

The Subcellular Distribution and Properties of Aldehyde Dehydrogenases in Rat Liver

By S. O. C. TOTTMAR, H. PETTERSSON and K.-H. KIESSLING
*Alcohol Research Group of the Swedish Medical Research Council,
Institute of Zoophysiology, University of Uppsala, Uppsala, Sweden*

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1. Kinetic experiments suggested the possible existence of at least two different NAD⁺-dependent aldehyde dehydrogenases in rat liver. Distribution studies showed that one enzyme, designated enzyme I, was exclusively localized in the mitochondria and that another enzyme, designated enzyme II, was localized in both the mitochondria and the microsomal fraction. 2. A NADP⁺-dependent enzyme was also found in the mitochondria and the microsomal fraction and it is suggested that this enzyme is identical with enzyme II. 3. The K_m for acetaldehyde was apparently less than 10 μM for enzyme I and 0.9-1.7 mM for enzyme II. The K_m for NAD⁺ was similar for both enzymes (20-30 μM). The K_m for NADP⁺ was 2-3 mM and for acetaldehyde 0.5-0.7 mM for the NADP⁺-dependent activity. 4. The NAD⁺-dependent enzymes show pH optima between 9 and 10. The highest activity was found in pyrophosphate buffer for both enzymes. In phosphate buffer there was a striking difference in activity between the two enzymes. Compared with the activity in pyrophosphate buffer, the activity of enzyme II was uninfluenced, whereas the activity of enzyme I was very low. 5. The results are compared with those of earlier investigations on the distribution of aldehyde dehydrogenase and with the results from purified enzymes from different sources.

Acetaldehyde is produced during ethanol metabolism and is oxidized by aldehyde dehydrogenase. There is much controversy in the literature on the distribution and properties of this enzyme and consequently about the oxidation site of acetaldehyde.

Walkenstein & Weinhouse (1953) and Glenn & Vanko (1959) reported a mitochondrial aldehyde dehydrogenase with broad substrate specificity in rat liver. Büttner (1965) and Deitrich (1966) found that aldehyde dehydrogenase was mainly localized in the cytoplasm in rat liver. The latter author also found that there was some activity in the mitochondria and in the microsomal fraction, and that the activities in the soluble and particular fractions could be attributed to two different proteins, as judged by gel-filtration experiments. Tietz *et al.* (1964) reported an enzyme in the soluble and microsomal fractions of rat liver which could oxidize long-chain aldehydes with either NAD⁺ or NADP⁺ as cofactor. Hedlund & Kiessling (1969) and Hassinen *et al.* (1970) found that mitochondria from rat liver could oxidize different aldehydes and that acetaldehyde is probably oxidized mainly in the mitochondria during ethanol metabolism. Smith & Packer (1972) found two locations for aldehyde oxidation in rat liver mitochondria, one associated with the respiratory chain and one

probably associated with the outer membrane. Marjanen (1972) found 80% in the mitochondrial fraction and 20% in the cytosol of the total aldehyde dehydrogenase activity in a rat liver homogenate. No activity was found in the microsomal fraction.

Aldehyde dehydrogenase has been purified from several different sources. Kraemer & Deitrich (1968) and Blair & Bodley (1969) purified and characterized aldehyde dehydrogenase from human liver. They found an apparent K_m of less than 1 μM for various aldehydes. The latter authors also reported the possible existence of another NAD⁺-linked aldehyde dehydrogenase, which was also reported by Erwin & Deitrich (1966), Sheppard *et al.* (1970) and Feldman & Weiner (1972) in their works on bovine brain, rat brain and mouse liver, and horse liver respectively.

Shum & Blair (1972) separated and partially purified two NAD⁺-dependent aldehyde dehydrogenases from rat liver cytosol. The two enzymes differed in substrate specificity, charge properties and pH optima. The apparent K_m for both enzymes was reported to be in the millimolar range.

The present work was carried out to find the distribution pattern of the total NAD⁺-dependent aldehyde dehydrogenase activity in rat liver and to evaluate the possible existence of more than one

enzyme, which could explain the discrepancies found in the literature about, first, the localization and properties of this enzyme and, secondly, the oxidation site of acetaldehyde during ethanol metabolism.

Materials and Methods

Chemicals

NAD⁺, NADP⁺, NADH, NADPH, cytochrome *c* (horse heart, type III), sodium deoxycholate, yeast alcohol dehydrogenase, ADP, *p*-nitrophenyl phosphate and α -oxoglutarate were supplied by Sigma Biochemical Co. (St. Louis, Mo., U.S.A.). NNN'N'-Tetramethyl-*p*-phenylenediamine dihydrochloride and pyrazole were supplied by Fluka AG, Buchs, Switzerland. All the other chemicals used were purchased from E. Merck AG, Darmstadt, Germany.

All spectrophotometric measurements were performed with a Shimadzu Spectronic 200 spectrophotometer, equipped with a Hitachi 165 recorder.

Fractionation of rat liver

All steps were carried out at +4°C. Livers from female Wistar albino rats were removed immediately after the rat had been killed and bled and put into an ice-cold buffer containing 0.25 M-sucrose–5 mM-Tris–HCl–0.5 mM-EDTA, pH 7.2 (referred to below as sucrose medium). The liver was cut into small pieces and rinsed several times to remove blood. A portion (5 g) of liver was homogenized in 20 ml of sucrose medium in an Potter–Elvehjem homogenizer, first with a loose-fitting and then with a tight-fitting Teflon pestle at 800 rev./min for 2 min in all. The homogenate was filtered through a double layer of cheesecloth to remove the connective tissue. This step was necessary to permit accurate activity measurements on the homogenate. The homogenate was made up to 10% (w/v) with sucrose medium and fractionated in principle by the method of de Duve *et al.* (1955). The homogenate was centrifuged for 10 min at 480 g_{av.}. The pellet was resuspended in 25 ml of sucrose medium and re-centrifuged as before. The final pellet was resuspended in 20 ml of sucrose medium and designated the nuclear fraction (N) [the nomenclature is adopted from de Duve *et al.* (1955)].

