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INTRACELLULAR LOCALISATION AND PROPERTIES OF ALDEHYDE DEHYDROGENASES FROM SHEEP LIVER

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SUMMARY

1. The distribution of aldehyde dehydrogenases in sheep liver was studied. Activity was found in the cytoplasm, mitochondria and microsomes.

2. Cross-contamination of activities from different subcellular fractions, during the isolation procedures used, was shown to be insignificant. Accordingly, the level of aldehyde dehydrogenase activity found in each fraction should reflect the distribution pattern in vivo.

3. Aldehyde dehydrogenases from the cytoplasm and mitochondria were isolated and some of their catalytic properties examined. The results show that the enzymes from the two fractions are not identical.

INTRODUCTION

In recent years, aldehyde dehydrogenase (aldehyde: NAD^+ oxidoreductase, EC 1.2.1.3) from various mammalian species has been isolated and some physical and catalytic properties have been reported [1–7]. Most of the reports have dealt with enzyme activities found in the soluble fraction of liver homogenates, although it appears that precautions were not taken to avoid contamination of the cytoplasm by mitochondrial enzymes released during the isolation procedures. Such precautions are necessary in view of reports of the presence of a mitochondrial aldehyde dehydrogenase in liver [1, 8, 9] as well as in brain tissue [10, 11]. Deitrich [1] claimed that 13% of the activity in rat liver homogenates is mitochondrial, while Marjanen [9] reported a value of 80%, and the results of Hassinen et al. [12] suggest that, in intact perfused rat liver, acetaldehyde is oxidised mainly in the mitochondria. Some of the kinetic properties of the soluble, but not the mitochondrial enzyme from liver have been previously studied [3, 5, 7].

The present investigation was planned to define more clearly the distribution of aldehyde dehydrogenase activities in the sub-cellular fractions of liver. The cytoplasmic and mitochondrial aldehyde dehydrogenases were purified separately and some of the kinetic properties of each enzyme were determined. It was considered

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that the data would demonstrate whether the activities in the two cellular fractions examined are due to the presence of the same enzyme or to two different aldehyde dehydrogenases.

Sheep liver was chosen for study on account of its availability and also in order to relate results to a concurrent and associated research project into the metabolism of ethanol and acetaldehyde by sheep.

MATERIALS

Sheep livers, obtained from the local freezing works, were kept in ice until the start of an enzyme preparation which was within 1 h of the death of the animal.

The following compounds and materials were obtained from the sources indicated: NAD^+, chloral hydrate and phenazine methosulphate: BDH Chemicals (Poole, Dorset, U.K.); acetaldehyde: Fluka AG (Buchs, Switzerland); D,L-glyceraldehyde and nitrotetrazolium blue: Koch-Light Laboratories (Colnbrook, Bucks., U.K.); progesterone: Sigma Chemical Co. (St. Louis, Mo.); diethyl stilbestrol: Boots Pure Drug Co. Ltd. (Nottingham, U.K.); enzyme grade $(NH_4)_2SO_4$: Nutritional Biochemicals Corp. (Cleveland, Ohio); DEAE-cellulose (Whatman microgranular DE32, standard grade): W. and R. Balston Ltd. (Maidstone, Kent, U.K.); Sephadex G-200: Pharmacia Fine Chemicals (Uppsala, Sweden).

METHODS

Protein estimation

Protein concentrations were determined by the method of Lowry et al. [13].

Enzyme assays

Aldehyde dehydrogenase was assayed by following the increase in absorbance at 340 nm due to the production of NADH. Concentrations in the normal assay system were pyrophosphate buffer, pH 9.3, 33 mM; NAD^+, 1.67 mM and acetaldehyde, 1.67 mM. The final volume, including the enzyme (added as a 0.1-ml aliquot), was 3 ml. One unit of activity was taken as the amount of enzyme required to cause a change of 0.001 absorbance unit at 340 nm per min, with a 1-cm light path, under these conditions. Kinetic experiments were carried out using the same assay system modified where appropriate; e.g. the K_m for NAD^+ was determined by varying the concentration of NAD^+ with the other concentrations maintained as described for the assay system. The effects of progesterone and diethyl stilbestrol were determined by assaying the enzyme in the presence of 10^{-5} M hormone which was added as a solution in aqueous ethanol (50%, v/v). The final concentration of ethanol was 1.67% (v/v) and this had no effect on the rate of reaction.

Lactate dehydrogenase (EC 1.1.1.27) was assayed according to the method of Wroblewski and LaDue [14], glutamate dehydrogenase (EC 1.4.1.3) was assayed according to the method of Robins et al. [15] and glucose-6-phosphatase (EC 3.1.3.9) was estimated by the method of Swanson [16].

