Intracellular Localization of Aldehyde Dehydrogenase in Rat Liver

By LEO MARJANEN

Research Laboratories of the State Alcohol Monopoly (Alko), Helsinki 10, Finland

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1. Distribution of aldehyde dehydrogenase activity in rat liver was studied by measuring the rate of disappearance of acetaldehyde in the presence of each of the subcellular fractions. These were obtained by rough separation of particulate fractions from the soluble portion of the cell, by differential centrifugation, and by isopycnic gradient centrifugation. 2. The maximal rate of acetaldehyde oxidation was $3.7 \mu\text{mol/min per g}$, with an apparent K_m value below 10^{-5} M . The highest rate of activity was observed in phosphate buffers of high P_i concentration (above 60 mM). 3. The activity measured was completely dependent on NAD^+ . 4. The microsomal fraction and the nuclei were inactive in the assay. Of the total activity 80% was found in the mitochondrial fraction and the remaining 20% in the cytoplasm. 5. The distribution pattern is important from the point of view of acetaldehyde oxidation during ethanol metabolism. The apparent discrepancy of the results obtained by different workers and the localization of acetaldehyde oxidation *in vivo* is discussed.

It seems to have been established that the first enzymic step in the oxidation of ethanol occurs in the cytoplasm (see Wallgren & Barry, 1970) but the site of the second step, from acetaldehyde to acetate, has not been unequivocally demonstrated, as shown by the discrepancy between the distribution patterns reported by different authors.

Walkenstein & Weinhouse (1953) described a mitochondrial enzyme from rat liver capable of oxidizing aliphatic aldehydes to the corresponding acids. From the mitochondrial fraction of rat liver homogenates Glenn & Vanko (1959) obtained a non-specific aldehyde dehydrogenase that showed marked activity towards acetaldehyde. Büttner (1965) found that an NAD^+ -dependent aldehyde dehydrogenase was mainly in the cytoplasm. McGuire (1965) demonstrated the presence of aldehyde dehydrogenase activity in a mitochondrial extract. Using indol-3-ylacetaldehyde as substrate, Deitrich (1966) found that the activity was mainly located in the cytoplasm. There was some evidence suggesting that two different enzymes might have been involved. The results of Hedlund & Kiessling (1969) and of Hassinen *et al.* (1970) indicated that the mitochondria were more important in the oxidation of acetaldehyde.

Besides rat liver other mammalian livers have been investigated. Two groups of workers (Kraemer & Deitrich, 1968; Blair & Bodley, 1969) have isolated an NAD^+ -dependent aldehyde dehydrogenase from human liver. Both these groups obtained this enzyme from the soluble portion of their crude homogenates. Sheppard *et al.* (1968) studied two inbred mouse strains for their capacity to oxidize indol-3-ylacetaldehyde and found most of the activity in the cytoplasm.

The presence of an aldehyde oxidase of low specificity in the mitochondria was reported by Horton & Packer (1970). A flavoprotein, hepatic aldehyde oxidase, has been studied by several workers, including Rajagopalan *et al.* (1962) and Johns (1967).

The purpose of the present work has been to gain further information about the site of acetaldehyde oxidation in rat liver cells. A definite pattern is important in connexion with the localization of the second step during ethanol metabolism. The results obtained indicated that 80% of the activity measured was confined to the mitochondrial fraction, the remaining activity being in the cytoplasm.

Materials and Methods

Chemicals

Acetaldehyde (BDH Chemicals Ltd., Poole, Dorset, U.K.) was redistilled before use. In many experiments the acetaldehyde used was distilled twice from paraldehyde (E. Merck A.-G., Darmstadt, Germany). The depolymerization of paraldehyde was catalysed by a small amount of conc. H_2SO_4 . The two products gave identical results. They kept well in the cold as molar solutions.

NAD^+ (Boehringer und Soehne, Mannheim, Germany, or Schick Pharmaceutical Inc., Seattle, Wash., U.S.A.) was dissolved in 0.154 M-sodium-potassium phosphate buffer and the pH adjusted to 7.4. Pyrazole (Fluka A.-G. Buchs, Switzerland) was dissolved in the same buffer.