The combined supernatants, designated the 'cytoplasmic extract' (E), were centrifuged for 7 min at 4200 g_{av.}. The new supernatant was carefully decanted and the pellet was gently resuspended in 25 ml of sucrose medium and washed twice at 4200 g_{av.} for 7 min. The final pellet was resuspended in 20 ml of sucrose medium and designated the mitochondrial fraction (M). The combined supernatants were centrifuged for 5 min at 19000 g_{av.}. The supernatant was decanted to the last drop and the pellet was resus-

pended in 20 ml of sucrose medium and designated the lysosomal fraction (L). The supernatant obtained after removal of the L fraction was centrifuged for 60 min at 76000 g_{av.} (30000 rev./min) in a MSE40 ultracentrifuge in an 8 × 50 ml angle rotor. The surface of the final pellet and the walls of the centrifuge tube were rinsed and the pellet was resuspended in 20 ml of sucrose medium and designated the microsomal fraction (P). The supernatant was combined with the washings and designated the final supernatant (S).

Samples of each fraction were taken and assayed within a few hours or, where possible, frozen and assayed the next day.

Determination of apparent K_m values for aldehyde dehydrogenase in the mitochondrial and the microsomal fractions

Initial-rate measurements were performed as described for the general assay, except that pyrazole could be omitted, as the mitochondrial and the microsomal fractions contained no alcohol dehydrogenase. All measurements were performed in duplicate, with a reproducibility of better than 2% at all the concentrations used. The results are presented as double-reciprocal plots and lines were fitted to the experimental points by eye or by the least-squares method.

Enzyme assays

Where not specifically indicated, the assays were performed at 23°C.

Aldehyde dehydrogenase (general assay). This was assayed spectrophotometrically with acetaldehyde as substrate by measuring the reduction of NAD⁺ at 340 nm. The assay mixture contained 50 mM-sodium pyrophosphate, pH 8.8, 0.5 mM-NAD⁺ or 2.5 mM-NADP⁺, 0.1 mM-pyrazole, 0.05–5 mM-acetaldehyde and 2 μ M-rotenone (in methanol). The reaction was started by addition of the substrate. Pyrazole was added to inhibit alcohol dehydrogenase, and rotenone to inhibit mitochondrial NADH oxidase. Rotenone was dissolved in methanol and added in a small volume (0.2% of the final volume). Sodium deoxycholate at a concentration of 0.25 mg/mg of protein was added to samples to release latent activity and to give clear solutions for the spectrophotometric measurement. To correct for a blank reaction that occurred in the assay of fractions containing large amounts of cytosolic enzymes, a blank cuvette was run simultaneously, with omission of the substrate.

Glutamate dehydrogenase. This was assayed spectrophotometrically as described by Schmidt (1970), with minor modifications. The reaction mixture contained 50 mM-KH₂PO₄, pH 7.5, 120 mM-

ammonium acetate, 0.15 mM-NADH, 1.5 mM-ADP and 8 mM- α -oxoglutarate. The sample was activated with 0.1% (v/v) Triton X-100 shortly before addition. The reaction was started by the addition of α -oxoglutarate.

Alcohol dehydrogenase. This was assayed in principle as described by Büttner (1965). The activity was measured by following the oxidation of NADH spectrophotometrically with acetaldehyde as the substrate. The reaction mixture contained 50 mM-KH₂PO₄, pH 7.5, 0.15 mM-NADH and 8 mM-acetaldehyde. The reaction was started by the addition of acetaldehyde.

Monoamine oxidase. This was assayed by a modification of the method of Tabor *et al.* (1954), as described by Schnaitman *et al.* (1967), by following the formation of benzaldehyde from benzylamine spectrophotometrically at 250 nm ($\epsilon = 13400$ litre \cdot mol⁻¹ \cdot cm⁻¹; Werner & Neupert, 1972) in an assay system containing 50 mM-KH₂PO₄, pH 7.5, and 2.5 mM-benzylamine.

NADPH-cytochrome *c* reductase. This was determined essentially as described by Phillips & Langdon (1962) in an assay mixture containing 50 mM-KH₂PO₄, pH 7.5, 0.05 mM-cytochrome *c*, 0.3 mM-KCN and 0.1 mM-NADPH. The reduction of cytochrome *c* was followed at 550 nm. The reaction was started by the addition of NADPH. $\epsilon = 18500$ litre \cdot mol⁻¹ \cdot cm⁻¹ was used for the reduction of cytochrome *c*.

Rotenone-insensitive NADH-cytochrome *c* reductase. This was assayed as NADPH-cytochrome *c* reductase, except for the substitution of NADH for NADPH and the addition of rotenone (in methanol) to a final concentration of 2 μ M, as described by Sottocasa *et al.* (1967).

Cytochrome *c* oxidase. This was measured at 25°C polarographically with a Clark oxygen electrode. The reaction mixture contained 50 mM-KH₂PO₄, pH 7.5, 0.5 mM-NNN'-N'-tetramethyl-*p*-phenylenediamine dihydrochloride, 2 mM-sodium ascorbate and 0.03 mM-cytochrome *c*. The reaction was started by the addition of cytochrome *c*.