Isolation of sub-cellular fractions

A 20% (w/v) homogenate of finely chopped liver in ice-cold 0.0025 M phos-

phate buffer, pH 7.3, 0.25 M in sucrose, was prepared using a glass–teflon Potter–Elvehjem homogeniser fitted to an electric motor. 3–5 strokes at 500 rev./min were required to homogenise the tissue fully. The homogenate was centrifuged in a Sorvall RC2B centrifuge at 500 × *g* for 5 min to remove whole cells and nuclei, and the supernatant was centrifuged at 20 000 × *g* for 30 min to sediment mitochondria. The supernatant at this stage was centrifuged at 34 000 × *g* for 2.5 h to sediment microsomes, finally giving a clear cytoplasmic supernatant. The sedimented pellets at each stage were resuspended in 0.0025 M phosphate buffer, pH 7.3, and the fractions were assayed for enzyme activities as shown in Table I.

Purification of cytoplasmic aldehyde dehydrogenase

All operations were performed at 0–4 °C. Buffers contained 0.1 % (v/v) mercaptoethanol. The cytoplasmic material from 160 g of liver was isolated as described above, and the solution was fractionated with $(NH_4)_2SO_4$. The precipitate obtained between 0.45 and 0.70 $(NH_4)_2SO_4$ saturation was dissolved in 0.01 M phosphate buffer (pH 7.3), and dialysed exhaustively against the same buffer. The cloudiness which appeared during dialysis was removed by centrifugation and the supernatant (60 ml) was applied to a DEAE-cellulose column (20 cm × 4 cm) which had been equilibrated with 0.01 M phosphate buffer (pH 7.3). The same buffer was used to wash the column until the effluent protein concentration dropped to approximately 0.1 mg/ml. The elution of aldehyde dehydrogenase was effected by a stepwise change of eluant to 0.05 M phosphate buffer (pH 7.3). Fractions containing activity were taken to an $(NH_4)_2SO_4$ saturation of 0.7, and the resultant precipitate redissolved in the minimum volume of 0.05 M buffer. After dialysis, the solution (6 ml) was applied to a Sephadex G-200 column (90 cm × 2.5 cm) which had been equilibrated with 0.05 M phosphate buffer (pH 7.3). The eluted fractions containing the highest specific activities were combined and stored in the cold.

Purification of mitochondrial aldehyde dehydrogenase

Liver (400 g) was homogenised in batches in a Waring blendor with a total volume of 2 l of 0.005 M phosphate buffer (pH 7.3) containing 0.9 % (w/v) KCl. The homogenate was centrifuged at 500 × *g* for 5 min and the supernatant recentrifuged at 20 000 × *g* for 1 h. The mitochondrial sediment was redispersed in 800 ml of 0.1 M citrate buffer (pH 4.8) and sonicated in 200-ml batches for 2 × 1-min periods using a MSE 100-Watt ultrasonic disintegrator. The mixture, after standing for 1 h, was centrifuged at 20 000 × *g* for 1 h to remove mitochondrial debris and protein insoluble at pH 4.8. Aldehyde dehydrogenase was obtained from the supernatant by $(NH_4)_2SO_4$ fractionations and DEAE-cellulose and Sephadex G-200 column chromatography as described for the cytoplasmic enzyme.

RESULTS

Sub-cellular distribution of aldehyde dehydrogenase

The results of the experiments to determine the subcellular location of sheep liver aldehyde dehydrogenase are shown in Table I. The level of activity in each cellular compartment is represented as a percentage of the total activity found in all fractions. Corresponding values for three other enzymes estimated simultaneously are also

TABLE I

SUB-CELLULAR DISTRIBUTION OF SHEEP LIVER ALDEHYDE DEHYDROGENASE

Activities in each fraction are represented as percentages of the total activities in all fractions. Included in the table are corresponding values for lactate dehydrogenase, glutamate dehydrogenase and glucose-6-phosphatase which were chosen as reference enzymes (see text). The values for aldehyde dehydrogenase are averages of six separate fractionations; the extreme values found are shown in brackets.

	Activities (% of total recovered activity)			
	Aldehyde dehydrogenase	Lactate dehydrogenase	Glutamate dehydrogenase	Glucose-6-phosphatase
500 × *g* sediment	19 (11–25)	6.4	31	27
Mitochondria	34 (26–45)	2.0	61	24
Microsomes	10 (5–18)	1.2	5	45
Cytoplasm	37 (30–45)	90.4	2.5	4.9
Recovery of activity	88%	81%	75%	126%

listed. Included in Table I are the recoveries of enzyme activity, given as percentages of the activity in the original homogenate. The recovery of protein was generally 100%.