Preparation of liver homogenate

Wistar albino rats (age 2–6 months) were used throughout the experiments. The animals were fed

on a normal laboratory diet until the start of the experiments. The rat was stunned by a blow on the head and decapitated immediately. The liver was removed and immersed in cold 0.154M-potassium phosphate buffer, pH 7.4, supplemented with 0.25M-sucrose and 3mM-MgCl₂. It was then washed. A 10% (w/v) homogenate was made in the same buffer in a Potter-Elvehjem Teflon-glass homogenizer at a speed of 700 rev./min with five strokes.

For zonal centrifugation the liver was perfused *in situ* with 0.154M-NaCl at room temperature. The liver homogenate was filtered through four layers of cheesecloth and in some cases cytoplasm was removed at 7500g (r_{av}) for 30 min in a Sorvall RC-2 centrifuge equipped with an SS-34 rotor.

Fractionation of the homogenate

Three methods were employed. At a preliminary stage the homogenate was roughly separated into a particulate and a soluble fraction. At the next stage a conventional differential-centrifugation scheme was used to separate the different subcellular fractions, and as the last method isopycnic gradient centrifugation in a zonal rotor was employed.

1. The homogenate was centrifuged at 7500g (r_{av}) for 30 min. The pellet containing the nuclei and the mitochondria was homogenized in the buffer. Both the pellet and the supernatant were adjusted to the volume of the original homogenate.

2. The homogenate was fractionated by differential centrifugation at 4°C. The nuclei and cell debris were removed at 460g (r_{av}) for 10 min in the Sorvall RC-2 centrifuge. The supernatant so obtained was separated at 7500g (r_{av}) for 30 min. The supernatant from this centrifugation was processed further at 79000g (r_{av}) for 60 min in a Spinco L-50 centrifuge equipped with a 30 rotor. All fractions so obtained were adjusted to the volume of the original homogenate.

In the purification of the nuclei the 460g pellet was washed twice and suspended in a small amount of 0.154M-potassium phosphate buffer, pH 7.4, and enough 2.4M-sucrose in the same buffer was added to give a final sucrose concentration of 2.2M (Chauveau *et al.*, 1956). The suspension was centrifuged at 4°C for 90 min at 50000g (r_{av}) in a Spinco L-50 centrifuge with a 30 rotor.

3. For isopycnic gradient centrifugation the Spinco L-50 centrifuge was equipped with a B-XV zonal rotor. The properties of this rotor have been described by Anderson *et al.* (1967). Loading and unloading were performed at 2°C at 4000 rev./min. First 250 ml of the buffer used was inserted, followed by 100 ml of 10% rat liver homogenate in the same buffer supplemented with 0.25M-sucrose. The gradient was formed by mixing solutions of 20 and 60% (w/w) sucrose dissolved in the buffer. Finally 100 ml

of 60% sucrose in the buffer was pumped in to form a 'cushion' at the rotor edge. The run was performed at a speed of 20000 rev./min for 3–5 h (5×10^6 g-min at the centre of the sample zone for 5-h run). Fractions (40 ml) were collected.

Enzyme assays

Aldehyde dehydrogenase activity was measured in samples incubated at 37°C. Normally the incubation mixture contained 0.5mM-pyrazole, 0.5mM-NAD⁺ and 0.5mM-acetaldehyde, with 10% homogenate buffered at pH 7.4 with 0.154M-KH₂PO₄–0.25M-sucrose–3mM-MgCl₂. The reaction vessel had three arms. To measure the O₂ pressure in the medium a Clark-type oxygen electrode was inserted in one arm. The pH was measured through the second arm with a Beckman combination electrode connected to a Beckman Expandometer pH-meter equipped with a scale expander. The third arm was used for adding and removing samples. It was closed with a pressure cap. Reagents were added before closing or through the cap. Pyrazole was added at the beginning of the 5-min preincubation period. During that time O₂ or N₂ (99.9%) was blown through the gas phase of the vessel while it was shaken at a rate of 120 strokes/min. NAD⁺ and acetaldehyde were added at the end of this period. The zero-time sample was drawn immediately into 0.6M-HClO₄. No spontaneous disappearance or formation of acetaldehyde occurred.