Acid phosphatase (total activity). This was assayed with *p*-nitrophenyl phosphate as the substrate by measuring the formation of *p*-nitrophenol.

A 0.1 ml portion of the sample was incubated (at 37°C in a shaking water bath) in 1.5 ml of 100 mM-sodium acetate buffer, pH 5.0. After 5 min, 0.5 ml of 20 mM-*p*-nitrophenyl phosphate was added, the mixture was incubated for 15 min and then 5 ml of 80 mM-NaOH was added to stop the reaction. The E_{400} was read against a blank taken through the procedure at the same time. The amount of *p*-nitrophenol formed was calculated from a standard curve constructed from experiments with known concentrations of *p*-nitrophenol. Samples were treated with 0.1% (v/v) Triton X-100, to release latent activity.

General methods

Standardization of acetaldehyde solutions. Solutions of acetaldehyde (10–200 mM) made from freshly distilled acetaldehyde were stable at +5°C for 2 weeks. Solutions stored at –23°C kept well for several weeks. The concentration of acetaldehyde was determined spectrophotometrically in an assay mixture containing 50 mM-KH₂PO₄, pH 7.0, 0.2 mM-NADH and 20 EC units of yeast alcohol dehydrogenase.

Protein. This was determined by the method of Lowry *et al.* (1951), with bovine albumin (fraction V powder) used as a standard. Samples were treated with 0.25 mg of sodium deoxycholate/mg of protein, which had no effect on the colour development at that concentration.

Results

Assay of aldehyde dehydrogenase

Blank reaction. The measurement of aldehyde dehydrogenase in fractions containing large amounts of cytosolic enzymes was disturbed by a large reduction of NAD⁺ in the absence of acetaldehyde. This blank reaction, which occurred between pH 7.5 and 9.6, could not be completely eliminated by dialysis or by desalting samples on Sephadex G-25 (coarse grade). By using a reference cuvette supplemented like the sample cuvette except for the substrate, this blank reaction was not recorded during the assay. No blank reaction was found in experiments on the mitochondrial or microsomal fractions. No reaction between NAD⁺ and acetaldehyde was detected at high pH values, such as has been reported to occur for certain aldehydes (Duncan & Tipton, 1971a). The reaction rate was linear for several minutes and proportional to the amount of sample added at all acetaldehyde concentrations, as shown in Fig. 1 from experiments on the 'cytoplasmic extract', the mitochondrial and the microsomal fractions. The blank rates for the 'cytoplasmic extract' were approx. 15 and 5% of the activities recorded at acetaldehyde concentrations of 0.05 and 5 mM respectively.

Interference by alcohol dehydrogenase. Fractions containing this enzyme could not be assayed in the absence of enzyme inhibitor. Pyrazole at a concentration of 0.1 mM completely inhibited alcohol dehydrogenase without affecting the activity of aldehyde dehydrogenase.

Effect of rotenone. In the absence of rotenone or other respiratory-chain inhibitors very little activity was found in fractions containing mitochondria, owing to their NADH oxidase activity. Rotenone at a concentration of 2 μ M completely inhibited this NADH oxidase activity.

Rotenone stabilized and activated the aldehyde dehydrogenase activity in the microsomal fraction

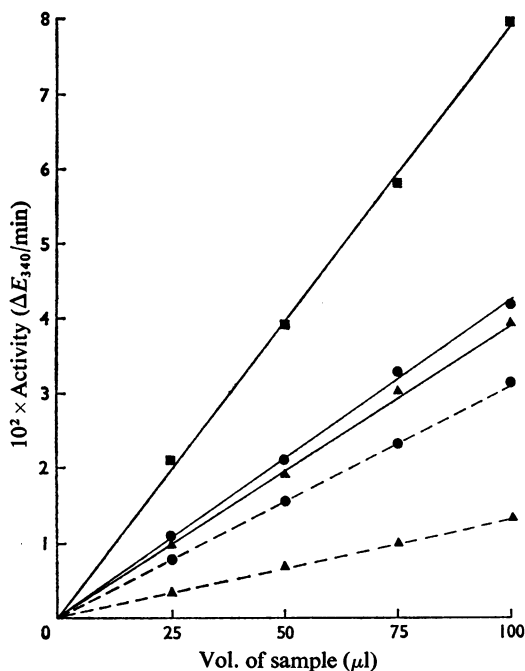


Fig. 1. Relationship between protein concentration and activity in the assay of NAD^+ -dependent aldehyde dehydrogenase in the 'cytoplasmic extract', the mitochondrial fraction and the microsomal fraction

The assay mixture contained 50mM-sodium pyrophosphate, pH 8.8, 0.5mM- NAD^+ , 0.1mM-pyrazole and 2 μ M-rotenone. Samples were treated with 0.25mg of sodium deoxycholate/mg of protein. The concentration of acetaldehyde was 5mM (—) or 0.05mM (----). ●, Mitochondrial fraction (5.0mg/ml); ■, microsomal fraction (3.8mg/ml); ▲, 'cytoplasmic extract' (6.0mg/ml). Values in parentheses refer to the protein concentration in each fraction.

by about 30%. Methanol, which was used as a solvent for rotenone, had no effect when added alone. As the microsomal fractions in this investigation are very little contaminated by mitochondria (see Table 1), the effect of rotenone cannot be explained as an inhibition of NADH oxidase activity. At the present time the effect of rotenone cannot be adequately explained.

