Eur J Cancer Clin Oncol, Vol. 19, No. 3, pp. 389–400, 1983.
Printed in Great Britain.

0277-5379/83/030389-12$03.00/0

The Subcellular Distribution and Properties of Aldehyde Dehydrogenase of Hepatoma AH-130*

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Abstract—*Aldehyde dehydrogenase subcellular distribution and activity were studied in the Yoshida hepatoma AH-130 and rat liver. NAD^+- and $NADP^+$-dependent dehydrogenase activities were lower in all hepatoma subfractions (except the cytosol) than in liver subfractions. In the presence of* 0.025 mM *substrate* 78–80% *of the liver NAD^+- or $NADP^+$-dependent aldehyde dehydrogenase was found in the mitochondria. With* 10 mM *substrate the enzyme activity was primarily in the mitochondria and microsomes. In the hepatoma a sharp increase of the soluble aldehyde dehydrogenase (either NAD^+- or $NADP^+$dependent) was observed at all substrate concentrations. The K_m of the different isoenzymes (either identified by their localization or coenzyme dependency) were of the same order for liver and hepatoma. However, a high K_m enzyme was present in liver mitochondria outer membranes but not in hepatoma. Hepatoma acetaldehyde dehydrogenase was inhibited, as was the liver enzyme, by diethyldithiocarbamate. The return of activity was slower for the hepatoma and neonatal liver than for the adult liver enzyme.*

INTRODUCTION

THE CARCINOSTATIC activity of different aldehydes has been well documented (see ref. [1] for review). The strong inhibition of growth by aldehydes could depend on their inhibitory effect on protein and nucleic acid synthesis [2, 3], as well as on energy processes [3–5]. According to Perin *et al.* [6] L-erythro-α,β-dihydrobutyraldehyde affects protein synthesis at concentrations that do not appreciably affect the cellular respiration.

The high sensitivity of tumours to aldehydes has been explained on the basis of a decrease of the catabolizing enzyme, aldehyde dehydrogenase, in tumour cells [6, 7]. In the presence of low (low-K_m enzyme) and high (high-K_m enzyme) substrate concentrations, a decrease of aldehyde dehydrogenase in primary rat hepatomas induced by 4-dimethylaminoazobenzene or ethionine as well as in transplanted tumours has been shown [6–9]. However, an increase of aldehyde dehydrogenase activity has been found in hepatomas induced in the same animal by 2-acetylaminofluorene or 4-dimethylaminoazobenzene [9, 10]. This increase was particularly evident when benzaldehyde and $NADP^+$ were used as a substrate and coenzyme respectively. Cytosolic isoenzymes, specific for hepatoma, which prefer benzaldehyde and $NADP^+$ have been identified on the basis of substrate and coenzyme specificity, inhibition by disulfiram and anti-(hepatoma aldehyde dehydrogenase) sera [9–14]. A cytosolic dehydrogenase which prefers benzaldehyde and $NADP^+$ has also been found in rat liver after induction with polycyclic aromatic hydrocarbons [15, 16]. A further characterization of aldehyde dehydrogenase isoenzymes was obtained by identifying their subcellular localization. In rat liver a low-K_m enzyme is located in the mitochondrial matrix, whereas high-K_m enzymes are present in a bound form in the endoplasmic reticulum and in the outer mitochondrial membrane, and in a soluble form in the cytosol [17–21]. At low aldehyde concentrations mitochondrial acetaldehyde dehydrogenase accounts for most of the cellular dehydrogenase activity. At high substrate concen-

Accepted 14 September 1982.
*This work was supported by a grant from Consiglio Nazionale delle Ricercche of Italy.
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trations the microsomal activity exceeds the mitochondrial activity [22].

Relatively high aldehyde concentrations were shown to induce metabolic damage and death of tumour cells [6, 23–27]. It would be interesting to know the role played by different tumour subcellular compartments in aldehyde catabolism. In the present communication the aldehyde dehydrogenase isoenzymes, identified by their subcellular localization, substrate affinity and coenzyme specificity, have been studied in Yoshida ascites hepatoma AH-130.