Succinate dehydrogenase activity was assayed by measuring the consumption in a Warburg apparatus. The reaction mixture contained 0.154M-KH₂PO₄–0.25M-sucrose–3mM-MgCl₂, and one of the fractions at a final concentration of 1–3% of the original wet weight of the tissue; 50mM-succinate adjusted to pH 7.4 with NaOH was used as substrate. Blank experiments consisted of vessels to which buffer was added instead of succinate.

To locate mitochondria after zonal centrifugation cytochrome oxidase was measured as described by Cooperstein & Lazarow (1951). However, cytochrome *c* (Merck A.-G.) was reduced with sodium dithionite, as shown by Schuel *et al.* (1964).

Measurement of acetaldehyde

A Perkin-Elmer F40 head-space gas-liquid chromatograph was used to measure the rate of disappearance of acetaldehyde. The standard deviation was 4%. In some experiments 2-methylpropan-2-ol was used as an internal standard, but usually it was omitted.

Other methods

The sucrose density gradient was analysed with a Zeiss refractometer at 20°C. Direct measurement of

u.v. extinction was not possible in zonal fractions because of their high turbidity. Every 40ml fraction was diluted with an equal amount of the buffer, pH 7.4, supplemented with iso-osmotic sucrose and Mg^{2+} . In these fractions protein was determined by the method of Lowry *et al.* (1951).

A Reichert Zetopan microscope with phase-contrast condenser was used throughout this work. The sonication procedures were performed at 0°C with a MSE sonifier at a setting of 6 μ m for 2.5 min in 30 s periods with 1–2 min interval for cooling.

Results

Aldehyde dehydrogenase assay system

Some factors relating to the general conditions during the assay were examined in greater detail.

Presence of oxygen. No difference in aldehyde dehydrogenase activity was found when either pure oxygen or air was used during the preincubation period. Normally, oxygen was used to equilibrate the medium. Table 1 shows that a small decrease in activity occurred under anaerobic conditions. The absolute amount of this decrease varied from one liver to another. After disruption of mitochondria this difference between aerobic and anaerobic conditions was no longer found. In both conditions the activity in the cytoplasmic fraction was the same.

Addition of pyrazole. The amount of pyrazole required to inhibit the alcohol dehydrogenase activity in the homogenates was established by following the formation of ethanol from acetaldehyde by g.l.c. This formation was completely inhibited by pyrazole at a concentration of 0.1 mM. This compound had no inhibitory effect on the activity of aldehyde dehydrogenase either in the cytoplasm or in the sonicated mitochondria. As expected, pyrazole was not necessary for the assay of the mitochondrial enzyme. Some experiments were done without added pyrazole, and the changes in the concentrations of acetaldehyde

and ethanol tallied well with those found when pyrazole had been added.

Requirement for NAD^+ . Several preparations showed an absolute requirement for added NAD^+ . In these samples activity was observed only after addition of NAD^+ . Other preparations exhibited a variable degree of activity in the absence of added NAD^+ .

Four homogenates showing a variable aldehyde dehydrogenase activity without addition of the coenzyme were tested further as follows. A sample was dialysed against a large volume of 0.154 M-potassium phosphate buffer, pH 7.4, containing iso-osmotic sucrose and Mg^{2+} . In 3 h the endogenous activity had virtually disappeared, but the activity on addition of NAD^+ was unaffected.

In another experiment the mitochondria were incubated at 37°C for 30 min to deplete endogenous NAD^+ (Purvis, 1968). They were separated by centrifugation and suspended in fresh buffer. Such mitochondria had completely lost their capacity to oxidize acetaldehyde without addition of NAD^+ . Again, the activity measured after addition of the coenzyme had not changed.

Apparent values for V_{max} and K_m . Ten animals were used in experiments aimed at measuring the K_m value for acetaldehyde in rat liver. The range of substrate concentration varied from 4 mM to 10 μ M. The latter value was the lowest concentration that could be measured by g.l.c. The concentration of the homogenate ranged from 0.5 to 10% and strict linearity was maintained between these values. However, the K_m value proved to be lower than 10^{-5} M, and maximal velocity was still retained at this substrate concentration.

