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SUBCELLULAR DISTRIBUTION AND PROPERTIES OF A CLOFIBRATE-INDUCED ALDEHYDE DEHYDROGENASE FROM RAT LIVER

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Abstract—The influence of hypolipidemic drug clofibrate on the activity of aldehyde dehydrogenase with different substrates was studied in subcellular fractions of rat liver homogenate. It was shown that under the action of clofibrate the content of the enzyme was increased 2–3-fold in purified peroxisomal fraction as well as in microsomes and mitochondria. No difference was found in the cytoplasmic fraction. Partial purification of clofibrate-induced aldehyde dehydrogenase from microsomes was undertaken. The enzyme is apparently membrane-bound. It has a molecular weight of 187,000 and a subunit size of 47,000, indicating that the molecule is a tetramer. An induced aldehyde dehydrogenase is active with several aliphatic and aromatic aldehydes but not with formaldehyde and glyceraldehyde. The enzyme has K_m-values in the millimolar range for acetaldehyde, propionaldehyde, benzaldehyde and phenylacetaldehyde and in the micromolar range for nonanal. Both NAD and NADP serve as coenzymes for the purified aldehyde dehydrogenase. According to subtrate specificity, kinetic and molecular properties clofibrate-induced aldehyde dehydrogenase appears to be identical to normal liver microsomal enzyme.

Administration of the hypolipidemic drug clofibrate has been shown to induce a marked proliferation of hepatic peroxisomes. The increase in peroxisomes is accompanied by a significant elevation of some peroxisome-associated enzymes: catalase, carnitine acetyltransferase, and peroxisomal fatty acyl-CoA oxidizing system [1, 2]. Recent studies have shown that clofibrate and other hypolipidemic agents with hepatic peroxisome proliferative properties induce hepatocellular carcinomas in rats and mice [3].

In rat liver several isozymes of aldehyde dehydrogenase (aldehyde:NAD(P) oxidoreductase, EC 1.2.1.3 and 1.2.1.5) with different molecular and kinetic properties and different subcellular distribution can be identified [4–6]. Some xenobiotics and chemical carcinogens increase the total aldehyde dehydrogenase activity due to appearance of at least two new cytosolic isozymes not detectable in normal liver. These isoenzymes consistently differ by a number of physical and functional properties from normal-liver aldehyde dehydrogenase [7, 8].

We have described earlier that the activity of peroxisomal and microsomal aldehyde dehydrogenase was significantly increased in rat liver following clofibrate administration [9]. In this report we present data on the subcellular distribution of rat liver aldehyde dehydrogenase activity after treatment with clofibrate. The induced aldehyde dehydrogenase was partially purified and characterized with an aim to clarify its relation to the enzyme normally present in rat liver microsomal fraction.

MATERIALS AND METHODS

Chemicals. Acetaldehyde, nonanal, glutaraldehyde and pyrazole were from the Aldrich Chemical Co. (Milwaukee, WI). NAD^+, NADH, EDTA, glucose-6-phosphate, cytochrome *c*, bovine serum albumin, 2-mercaptoethanol, rotenon, nitro blue tetrazolium, phenazine methosulphate, tetraethylthiuram disulphide (disulphiram), benzaldehyde, substituted benzaldehydes, phenylacetaldehyde, molecular weight standards were from the Sigma Chemical Co. (St. Louis, MO). Dithiothreitol (DTT), 5,5′-dithiobis-(2-nitrobenzoic) acid (DTNB), Tris, Triton X-100, sodium dodecyl sulphate, propionaldehyde and clofibrate (ethyl-α-p-chlorophenoxyisobutyrate) were from the Serva Feinbiochemica (Heidelberg, F.R.G.). DEAE-Cellulose DE-52 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden) and Ultragel AcA-22 or AcA-34 from LKB Instruments Inc. Reagents for polyacrylamide gel electrophoresis and sodium deoxycholate were from Reanal. Acetaldehyde and propionaldehyde were redistilled before use. All other chemicals were of reagent grade.

Tissue fractionation. Male Wistar rats (200–250 g) were injected i.p. with saline (control) or clofibrate in a dose of 400 mg/kg daily for 10 days. Animals were anesthetized with light ether and killed by decapitation after 16–18 hr starvation. The livers were immediately removed and homogenized with a Potter–Elvehjem type Teflon-glass homogenizer in 0.25 M sucrose containing 20 mM Tris–acetate, pH 7.2 and 1 mM DTT (isolation medium) to make a 10% (w/v) homogenate. The subcellular fractionation was performed by differential centrifugation as described [10, 11] with slight modifications. The homogenate was centrifuged for 10 min at 2500 *g* (max). The pellet was resuspended in the isolation medium and centrifuged for 10 min at 700 *g*. The final pellet obtained was designated as the nuclear fraction. The combined supernatants were centrifuged for 10 min at 4200 *g* and the mitochondrial

pellet was gently resuspended and recentrifuged as before. The combined postmitochondrial supernatant was centrifuged for 30 min at 5600 *g*. The pellet obtained was designated as the lysosomal fraction. The supernatant was centrifuged for 60 min at 100,000 *g*. The final supernatant was used as the soluble fraction and the pellet which contained a microsomal fraction was suspended in isolation medium. In some experiments peroxisomes and mitochondria were fractionated by isopycnic sucrose density gradient centrifugation of the lysosomal fraction [9, 11]. Marker enzyme assays for the various subcellular fractions were performed as described [9, 12]. Catalase (EC 1.11.1.6) and urate oxidase (EC 1.7.3.3) were assayed as peroxisomal markers; glutamate dehydrogenase (EC 1.4.1.2) as mitochondrial; acid phosphatase (EC 3.1.3.2) as lysosomal and glucose-6-phosphatase (EC 3.1.3.9) as microsomal. The activity of the enzymes (except for catalase) was expressed in nmoles/min. The catalase activity was calculated as described earlier [11].