MATERIALS AND METHODS

Animals and tumour

Male Wistar rats, weighing 200–250 g, were used as a source of normal liver or to transplant the Yoshida ascites hepatoma. This hepatoma, originally induced by Yoshida [28], is maintained in Wistar rats through i.p. serial passages of the tumour cell suspension (1:10, w:v) carried out at 6- to 7-day intervals for many years. Neonatal liver was obtained from 6-day-old Wistar rats. Adult animals were housed no more than four to a cage and given tap water *ad libitum*. They were maintained on a semi-synthetic diet (Piccioni, Brescia, Italy).

Fractionation procedures

Livers were removed after the rats were killed by decapitation; then the livers were chilled in a medium containing 220 mM mannitol, 70 mM sucrose, 20 mM Tris-Cl buffer (pH 7.4), 2 mM EDTA and 0.1% (w:v) bovine serum albumin (Sigma Chemical Co., fraction V, defatted). The minced tissue was suspended with amounts of the above ice-cold medium equivalent to 2.5 times the weight of the tissue. Six- or seven-day-old hepatoma cells were separated from the ascitic fluid by low-speed centrifugation, washed in ice-cold medium and re-suspended in a volume of medium corresponding to their weight. Homogenates were prepared in a Potter–Elvehjem homogenizer with 4–5 strokes of a tightly fitting pestle. For fractionation or polarographic experiments the tissues were homogenized, as previously described [29], by one stroke of a loosely fitting pestle running at 1500 rev/min. The homogenates were diluted to 20% with the isolation medium and centrifuged at 10,000 g_{min}. The pellets were washed twice by centrifugation at 6000 g_{min}. From the collected supernatants, designated as cytoplasmic extract, the subcellular fractions were isolated according to De Duve *et al.* [30]. In some experiments a mitochondrial fraction isolated at 133,000 g_{min} was used [31]. Microsomes were isolated from the post-mitochondrial supernatants as previously described [32]. Mitochondrial and microsomal fractions were washed three times and one time respectively before use. Post-microsomal supernatants from 20% homogenates were used to determine cytosolic enzyme activities.

Enzyme assays

The cytochrome oxidase was determined according to De Duve *et al.* [30], malate dehydrogenase and adenylate kinase according to Schnaitman and Greenawalt [33], β-galactosidase according to Sellinger *et al.* [34] and lactate dehydrogenase according to Kornberg [35]. Monoamine oxidase was determined polarographically, according to Sawyer and Greenawalt [36], using 5-hydroxytryptamine as a substrate. NADPH-cyt. *c* reductase and NADH-cyt. *c* reductase (rotenone-insensitive) were determined as previously described [37]. The activity of aldehyde dehydrogenase was determined in 1 ml of a reaction mixture containing: [38] 50 mM sodium pyrophosphate (pH 8.8), 1 mM pyrazole, 1 mM NAD^+, 0.002 mM rotenone and homogenate or subcellular fractions. In some experiments 2.5 mM $NADP^+$ was used in place of NAD^+. The homogenate, mitochondria and microsomes were incubated 5 min at room temperature with 0.2% deoxycholate and then centrifuged for 70 min at 106,000 *g*. Aliquots (5–20 μl) of supernatants, corresponding to 0.1–0.25 mg of protein for homogenate, 0.25–0.4 mg for mitochondria and 0.6–0.8 mg for microsomes, were used for the aldehyde dehydrogenase assay. In the case of cytosol the amounts used were 5–15 μl, corresponding to 0.35–0.5 mg of protein. The reaction was started by the addition of the indicated amounts of various aldehydes. The reference cuvettes were supplemented like the sample cuvettes except for the substrate. All enzyme activities were determined at 25°C.

Determination of rotenone-insensitive aldehyde oxidation

Mitochondrial respiration, in the presence of the indicated concentrations of acetaldehyde, was measured polarographically at 25°C in 2 ml of a reaction medium containing: 80 mM KCl, 5 mM Tris-Cl (pH 7.4), 10 mM sodium phosphate and 5 mM $MgCl_2$ [29]. The reaction was started by the addition of mitochondria (2–3 mg of protein). The following reagents were then added in this order: 0.4 mM ADP, 0.002 mM rotenone, 0.5 mM NAD^+ and 0.03 mM cytochrome *c*. The respiratory increments following this latter addition were considered a measure of the rotenone-insensitive aldehyde oxidation [22].