Apparent V_{max} values obtained at the 0.5 mM substrate concentration ranged from 3.0 to 4.2 μ mol/min per g of fresh tissue (mean 3.7). A total of 45 animals was used to measure the range, and no sex difference was found.

Table 1. Mitochondrial and cytoplasmic aldehyde dehydrogenase activity in aerobic and anaerobic conditions

A 10% rat liver homogenate was prepared as described in Fig. 2 at pH 7.4 and fractionated by differential centrifugation. Assay conditions were as in Fig. 1. Pure oxygen or nitrogen was used to equilibrate the reaction mixture. Mitochondrial recovery is not included in the calculations. The lower activity after sonication is purely coincidental, as shown in the Discussion section.

Enzyme source	Enzyme activity (μ mol/min per g of fresh tissue)	
	Aerobic conditions	Anaerobic conditions
Original homogenate	3.8	3.0
Mitochondrial fraction		
Before sonication	1.1	0.9
After sonication	0.8	0.8
Cytoplasmic fraction	0.6	0.6

Fig. 1. Stimulation of aldehyde dehydrogenase activity by P_i

A 10% rat liver homogenate was made in 0.25M-sucrose-3mM-MgCl₂ and was incubated at 37°C under aerobic conditions in the presence of 0.5mM concentrations of pyrazole, NAD⁺ and acetaldehyde at pH 7.4. KH₂PO₄ adjusted with NaOH was used to give the required P_i concentration. The acetaldehyde disappearance rate was followed by g.l.c.

Other aspects. Among the three buffer systems tested (phosphate, pyrophosphate and tris) the highest rate of acetaldehyde disappearance was found in phosphate buffers; 0.154M-sodium- or potassium-based phosphate buffers gave identical results. Marked stimulation by P_i was found (Fig. 1). pH optimum was established between pH 7.4 and 7.8 in 0.154M-potassium phosphate buffer (Fig. 2).

Intracellular localization

Rough separation. The distribution of aldehyde dehydrogenase activity between particulate and soluble fractions after high-speed centrifugation showed that 80% (range 77–85% in ten experiments) of the total activity was located in the sediment. Recovery was 89%.

Differential centrifugation. Mitochondrial contamination of the nuclear fraction was high. This could gradually be decreased by repeated washings, but the method was inefficient. To overcome this difficulty the effectiveness of the fractionation was tested by measuring the activity of succinate dehydrogenase as well as that of aldehyde dehydrogenase. When the ratios of the two enzyme activities in the nuclear and mitochondrial fractions were calculated, essentially the same value was obtained (Table 2). This can be interpreted to mean that all the activity in the nuclear pellet was due to mitochondrial contamination. Further support for this view was obtained when

Fig. 2. Effect of pH on aldehyde dehydrogenase activity

A 10% rat liver homogenate was made in 0.154M-KH₂PO₄-0.25M-sucrose-3mM-MgCl₂, the pH being varied over the range required. The assay conditions were as in Fig. 1.

the nuclear fraction was repeatedly washed. The gradual decrease in succinate dehydrogenase activity paralleled the loss of aldehyde dehydrogenase activity from this fraction. The purified nuclei did not show any aldehyde dehydrogenase activity. The microsomal fraction obtained from post-mitochondrial supernatant by high-speed centrifugation (79 000g) was also inactive in this respect. This clearly indicates that all the activity found in the particulate fraction (from rough separation) was due to the mitochondria. The existence of other particles in the mitochondrial fraction is probably less important, because the general nature of the enzymes concentrated in these particles is different (de Duve, 1964).

Isopycnic gradient centrifugation. When the run was performed in 0.154M-potassium phosphate buffer, pH 7.4, large aggregates appeared in front of the mitochondrial population. These aggregates contained intact cells surrounded by nuclei and some smaller particles. In 5mM-tris-1mM-EDTA, pH 7.4, no such phenomenon occurred (Fig. 3). The aldehyde dehydrogenase activity exactly coincides with the cytoplasmic and mitochondrial fractions. No activity was evident in the other fractions. The distribution of the total aldehyde dehydrogenase activity was such that 75% of the activity recovered was located under the mitochondrial peak.