Enzyme purification. The microsomal fraction from three rat livers was used for partial purification of aldehyde dehydrogenase. Microsomes were resuspended in 0.1 M Tris–HCl buffer (pH 7.6) containing 1 mM EDTA and 1 mM DTT. On the first step the enzyme was solubilized with sodium deoxycholate and Triton X-100 in a final concentration of 0.1 and 0.3% (w/v) respectively. The resulting preparation was left to stand at 4° for 30 min and then centrifuged for 60 min at 120,000 *g*. Clear supernatant was fractionated by ammonium sulphate between 40 and 70% saturation. The relevant precipitate was dissolved in a minimal volume of 0.05 M Tris–HCl buffer (pH 7.6) containing 20% (w/v) glycerol, 1 mM EDTA, 1 mM DTT and 0.2% Triton X-100. The resulting solution was applied to Ultragel AcA-34 column (1.6 × 73 cm) equilibrated with the same buffer. The active fractions were pooled and applied to DEAE-cellulose DE-52 column (2.6 × 6.1 cm) equilibrated with the buffer indicated above. The bulk of aldehyde dehydrogenase activity did not bind to the resin under such conditions and was eluted with the starting buffer. Pooled active fractions were used for further investigations. In some experiments the affinity of the enzyme to DEAE-cellulose was studied under conditions similar to those earlier described [4]. In this case the enzyme solubilized from microsomes or its precipitate after ammonium sulphate fractionation was dialysed overnight against 20 vol. of 10 mM Tris–HCl, pH 7.6, containing 1 mM EDTA and 1 mM DTT, with two changes of buffer. The obtained preparation was applied to DEAE-cellulose DE-52 column (2.6 × 10.5 cm) equilibrated with the dialysis buffer. A linear concentration gradient from 50 to 500 mM NaCl (with or without 0.2% Triton X-100) was then started and the activity of aldehyde dehydrogenase with different substrates was assayed in the effluent fractions.

Molecular weight determination. The molecular weight of the microsomal aldehyde dehydrogenase was estimated by gel filtration on Ultragel AcA-34 column (1.6 × 73 cm) equilibrated with 50 mM Tris–HCl buffer (pH 7.6) containing 1 mM EDTA and 1 mM DTT. In some experiments the elution buffer contained 0.2% Triton X-100. The detergent did not affect the aldehyde dehydrogenase mobility. The following standards were used for calibration of the column: catalase, lactate dehydrogenase (EC 1.1.1.27), bovine serum albumin, ovalbumin, and tripsin inhibitor from soybean. A linear sucrose gradient ultracentrifugation technique according to Martin and Ames [13] was used to determine the sedimentation constants using catalase, lactate dehydrogenase, and malate dehydrogenase (EC 1.1.1.37) as standard proteins. The sucrose concentration gradient from 5 to 30% (w/v) was formed in 50 mM Tris–HCl buffer (pH 8.0), 1 mM EDTA, 1 mM DTT and 0.2% sodium deoxycholate. Bovine serum albumin, ovalbumin and tripsin inhibitor were determined by measuring their absorbance at 280 nm or by Lowry's method, while the other marker proteins were determined enzymatically.

Analytical gel electrophoresis. Polyacrylamide gel electrophoresis was performed at 0–4° under alkaline conditions [4]. In some experiments electrophoresis was performed after previous treatment of the samples with 0.3% Triton X-100 and 4 M urea to prevent the aggregation of solubilized aldehyde dehydrogenase. Enzyme activity was detected directly by incubating the gels in 50 mM sodium phosphate buffer (pH 7.5) containing 5 mM aldehydes, 2 mM NAD^+, 5 mM nitro blue tetrazolium and 0.13 mM phenazine methosulfate at 37° in the dark. Electrophoresis in the presence of sodium dodecyl sulphate was performed according to Weber and Osborn [15] using 10% polyacrylamide gels. For calibration of the gels the molecular weight standard proteins from Sigma Chemical Co were used. Gels were fixed and stained with Coomassie Brilliant Blue R-250.

Enzyme assay. Aldehyde dehydrogenase activity was measured at 37° by following NAD^+ reduction at 340 nm (Gilford, model 250). According to [16] the standard assay mixture (1 ml) contained 50 mM sodium pyrophosphate buffer (pH 9.0), 0.5 mM NAD^+ (2.5 mM $NADP^+$) and 10–50 μl of enzyme. Pyrazole (0.1 mM final concentration) and 2 μM rotenone were included to inhibit alcohol dehydrogenase and NADH oxidase respectively. The reaction was started by addition of aldehyde. Blank reactions without substrate and with certain aldehyde without enzyme were followed simultaneously. One unit of activity is defined as the amount of enzyme which catalysed the formation of 1 nmole of NADH per min under the above conditions. Effect of some inhibitors or activators on the partially purified microsomal aldehyde dehydrogenase was determined by mixing 50 μl of the effector stock solution with 0.9 ml of assay mixture containing sample, but no substrate. After a 5-min preincubation (37°), the reaction was initiated by the addition of 50 μl of substrate solution. For these experiments DTT was removed from enzyme solutions by dialysis against appropriate buffers for 14–16 hr. Disulphiram was added dissolved in methanol (10 μl). The same amount of methanol was added into controls and had no effect on aldehyde dehydrogenase activity.