Purification of aldehyde dehydrogenases

The enzymes from cytoplasm and mitochondria were separately purified as described above and activities at individual steps are given in Table II. Polyacrylamide-gel electrophoresis of the final cytoplasmic enzyme preparation showed one predominant protein band together with one or two minor bands. The major band

TABLE II

PURIFICATION OF CYTOPLASMIC AND MITOCHONDRIAL ALDEHYDE DEHYDROGENASES FROM SHEEP LIVER

Enzyme activities were assayed as described in the text and the protein concentration was estimated by the method of Lowry et al. [13].

Step	Total activity (units)	Protein concentration (mg/ml)	Spec. act. (units/mg)
Cytoplasmic enzyme			
Initial cytoplasmic extract	95 000	12.5	13
$(NH_4)_2SO_4$ ppt	60 500	49.5	22
DEAE-cellulose	56 800	2.25	110
$(NH_4)_2SO_4$ ppt	42 000	22.5	178
Sephadex G-200	11 200	2.2	255
Mitochondrial enzyme			
Sonicated mitochondrial extract	92 000	4.0	29
$(NH_4)_2SO_4$ ppt.	62 500	5.0	50
DEAE-cellulose/$(NH_4)_2SO_4$ ppt.	26 200	22.5	116
Sephadex G-200	12 200	1.0	325

TABLE III

SOME KINETIC CONSTANTS OF CYTOPLASMIC AND MITOCHONDRIAL ALDEHYDE DEHYDROGENASES

The values for Michaelis constants are averages of at least four determinations using different enzyme preparations; the extreme values are shown in brackets. The K_i value for chloral hydrate was determined using two different enzyme preparations in each case. K_m for NAD^+ was determined with acetaldehyde as the substrate and K_i for chloral hydrate was obtained with D,L-glyceraldehyde as the substrate.

	Kinetic constants (10^{-6} M)	
	Cytoplasmic enzyme	Mitochondrial enzyme
K_m, NAD^+	12.5 (10.4–14.4)	77 (64–95)
K_m, D,L-glyceraldehyde	174 (152–222)	210 (200–230)
K_i, chloral hydrate	5.7, 7.3	4.5, 4.7

coincided with the activity stain, based on the production of formazan from nitrotetrazolium blue in the presence of NADH and phenazonium methosulphate. After electrophoresis under identical conditions, however, mitochondrial enzyme preparations responded comparitively weakly to this activity stain. The reason for this is not known.

Properties of the cytoplasmic and mitochondrial enzymes

After the two enzymes were purified to a similar extent, some of their properties were compared.

Certain kinetic constants for the enzymes are shown in Table III. Chloral hydrate was found to be a potent competitive inhibitor of both enzymes. Table IV shows the specificity of each enzyme towards a series of aldehyde substrates. The concentration of the aldehyde in each case was 1.67 mM and the concentration of enzyme was adjusted so that the absolute rate was the same for both enzymes with

TABLE IV

SUBSTRATE SPECIFICITIES OF CYTOPLASMIC AND MITOCHONDRIAL ALDEHYDE DEHYDROGENASES

The activity with acetaldehyde as substrate is set at 100. Values listed are averages for four separate enzyme preparations; the extreme values obtained are shown in brackets. The concentration of NAD^+ and of each aldehyde were as described for the normal assay system.

Substrate	Relative activities	
	Cytoplasmic enzyme	Mitochondrial enzyme
Acetaldehyde	100	100
Propionaldehyde	98 (81–108)	94 (91–96)
Benzaldehyde	10 (7–13.5)	18.5 (16–21)
Pyruvic aldehyde	24 (20–26)	21.5 (17–24)
D,L-Glyceraldehyde	85 (83–88)	131 (127–136)
Glycolaldehyde	76 (73–80)	150 (144–159)

acetaldehyde as substrate. These data are not corrected for the possible effects of substrate inhibition. The effect of phosphate concentration on the activity of the enzymes was determined. The level of activity of the cytoplasmic enzyme (estimated for three different enzyme preparations) in 0.25 M phosphate buffer (pH 8.0) fell in the range 407–440% relative to the activity in 0.005 M phosphate buffer, arbitrarily set at 100%. The corresponding figure for the mitochondrial enzyme is 224–292%. Similarly, the effects of progesterone and the steroidal hormone analogue, diethyl stilbestrol, on three preparations of each enzyme were investigated. For the cytoplasmic enzyme, 10^{-5} M progesterone lowered the activity to between 36 and 49% of the control reaction without hormone. The same concentration of diethyl stilbestrol raised the rate to 142–149% of the control. The corresponding figures for the mitochondrial enzyme were 88–94% and 110–112% respectively.