Latency and stability of aldehyde dehydrogenase. Sonication, freezing-and-thawing and treatment with detergents (sodium deoxycholate, Triton X-100) caused a considerably higher activity of aldehyde dehydrogenase in mitochondrial fractions by releasing latent activity. Treatment with detergents was most effective in this respect and also gave clear solutions for the spectrophotometric measurements.

Sodium deoxycholate was chosen as a convenient solubilizer and clearing agent. To release all aldehyde dehydrogenase activity, samples had to be treated with 0.25mg of sodium deoxycholate/mg of protein.

No latent activity was found in the microsomal fraction, and sodium deoxycholate was only added to give a clear suspension. Aldehyde dehydrogenase was very stable in sodium deoxycholate-treated samples.

Aldehyde dehydrogenase in all fractions was very stable when stored at -23°C . No activity was lost after 3–5 days and only about 25% after 4 weeks.

Apparent K_m

NAD^+ -dependent aldehyde dehydrogenase. When activity measurements were performed on a homogenate or mitochondria it was found that, at a saturating concentration of NAD^+ , a high concentration (5mM) of acetaldehyde was needed for maximum activity and that 30–50% of the activity persisted at very low concentrations (less than 10 μ M). The Lineweaver–Burk plots obtained from experiments on mitochondria were curved, suggesting the possible presence of more than one enzyme (Fig. 2a). The activity remained constant when the concentration of acetaldehyde was varied between 0.01 and 0.05mM, suggesting the presence of an enzyme (designated enzyme I) with a K_m apparently well below 10 μ M for acetaldehyde.

According to Reiner (1969) the K_m values for two similar enzymes acting on the same substrate can be calculated from the resulting curved double-reciprocal plot as follows. At high substrate concentrations the curve is approximately linear with a slope $(V_1K_1 + V_{11}K_{11})/V^2$ (V and K refer to maximum velocity and K_m , the subscripts refer to enzyme I and enzyme II and $V = V_1 + V_{11}$). The intercept of the curve on the vertical axis ($1/v$) is $1/V$. The tangent at the point where the curve intersects the vertical axis itself intersects the horizontal axis at $1/s = -(V_1 + V_{11})/(K_1V_1 + K_{11}V_{11})$. This may be simplified to $s = -(K_{11}V_{11})/(V_1 + V_{11})$ when $K_1 \ll K_{11}$. The value obtained from the intercept on the horizontal axis on correction for the factor $V_{11}/(V_1 + V_{11})$ is the value of K_{11} . Re-plotting data from the linear portion of the curve in Fig. 2(a) on a larger scale (not shown here) gives on extrapolation $s = -0.7\text{mM}$ and $V = 69$. The value obtained from the intercept of the asymptote on the vertical axis in Fig. 2(b) should correspond approximately to V_1 . This value is 24, which gives $V_{11} \sim 45$ (V , V_1 and V_{11} are expressed in nmol/min per mg of protein). From these data an apparent K_m of 1.2mM can be calculated for enzyme II.

When the rate of enzyme I (V_1) is subtracted from the total rates at acetaldehyde concentrations

Table 1. Intracellular distribution of aldehyde dehydrogenases and some marker enzymes in rat liver

Enzyme assays and protein determinations were performed as described in the Materials and Methods section. Absolute values for total homogenate (N+E) are expressed as $\mu\text{mol}/\text{min}$ per g wet wt. of liver for enzyme activities and as mg/g wet wt. of liver for protein. N, Nuclear fraction; E, cytoplasmic extract; M, mitochondrial fraction; L, lysosomal fraction; P, microsomal fraction; S, final supernatant. Means \pm s.d. are given.

Enzyme	No. of experiments	Absolute values (N+E)	Percentage values						Recovery
			N+E	N	M	L	P	S	
Aldehyde dehydrogenase (total activity)	5	5.30 \pm 0.44	100	9.7 \pm 2.5	35.0 \pm 2.1	19.8 \pm 2.7	26.4 \pm 2.4	4.2 \pm 0.8	95.1 \pm 3.6
Enzyme I	5	1.32 \pm 0.17	100	14.8 \pm 2.4	60.1 \pm 2.8	14.9 \pm 2.3	1.7 \pm 0.5	1.9 \pm 0.6	93.6 \pm 3.9
Enzyme II (total -I)	5	3.98 \pm 0.34	100	7.0 \pm 3.0	24.0 \pm 1.6	21.9 \pm 2.0	35.6 \pm 1.8	5.7 \pm 0.7	94.1 \pm 3.6
Aldehyde dehydrogenase (NADP ⁺ -dependent activity)	3	1.14 \pm 0.09	100	9.4 \pm 0.8	21.9 \pm 2.0	26.3 \pm 2.8	37.5 \pm 2.5	2.5 \pm 1.1	97.6 \pm 1.4
Glutamate dehydrogenase	5	156.2 \pm 0.7	100	15.0 \pm 2.2	62.6 \pm 1.3	17.0 \pm 1.3	1.4 \pm 0.2	1.5 \pm 0.2	97.5 \pm 2.6
Cytochrome c oxidase	5	86.4 \pm 3.2	100	15.1 \pm 3.3	61.6 \pm 1.9	19.9 \pm 3.1	2.0 \pm 0.6	1.5 \pm 0.3	99.0 \pm 3.7
Monoamine oxidase	4	0.39 \pm 0.02	100	13.1 \pm 3.9	61.0 \pm 2.8	17.5 \pm 3.1	6.3 \pm 1.1	2.1 \pm 0.7	100.0 \pm 2.4
Acid phosphatase (total activity)	4	164 \pm 2.0	100	5.7 \pm 2.2	20.0 \pm 2.2	30.9 \pm 3.3	8.8 \pm 0.5	24.6 \pm 1.7	92.3 \pm 3.6
NADPH-cytochrome c reductase	5	3.59 \pm 0.33	100	7.2 \pm 0.9	7.6 \pm 0.7	20.4 \pm 1.4	60.1 \pm 2.0	3.5 \pm 0.3	98.7 \pm 3.0
NADH-cytochrome c reductase (rotenone-insensitive)	3	102 \pm 9	100	9.5 \pm 1.0	20.7 \pm 1.7	27.6 \pm 3.2	38.7 \pm 2.9	2.0 \pm 0.8	98.5 \pm 3.2
Alcohol dehydrogenase	5	7.76 \pm 0.90	100	1.7 \pm 0.7	0.0	0.5 \pm 0.5	1.0 \pm 0.2	94.8 \pm 2.1	97.5 \pm 1.6
Protein	5	152.4 \pm 3.0	100	18.9 \pm 3.5	22.5 \pm 4.7	9.4 \pm 2.1	10.0 \pm 0.8	39.3 \pm 5.1	100.1 \pm 5.0