Protein concentration was determined by the method of Lowry *et al.* [17] with bovine serum albumin as the standard.

Results are expressed as means ± S.D. The stat-

Table 1. Aldehyde dehydrogenase and marker enzymes activities in subcellular fractions of (a) the normal rat liver and (b) the clofibrate-treated rat liver

Enzyme	Activity (μmoles/min per g liver wet weight)					
	Homogenate	Fractions Nuclear	Mitochondrial	Lysosomal	Microsomal	Cytoplasmic
(a)						
Catalase	29.50 ± 0.29	1.84 ± 0.36	5.35 ± 0.65	4.90 ± 0.41	0.99 ± 0.21	16.34 ± 0.65
Urate oxidase	1.84 ± 0.15	0.39 ± 0.09	0.69 ± 0.08	0.51 ± 0.07	0.19 ± 0.04	0.04 ± 0.01
Glutamate dehydrogenase	154.0 ± 8.0	64.4 ± 10.3	78.6 ± 11.2	3.2 ± 0.8	4.0 ± 0.7	4.2 ± 1.0
Glucose-6-phosphatase	8.71 ± 0.92	2.11 ± 0.22	1.52 ± 0.18	1.28 ± 0.17	3.42 ± 0.25	0.35 ± 0.07
Acid phosphatase	7.86 ± 0.93	0.95 ± 0.09	3.15 ± 0.17	2.06 ± 0.16	1.17 ± 0.07	0.81 ± 0.13
Aldehyde dehydrogenase						
Acetaldehyde NAD^+						
High K_m	3.29 ± 0.27	0.76 ± 0.04	1.27 ± 0.06	0.21 ± 0.02	1.25 ± 0.04	0.15 ± 0.04
Low K_m	0.59 ± 0.03	0.18 ± 0.02	0.32 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.05 ± 0.01
Benzaldehyde NAD^+	1.36 ± 0.13	0.25 ± 0.03	0.44 ± 0.06	0.09 ± 0.02	0.84 ± 0.05	0.03 ± 0.01
Propionaldehyde $NADP^+$	1.20 ± 0.08	0.14 ± 0.03	0.34 ± 0.03	0.06 ± 0.01	0.49 ± 0.07	0.10 ± 0.03
Protein (mg/g liver)	167.6 ± 6.0	38.0 ± 0.7	45.3 ± 0.3	7.2 ± 0.5	17.3 ± 0.7	66.0 ± 4.7
(b)						
Catalase	47.06 ± 1.78	3.81 ± 0.46	7.23 ± 0.62	4.29 ± 0.67	0.79 ± 0.21	30.60 ± 1.92
Urate oxidase	2.53 ± 0.24	0.61 ± 0.10	1.18 ± 0.06	0.49 ± 0.04	0.08 ± 0.02	0.02 ± 0.01
Glutamate dehydrogenase	139.3 ± 6.9	53.0 ± 3.4	83.3 ± 9.5	3.9 ± 0.9	3.7 ± 1.0	4.5 ± 1.7
Glucose-6-phosphatase	7.29 ± 1.31	1.24 ± 0.33	1.62 ± 0.23	1.49 ± 0.07	2.95 ± 0.07	0.35 ± 0.09
Acid phosphatase	8.75 ± 0.45	1.44 ± 0.11	3.12 ± 0.45	2.11 ± 0.37	0.82 ± 0.08	1.32 ± 0.11
Aldehyde dehydrogenase						
Acetaldehyde NAD^+						
High K_m	5.35 ± 0.28*†	0.72 ± 0.07	1.87 ± 0.20*	0.33 ± 0.01†	1.83 ± 0.19*	0.16 ± 0.06
Low K_m	0.58 ± 0.04	0.15 ± 0.01	0.31 ± 0.02	0.02 ± 0.01	0.04 ± 0.01	0.06 ± 0.01
Benzaldehyde NAD^+	2.26 ± 0.25*	0.32 ± 0.03	0.86 ± 0.10*	0.16 ± 0.01†	1.19 ± 0.12*	0.04 ± 0.02
Propionaldehyde $NADP^+$	1.87 ± 0.21*	0.18 ± 0.03	0.75 ± 0.12*	0.11 ± 0.03*	0.84 ± 0.11*	0.08 ± 0.02
Protein (mg/g liver)	188.8 ± 4.5	34.5 ± 3.2	58.7 ± 7.4	9.9 ± 0.3	16.3 ± 1.1	70.0 ± 2.1

Subcellular fractions were obtained by differential centrifugation of homogenate. Aldehyde dehydrogenase was assayed with 5 mM aldehyde (high K_m activity for acetaldehyde) and 0.5 mM NAD^+ (2.5 mM $NADP^+$). Low K_m activity was measured with 0.05 mM acetaldehyde. Catalase activity was expressed as described [11]. Each value represents means ± S.D., N = 4. * $P < 0.05$, † $P < 0.01$ for difference between aldehyde dehydrogenase activities in clofibrate-treated and control groups.