Analytical determinations

Proteins were determined by a biuret procedure [39].

RESULTS

Aldehyde dehydrogenase activity in homogenate and subcellular fractions

The data in Table 1 report the aldehyde dehydrogenase activity in liver and hepatoma in the presence of different aldehydes. It appears that the enzyme-specific activities of the hepatoma homogenate were 28–54% lower than those of liver homogenate with all the aldehydes tested in the presence of NAD^+ as a coenzyme. The difference between liver and hepatoma was even larger (61–75%) when a comparison between the enzyme activities per gram of tissue was made. This depended on the existence of a lower protein content in the hepatoma homogenate compared to the liver homogenate (71 ± 6 mg/g of wet wt in hepatoma, and 144 ± 20 mg/g of wet wt in liver). When $NADP^+$ was used with acetaldehyde or benzaldehyde as substrates the specific activities were 84–90% lower and the activities per gram were 93–98% lower in hepatoma than in normal liver.

Table 2 shows the activity of acetaldehyde and benzaldehyde dehydrogenase in the homogenate and subcellular fractions of liver and hepatoma at low and high substrate concentrations. NAD^+-dependent acetaldehyde dehydrogenase activity was lower, at all substrate concentrations, in hepatoma than in liver subfractions, except for the cytosol, where no differences between the two tissues were observed. In the presence of $NADP^+$ and 0.025 mM acetaldehyde a specific activity 6 times lower than in liver mitochondria was detected in the hepatoma organelles. No activity was found in the other hepatoma subfractions. At 10 mM acetaldehyde the $NADP^+$-dependent enzyme activity was very low in all of the hepatoma subfractions, except for the cytosol again, where a specific activity as high as that of liver cytosol was recorded. There was similar behaviour with 10 mM benzaldehyde as a substrate in the presence of either NAD^+ or $NADP^+$. However, no $NADP^+$-dependent benzaldehyde dehydrogenase was detected in all hepatoma subfractions at 0.025 mM substrate. Also, no differences between liver and hepatoma as concerns the NAD^+-dependent benzaldehyde dehydrogenase were found at this aldehyde concentration.

Subcellular distribution of the aldehyde dehydrogenase activity

The subcellular distribution of acetaldehyde, maleylaldehyde and benzaldehyde dehydrogenase and seven marker enzymes in liver and hepatoma is shown in Table 3. The activities and recoveries were referred to the activities found in the cytoplasmic extract rather than the crude homogenate, as is frequently done. This method was adopted because the nuclear fraction was composed largely of unbroken cells, particularly in the hepatoma.

The cytochrome oxidase, malate dehydrogenase, adenylate kinase and monoamine oxidase were used as markers of the mitochondrial inner membrane, matrix, inter-membrane and outer membrane compartments respectively. β-Galactosidase and NADPH-cyt. *c* reductase were used as markers for lysosomes and microsomes respectively, and lactate dehydrogenase as a marker for cytosol. It appears that no large contamination of mitochondrial inner membrane was present in the microsomes of liver and hepatoma. The leakage of matrix material into the microsome and supernatant fractions was very low in both tissues. By contrast, relatively large amounts of adenylate kinase were found in the supernatants, indicating that rupture or permeabilization of liver and hepatoma mitochondrial outer membrane occurred. However, the determination of monoamine oxidase in the liver and hepatoma subfractions indicated that low amounts of mitochondrial outer membrane sedimented together with microsomes. The β-galactosidase was present primarily in the heavy and light mitochondrial fractions of liver and hepatoma, while the activity in the supernatants was low. Thus it appears that there has been a very small breakage of lysosomes during the subfractionation. As expected, the NADPH-cyt. *c* reductase activity was found principally in liver and hepatoma microsomal fractions and lactate dehydrogenase exclusively in the soluble fraction.

