Biochimica et Biophysica Acta, 397 (1975) 9—23

BBA 67539

DIFFERENT FORMS OF RAT LIVER ALDEHYDE DEHYDROGENASE AND THEIR SUBCELLULAR DISTRIBUTION

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(Received December 12th, 1974)

Summary

1. The properties and distribution of the NAD-linked unspecific aldehyde dehydrogenase activity (aldehyde:NAD^+ oxidoreductase EC 1.2.1.3) has been studied in isolated cytoplasmic, mitochondrial and microsomal fractions of rat liver. The various types of aldehyde dehydrogenase were separated by ion exchange chromatography and isoelectric focusing.

2. The cytoplasmic fraction contained 10—15, the mitochondrial fraction 45—50 and the microsomal fraction 35—45% of the total aldehyde dehydrogenase activity, when assayed with 6.0 mM propionaldehyde as substrate.

3. The cytoplasmic fraction contained two separable unspecific aldehyde dehydrogenases, one with high K_m for aldehydes (in the millimolar range) and the other with low K_m for aldehydes (in the micromolar range). The latter can, however, be due to leakage from mitochondria. The high-K_m enzyme fraction contained also all D-glucuronolactone dehydrogenase activity of the cytoplasmic fraction. The specific formaldehyde and betaine aldehyde dehydrogenases present in the cytoplasmic fraction could be separated from the unspecific activities.

4. In the mitochondrial fraction there was one enzyme with a low K_m for aldehydes and another with high K_m for aldehydes, which was different from the cytoplasmic enzyme.

5. The microsomal aldehyde dehydrogenase had a high K_m for aldehydes and had similar properties as the mitochondrial high-K_m enzyme. Both enzymes have very little activity with formaldehyde and glycolaldehyde in contrast to the other aldehyde dehydrogenases. They are apparently membrane-bound.

Introduction

Oxidation of aldehydes in liver is considered to be mainly due to the unspecific NAD-linked aldehyde dehydrogenases (EC 1.2.1.3), which has been

generally supposed to be located mainly in the soluble fraction of the cell [1—3]. During the recent years, however, increasing evidence has appeared in the literature, which shows that this enzyme activity in rat liver is for the most part located in mitochondria [4—11], and also microsomal aldehyde dehydrogenase activity with a high K_m value for aldehydes has been described [10—12].

Most enzymological studies on rat liver aldehyde dehydrogenase have been made with the soluble fraction. Two different NAD-linked aldehyde dehydrogenases have been separated by chromatographic methods [13,14] and Deitrich et al. [15] have also described two enzymes in rat liver soluble fraction with different inducibility by phenobarbital and with different substrate specificities.

We have studied the properties of the different types of unspecific aldehyde dehydrogenase present in rat liver using partially purified enzymes prepared from isolated subcellular fractions. The results show that there are several different enzymes, which can catalyze the NAD-linked oxidation of various aldehydes. These differ in their subcellular distribution, isoelectric points, substrate specificities and K_m values for aldehydes. In addition there is a specific formaldehyde dehydrogenase (EC 1.2.1.1) in the soluble fraction and a specific betaine aldehyde dehydrogenase (EC 1.2.1.8) both in the soluble and mitochondrial fractions. A preliminary abstract of this work has appeared [12].

Materials and Methods

Chemicals

NAD^+, $NADP^+$ and glucose 6-phosphate were purchased from Boehringer, Mannheim, Germany. Pyrazole, butyraldoxime, 2,2-diethoxy-ethyltrimethylammonium iodide and phenylacetaldehyde were obtained from Aldrich-Europe, Beerse, Belgium. Benzaldehyde and anisaldehyde were from BDH Chemical Ltd., Poole, Dorset, U.K. and all other aldehydes from Fluka AG Buchs, Switzerland. Chloral hydrate, 2-mercaptoethanol and all other chemicals used were products of E. Merck, Darmstadt, Germany. The Ampholines 8141, 8143, 8152 and 8155 were obtained from LKB, Bromma, Sweden. DEAE-cellulose (DE 22) and CM-cellulose (CM 22) were products of Whatman Biochemicals Ltd., Maidstone, Kent, U.K. and were prepared for use as directed by the manufacturer. Sephadex G-200 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Synthesis of betaine aldehyde

Betaine aldehyde chloride hydrate was synthesized from 2,2-diethoxy-ethyltrimethylammonium iodide according to the principle of Bergel et al. [16]. A stock solution of betaine aldehyde was made by dissolving the synthesized crystalline product in water and by bringing the pH of the solution to 7.0 with addition of $NaHCO_3$. The concentration of betaine aldehyde was determined by the method of Rotschild and Barron [17].

Assay methods

If not indicated otherwise, the enzyme assays were performed at 25°C.

Aldehyde dehydrogenase activity was routinely measured spectrophotometrically by following the NADH production at 340 nm with a Gilford model 2000 attachment for the Beckman DU monochromator. The standard assay mixture contained 70 mM sodium pyrophosphate buffer, pH 8.0, 1.33 mM NAD, 1.67 mM pyrazole and 6 mM propionaldehyde. In the activity localization analysis of column chromatographies samples of the effluent fractions were incubated for 30 min at 37 °C with the assay mixture. In the K_m determinations the NADH production was followed fluorimetrically with a Farrand fluorometer. One unit of activity is defined as the amount of enzyme, which catalyzes the formation of 1 μmol of NADH per min under the above conditions. A molar extinction of $6.22 \cdot 10^3\ M^{-1} \cdot cm^{-1}$ was used for NADH [18]. The reactions were started by addition of the coenzyme. Blank reactions without substrate and with certain aldehydes without enzyme were followed simultaneously.