Table 2. Aldehyde dehydrogenase activity in peroxisomes isolated from normal and clofibrate-treated rats

Enzyme activity or protein concentration in peroxisomal fraction	Control rats	Clofibrate-treated rats
Total activity (nmoles/min per ml fraction)	17.3 ± 4.2	55.1 ± 4.9*
Specific activity (nmoles/min per mg protein)	34.5 ± 5.8	37.0 ± 4.3
Protein concentration (mg/ml fraction)	0.54 ± 0.10	1.58 ± 0.31†

Peroxisomes were isolated by isopycnic subfractionation of lysosomal fraction in sucrose gradient. Aldehyde dehydrogenase was assayed with 5 mM acetaldehyde and 0.5 mM NAD^+. Values represent means ± S.D.. N = 6. * $P < 0.01$; † $P < 0.05$.

istical comparisons between the groups were made with Student's *t*-test.

RESULTS

Administration of clofibrate to male rats caused an elevation of the total aldehyde dehydrogenase activity in liver homogenate as a result of significant increase in the activity of isozyme with high K_m for acetaldehyde (Table 1). When the acetaldehyde concentration was lowered from 5 mM to 0.05 mM the activities were similar in both groups. Using benzaldehyde (5 mM; NAD^+) and propionaldehyde (5 mM; $NADP^+$) as substrates also demonstrated a 1.5- and 1.7-fold induction of activity respectively in homogenate from clofibrate-treated rats compared to their littermates. Differential centrifugation of homogenate showed the elevation of the total aldehyde dehydrogenase activity (units/g liver wet weight) in mitochondria and in lysosomal fraction enriched with peroxisomes and lysosomes. In addition, significant increase in aldehyde dehydrogenase content was observed in microsomes. The data on the distribution of the peroxisomal marker urate oxidase demonstrate that microsomal fraction contains 3–10% of the total amount of peroxisomes in liver cells (Table 1a, b). At the same time, the recovery of aldehyde dehydrogenase with a high K_m for acetaldehyde in microsomal fraction reached 40% with both normal and clofibrate-induced rat livers. The specific activity of this enzyme in microsomes is 2–3 times higher than in purified peroxisomal fraction (Tables 1 and 2). These results suggest that elevation by clofibrate of the aldehyde dehydrogenase activity in microsomes cannot be accounted for by peroxisomal contamination. In contrast, mitochondrial fraction remains significantly contaminated with peroxisomes especially following administration of clofibrate when the relative amounts of the peroxisomal impurities increase. In addition, a large part of the microsomal marker glucose-6-phosphatase also resides in mitochondria (Table 1). Therefore, the differential centrifugation data does not exclude the possibility that the induction of aldehyde dehydrogenase observed in mitochondria can be attributed to microsomal and peroxisomal contamination. There were no differences between the control and clofibrate-treated rats in the aldehyde dehydrogenase activity of nuclear or cytoplasmic fractions with all the aldehydes tested (Table 1).

Isopycnic centrifugation of the lysosomal fraction revealed an increase in the total aldehyde dehydrogenase activity in purified peroxisomes and mito-

Table 3. Substrate specificity of microsomal aldehyde dehydrogenase

Aldehyde and coenzyme	Microsomes				Partially purified enzyme*
	Control	Clofibrate	Control	Clofibrate	
Experiment I					
Propionaldehyde NAD^+	64.1 ± 1.2	128.1 ± 18.0	100	100	100
$NADP^+$	20.5 ± 1.0	42.6 ± 5.4	31.9	33.1	26.3
Acetaldehyde NAD^+	50.5 ± 3.2	99.2 ± 11.6	78.8	77.5	89.5
Benzaldehyde NAD^+	50.3 ± 3.3	99.7 ± 15.1	78.1	77.1	80.4
$NADP^+$	8.4 ± 0.8	19.5 ± 1.4	13.1	15.2	12.4
Glutaraldehyde NAD^+	15.2 ± 1.0	26.2 ± 3.5	23.7	20.5	15.6
Experiment II					
Propionaldehyde NAD^+	54.4 ± 10.0	87.4 ± 1.3	100	100	100
Acetaldehyde NAD^+	39.7 ± 9.3	66.2 ± 3.5	73.0	75.7	76.4
Phenylacetaldehyde NAD^+	58.8 ± 11.9	104.4 ± 3.9	108.1	119.5	137.5
Nonanal NAD^+	159.4 ± 32.1	233.5 ± 16.3	294.4	267.2	305.9

Aldehyde dehydrogenase activity was determined using 5 mM aldehyde, except for nonanal (0.5 mM) and phenylacetaldehyde (0.5 mM), and 0.5 mM NAD^+ (2.5 mM $NADP^+$). Specific activity is defined as nmoles/min per mg of microsomal protein, N = 6. For relative activities the activity with propionaldehyde (NAD^+) is set equal to 100 as standard for each fraction.

* Partially purified enzyme from DEAE-cellulose step was used (clofibrate-treated rats).

chondria under the action of clofibrate. In peroxisomal fraction total activity was elevated threefold though specific activity of aldehyde dehydrogenase (units/mg fraction protein) was not changed (Table 2). Such a difference can be attributed to a significant increment in peroxisomal protein recovery as a result of a clofibrate-induced elevation in the number of peroxisomes in liver cells [1–3]. According to enzymatic analysis performed earlier [9], peroxisomal fraction contained insignificant quantities of mitochondria or microsomes. The content of peroxisomal protein in the purified fractions from intact or clofibrate-treated rat livers amounted to 75–80% [2, 9, 11]. The method used in this study for the isolation of peroxisomes does not enable us to obtain highly purified mitochondria. This fraction still remains contaminated with peroxisomes and other subcellular particles [9]. The latter makes the interpretation of results on the elevation of aldehyde dehydrogenase activity in mitochondria difficult. In this case increase in the total enzyme activity can also be associated with the induction of peroxisomal isozyme.