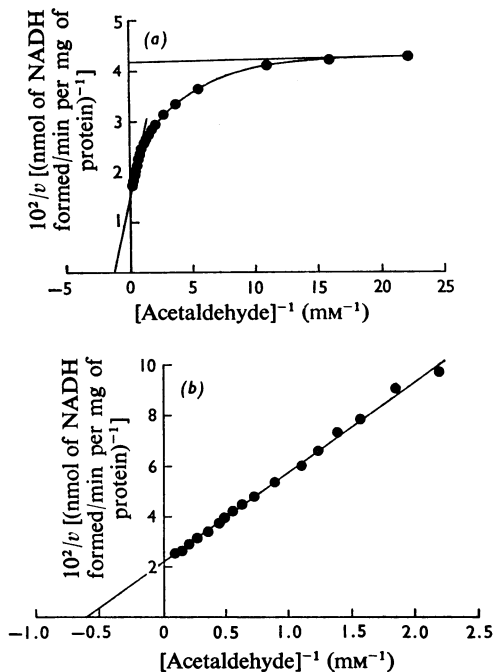


Fig. 2. Double-reciprocal plot for the oxidation of acetaldehyde by mitochondria

(a) The activity was measured at 23°C in 50mM-sodium pyrophosphate buffer, pH 8.8, at a fixed concentration of NAD^+ (0.5 mM). The concentration of acetaldehyde was varied between 0.04 mM and 8 mM. Rotenone was present at a concentration of 2 μ M. The amount of protein used in the assay was 0.8 mg, in a final volume of 2.3 ml. (b) Data from (a) were used to calculate the differences between the total aldehyde dehydrogenase activity at high acetaldehyde concentrations and the activity obtained from the intercept of the asymptote on the vertical axis in (a).

between 0.45 and 9 mM, a linear double-reciprocal plot is obtained when the differences are plotted as shown in Fig. 2(b). From this plot an apparent K_m of 1.7 mM can be calculated for enzyme II.

Plots obtained from experiments on the microsomal fraction were linear (Fig. 3) and no activity was detected at an acetaldehyde concentration of 0.05 mM. The apparent K_m value for acetaldehyde was 0.9 mM, which suggested that the microsomal enzyme might be identical with the mitochondrial enzyme II and, for the sake of simplicity, this enzyme will be referred to as the microsomal enzyme II.

K_m values for NAD^+ were determined as described in the Materials and Methods section. The concen-

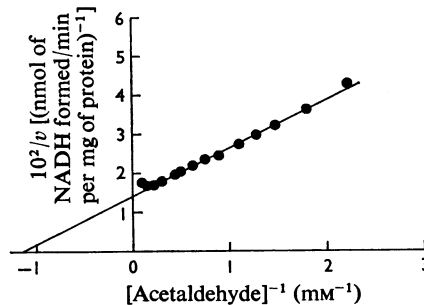


Fig. 3. Double-reciprocal plot for the oxidation of acetaldehyde by the microsomal fraction

The assays were performed exactly as described in Fig. 2. The amount of protein used in the assay was 0.4 mg, in a final volume of 2.3 ml.

tration of NAD^+ was varied between 0.01 and 5 mM and acetaldehyde was kept at fixed concentrations of 0.05 and 5 mM for the mitochondrial enzyme I and the microsomal enzyme II respectively. The apparent K_m values for enzyme I and enzyme II were 24 and 35 μ M respectively. Owing to the presence of both enzymes in the mitochondria, no accurate determinations of the K_m for the mitochondrial enzyme II could be obtained.

NADP⁺-dependent aldehyde dehydrogenase. The Lineweaver-Burk plots from experiments on mitochondria and the microsomal fraction were linear, both for acetaldehyde and $NADP^+$, indicating the presence of only one enzyme in each fraction. The apparent K_m values for acetaldehyde and $NADP^+$ (measured at various concentrations of one reactant and a constant saturating concentration of the other) were 0.5 and 3.1 mM respectively for the mitochondrial enzyme and 0.6 and 2.5 mM respectively for the microsomal enzyme. The $NADP^+$ preparation used was stated by the manufacturer to be free from NAD^+ , and this was confirmed in a test with yeast alcohol dehydrogenase, which is absolutely NAD^+ -specific (Dalziel & Dickinson, 1965).