For the assay of alcohol dehydrogenase activity the reaction mixture contained 70 mM NaOH/glycine buffer, pH 9.6, 0.67 mM NAD and 10 mM ethanol. The initial rates of NADH production were measured spectrophotometrically as in the aldehyde dehydrogenase assay. Glucose-6-phosphatase activities were measured in homogenates and subcellular fractions by the method of Harper [19]. Cytochrome *a* concentration was determined by the method of Schollmeyer and Klingenberg [20]. Difference spectra at 630—605 nm were measured with a Beckman DK 2 spectrophotometer. Formaldehyde dehydrogenase was assayed as described before [21].

Protein was determined in crude preparations by the biuret method [22] and in purified preparations by the method of Lowry et al. [23]. Dry bovine serum albumin (Armour) was used as standard. Corrections for the detergent, sucrose and mercaptoethanol contents of the samples were made by adding appropriate amounts of these to the protein standard solutions. Protein in the effluent of chromatography columns was monitored at 280 nm with Uvicord II (LKB, Bromma, Sweden).

Isoelectric focusing

Isoelectric focusing was conducted using a 110 ml column model 8101 of LKB (Bromma, Sweden) according to the instructions of the manufacturer. pH gradients 3—10, 4—6, 5—8 and 7—9 were used with 1% (w/v) ampholyte concentration. The samples were added to the light solution. Focusing was continued for 48 h at 4 °C. The column contents were collected fractionally and the pH of each fraction was determined at 4 °C. The aldehyde dehydrogenase activities of the fractions were measured as described above.

Subcellular fractionation

Livers from female Wistar albino rats (weight 200—250 g) were removed immediately after decapitation, and put into an ice-cold 0.25 M sucrose solution containing 10 mM sodium phosphate pH 7.4 and 2 mM mercaptoethanol (referred as sucrose solution). All further steps were carried out at 4 °C. The livers were homogenized in the sucrose solution in a Potter-Elvehjem type homogenizer to make a 10% (w/v) homogenate. This was centrifuged for 5 min at $700 \times g$ and the pellet was washed once by resuspending it in 30 ml of

sucrose solution and centrifuging as before. The combined supernatants were centrifuged for 10 min at 4500 × *g* and the pellet containing mitochondria was washed twice with 30 ml of sucrose solution. The washed mitochondria were suspended in a volume of 0.16 M KCl twice the original liver weight and the suspension was treated with 0.3% (w/v) sodium deoxycholate (final concentration) corresponding to 0.20—0.30 mg/ml mitochondrial protein. The suspension was centrifuged for 60 min at 106 000 × *g* in a Spinco preparative ultracentrifuge L and the supernatant was used for studies on the mitochondrial aldehyde dehydrogenase activity.

The combined postmitochondrial supernatants were centrifuged for 15 min at 12 000 × *g*. The pellet which contained most of the lysosomes was discarded. The microsomal fraction was sedimented by centrifuging the supernatant for 60 min at 106 000 × *g*. The clear supernatant was sucked out from below the lipoprotein layer and designated the cytoplasmic fraction. After rinsing the final tight pellet with sucrose solution it was resuspended in a volume of sucrose solution twice the original liver weight and treated with 0.3% (w/v) sodium deoxycholate (final concentration). After centrifuging for 60 min at 106 000 × *g* the supernatant was used for studies of the microsomal activity.

For the studies on the subcellular distribution of aldehyde dehydrogenase activity mitochondrial and microsomal suspensions which were treated with deoxycholate as described, were used without centrifugation. These same suspensions and the original homogenate treated with deoxycholate (final concentration 0.3% w/v) were used also for the determinations of cytochrome *a* content and glucose-6-phosphatase activity.

In the experiments on the solubilization of the membrane bound activities also Triton X-100 (0.70 mg/mg protein) and digitonin (0.22 mg/mg protein) were used as detergents. Three freezing and thawing cycles with solid carbon dioxide/ethanol mixture were also used in these experiments.

Purification of enzymes

The cytoplasmic, mitochondrial and microsomal fractions from five rat livers were pooled at a time for partial purification of their aldehyde dehydrogenase activities. The first step was ammonium sulphate fractionation between 30 and 80% saturation (at 0°). Ammonium sulphate was added as solid salt and the pH kept at 7.2 by addition of 2 M NH_4OH. The relevant precipitate was dissolved in a minimal volume of 10 mM sodium phosphate buffer, pH 7.4 containing 2 mM mercaptoethanol and dialyzed overnight against 20 volumes of the same buffer, with two changes of buffer.

The dialyzed enzyme solution was applied to a DEAE-cellulose column (DE 22, 2.0 cm × 40 cm) equilibrated with the dialysis buffer and the column was eluted with three column volumes of the starting buffer. A linear concentration gradient from 10 to 150 mM sodium phosphate pH 7.4 (2 × 600 ml) was then started. All buffers contained 2 mM mercaptoethanol. The fractions were analyzed for alcohol and aldehyde dehydrogenase activities using ethanol, propionaldehyde, glycolaldehyde, D-glucuronolactone and betaine aldehyde as substrates. The fractions containing aldehyde dehydrogenase activity were pooled.

The first peak of cytoplasmic aldehyde dehydrogenase activity, which was

not bound to the DEAE-cellulose column, was purified further with CM-cellulose chromatography and Sephadex G-200 gel chromatography. The pooled fractions were dialyzed for 10 h against 10 mM sodium phosphate buffer pH 6.2 containing 2 mM mercaptoethanol. The enzyme solution was then applied to a CM-cellulose column (CM 22, 1.5 cm × 30 cm) equilibrated with the dialysis buffer. After elution with three column volumes of the starting buffer, a linear concentration gradient from 10 mM to 150 mM sodium phosphate pH 6.2 (2 × 400 ml) was run. All buffers contained 2 mM mercaptoethanol. The fractions were analyzed for aldehyde dehydrogenase activity as above and active fractions were collected and concentrated with ultrafiltration in an Amicon cell with PM 10 membrane.