DISCUSSION

The results shown in Table I indicate that aldehyde dehydrogenase activity may be found consistently in the cytoplasmic, mitochondrial and, to a lesser extent, microsomal fractions of sheep liver homogenate. Any ambiguities regarding this finding are eliminated by consideration of the results obtained with the reference enzymes. Thus, it is evident from the distribution of the cytoplasmic enzyme, lactate dehydrogenase [17], that the mitochondria and microsomes are virtually uncontaminated by cytoplasm. Furthermore, the observation that only very low levels of glutamate dehydrogenase activity (a non-membrane bound mitochondrial enzyme [18, 19]) and glucose-6-phosphatase activity (a microsomal enzyme [20]) are found in the cytoplasmic fraction shows that during the procedures used in the present experiments there is negligible contamination of the cytoplasmic fraction by the contents of ruptured mitochondria or by microsomes. Thus the figure of 37% in Table I can be taken as an average value of the actual level of aldehyde dehydrogenase activity in the cytoplasm. The possibility of this figure being spuriously high because of the presence of activity from other sub-cellular fractions is considered to be unlikely.

In addition, it may be claimed that since very little glutamate dehydrogenase is found in the microsomal fraction, the aldehyde dehydrogenase detected in that fraction is not due, in the main, to contamination by mitochondria. Aldehyde dehydrogenase activity can be readily released from microsomes by treatment with a detergent, but in comparison with the mitochondrial enzyme it is released relatively slowly during sonication. This supports the findings of Deitrich [1] using rat liver. Marjanen [9], however, found no aldehyde dehydrogenase activity in rat liver microsomes. Further work is proceeding on the microsomal enzyme from sheep liver.

The activity found in the 500 $\times$ *g* sediment arises in part from whole cells and also probably from some mitochondria co-sedimenting with the heavier material. Thus it can be assumed that 34% represents the minimum value for the fraction of the total activity which is mitochondrial; the actual level may be a few percent higher.

A conclusion which can be drawn from these experiments is that sheep liver cytoplasm and mitochondria contain comparable levels of aldehyde dehydrogenase activity. Only limited quantitative comparisons may be made between these results and previously published values, partly because there may be interspecies differences in this respect between sheep and the other animals studied (mainly rat) and partly

because of the very large discrepancies in literature data, referred to in the Introduction. Deitrich [1] and Buttner [21] have found most aldehyde dehydrogenase activity in the cytoplasm, while the results of Marjanen [9], Hedlund and Kiessling [22], and Hassinen et al. [12] indicate that the mitochondria contain most of the total cellular activity. In the present study, the use of 'marker' enzymes to confirm the effective separation of sub-cellular fractions ensures the reliability of the results reported here.

The aldehyde dehydrogenase activities found in the various cellular fractions could be due to either a single enzyme or to more than one enzyme with different properties. To resolve this question, the enzymes from cytoplasm and mitochondria were separately isolated and some of the characteristics of the purified enzymes determined and compared. It has been demonstrated that the K_m values for NAD^+ of the two enzymes differ by a factor of approximately 6 (Table III), while relatively insignificant differences in the K_m values for D,L-glyceraldehyde or K_i values for chloral hydrate (an inhibitor which competes for the aldehyde binding site) were found. Notable differences in the relative maximal activities of the enzymes towards D,L-glyceraldehyde, glycolaldehyde and possibly benzaldehyde were demonstrated (Table IV). Increasing the concentration of phosphate caused an increase in activity with the cytoplasmic enzyme greater than that observed for the mitochondrial enzyme (see Results). A similar observation was reported by Deitrich [1] with enzymes isolated from rat liver. Since steroid hormones affect the activity of some aldehyde dehydrogenases [23, 24], the two enzyme activities being examined here were tested for possible effects of such compounds. The results are qualitatively similar; progesterone lowers the activity while diethyl stilbestrol raises it. However, the quantitative effect is consistently greater in each case for the cytoplasmic enzyme (see Results).

The results have shown that although the enzymes from cytoplasm and mitochondria are similar, they do exhibit some appreciable and reproducible differences. Explanations for such differences, and indeed for the presence of aldehyde dehydrogenase activity in more than one sub-cellular compartment must be speculative at this stage. As Jakoby [25] has pointed out, the ubiquitous distribution of the enzyme may simply reflect an efficient system for the removal of toxic aldehydes from the cell. Marjanen [9] has suggested that in the presence of ethanol, the amount of NAD^+ in the cytoplasm may become limited and most of the acetaldehyde oxidation may then take place in the mitochondria. If this is so, it is not surprising that K_m for NAD^+ of the cytoplasmic enzyme should be smaller than that of the mitochondrial species, since the former must compete with alcohol dehydrogenase for available NAD^+.

Studies are continuing on the aldehyde dehydrogenases from sheep liver, with particular reference to kinetic properties and the mechanism of action of steroid hormones on the two enzymes.

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