The highest specific activity of the clofibrate-induced aldehyde dehydrogenase with high K_m for acetaldehyde was observed in microsomes. At the same time this fraction contained only negligible amounts of low-K_m activity which was likely to represent a mitochondrial contamination (Table 1). Drug treatment leads to approximately 1.5–2.0-fold increase in microsomal aldehyde dehydrogenase specific activity with different substrates (Table 3). With an aim to study properties of the clofibrate-induced aldehyde dehydrogenase partial purification of enzyme from microsomal fraction was taken. Isolation procedure included enzyme solubilization, ammonium sulphate fractionation, gel filtration and ion-exchange chromatography. Isolation yielded 18–20-fold increase in aldehyde dehydrogenase specific activity in comparison with the activity in microsomes. Enzyme activities with acetaldehyde, nonanal and phenylacetaldehyde as substrates were found in the same fractions at all purification steps (Table 4). The activity ratio remained constant during the enzyme purification. The similar level of purification was obtained for the enzyme from control liver microsomes. In both cases preparation of the purified enzyme was very labile and rapidly lost activity when stored at −18° even in the presence of glycerol and DTT. High lability of microsomal aldehyde dehydrogenase during isolation and storage has been noted in the earlier reports [4, 6].

Gel filtration on Ultragel yielded one symmetrical peak of aldehyde dehydrogenase activity which corresponded to molecular weight of about 187,000 (Fig. 1). The elution patterns of both enzyme preparations from the clofibrate-induced and control liver microsomes were identical. Estimation of the molecular weight was also performed by sucrose-density-gradient centrifugation. A value of 170,000–180,000 was calculated for the partially purified enzyme on the ground of direct relationship between the molecular weights of standard proteins and their mobility during centrifugation (data not shown).

A single peak of activity was eluted from DEAE-cellulose column with the starting buffer containing

Table 4. Partial purification of aldehyde dehydrogenase from clofibrate-treated animals

Purification step	Total protein (mg)	Total activity (nmoles/min)			Specific activity (nmoles/min/mg protein)			Fold purification		
		I	II	III	I	II	III	I	II	III
Microsomes	194.0	15573	42614	17668	80	220	91	—	—	—
$(NH_4)_2SO_4$ 40–70% saturated	36.0	8935	29408	9263	248	817	257	3.1	3.7	2.8
Ultragel AcA-34	9.8	7137	22848	7099	728	2331	724	9.1	10.6	8.0
DEAE-cellulose	3.0	4334	13081	4914	1445	4360	1639	18.1	19.8	18.0

Various fractions obtained as described in the text were assayed with 5 mM acetaldehyde (I), 0.5 mM nonanal (II), 0.5 mM phenylacetaldehyde (III) and 0.5 mM NAD^+.

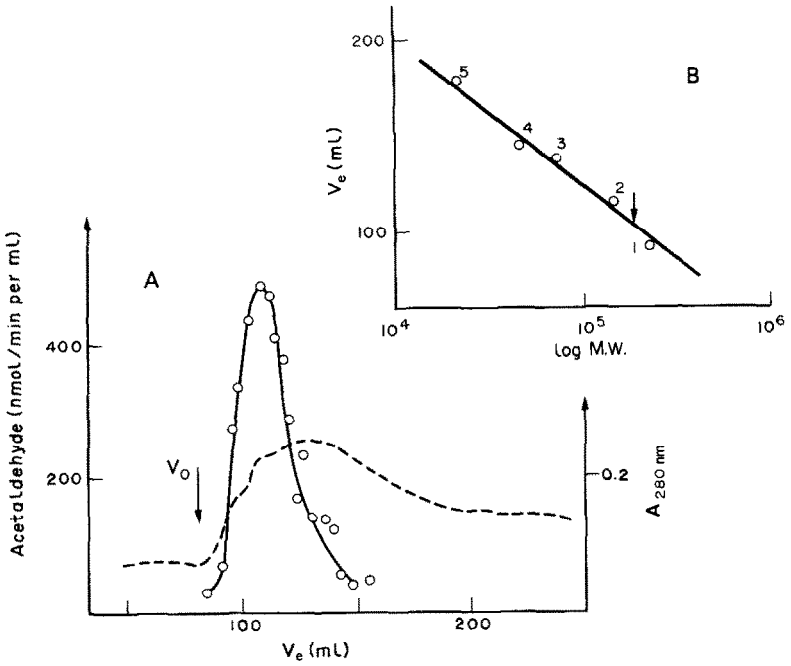

Fig. 1. (A): elution of the microsomal aldehyde dehydrogenase from Ultragel AcA-34 column. Enzyme from the $(NH_4)_2SO_4$ fractionation step was placed on the column (1.6 × 78 cm), equilibrated with 50 mM Tris–HCl (pH 7.6), 1 mM EDTA and 1 mM DTT. Fractions were analysed for aldehyde dehydrogenase activity with 5 mM acetaldehyde as substrate and 0.5 mM NAD^+ (○), and for protein concentration (-----, absorbance at 280 nm). (B): Estimation of the molecular weight of aldehyde dehydrogenase. Elution volume (V_e) is plotted against the logarithm of the molecular weight (log M.W.) of the indicated proteins: 1, catalase (240,000); 2, bovine lactate dehydrogenase (140,000); 3, bovine serum albumin (67,000); 4, ovalbumin (45,000); 5, tripsin inhibitor from soybean (21,500). The arrow indicates the position of aldehyde dehydrogenase.