A sample of the concentrated enzyme was passed through a Sephadex G-200 column (2.5 cm × 40 cm), equilibrated with 10 mM sodium phosphate buffer pH 7.4 containing 2 mM mercaptoethanol. Fractions of 2 ml were collected and analyzed for aldehyde dehydrogenase activity. Those with at least one third of the highest activity were pooled. During this step the enzyme became so labile, that further purification was not attempted. For the same reason most of the measurements for characterization were made with the preparation obtained after the CM-cellulose step.

The cytoplasmic fraction of aldehyde dehydrogenase activity, which was not bound to DEAE-cellulose was not purified further beyond this step, neither were the mitochondrial nor microsomal fractions.

Results

Subcellular distribution of aldehyde dehydrogenase activity

The purity and yield of the isolated subcellular fractions were evaluated by measurements of the cytochrome *a* content and the glucose-6-phosphatase and alcohol dehydrogenase activities of both the total homogenate and the various fractions. The mitochondrial and microsomal fractions were free from alcohol dehydrogenase activity. About 60—70% of the cytochrome *a* content of the original homogenate was recovered in the mitochondrial fraction. Cytoplasmic and microsomal fractions did not contain any measurable amounts of cytochrome *a*. The values for glucose-6-phosphatase activity found in the cytoplasmic, mitochondrial and microsomal fractions were about 10, 10 and 65% of the activity in the original homogenate, respectively. The distribution of aldehyde dehydrogenase activity was calculated, therefore, after correction for 60% yield in the mitochondrial and for 65% yield in the microsomal fraction. Thus the cytoplasmic fraction contained 10—15%, the mitochondrial fraction 45—50% and the microsomal fraction 35—45% of the total aldehyde dehydrogenase activity, when assayed with 6.0 mM propionaldehyde as the substrate (see Methods).

Purification

Cytoplasmic fraction (Fig. 1). DEAE-cellulose chromatography of the cytoplasmic fraction revealed two constantly separable unspecific aldehyde dehydrogenase peaks. The first one, referred here as cytoplasmic enzyme I was not bound to the column equilibrated with 10 mM sodium phosphate pH 7.4.

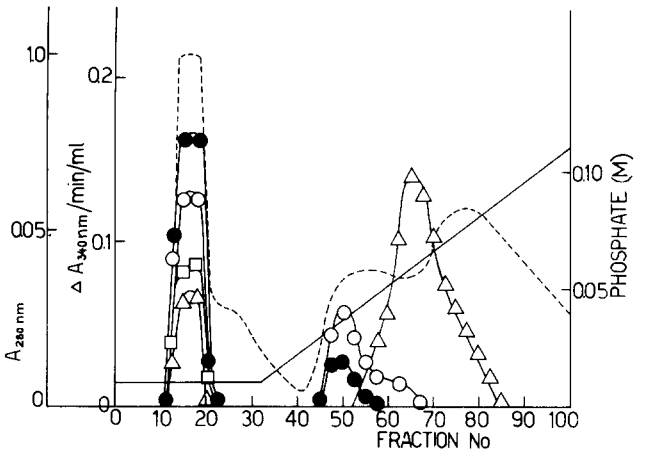

Fig. 1. DEAE-cellulose chromatography of rat liver cytoplasmic fraction. The experimental procedure and assay methods are described in the text. The fractions were analyzed for aldehyde dehydrogenase activity with 2 mM propionaldehyde (●), 1.0 mM glycolaldehyde (○), 18 mM D-glucuronolactone (□) or 0.5 mM betaine aldehyde (△) as substrates and for protein concentration (- - - - -, absorbance at 280 nm).

The same fractions contained also all of the alcohol and formaldehyde dehydrogenase activities present in the liver homogenate. During further purification with CM-cellulose chromatography these activities were removed from the aldehyde dehydrogenase preparation. The D-glucuronolactone dehydrogenase activity, which was also present only in the cytoplasmic fraction, was found in the same fractions as cytoplasmic enzyme I in all purification steps used.

The second cytoplasmic aldehyde dehydrogenase activity, cytoplasmic enzyme II, was eluted as a symmetrical peak of activity at about 50—60 mM phosphate concentration at pH 7.4. Aldehyde dehydrogenase activities, when measured either with propionaldehyde or glycolaldehyde as substrate were located in the same fractions. A third peak of activity, reacting only with betaine aldehyde was eluted immediately after the cytoplasmic enzyme II. It represents obviously the specific cytoplasmic betaine aldehyde dehydrogenase.

After the partial purification the activities of both cytoplasmic enzymes diminished very rapidly. The specific activity of the cytoplasmic enzyme I increased in the three purification steps used (DEAE- and CM-cellulose chromatographies and Sephadex G-200 gel chromatography) from 4.66 mU/mg protein to 40.2 mU/mg protein, corresponding to a 10-fold purification. The specific activity of the cytoplasmic enzyme II was 1.72 mU/mg protein after the one DEAE-cellulose chromatography used.