The data in Table 3 also show that the liver acetaldehyde dehydrogenase activity was primarily found in the mitochondrial and microsomal fractions, with only the 28% of the activity in the supernatant. A similar behaviour was observed for the $NADP^+$-dependent as well as NAD^+-dependent (not shown) benzaldehyde dehydrogenase. In the case of maleylaldehyde dehydrogenase the activity linked to microsomes was relatively low (18.6%), while 47% of the enzymatic activity was found in the supernatant.

A profoundly different distribution of the three aldehyde dehydrogenases was observed in the hepatoma. The enzymatic activities were mostly present in the supernatant, less than 20% being linked to the subcellular organelles. The activity linked to mitochondria (H plus L) was 11.7% for acetaldehyde dehydrogenase, 7.5% for maleylaldehyde dehydrogenase and 5.1% for benzaldehyde dehydrogenase, against 40.4, 32.2 and 37.3%

for the same dehydrogenases in liver mitochondria. Hepatoma microsomes contained only 6% of the acetaldehyde and maleylaldehyde dehydrogenases and 13% of benzaldehyde dehydrogenase. NAD^+-dependent benzaldehyde dehydrogenase was distributed among the liver and hepatoma subcellular fractions in the same way as the $NADP^+$-dependent isoenzyme (not shown).

The above results were obtained with 10 mM aldehydes. When 0.025 mM acetaldehyde was used, about 80 and 55% of the enzymatic activity was present in liver and hepatoma mitochondria (H plus L) respectively. Less than 6% for liver and 0.5% for hepatoma of the activity was found in the microsomal fraction, the remaining activity being in the supernatant fraction.

Apparent K_m

In order to investigate the presence of aldehyde dehydrogenases with different K_ms in the hepatoma, as already known for the liver [18], aldehyde dehydrogenase activity was determined in the various subfractions in the presence of increasing concentrations of substrate at saturating NAD^+ concentrations.

The data in Fig. 1A show that the Lineweaver–Burk plot obtained in experiments of liver mitochondria (H plus L) was curved. The activity increased slightly when the acetaldehyde concentration varied between 0.01 and 0.33 mM. This activity clearly depends on the presence of an

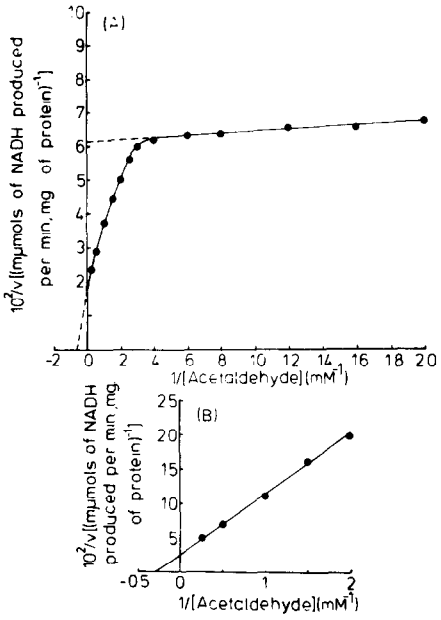

Fig. 1. Double-reciprocal plot for the oxidation of acetaldehyde by liver mitochondria. (A) The enzymatic activity was measured in the presence of NAD^+. The acetaldehyde concentrations varied from 0.005 to 10 mM. (B) Data from (A) were used to calculate the differences between the total aldehyde dehydrogenase activity at high substrate concentrations and the activity obtained from the intercept of the asymptote on the vertical axis in (A).