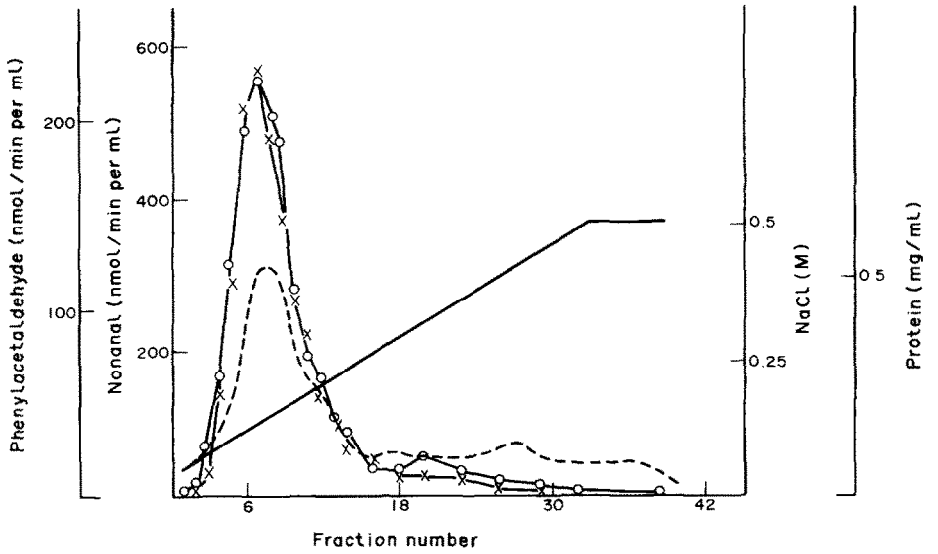

Fig. 2. DEAE-cellulose chromatography of the microsomal aldehyde dehydrogenase. The dialysed supernatant from detergent-treated microsomal fraction was applied to a DEAE-cellulose column. Some protein without aldehyde dehydrogenase activity was eluted with two column volumes of the starting buffer. A linear concentration gradient from 50 to 100 mM NaCl was then started. Both solutions contained 0.2% Triton X-100. Fractions were analysed for aldehyde dehydrogenase activity (NAD^+) with 0.5 mM nonanal (○) or 0.5 mM phenylacetaldehyde (×) as substrates and for protein concentration (----, mg/ml).

nonionic detergent Triton X-100 (Fig. 2). This fraction was of the same relative magnitude as that found in similar experiments with control liver material. The obtained fraction was active with acetaldehyde, nonanal and phenylacetaldehyde.

Polypeptide content of samples obtained on the different purification steps was analysed using polyacrylamide gel electrophoresis in the presence of sodium dodecysulphate. Assessment of the changes in the relative amounts of polypeptide bands revealed successive elevation in the content of one polypeptide corresponded to 47,000. On the final step of enzyme purification (DEAE-cellulose) the relative content of this polypeptide constituted about 30–35% of the total amount of protein in the gel. So, we supposed that it represented a subunit of aldehyde dehydrogenase molecule. A similar result was obtained by Nakayasu *et al.* [61] who determined the subunit molecular weight of the microsomal aldehyde dehydrogenase from normal liver to be about 51,000. These data speak in favour of the tetrameric structure of the microsomal aldehyde dehydrogenase molecule.

As shown in Table 4, the relative velocities of enzyme reaction with different aldehydes are similar in control and clofibrate-treated microsomes as in partially purified aldehyde dehydrogenase from drug-treated rat livers. The enzyme had the highest activity with nonanal from all the aldehydes tested. It also oxidizes *o*-, *m*- and *p*-nitrobenzaldehyde at rates not significantly different from benzaldehyde. Control and clofibrate-induced aldehyde dehydrogenase did not react with formaldehyde, glyceraldehyde, glyoxal and glyoxylic acid. The apparent K_m and V_{max} for various aldehydes are represented in Table 5. The K_m value for nonanal was calculated from the double-reciprocal plots to be 16–18 μM. The high apparent K_ms were obtained for benzaldehyde, acetaldehyde and propionaldehyde, and a somewhat lower value (290 μM), for phenylacetaldehyde. The maximal rate of the partially purified enzyme with phenylacetaldehyde as substrate was achieved at pH 8.0. The optimal pH for other aldehydes tested was about 8.8 (Table 5). Below pH 6.0 the enzyme was inactive.

Table 5. Kinetic constants of microsomal aldehyde dehydrogenase for various aldehydes

Substrates	K_m (μM)	V_{max}	pH Optimum
Acetaldehyde	1700	0.7	8.8–9.2
Propionaldehyde	1300	1.0	8.6
Benzaldehyde	2300	0.7	8.6–9.2
Phenylacetaldehyde	290	1.2	8.0–8.2
Nonanal	16–18	3.7	8.6–9.0

Michaelis constants (K_m) and maximal velocities (V_{max}) were determined from Lineweaver–Burk plots. V_{max} is expressed as relative velocities, the activity with propionaldehyde being taken as 1.0. Effect of pH on enzyme activity was determined in 50 mM sodium pyrophosphate buffer with 0.5 mM phenylacetaldehyde or 0.5 mM nonanal. Concentration of other aldehydes tested was 5 mM. NAD^+ concentration was 0.5 mM. Partially purified enzyme from clofibrate-treated rats was used.