Mitochondrial fraction (Fig. 2a). In the DEAE-cellulose chromatography of the detergent-treated and dialysed mitochondrial fraction all aldehyde dehydrogenase activity was bound to the column equilibrated with 10 mM sodium phosphate at pH 7.4. The main part of the activity, when measured with 2 mM propionaldehyde as the substrate was eluted with 50—70 mM phosphate. The peak of activity was, however, not symmetrical and some activity was eluted from the column up to about 150 mM phosphate. When the activity was measured with 2 mM glycolaldehyde as substrate a symmetrical peak of activity

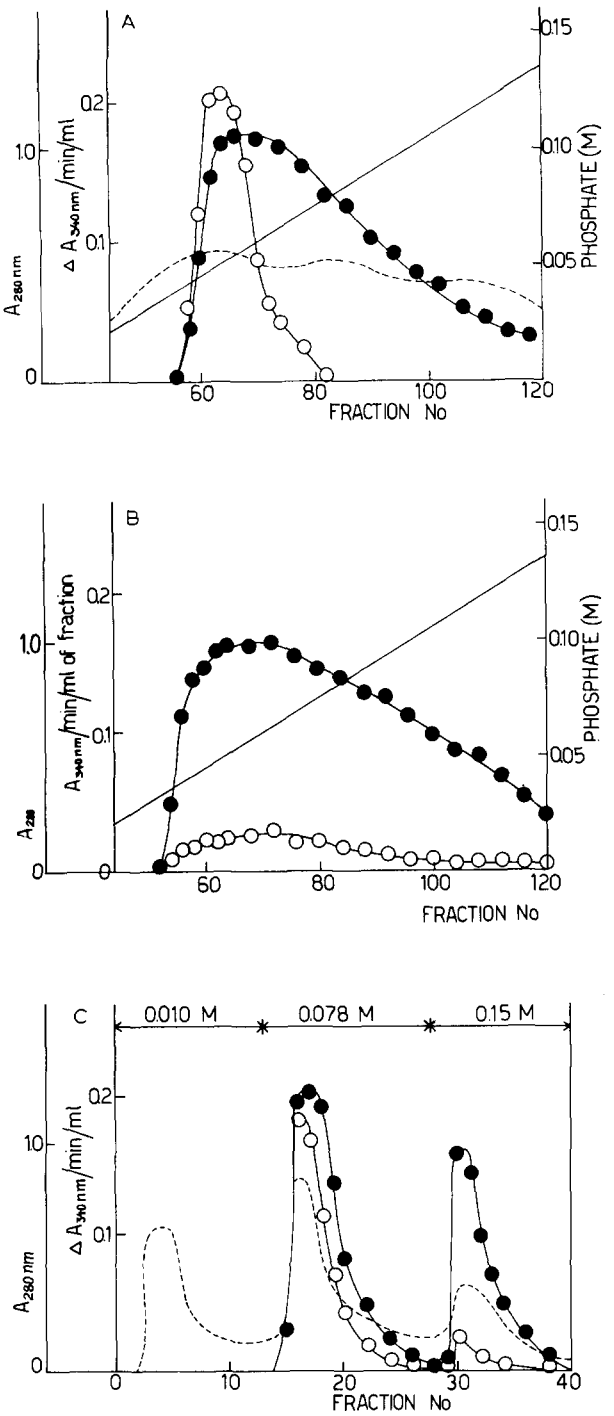

Fig. 2. DEAE-cellulose chromatography of rat liver (a) mitochondrial, (b) microsomal and (c) combined mitochondrial and microsomal fractions. A linear phosphate gradient was used for elution in (a) and in (b) as described in the text, in (c) there was a stepwise elution. Fractions were analyzed for aldehyde dehydrogenase activity with 2.0 mM propionaldehyde (•) or 1.0 mM glycolaldehyde (○) as substrates and for protein concentration (- - - - -, absorbance at 280 nm).

was obtained with no tailing. This main part of the activity reacting both with propionaldehyde and glycolaldehyde was designated mitochondrial enzyme I and the other part reacting only with propionaldehyde was designated mitochondrial enzyme II. The lack of a symmetrical constant elution pattern for mitochondrial enzyme II is probably due to particulate nature of this enzyme.

Microsomal fraction (Fig. 2b). The microsomal aldehyde dehydrogenase activity gave a similar elution pattern in DEAE-cellulose chromatography than the mitochondrial enzyme II. There was also no activity with 2 mM glycolaldehyde as substrate. The probable particulate nature of this activity could be demonstrated by centrifuging the detergent treated microsomal suspension in ultracentrifuge; after centrifugation for 4 h at 106 000 × *g* there was only one third of the activity remaining in the supernatant when compared to the activity present after one h centrifugation at the same speed.

