activity are limited to a narrow range of enzyme con-

centrations. The ester undergoes spontaneous hydrolysis at a slow rate, and as enzyme concentrations are lowered this 'blank' rate becomes more significant. The experiment with 0.09 μM enzyme required a 12% correction of the steady-state rate, whereas that with 0.9 μM enzyme required none.

The simplest explanation of the results presented above appears to be that the enzyme dissociates slowly in assays to yield a more active species. The enzyme is known (Hart & Dickinson, 1977) to exist as a tetramer of M_r 200000 at relatively high concentrations (>5 μM). One might suggest then that the enzyme may dissociate to dimers, or even monomers, at very low concentrations and that these are intrinsically more active than the tetramer. The steady-state rate achieved in assays such as those of Fig. 3 is then a function of the concentrations of tetramer and its dissociated product, the position of equilibrium between the enzyme species and its rate of attainment being determined by the enzyme concentration taken. The fact that the initial rate in assays is apparently proportional to enzyme concentration (see above) seems to support the proposal. Enzyme in stock solutions would be present as almost all tetramer. The true initial rate than represents the activity of the tetramer before dissociation to more-active species has begun. Again a system of the kind $A_4 \rightleftharpoons 2A_2$ would be expected to yield curved plots when analysed as described above. The curvature might well be slight, however. Such a process would show limited concentration-dependence. At high concentrations little (<10%) dissociation would occur, whereas at low concentrations complete dissociation (>90%) would occur. It would only be in the intermediate ranges that the concentration-dependence would be noted. Our results seem to be broadly in agreement with these expectations.

Gel-filtration experiments

In an attempt to obtain independent evidence for enzyme dissociation, we have subjected purified samples to gel filtration on Sephadex G-200. Fig. 5 shows the elution profiles from two such experiments where samples were added to the column at concentrations of 70 μM and 2.5 μM. With the higher concentration, enzyme was eluted in a fairly sharp band, the elution volume indicating a M_r of about 200000, as expected. The more dilute sample was eluted in a much broader peak, the elution volume indicating an average M_r of about 145000. These experiments proved to be reproducible. It was not possible to work at significantly lower enzyme concentrations to determine what the smallest molecular species might be because of the low intrinsic activity of the enzyme. With 1 mM-NAD^+ included in the elution buffer, the behaviour with the high enzyme concentration (initially 70 μM) was unaffected, but at the low concentration (initially 2.5 μM) the peak was moved forward and the enzyme emerged almost as early as in runs with the high enzyme concentration. Preincubation of enzyme with NAD^+ was the only

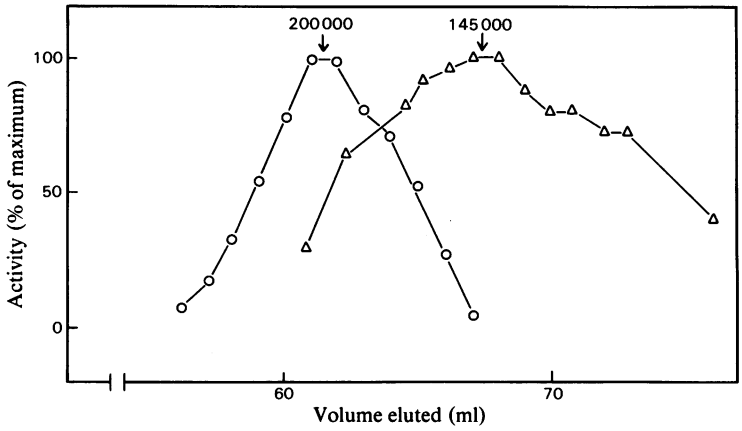

Fig. 5. *Gel-filtration behaviour of mitochondrial aldehyde dehydrogenase*

Enzyme fractions (0.3 ml) were added to a Sephadex G-200 column (1.0 cm × 60 cm) equilibrated with 50 mM-phosphate buffer, pH 7.4, containing 0.3 mM-EDTA and 100 μM-dithiothreitol. The enzyme was applied to the column at concentrations of 70 μM (○) and 2.5 μM (△). The M_r values indicated corresponding to the peak volumes were obtained by interpolation on a plot of log M_r versus V_e/V_0: V_e is the peak elution volume with the protein in question and V_0 the void volume obtained with Blue Dextran. The M_r markers used in calibrating the column were cytochrome *c* (12500), catalase (240000), bovine serum albumin (68000), yeast alcohol dehydrogenase (140000) and ferritin (450000).

treatment observed to alter materially the lag phase in assays (Hart & Dickinson, 1978).

The experiments suggest that dissociation of the mitochondrial enzyme does occur at low concentration, and this conclusion is reinforced by comparing the behaviour of mitochondrial and cytosolic enzymes. The latter enzyme behaved as a species of M_r 200000 when passed through the gel-filtration column at high (initially 70 μM) or low (initially 2.5 μM) concentration and was unaffected by the presence of NAD^+ in elution buffers. The cytosolic enzyme shows neither lag phases nor variable specific activity in assays. It is notable that the mitochondrial enzyme is much more sensitive to inactivation by urea than is the cytosolic enzyme (see above). This could well be due to the much greater tendency of the mitochondrial enzyme to dissociate.

Conclusion

Dissociation of the mitochondrial enzyme seems to provide a satisfactory basis for interpretation of assay data, but there are problems which are not yet explained. The gel-filtration experiments indicate that dissociation occurs, but we do not know what dissociated species are involved. The gel-filtration experiments could not be conducted at the very low enzyme concentrations that were used in some of the assays. Although dissociation seems to occur spontaneously at low concentrations, this does not fully explain what happens in assays. When enzyme is preincubated in buffer and the reaction is then initiated with NAD^+/acetaldehyde mixture, the lag is just the same in both length and amplitude as if enzyme had been used to initiate the reaction. It would appear from assays and gel-filtration experiments that incubation with NAD^+ can exert some influence on the process, but, since preincubation with NAD^+ does not eliminate lag phases and NAD^+ is not at all involved in the esterase assays, this aspect of the work is ill-understood. So far the only combination of factors which results in full activation of the enzyme is turnover in the assay (either dehydrogenase or esterase) at the appropriate enzyme concentration.

What is proposed above appears to be broadly similar to what has been proposed for horse mitochondrial aldehyde dehydrogenase. The detailed behaviour of the sheep and horse enzymes seem to be quite different, however. According to Takahashi & Weiner (1980) and Takahashi *et al.* (1980) the horse pI 5 isoenzyme (mitochondrial) is dissociated into dimers by 0.5–1 mM-Mg^{2+}. The dissociation results in a 2-fold increase in the number of active sites and of the specific activity. For the sheep enzyme, inclusion of 1 mM-$MgCl_2$ in elution buffers had no effect on the elution position from Sephadex G-200 when various initial enzyme concentrations were used. Inclusion of 1 mM-$MgCl_2$ in normal assays in phosphate buffer at pH 8.0 gave no activation, but about 30% inhibition, whereas inclusion of 1 mM-$MgCl_2$ in assays of the type used by Takahashi & Weiner (1980) in phosphate buffer, pH 7.5, gave some 40% activation. Lower concentrations gave less activation, and higher concentrations (5–20 mM) gave strong inhibition. The inclusion of Mg^{2+} in assays of the sheep enzyme did not seem to alter the duration and amplitude of lag phases. One may note that Takahashi & Weiner (1980) did not report lags in the assay of the horse enzyme in the presence or absence of Mg^{2+}. Apparently for that enzyme steady-state rates are established rapidly.

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