Table 6. Effect of various agents on the induced aldehyde dehydrogenase

Inhibitor or activator	Concentration (μM)	Relative activity
Disulfiram	1	100
	10	68
	50	48
DTNB	10	65
	100	56
N-ethylmaleimide	1	59
	10	24
	100	0.0
Sodium arsenite	500	74
	5000	43
KCN	10	68
	100	42
	1000	0.0
EDTA	500	100
Dithiothreitol	2000	93
2-Mercaptoethanol	2000	98

The partially purified enzyme from the DEAE-cellulose step was used. Aldehyde dehydrogenase was assayed in 50 mM sodium pyrophosphate buffer, pH 9.0 with 0.5 mM NAD^+ and 5 mM acetaldehyde at 37°. The activity with no effectors was defined as 100.

The most potent effectors of the microsomal aldehyde dehydrogenase activity were thiol reagents (Table 6). Disulphiram (50 μM) decreased the activity of the purified enzyme by 50%. The similar level of inactivation was achieved with 1 μM *N*-ethylmaleimide and 5 mM sodium arsenite. The enzyme was also sensitive towards DTNB and cyanide. KCN inhibited the aldehyde dehydrogenase activity not only in the partially purified enzyme but also in microsomal fraction. In the latter case a complete inactivation was achieved at 3 mM concentration regardless of the substrate used (acetaldehyde, nonanal, phenylacetaldehyde, etc.). At the same time 2-mercaptoethanol and dithiothreitol did not affect the enzyme activity. Aldehyde dehydrogenase appears to be tightly bound with microsomal membrane. Triton X-100 (1 mg per mg of microsomal protein) caused the release of about 60% of the total enzyme activity in the supernatant fluid. The higher concentrations of the detergent led to a significant inactivation of the aldehyde dehydrogenase in microsomes from control and clofibrate-treated rats. In addition, heat denaturation studies did not demonstrate any difference between both enzyme preparations.

Taking into account that clofibrate administration leads to induction of the total enzyme activity in hepatocytes, it can be supposed that this drug, like some other carcinogens, would also change the isozyme pattern of aldehyde dehydrogenases. For additional clarification of this question the electrophoretic separation of microsomal proteins and enzyme preparations obtained in the course of purification of aldehyde dehydrogenase was undertaken. The gels were stained for enzyme activity with different aldehydes and NAD^+. This study revealed

that only one isoform of aldehyde dehydrogenase was detectable in microsomes. Besides, the enzyme from control and drug-induced liver microsomes possessed equal mobility in the gel (data not shown).

DISCUSSION

Recently it has been shown that prolonged administration of hypolipidemic peroxisome proliferators (clofibrate, nafenopin, di(2-ethylhexyl)-phthalate) induce hepatocellular carcinomas in rats and mice. This fact warranted the distinguishing of clofibrate and its analogs to a novel class of chemical carcinogens [18]. The mechanism of the neoplastic action of hypolipidemic drugs is unclear. Unlike the other carcinogens they do not possess mutagenic activity [19]. According to the view of Reddy *et al.* [20] the carcinogenic effect of hypolipidemics is associated with the proliferation of peroxisomes and activation of the peroxisomal hydrogen peroxide-generating oxidases. This process is supposed to lead to accumulation of toxic hydrogen peroxide in the cell causing the genetic change which may be necessary for the initiation of peroxisome proliferator-induced hepatocarcinogenesis.

In hepatomas inducible by various carcinogens particular isoenzymes of aldehyde dehydrogenase can be identified differing from the normal liver isoenzymes by their substrate specificity, electrophoretic mobility, isoelectric points and immunochemical properties [21–23]. All these unique species of aldehyde dehydrogenase are detectable in the soluble fraction of the cell. In addition administration of some xenobiotics and carcinogens evokes the expression of aldehyde dehydrogenase phenotype not observable in normal liver or hepatoma cytosol [7, 8, 24]. As has been found recently clofibrate similar to other carcinogens induces the aldehyde dehydrogenase activity in rat liver [9, 25]. In the present work we have conducted a study of the intracellular localization and properties of the clofibrate-induced aldehyde dehydrogenase to ascertain whether it differs from the normal liver enzyme.

The clofibrate-activated aldehyde dehydrogenase (high K_m) is distributed among the mitochondrial, lysosomal and microsomal fractions and is equally induced in all of these fractions regardless of the substrate used (Table 1). The differing results were obtained for the aldehyde dehydrogenase activity with a low K_m for acetaldehyde which was primarily located in mitochondria and was not enhanced by clofibrate treatment. When lysosomal fraction was separated by isopycnic centrifugation the clofibrate-induced aldehyde dehydrogenase was found in peroxisomes (Table 2). Similar to the case with microsomes the peroxisomal enzyme appeared to be tightly bound to a membrane (unpublished results). At the same time we failed to demonstrate the induction of the cytosolic aldehyde dehydrogenase under the action of clofibrate. This fact provides evidence for a discrepancy in the effects of clofibrate and other carcinogens (for example aromatic amines [22, 23] and nitrosamines [26]) which will induce predominantly the cytosolic isoenzymes.