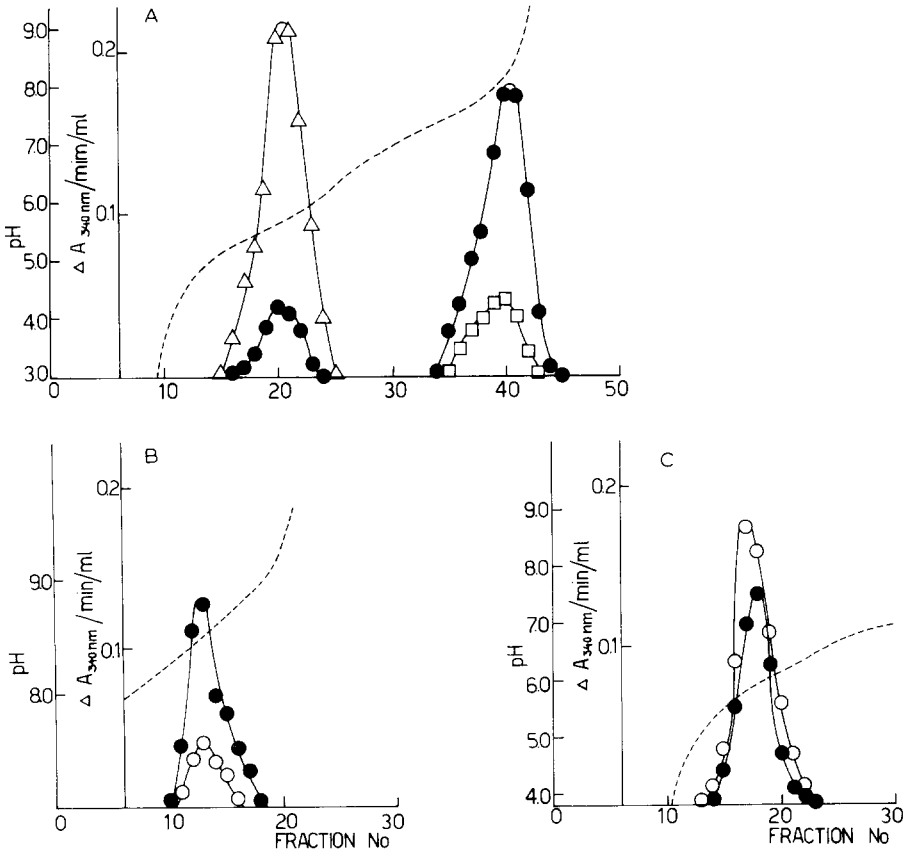

Fig. 3. Isoelectric focusing of rat liver (a) cytoplasmic fraction (b) cytoplasmic DEAE-fraction I and (c) cytoplasmic DEAE-fraction II. The experimental procedure and assay methods are described in the text. Fractions were analyzed for aldehyde dehydrogenase activity with 2.0 mM propionaldehyde (●), 1.0 mM glycolaldehyde (○), 18 mM D-glucuronolactone (□) and 0.5 mM betaine aldehyde (△) as substrates, and for pH (- - - - - -).

Isoelectric focusing

Two active peaks could be separated by isoelectric focusing of the soluble fraction, as was expected on the basis of the ion exchange chromatography (Fig. 3a). The isoelectric points of these fractions were 8.5 and 5.8 corresponding to the cytoplasmic enzymes I and II, respectively. If the partially purified cytoplasmic enzymes I and II were focused separately the same isoelectric points were obtained (Figs 3b and 3c). D-Glucuronolactone dehydrogenase activity was found in the same fractions as enzyme I. Betaine aldehyde dehydrogenase activity was present only in the peak focused at pH 5.8.

Isoelectric focusing of the mitochondrial fraction gave only one major peak with aldehyde dehydrogenase activity when measured with propionaldehyde or glycolaldehyde as substrates (Fig. 4a). The isoelectric point was about 5.6 for this activity, which corresponds to the mitochondrial enzyme I of the DEAE-cellulose chromatography. There was no activity peak corresponding to the mitochondrial enzyme II and no activity with betaine aldehyde.

The microsomal fraction gave by focusing a cloudy layer at about pH 4.0 of the gradient (Fig. 4b). A relatively low aldehyde dehydrogenase activity was observed at this area and no activity was found elsewhere in the gradient.

Substrate specificity

Because of the heterogeneity of the activity in the crude preparations only the partially purified enzyme preparations were investigated for their substrate specificity. The maximal velocities for various aldehydes were calculated on the basis of Lineweaver-Burk plots [24] with four or more different concentrations of aldehyde. The constant level of NAD was 1.33 mM, which is about 50-fold its K_m value. All of the studied fractions could oxidize a rather broad spectrum of both aliphatic and aromatic aldehydes (Table I). The microsomal enzyme and the mitochondrial enzyme II had similar substrate specificities and oxidized glycolaldehyde and formaldehyde only very slowly in contrast to other

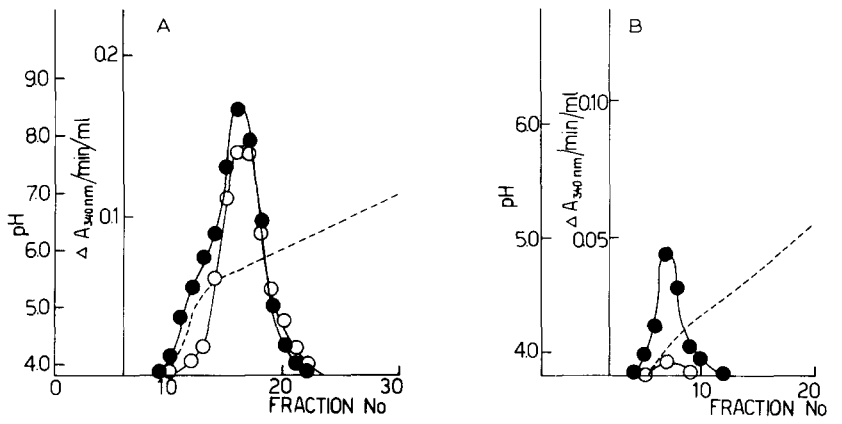

Fig. 4. Isoelectric focusing of rat liver (a) mitochondrial and (b) microsomal fractions. The experimental procedure and assay methods are described in the text. Fractions were analyzed for aldehyde dehydrogenase activity with 2.0 mM propionaldehyde (●) and 1.0 mM glycolaldehyde (○) as substrates and for pH (- - - - -).

TABLE I

SUBSTRATE SPECIFICITY OF RAT LIVER ALDEHYDE DEHYDROGENASES

Maximal velocities were determined from Lineweaver-Burk plots [33]. NAD^+ concentration was 1.33 mM. The results are expressed as relative activities. The activity with propionaldehyde is set equal to 1.0 as standard for each fraction.