Effect of pH and different buffer systems

The variation of the NAD^+ -dependent activity in relation to pH was studied in 50mM-sodium pyrophosphate, 50mM-potassium phosphate and 10mM-Tris-HCl buffers with acetaldehyde as substrate. Enzyme I in the mitochondrial fraction was assayed at 0.05mM-acetaldehyde. Enzyme II in the microsomal fraction was assayed at 5 mM-acetaldehyde.

As can be seen from Figs. 4 and 5, both enzymes show pH optima at about 9 in pyrophosphate and phosphate buffers, but no sharp pH optimum was found in Tris-HCl buffer, in which the activity

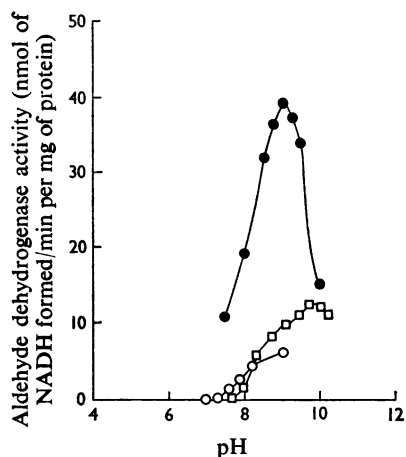


Fig. 4. Influence of pH on the activity of mitochondrial aldehyde dehydrogenase (enzyme I)

The activity was measured in the following buffers: ●, 50mM-sodium pyrophosphate; ○, 50mM-potassium phosphate; □, 10mM-Tris-HCl. The concentrations of NAD^+ and acetaldehyde were 0.5 and 0.05mM respectively. The amount of protein used in the assay was 0.8mg, in a final volume of 2.2ml.

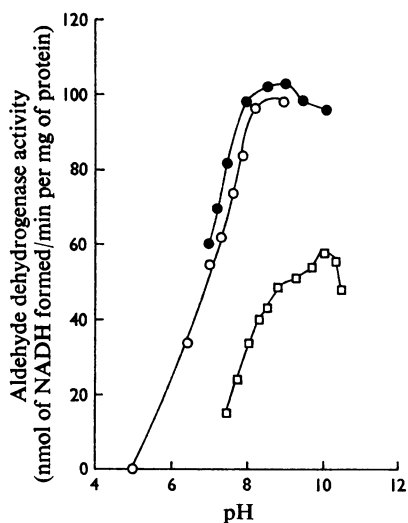


Fig. 5. Influence of pH on the activity of microsomal aldehyde dehydrogenase (enzyme II)

The activity was measured in the same buffers, identified as given for Fig. 4, and the conditions were the same as in Fig. 4, except that the concentration of acetaldehyde was 5mM. The amount of protein used in each assay was 0.4mg, in a final volume of 2.2ml.

increases up to pH10 and then declines, probably owing to denaturation. At concentrations of Tris-HCl higher than 10mM the activity of enzyme II declined drastically, but the activity of enzyme I was unaffected in the concentration range 10–50mM. Both enzymes showed lower activity in Tris-HCl than in pyrophosphate buffer.

The activity at the pH optimum of enzyme II in phosphate and pyrophosphate buffers was of the same order (Fig. 5), whereas the activity of enzyme I in 10–50mM-phosphate buffer was only 15–20% of the activity in 50mM-pyrophosphate buffer and was zero at pH7.2 (Fig. 4).

This effect of phosphate has not been further investigated. The activities of enzyme I and enzyme II were the same in sodium phosphate as in potassium phosphate buffer and were slightly stimulated by an increasing concentration of phosphate up to 50mM.

The variation of the NADP^+ -dependent activity with pH was not studied.

Distribution of marker enzymes and aldehyde dehydrogenases

General comments. After a careful standardization of the fractionation procedure, five complete experiments were performed. Nine enzyme activities were determined in each isolated fraction (see the Materials and Methods section for the designation of the different fractions).

The activities were calculated as percentages of the sum of activities found in the nuclear fraction (N) and the cytoplasmic extract (E) (Table 1). Calculations as percentages of the activity in the original homogenate proved to be very inaccurate and were not reproducible for several enzymes. The reason for this is not known, but it may be due to aggregation in the homogenate, as pointed out by de Duve *et al.* (1955).

Recoveries were excellent and ranged between 95 and 100%. The percentage distribution of the marker enzymes and protein and the absolute values in fractions N+E for the different enzyme activities corresponded to the results obtained by others (de Duve *et al.*, 1955; Scholte, 1969; Van Tol & Hülsmann, 1969).

The specific enzyme activities obtained for the marker enzymes in the different subcellular fractions are not presented but corresponded well to the data reported in the literature.

Mitochondrial marker enzymes. Homogenization of liver tissue causes leakage of soluble matrix and intermembrane enzymes and rupture of the outer membrane. The extent of this damage to the mitochondria was studied by using suitable marker enzymes.

Glutamate dehydrogenase, cytochrome *c* oxidase and monoamine oxidase were chosen as markers for

the matrix, the inner membrane and the outer membrane respectively. No marker was used for the intermembrane compartment. Table 1 shows that the distributions of these enzymes were very similar. The percentage activity for glutamate dehydrogenase in the final supernatant (fraction S) was very low and equal to cytochrome *c* oxidase, indicating no or very little leakage of matrix enzymes. The activity of monoamine oxidase in the microsomal fraction (P) was higher than the activity of cytochrome *c* oxidase and glutamate dehydrogenase, indicating some rupture of the outer mitochondrial membrane. The extent of the contamination of the microsomal fraction with outer membrane was, however, low, considering the relatively extensive homogenization of the liver.