The highest specific activity of the clofibrate-induced aldehyde dehydrogenase was observed in microsomes. This fraction was employed for partial purification of the enzyme to study some of its properties. The microsomal aldehyde dehydrogenase was purified 18–20-fold. It appears that according to its main properties the clofibrate-induced enzyme is similar to the microsomal aldehyde dehydrogenase from normal liver. Indeed, both enzymes have similar molecular weight and substrate specificity. They are identical by their electrophoretic mobility, elution patterns during DEAE-cellulose chromatography and gel filtration and by their thermostability. Further, they respond similarly to the action of various inhibitors or activators. Both the normal and induced microsomal enzymes are tightly bound to membrane and rapidly loose their activity in the presence of ionic or non-ionic detergents. Thus, the data obtained speak against the supposition about induction in the liver of any new isoenzyme of aldehyde dehydrogenase by clofibrate. It is likely that this drug induces membrane-bound aldehyde dehydrogenase with a high K_m for acetaldehyde which was delineated earlier in normal rat liver. The conclusion made is further supported by a comparison of properties of the clofibrate-induced enzyme described in the present work and those reported for the normal liver microsomal aldehyde dehydrogenase. Our results concerning substrate specificity, affinity to DEAE-cellulose and sensitivity of the microsomal enzyme towards detergents and sulfhydryl reagents are in good agreement with the data of Koivula and Koivusalo [4]. In the study of Nakayasu *et al.* [6] the subunit molecular weight of the microsomal aldehyde dehydrogenase purified to homogeneity was estimated to be 51,000. In aqueous solution the purified, detergent-free enzyme formed large polymeric aggregates. Consequently the authors were unable to determine its molecular weight following sucrose density gradient centrifugation. According to our observations the partially purified aldehyde dehydrogenase does not form aggregates in the presence of detergents (Triton X-100, sodium deoxycholate). Its molecular weight estimated by gel filtration and ultracentrifugation amounts to 170,000–190,000. It is conceivable that native molecule of the microsomal enzyme consists of four subunits each equal to about 50,000.

Reports from different laboratories argue that only one aldehyde dehydrogenase isoenzyme with a high K_m for acetaldehyde is normally present in liver microsomes [4, 6]. On the contrary Lindahl [23, 27] managed to distinguish two microsomal isoenzymes by virtue of their different sensitivity towards disulphiram with NAD^+ or $NADP^+$. Nevertheless other properties of these supposed isoforms including isoelectric point, coenzyme requirements and K_m for acetaldehyde were identical. The author indicated that disulphiram-sensitive aldehyde dehydrogenase would preferentially react with aromatic aldehydes (benzaldehyde, phenylacetaldehyde) [23]. It should be noted, however, that purification or separation of microsomal isoenzymes were not attempted in the described investigation. Our data speak in favour of only one isoenzyme of aldehyde dehydrogenase being present in either of normal and clofibrate-induced rat liver microsomes. This conclusion is evi-

denced by a similar level of induction of the enzyme activity with a number of substrates (including aromatic aldehydes) under the influence of clofibrate (Table 3). In addition, the ratio of aldehyde dehydrogenase activity with acetaldehyde, nonanal and phenylacetaldehyde remained constant during all purification steps (Table 4) and the final enzyme preparation exhibited the same relative velocities with various aldehydes as were observed in crude microsomes (Table 3). Chromatography on DEAE-cellulose or gel filtration yielded a single symmetrical peak of the microsomal aldehyde dehydrogenase (Figs. 1 and 2). Similarly polyacrylamide gel electrophoresis revealed only one band of activity with different substrates (acetaldehyde, propionaldehyde, benzaldehyde, nonanal, phenylacetaldehyde) and with NAD^+ as coenzyme. In apparent disagreement with the study of Lindahl [23, 27], we found that disulphiram was equally effective in inhibition of the microsomal aldehyde dehydrogenase with propionaldehyde regardless of whether NAD^+ or $NADP^+$ were used as coenzymes. When the reaction was maintained at 37° the enzyme activity in microsomes decreased by 80–90% following the addition of disulfiram (100 μM), while at 20° the level of inactivation amounted only to 25–30%.

The biological role of the microsomal and peroxisomal aldehyde dehydrogenases remains unclear. The microsomal enzyme is most active with long-chain aliphatic aldehydes [6] and exhibits the highest affinity towards nonanal from all the aldehyde tested (Table 5). According to the view of Nakayasu *et al.* [6] the microsomal aldehyde dehydrogenase may be implicated in the detoxication of water-insoluble aldehydes forming during lipid metabolism, for example in the reaction catalysed by a microsomal alkyl glyceryl ether cleavage system. The other plausible function of the membrane-bound aldehyde dehydrogenase may consist in the utilization of toxic aldehydes which are amply produced during lipid peroxidation. As has been shown by Esterbauer *et al.* [28] the lipid peroxidation in the microsomal membrane engenders a bulk of highly active aldehydes with chain lengths from C_6 to C_{12} together with the malonic dialdehyde. These products are able to upset a number of intracellular processes including DNA and protein synthesis, metabolism of the thiol compounds and function of various enzymes [29–31]. Administration of clofibrate leads to hydrogen peroxide toxicity [20], which may eventually lead to the development of membrane lipid peroxidation and accumulation of long-chain aldehydes. Thus, clofibrate-dependent induction of the microsomal and peroxisomal aldehyde dehydrogenases can play a protective role against the adverse action of lipid peroxidation on the cellular components.

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