Aldehyde	Cytoplasmic		Mitochondrial		Microsomal
	I	II	I	II	
Formaldehyde	0.7	0.8	0.8	0.0	0.0
Acetaldehyde	1.1	0.7	0.9	0.7	0.7
Propionaldehyde	1.0	1.0	1.0	1.0	1.0
Glycolaldehyde	0.6	1.2	0.9	0.2	0.1
D-Glyceraldehyde	0.3	1.0	0.6	0.7	0.8
Benzaldehyde	0.4	0.6	0.6	0.7	0.7
Anisaldehyde	0.3	0.4	0.4	0.7	0.8
Phenylacetaldehyde	1.0	1.6	1.2	1.3	1.3

fractions. Cytoplasmic enzyme I had a rather high activity with formaldehyde but a low one with aromatic aldehydes. Cytoplasmic enzyme II and mitochondrial enzyme I were very similar in their substrate specificities. D-Glucuronelactone was oxidized only by the cytoplasmic enzyme I. Betaine aldehyde was oxidized only by the cytoplasmic betaine aldehyde dehydrogenase and by the crude mitochondrial fraction. After purification of the mitochondrial fraction no activity with betaine aldehyde could be found in any fractions. The instability of mitochondrial betaine aldehyde dehydrogenase has been noted also by earlier authors [25].

K_m values

Determinations of apparent K_m values for unpurified cytoplasmic and mitochondrial fractions yielded two different values for propionaldehyde but not for the coenzyme NAD. One value was in the millimolar and the other in micromolar range. The microsomal fraction yielded one K_m value for propionaldehyde and it was in the millimolar range. K_m values for NAD of all crude subcellular fractions were of the same order of magnitude, 20—40 μM. For NADP a high K_m value in the millimolar range was observed both in the mitochondrial and microsomal fractions. The cytoplasmic fraction was inactive with NADP.

The partially purified enzyme preparations gave only one K_m value for acetaldehyde and propionaldehyde (Table II). All K_m values were lower for propionaldehyde than for acetaldehyde. They were not corrected for the effect of hydration of the aldehydes. K_m values for D-glucuronolactone of the cytoplasmic enzyme I was 5 mM. Activities of the partially purified enzymes with NADP as coenzyme were negligible even in the case of mitochondrial and microsomal fractions.

Betaine aldehyde dehydrogenase of the soluble fraction had an apparent K_m value of 140 μM (NAD 1.33 mM) for betaine aldehyde and 130 μM (betaine aldehyde 1 mM) for NAD. The crude mitochondrial fraction gave

TABLE II

APPARENT MICHAELIS CONSTANTS FOR PARTIALLY PURIFIED RAT LIVER ALDEHYDE DEHYDROGENASE FRACTIONS

In the determinations with variable aldehyde concentration NAD^+ was kept at 1.33 mM and in those with variable NAD^+ concentration the aldehyde concentration was 6 mM. I = cytoplasmic enzyme I, II = cytoplasmic enzyme II, III = mitochondrial enzyme I, IV = mitochondrial enzyme II, V = microsomal enzyme.

Substrate	K_m (μM)				
	I	II	III	IV	V
Acetaldehyde	1000	0.7	0.7	2000	2000
Propionaldehyde	200	0.3	0.4	500	500
D-Glucuronolactone	5000	—	—	—	—
NAD^+ (with propionaldehyde)	20	40	30	30	30

similar K_m values for betaine aldehyde dehydrogenase than the soluble fraction.

Effect of inhibitors

The effects of some potential inhibitors were studied with all of the isolated aldehyde dehydrogenase fractions. Propionaldehyde (6 mM) was used as substrate. Pyrazole and butyraldoxime were not inhibitory to any fraction at least up to 10 mM concentration. The results from experiments with chloral hydrate and disulfiram are presented in Table III. Chloral hydrate was a potent inhibitor of the cytoplasmic enzyme I. The other enzyme fractions needed 300–600-fold higher inhibitor concentrations for 50% inhibition. Disulfiram inhibited effectively all fractions.

Efficiency of different solubilization techniques

Different solubilization techniques of the mitochondrial and microsomal fractions were found to release the high K_m and low K_m activities in different ratios (Table IV). Triton X-100, digitonin and deoxycholate solubilized in the

TABLE III

EFFECT OF INHIBITORS ON ALDEHYDE DEHYDROGENASE ACTIVITY

The assay mixture contained 0.07 M sodium pyrophosphate pH 8.0, 1.33 mM NAD and 6 mM propionaldehyde. The inhibitors were added to the standard assay mixture from which pyrazole was omitted. Disulfiram was added dissolved in ethanol. The same amount of ethanol was added into controls and had no effect on aldehyde dehydrogenase activity.

Enzyme fraction	Inhibitor concentration for 50% inhibition (M)	
	Chloral hydrate	Disulfiram
Cytoplasmic I	$5 \cdot 10^{-5}$	$4 \cdot 10^{-6}$
Cytoplasmic II	$3 \cdot 10^{-2}$	$2.5 \cdot 10^{-5}$
Mitochondrial I	$2 \cdot 10^{-2}$	$3 \cdot 10^{-5}$
Mitochondrial II	$1.5 \cdot 10^{-2}$	$7 \cdot 10^{-6}$
Microsomal	$1.5 \cdot 10^{-2}$	$5 \cdot 10^{-6}$

TABLE IV

EFFECT OF DIFFERENT SOLUBILIZATION METHODS ON THE RELEASE OF ALDEHYDE DEHYDROGENASE ACTIVITY FROM MITOCHONDRIAL AND MICROSOMAL FRACTIONS OF RAT LIVER

The results are expressed as per cent of the activity of the original suspension found in the supernatant after centrifugation for 60 min at 100000 × *g*.