Lysosomal marker enzyme. Acid phosphatase was used as marker. Only the total activity was assayed and not the free activity (which, when assayed, gives the distribution of enzyme released from damaged lysosomes), which, anyway, would correspond to the activity found in the soluble fraction. Acid phosphatase is concentrated in the lysosomal fraction (L). High activities were also found in the mitochondrial and the soluble fractions.

Microsomal marker enzyme. NADPH-cytochrome *c* reductase was used as marker, and in a few experiments glucose 6-phosphatase was used. The distributions (not shown for glucose 6-phosphatase in Table 1) for these markers were identical. In our hands, NADPH-cytochrome *c* reductase proved to be the most suitable marker, because it provided a quick and reliable test and gave a good recovery.

Marker enzyme for the cytosol. Alcohol dehydrogenase was found to be an acceptable marker, although some difficulties were encountered in the assay of this enzyme in crude fractions (with little activity), owing to turbidity in the spectrophotometric cuvettes. The addition of clearing agents (sodium deoxycholate or Triton X-100) caused inactivation of the enzyme and could not be used. Very little or no activity was found in the particulate fractions.

Distribution of aldehyde dehydrogenases

It was found from activity measurements at a saturating concentration of acetaldehyde (5 mM) that the distribution was bimodal (aldehyde dehydrogenase, total activity in Table 1). The distribution patterns for mitochondrial and microsomal marker enzymes suggest that the aldehyde dehydrogenase activity found is confined to the mitochondria and the microsomal fraction.

Very little activity was found in the soluble fraction (S) and was probably caused by enzymes released from the mitochondria and the microsomal fraction as is indicated by the marker enzymes. The specific activity for the total activity was about 4 nmol of

Table 2. *Specific activities for the NAD⁺- and NADP⁺-dependent aldehyde dehydrogenases in mitochondria and the microsomal fraction*

Activities were measured as described in the Materials and Methods section. Values are the means \pm S.D.; the numbers of preparations used for the determinations are given in parentheses.

Enzyme	Specific activities (nmol of NADH formed/ min per mg of protein)	
	Mitochondria	Microsomal fraction
Aldehyde dehydrogenase (total activity) (7)	61.9 \pm 6.3	90.3 \pm 11.6
Enzyme I (7)	30.3 \pm 2.8	1.1 \pm 0.4
Enzyme II (total-I) (7)	31.6 \pm 6.6	89.3 \pm 11.6
Aldehyde dehydrogenase (NADP ⁺) (3)	23.0 \pm 3.5	56.1 \pm 10.5

NADH formed/min per mg of protein, which is similar to the results reported by others (Hassinen *et al.*, 1970).

The results of the kinetic experiments revealed the possible existence of at least two enzymes, which could be assayed separately from each other by using NADP⁺ instead of NAD⁺ or different concentrations of acetaldehyde. When the activity was determined at a low acetaldehyde concentration (0.05 mM), the distribution pattern followed exactly those of the mitochondrial marker enzymes, suggesting an enzyme exclusively localized in the mitochondria (enzyme I in Table 1). When the difference between the total activity and the activity for enzyme I was calculated, a distribution pattern was obtained (enzyme II in Table 1) that was very similar to that of rotenone-insensitive NADH-cytochrome *c* reductase, which is known from the work of Sottocasa *et al.* (1967) to occur in both the mitochondria and the microsomal fraction. The same distribution pattern as that for enzyme II was obtained for the NADP⁺-dependent activity, which means that enzyme II and the NADP⁺-dependent enzyme may be identical. By using data from Table 1, mitochondria and the microsomal fraction were calculated to contain 20–30% and 70–80% respectively of the total activity of enzyme II.

The specific activities obtained for NAD⁺- and NADP⁺-dependent aldehyde dehydrogenase activities are shown in Table 2.

No significant sex differences were found in the kinetics or distribution of the aldehyde dehydrogenase. Experiments performed on Sprague-Dawley rats give similar results.

Discussion

The reports on aldehyde dehydrogenase obtained from rat liver do not agree on the molecular properties and the subcellular localization. Most studies on purified aldehyde dehydrogenase have been performed on preparations from sources other than rat liver. However, Shum & Blair (1972) isolated two different aldehyde dehydrogenases from rat liver cytosol. These two enzymes differed in some respects (see the introduction), but both had apparent K_m values for acetaldehyde in the millimolar range. Although comparisons between the properties of an enzyme in a purified condition and in a crude fraction are susceptible to error, a few remarks seem to be justified. Shum & Blair (1972) give for their enzyme I a K_m of 1 mM for acetaldehyde and $34 \mu\text{M}$ for NAD^+ and pH optimum between 8 and 9.5, which are similar to the data for enzyme II in our report. Unlike our results for enzyme II, none of their enzymes was reactive with NADP^+ as cofactor. Their enzyme II exhibited more restricted substrate specificity than enzyme I. Substrate specificities were not tested in the present investigation.

Lundquist *et al.* (1959) suggested, from experiments on rat liver homogenate, an apparent K_m for acetaldehyde less than $1 \mu\text{M}$ for NAD^+ -dependent aldehyde dehydrogenase, and Marjanen (1972) found a value of less than $10 \mu\text{M}$ for the disappearance rate of acetaldehyde in a homogenate.