Table 1. Aldehyde dehydrogenase activity in liver and hepatoma homogenates

Substrate, coenzyme	Liver			Hepatoma			*t*-test*	
	No. of experiments	specific activity	Activity (g^{-1})	No. of experiments	specific activity	Activity (g^{-1})	Specific activity	Activity (g^{-1})
Formaldehyde, NAD^+	6	4.41 ± 0.56	618 ± 86	5	2.60 ± 0.41	178 ± 31	$P<0.001$	$P<0.001$
Maleylaldehyde, NAD^+	7	4.47 ± 0.55	854 ± 117	8	2.23 ± 0.47	244 ± 70	$P<0.001$	$P<0.001$
Methylglyoxal, NAD^+	4	5.20 ± 1.27	812 ± 215	8	3.29 ± 1.10	205 ± 65	$P<0.025$	$P<0.001$
Malonaldehyde, NAD^+	15	7.72 ± 1.41	1151 ± 267	9	5.52 ± 1.09	448 ± 141	$P<0.001$	$P<0.001$
Glutaraldehyde, NAD^+	6	7.08 ± 1.79	983 ± 157	6	3.27 ± 1.18	292 ± 46	$P<0.005$	$P<0.001$
Acetaldehyde, NAD^+	7	12.58 ± 1.99	2911 ± 385	7	7.45 ± 0.96	923 ± 126	$P<0.001$	$P<0.001$
Acetaldehyde, $NADP^+$	2	6.81	1004	2	1.06	110		
Benzaldehyde, NAD^+	3	8.89 ± 1.08	1288 ± 126	3	4.19 ± 0.60	282 ± 40	$P<0.005$	$P<0.001$
Benzaldehyde, $NADP^+$	3	4.00 ± 0.26	578 ± 43	3	0.39 ± 0.03	20 ± 2	$P<0.001$	$P<0.001$

Substrate concentration, 10 mM. Specific activity (nmol of NADH or NADPH produced per min per mg of protein) is the mean ± standard deviation for the numbers of experiments indicated.
*Hepatoma vs liver.

Table 2. Acetaldehyde and benzaldehyde dehydrogenase activities in different subcellular fractions of liver and hepatoma

Subfractions	Substrate, coenzyme	Liver Substrate concentration (mM)		Hepatoma Substrate concentration (mM)		*t*-test* Substrate concentration (mM)	
		0.025	10	0.025	10	0.025	10
Homogenate	ACA, NAD^+	6.4† ± 1.2	16.0 ± 0.2	4.2 ± 1.3	5.0 ± 0.5	$P<0.025$	$P<0.001$
	ACA, $NADP^+$	1.7	6.8	ND	1.2		
	BA, NAD^+	2.1 ± 0.6	8.9 ± 1.4	2.2 ± 0.2	5.0 ± 0.7	NS	$P<0.001$
	BA, $NADP^+$	1.1 ± 0.1	4.0 ± 0.8	ND	0.4 ± 0.1		$P<0.001$
Mitochondria	ACA, NAD^+	22.9 ± 2.0	35.2 ± 10.0	11.2 ± 0.8	5.0 ± 0.6	$P<0.001$	$P<0.001$
	ACA, $NADP^+$	12.0	16.1	2.0	0.3		
	BA, NAD^+	3.0 ± 0.7	8.9 ± 2.0	2.7 ± 0.7	2.3 ± 0.6	NS	$P<0.001$
	BA, $NADP^+$	0.4 ± 0.1	1.4 ± 0.3	ND	0.3 ± 0.1		$P<0.001$
Microsomes	ACA, NAD^+	3.1 ± 0.6	29.0 + 3.1	1.0 ± 0.2	2.1 ± 0.4	$P<0.001$	$P<0.001$
	ACA, $NADP^+$	1.4	16,7	ND	0.6		
	BA, NAD^+	2.7 ± 0.8	13.9 ± 2.3	2.2 ± 0.1	4.4 ± 0.2	NS	$P<0.001$
	BA, $NADP^+$	2.2 ± 0.6	6.7 ± 1.6	ND	0.2 ± 0.1		$P<0.001$
Cytosol	ACA, NAD^+	6.0 ± 0.5	13.6 ± 0.9	7.0 ± 1.0	14.4 ± 0.5	NS	NS
	ACA, $NADP^+$	3.1	7.4	ND	6.8		
	BA, NAD^+	2.1 + 0.8	5.1 ± 1.2	2.2 ± 0.3	5.9 ± 0.7	NS	NS
	BA, $NADP^+$	0.5 ± 0.1	1.1 ± 0.4	ND	0.7 ± 0.1		NS

The data are means ± standard deviation of at least 4 experiments or means of 2 experiments. Abbreviations: ACA, acetaldehyde; BA, benzaldehyde; ND, not detectable; NS, not significant.

*Hepatoma vs liver.