Treatment	9 mM propionaldehyde	4.5 mM propionaldehyde	0.09 mM propionaldehyde
Mitochondria			
Freezing and thawing	55	57	83
Digitonin (0.22 mg/mg protein)	84	86	87
Deoxycholate (0.20 mg/mg protein)	83	84	84
Triton X-100 (0.70 mg/mg protein)	91	90	86
Microsomal fraction			
Freezing and thawing	4		
Digitonin (0.22 mg/mg protein)	59		
Deoxycholate (0.20 mg/mg protein)	90		
Triton X-100 (0.70 mg/mg protein)	89		

concentrations used effectively both types of activity. The freezing and thawing method was much less effective in the case of the high K_m activity than the detergents and released mostly low K_m activity.

Discussion

Rat liver unspecific aldehyde dehydrogenase activity is apparently due to several different enzymes, which are present in the cytoplasmic, mitochondrial and microsomal subcellular fractions. The purpose of this work has been to separate and characterize the main types of this enzyme by partial purification starting from isolated subcellular fractions. The considerable lability of these activities in rat liver together with binding to mitochondrial and microsomal membranes makes extensive purification of these enzymes difficult. Our results are in good agreement with those of Tottmar et al. [10] demonstrating the presence of considerable membrane bound aldehyde dehydrogenase activity with high K_m for aldehyde in the mitochondrial and microsomal fractions in addition to the low K_m-activity in mitochondria. The relative activity of the cytoplasmic fraction obtained in the present investigation was, however, markedly higher than that found by Tottmar et al. [10,11] accounting to about 10% of the total aldehyde dehydrogenase activity when measured at millimolar aldehyde concentration.

The cytoplasmic fraction of rat liver as isolated in the present study contains two different unspecific aldehyde dehydrogenases and the specific betaine aldehyde and formaldehyde dehydrogenases. The cytoplasmic enzyme I, which is not retained by the DEAE-cellulose column at the conditions used, is similar to the cytoplasmic enzyme I of Shum and Blair [13] and also to the inducible aldehyde dehydrogenase activity described by Deitrich [8]. All of the cytoplasmic D-glucuronolactone dehydrogenase activity is found in this

fraction even when purified further by CM-cellulose and Sephadex G-200 chromatographies. This gives support to the present suggestions that the D-glucuronolactone dehydrogenase actually is identical with a nonspecific aldehyde dehydrogenase [26,27]. The K_m values of the cytoplasmic enzyme I for propion- and acetaldehydes and also for D-glucuronolactone are in the millimolar range. The isoelectric point 8.4 for this enzyme is much more alkaline than those of the other aldehyde dehydrogenase fractions and there are also clear differences in the relative substrate specificity. The cytoplasmic enzyme I evidently accounts for most of the unspecific aldehyde dehydrogenase activity of rat liver cytoplasmic fraction, when measured at millimolar concentration of aldehyde.

The cytoplasmic enzyme II has different properties from the enzyme I and resembles very much the low K_m enzyme from mitochondria. They have similar chromatographic behaviour, isoelectric points and K_m values for propion- and acetaldehyde (in the millimolar range). Their substrate specificities are also very much alike. The relative activity of enzyme II in the cytoplasmic fraction varied moderately from experiment to experiment and was apparently influenced by slight variations in the rapidity of the subcellular fractionation. It is possible therefore that it is not a true cytoplasmic enzyme but is released from the mitochondria during homogenization. A variability in the mitochondrial leakage can also be one explanation for the differing results in the literature concerning the cytoplasmic aldehyde dehydrogenase activity.

There is some discrepancy in the K_m values of the rat liver cytoplasmic aldehyde dehydrogenases reported earlier. Only high K_m enzymes have been found by Shum and Blair [12] and Tottmar [10,11], but Marjanen [28] has reported an intermediate K_m for acetaldehyde (30 μM) for his preparation which chromatographically resembled the enzyme I described in this report. There is no evident explanation for these differences.

The existence of two types of aldehyde dehydrogenase with markedly different K_m values for aldehydes in rat liver mitochondria has been reported earlier [10,11,29]. Based mainly on kinetic data Tottmar [11] suggested the existence of at least two enzymes, the one with K_m located in the matrix and the other with high K_m in the outer membrane fraction of mitochondria. Using DEAE-cellulose chromatography we have isolated these enzymes practically free from each other. The low K_m activity was readily released from mitochondria by freezing and thawing but the high K_m activity required addition of detergents for solubilization and even then the enzyme was apparently still partially particle bound.

A specific betaine aldehyde dehydrogenase was present in the cytoplasmic fraction and it had similar properties as has been described earlier [17,25,30]. The incapability of isoelectric focusing to separate this activity from the cytoplasmic aldehyde dehydrogenase II was probably due to the relatively large pH scale used and the close isoelectric points of these two enzyme proteins. It has been claimed that the mitochondrial betaine aldehyde oxidation is due to the unspecific aldehyde dehydrogenase [31]. The present results suggest that there is, however, a specific enzyme also in the mitochondrial fraction which is similar to the cytoplasmic enzyme, although its lability [25] prevented its partial purification.