Our kinetic experiments suggest the presence of at least two aldehyde dehydrogenases, with strikingly different affinities for acetaldehyde. One of the enzymes, enzyme I, probably corresponds to the aldehyde dehydrogenase isolated from horse, human and mouse liver, although the conclusive confirmation of the presence of an enzyme of low K_m in rat liver mitochondria must await purification and characterization. The K_m for enzyme I for NAD^+ is similar to the values ($30\text{--}80 \mu\text{M}$) obtained by others (Lundquist *et al.*, 1959; Shum & Blair, 1972; Feldman & Weiner, 1972).

No detailed kinetic experiments were performed on enzyme I to study the possible substrate activation, which was reported for brain aldehyde dehydrogenase by Duncan & Tipton (1971*b*), or the substrate inactivation for horse liver aldehyde dehydrogenase reported by Feldman & Weiner (1972). These effects would, anyway, be difficult to study in our activity measurements on unpurified preparations, and further studies must be performed on purified extracts. Weak substrate inhibition was observed, however, for enzyme II in the microsomal fraction at acetaldehyde concentrations greater than 5 mM. Substrate activation may give rise to double-reciprocal plots, like that in Fig. 2(*a*), which has also been considered by Erwin & Deitrich (1966) and Duncan & Tipton (1971*b*). Abrupt transitions in the double-reciprocal plot are known for some single enzymes, e.g. gluta-

mate dehydrogenase (Engel & Ferdinand, 1973). The apparent presence of an enzyme of low K_m may be due to a non-enzymic blank rate, and a sharp break in the double-reciprocal plot would therefore be expected. However, the large differences between the K_m values calculated from Fig. 2, the complete absence of any blank rates in experiments on the mitochondria, the results of the distribution study, and the reports by others (see the introduction) of isolated dehydrogenases with K_m for acetaldehyde less than $1 \mu\text{M}$ do not support these other possible explanations. Further, the results reported by Smith & Packer (1972) and results from our own laboratory (S. O. C. Tottmar, unpublished work) show the presence of two differently located aldehyde dehydrogenases in rat liver mitochondria.

High apparent K_m values (0.7–1.5 mM) have been found in experiments on crude liver preparations (Deitrich, 1966) and in liver-perfusion experiments for the disappearance rate of acetaldehyde (Lindros *et al.*, 1972). Our results suggest that these high apparent K_m values can be explained by the presence of enzyme II.

The high optima (pH 9–10) found in our assay system are similar to the results reported by Feldman & Weiner (1972), Blair & Bodley (1969) and Racker (1949) in their papers on horse liver, human liver and ox liver enzymes respectively. Marjanen (1972), on the other hand, reported a pH optimum of 7.8 at 37°C for the disappearance of acetaldehyde.

Tris-HCl buffers were found to inhibit both enzyme I and enzyme II. Feldman & Weiner (1972) also reported this inhibition by Tris of purified horse liver aldehyde dehydrogenase and suggested that the formation of acetaldehyde complex rather than a direct effect on the enzyme was responsible for this inhibition.

The presence of an NADP^+ -dependent aldehyde dehydrogenase has been reported for the microsomal fraction (Tietz *et al.*, 1964) but not for the mitochondria. The kinetic experiments show that the activities found in the microsomal fraction and the mitochondria are probably caused by the same enzyme, as was also suggested for the NAD^+ -dependent enzyme II in the Results section. Further, the similar distribution patterns for enzyme II and the NADP^+ -dependent activity suggest that only one enzyme may be involved. Other NAD^+ -dependent dehydrogenase with low affinities for NADP^+ compared with NAD^+ but with similar maximum rates with both cofactors have been reported (Dalziel & Dickinson, 1965; Mehler *et al.*, 1948), which is consistent with our results and suggestions.

The discrepant results in the literature about the distribution of aldehyde dehydrogenase in rat liver can be explained partly by the possible presence of two or more different enzymes and partly by incomplete fractionation studies. To obtain a complete

distribution pattern of aldehyde dehydrogenase, the fractionation was performed with suitable marker enzymes for the different subcellular entities, so that the purity of each fraction and subsequently the true distribution of aldehyde dehydrogenase could be established.

Our results show that enzyme I is exclusively localized in the mitochondria and that at least one more enzyme is present in the mitochondria. The presence of aldehyde dehydrogenase in rat liver microsomal fraction has been mentioned in a few reports. Deitrich (1966) found that the microsomal fraction contained only 3.7% of the total activity of a liver homogenate, 85% was found in the soluble fraction and the rest in the mitochondrial fraction. Marjanen (1972), on the other hand, found no activity in the microsomal fraction.

Our results show that rat liver cytosol contains none or very little of the total aldehyde dehydrogenase activities and that the activities found in the soluble fraction are partly caused by enzymes released from the mitochondria and the endoplasmic reticulum during homogenization.

The present study was undertaken to gain a better understanding of the possible oxidation sites for acetaldehyde during ethanol metabolism. Only the NAD⁺- and NADP⁺-dependent aldehyde dehydrogenases have been studied, as other possible oxidation pathways, for example aldehyde oxidase and xanthine oxidase, are of minor importance (Lundquist *et al.*, 1962; Lundquist, 1971).

The existence of several aldehyde dehydrogenases in rat liver does not invalidate the suggestion, put forward by Hedlund & Kiessling (1969) and Hassinen *et al.* (1970), that acetaldehyde is mainly oxidized in the mitochondria during ethanol metabolism. The exclusive mitochondrial localization of enzyme I and the high affinity to acetaldehyde are in fact evidence in favour of their suggestion. At the present time the role of the other NAD⁺- or NADP⁺-dependent aldehyde dehydrogenases cannot be evaluated.

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