Solubility of the mitochondrial aldehyde dehydrogenase. After normal differential centrifugation the mitochondrial pellet was washed several times to test the possibility that the activity in the cytoplasm could be derived from the mitochondria. A barely significant decrease in activity was found. When the

sonicated mitochondria were centrifuged at 79000g (r_{av}) for 30min, the clear supernatant contained all the activity.

Discussion

Several enzyme systems have been considered to carry out the aldehyde oxidation (Lundquist *et al.*, 1959, 1962; Dalziel & Dickinson, 1965) but the high K_m values obtained for these enzymes make them rather unimportant *in vivo*. It seems to be well established that most of the oxidation of acetaldehyde is catalysed by NAD⁺-dependent aldehyde dehydrogenase (see Wallgren & Barry, 1970). The results obtained in the present study very definitely imply that all the activity measured was coupled with NAD⁺. The experimental conditions covered do not rule out the possibility that other enzyme systems are capable of oxidizing acetaldehyde but their importance is questionable, especially in view of the low substrate concentration (0.5mM) used throughout the work.

With intact mitochondria, the disappearance rate for acetaldehyde was higher in aerobic than in anaerobic conditions (Table 1). This could be interpreted to mean the existence of O₂-dependent enzyme activity in the preparation. After sonication of the mitochondria the same activity was obtained in both conditions. This observation, taken with what was said above about NAD⁺-dependence, rules out the possibility that the activity is coupled

to molecular O₂. On the other hand, the decrease in activity could imply the impairment of NAD⁺ transfer to the mitochondria under anaerobic conditions. Two known facts have some importance in this respect. It has been shown by several workers that P_i induces swelling of mitochondria (see Chappell & Greville, 1963). Hunter *et al.* (1959b) have shown that this phenomenon can be inhibited by anaerobiosis. In addition, it is known that the mitochondrial membrane, if intact, is fairly impermeable to nicotinamide nucleotides (Birt & Bartley, 1960; Purvis & Lowenstein, 1961). Attempts to incorporate NAD⁺ into such mitochondria have failed, but once the mitochondria have undergone swelling in the presence of P_i they can incorporate several times the amount of NAD⁺ originally present (Hunter *et al.*, 1959a). These facts could offer an explanation for the difference observed in the present study.

In sonicated mitochondria the activity was somewhat lower than in fresh preparations (Table 1). Later experiments showed that this decrease was due to the slight warming that occurred during the sonication. There was no further decrease in the cold, but at 37°C there was a measurable decline, which was not abolished by higher NAD⁺ concentrations. Since no such decline occurred when intact mitochondria were used, it was concluded that liberation of lysosomal enzymes during the sonication was responsible.

No latent activity was found in the mitochondria. The experimental system worked as well with freshly

Table 2. *Distribution of aldehyde dehydrogenase and succinate dehydrogenase activities in subcellular fractions*
Experimental conditions were as in Table 1. Activities were measured under aerobic conditions with fresh unsonicated preparations. The distribution of a mitochondrial marker enzyme, succinate dehydrogenase, is measured for the calculation of mitochondrial recovery in each fraction.

Enzyme source	Enzyme activities	
	Aldehyde dehydrogenase (μ mol/min per g of fresh tissue)	Succinate dehydrogenase (μ l of O ₂ /min per g of fresh tissue)
Original homogenate	3.2	162
Nuclear fraction	1.7	119
Mitochondrial fraction	0.6	39
Cytoplasmic fraction	0.4	2
Microsomal fraction	0.0	3
Purified nuclei	0.0	—
Activity recovered	2.7	163
Sum of nuclear and mitochondrial fractions	2.3	158
Mitochondrial fraction (corrected for recovery)	2.4	
Rough separation:		
Particulate fraction	2.3	162
Soluble fraction	0.4	9