The present results confirm those of Tottmar et al. [10,11] in showing that there is also considerable aldehyde dehydrogenase activity in the microsomal fraction of rat liver. It has a high K_m for aldehydes in the millimolar range, which is apparently one possible reason why in the earlier investigations only very low relative activities were found in the liver microsomal fraction [2,6]. According to the present results this activity may account for up to 40 per cent of the total aldehyde dehydrogenase activity when measured at a high aldehyde concentration. It has properties which are similar to those of the mitochondrial high K_m enzyme. It is tightly membrane bound and detergents are needed to obtain an apparent solubilization. Its substrate specificity and behaviour in ion-exchange chromatography are also similar to mitochondrial enzyme II. It is, however, unlikely that the mitochondrial high K_m activity is due solely to microsomal contamination, if the results obtained with marker enzymes [10] are taken into consideration.

The mitochondrial low K_m aldehyde dehydrogenase is apparently the main enzyme responsible for the oxidation of the relatively low acetaldehyde concentrations formed during metabolism of ethanol [8,11,32]. The role of the high K_m enzymes and especially that of the microsomal enzyme in the metabolism of aldehydes remains to be elucidated. The high K_m for acetaldehyde does not suggest a significant role for these enzymes in metabolism of ethanol. Alcohol preferring genetically selected rat strains have, however, significantly higher microsomal and mitochondrial high K_m aldehyde dehydrogenase activities in liver than alcohol avoiding strains without differences in the mitochondrial low K_m or cytoplasmic activities [33]. The microsomal aldehyde dehydrogenase activity seems not to be induced by treatment with phenobarbital like the cytoplasmic activity (Koivula, unpublished). Further work on the purification and properties of the microsomal and cytoplasmic high K_m enzymes is in progress.

Acknowledgements

This investigation was supported by grants from the Sigrid Jusélius Foundation, the National Research Council for Medical Sciences and the Foundation for Studies on Alcohol, Finland. We are indebted to Mrs Eija Haasanen and Mrs Ritva Leponiemi for the skilful technical assistance.

References

1 Büttner, H. (1965) Biochem. Z. 341, 300–314
2 Deitrich, R.A. (1966) Biochem. Pharmacol. 15, 1911–1922
3 Lundquist, F. (1971) Biological Basis of Alcoholism (Israel, Y. and Mardones, J.J., eds), pp. 1–55, Wiley-Interscience, New York
4 Hedlund, S.G. and Kiessling, K.-H. (1969) Acta Pharmacol. Toxicol. 27, 381–396
5 Hassinen, I.E., Ylikahri, R.H. and Kähönen, M.T. (1970) Ann. Med. Exp. Biol. Fenn. 48, 176–183
6 Marjanen, L. (1972) Biochem. J. 127, 633–639
7 Grunnet, N. (1973) Eur. J. Biochem. 35, 236–243
8 Deitrich, R.A. (1971) Science 173, 334–336
9 Smith, L. and Packer, L. (1972) Arch. Biochem. Biophys. 148, 270–276
10 Tottmar, S.O.C., Pettersson, H. and Kiessling, K.-H. (1973) Biochem. J. 135, 577–586
11 Tottmar, O. (1974) Acta Universit. Upsal. 289, 1–45
12 Koivula, T. and Koivusalo, M. (1973) 9th Int. Congr. Biochem. Abstr. 2d15, Stockholm

13 Shum, G.T. and Blair, A.H. (1972) Can. J. Biochem. 50, 741—748
14 Marselos, M. and Hänninen, O. (1974) Biochem. Pharmacol. 23, 1457—1466
15 Deitrich, R.A., Collins, A.C. and Erwin, V.G. (1972) J. Biol. Chem. 247, 7232—7236
16 Bergel, F., Cohen, A. and Hindley, N.C. (1950) J. Chem. Soc. 1439—1443
17 Rothschild, H.A. and Barron, E.S.G. (1954) J. Biol. Chem. 209, 511—523
18 Horecker, B. and Kornberg, A. (1948) J. Biol. Chem. 175, 385—390
19 Harper, A.E. (1963) Methods of Enzymatic Analysis (Bergmeyer, H.-U., ed.), pp. 788—792, Verlag Chemie, Weinheim
20 Schollmeyer, P. and Klingenberg, M. (1962) Biochem. Z. 335, 426—439
21 Koivusalo, M. and Uotila, L. (1974) Anal. Biochem. 59, 34—45
22 Gornall, A.G., Bardawill, C.J. and David, M.M. (1949) J. Biol. Chem. 177, 751—766.
23 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
24 Lineweaver, H. and Burk, D. (1934) J. Am. Chem. Soc. 56, 658—666
25 Wilken, D.R., McMacken, M.L. and Rodriquez, A. (1970) Biochim. Biophys. Acta 216, 305—317
26 Aarts, E.M. and Hinnen-Bouwmans, C. (1972) Biochim. Biophys. Acta 268, 21—29
27 Tonkes, P.G. and Marsh, C.A. (1973) Aust. J. Biol. Sci. 26, 839—849
28 Marjanen, L.A. (1973) Biochim. Biophys. Acta 327, 238—246
29 Deitrich, R.A. and Siew, C. (1974) Alcohol and Aldehyde Metabolizing Systems (Thurman, R.G., Yonetani, T., Williamson, J.R. and Chance, B., eds), pp. 125—135, Academic Press, New York
30 Goldberg, A.M. and McCaman, R.E. (1968) Biochim. Biophys. Acta 167, 186—189
31 Glenn, J.L. and Vanko, M. (1959) Arch. Biochem. Biophys. 82, 145—152
32 Parrilla, R., Ohkawa, K., Lindros, K.O., Zimmerman, U.-J.P., Kobayashi, K. and Williamson, J.R. (1974) J. Biol. Chem. 249, 4926—4933
33 Koivula, T., Koivusalo, M. and Lindros, K.O. (1975) Biochem. Pharmacol., in press