Fig. 3. Distribution of aldehyde dehydrogenase activity in zonal centrifugation

A sample (100ml) of 10% rat liver homogenate in 5mM-tris-1mM-EDTA-0.25M-sucrose, pH 7.4, was used in the B-XV zonal rotor with a 20–60% sucrose gradient. The run was performed for 5 h at 2°C and 20000 rev./min. Fractions (40ml) were collected and the concentration of sucrose was measured. Each fraction was diluted and the particulate material was spun down. Both the sediments and the supernatants were analysed for protein, aldehyde dehydrogenase and cytochrome oxidase activity. The sediments were observed by phase-contrast microscopy. ○, Sucrose concentration; △, E_{280} (the supernatants); ▲, E_{750} (protein in the sediments; Lowry *et al.*, 1951); □, $\Delta E_{550}/\text{min}$ (cytochrome oxidase activity in the sediments); ●, aldehyde dehydrogenase activity from the supernatants and the sediments. The E_{280} and E_{750} curves are not on a comparable scale. Their only purpose is to show the position of each subcellular entity after the run.

prepared fractions as with sonicated preparations in aerobic conditions at high P_i concentration. The increase of NAD^+ concentration above that used (0.5mM) did not reveal any further activity and the apparent need for NAD^+ was much lower.

Distribution of the activity. The results obtained by differential centrifugation seemed to be in disagreement with those of the other methods used. But when the percentage recovery of mitochondria was included in the calculations, the picture agreed with that obtained for the sediment after centrifugation at 7500g and with the results of zonal centrifugation. A slightly lower activity in mitochondria was found in zonal runs. However, if a sample in such a run did not contain the cytoplasmic fraction, a peak of a few per cent of the normal value was still formed in the cytoplasmic area, for which a small leakage of mitochondrial activity to the cytoplasmic portion was the most likely explanation. This would raise the activity in the cytoplasmic portion at the expense of the mitochondria. It is reasonable to conclude that four-fifths of the activity was confined to the mitochondrial fraction.

Several distribution patterns for aldehyde dehydrogenase enzymes have been reported, as outlined in the introduction. Even for the same tissue, rat liver, conflicting results have been reported. One reason for the variability could be the use of substrates other than acetaldehyde, such as indol-3-ylacetaldehyde (Deitrich, 1966; Sheppard *et al.*, 1968) or betaine aldehyde (Wilken *et al.*, 1970). If two different enzymes were involved, their affinities towards different aldehydes might differ enough to change the pattern. Further, these enzymes might have different responses to changes in the assay conditions. Another difference that might be meaningful is the strain of rat used. Büttner (1965) and Deitrich (1966) used the Sprague-Dawley strain whereas I used the Wistar strain like Hedlund & Kiessling (1969) and Hassinen *et al.* (1970). As mentioned above, these two strains appear to give discrepant results.

To check the last possibility three male and three female Sprague-Dawley rats were tested (Orion Oy, Espoo, Finland). In all animals the same total activity was found, $3.4 \mu\text{mol}/\text{min}$ per g wet weight. The subcellular particles were sedimented at 7500g

($r_{av.}$) for 30 min. The recovery of the activity was 88%, and 76% of this activity was found in the sediment. No sex difference existed. This means that other factors have to be called on to explain the differences in the values reported.

One has to be careful in assigning an enzymic phenomenon to a definite compartment of the cell. *In vivo* the NAD⁺-dependent aldehyde dehydrogenase activity will depend on the functional NAD⁺ pool available. The fact that the enzyme is available in two compartments may permit the existence of a dynamic state between the two compartments involved. During an ethanol load the amount of NAD⁺ in the cytoplasm may become limited and most of the acetaldehyde oxidation may take place in the mitochondria, thus decreasing the load on the normal NADH-shuttle system. The results of Hassinen *et al.* (1970) suggested that in a perfused liver acetaldehyde is oxidized mainly in the mitochondria and does not compete with the ethanol oxidation taking place in the cytoplasmic compartment. If this occurs in a perfused liver it could well do so *in vivo*, and emphasizes the importance of the mitochondria in the oxidation of acetaldehyde in the rat liver.

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