†*nmol of NADH or NADPH produced per min per mg of protein.*

Table 3. Intracellular distribution of aldehyde dehydrogenase and some marker enzymes in liver and hepatoma

Enzyme	*n*		Recovery		Absolute values		Percentage values							
							H		L		P		S	
	Liver	Hepatoma	Liver	Hepatoma	Liver	Hepatoma	Liver	Hepatoma	Liver	Hepatoma	Liver	Hepatoma	Liver	Hepatoma
Cytochrome oxidase	6	7	93.7 ±14.5	88.0 ±15.0	17.20 ±4.81	8.15 ±2.47	76.2 ±2.8	60.6 ±6.4	21.0 ±3.4	36.9 ±7.9	2.6 ±0.9	1.9 ±0.6	0.0	0.0
Malate dehydrogenase	2	2	96.0	100.0	8.01	10.35	73.3	65.5	19.9	28.5	3.5	1.1	2.8	1.9
Adenylate kinase	3	3	106.7 ±3.0	97.8 ±4.8	8.83 ±1.21	1.84 ±0.07	23.0 ±2.7	19.9 ±1.9	23.6 ±3.3	21.6 ±4.4	10.5 ±1.1	11.6 ±3.1	42.8 ±8.5	51.5 ±4.9
Monoamine oxidase	2	2	100.0	98.7	0.507	0.130	57.7	55.0	31.8	33.1	10.5	9.1	0.0	0.0
β-galactosidase	4	3	94.2 ±6.0	98.0 ±5.3	0.975 ±0.07	0.027 ±0.01	32.5 ±3.7	39.6 ±4.1	47.3 ±9.2	49.3 ±8.2	11.9 ±4.0	6.0 ±1.6	3.0 ±0.9	11.0 ±4.8
NADPH-cyt. *c* reductase	6	6	95.6 ±2.1	99.0 ±6.0	2.27 ±0.55	0.219 ±0.04	5.9 ±1.5	4.5 ±1.5	10.8 ±3.8	8.7 ±3.5	78.7 ±9.3	77.8 ±8.1	4.4 ±1.9	4.9 ±0.8
Lactate dehydrogenase	2	2	97.3	90.0	250.5	367.5	0.1	0.0	0.1	0.0	0.8	0.0	99.0	99.2
Acetaldehyde dehydrogenase*	10	12	95.3 ±7.6	97.1 ±8.2	2.28 ±1.54	0.385 ±0.05	29.8 ±5.9	4.5 ±0.6	10.6 ±3.1	7.0 ±0.7	28.6 ±4.1	6.0 ±0.6	28.2 ±4.4	81.3 ±7.7
Maleylaldehyde dehydrogenase*	10	12	99.5 ±7.6	96.0 ±5.8	0.530 ±0.01	0.260 ±0.06	23.5 ±2.9	3.5 ±0.8	9.3 ±0.9	4.0 ±0.6	18.6 ±5.5	6.2 ±0.6	47.3 ±2.7	86.6 ±8.1
Benzaldehyde dehydrogenase ($NADP^+$-dep.)*	4	4	97.0 ±1.2	93.0 ±0.1	0.154 ±0.02	0.010 ±0.00	29.4 ±1.6	2.7 ±0.1	7.9 ±0.8	2.4 ±0.0	40.4 ±6.8	13.0 ±4.0	22.2 ±4.3	82.7 ±8.4
Protein	10	12	100.3 ±9.2	100.6 ±5.1	129.6 ±9.7	39.9 ±9.9	28.9 ±4.8	9.4 ±1.1	12.0 ±2.0	4.5 ±2.6	16.3 ±4.6	17.1 ±2.6	46.1 ±5.0	68.0 ±9.4

Absolute values are for the cytoplasmic extract (E). They are expressed as μmol per min per g of wet wt. of liver or hepatoma for enzyme activities, and as mg/g of wet wt. for protein. Mean percentage recovery is calculated from the total activity or protein in H+L+P+S divided by the activity or protein in E × 100. Data are means ± standard deviation. Abbreviations: H, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; S, final supernatant.

*Substrate concentration, 10 mM. Coenzyme: NAD^+ for acetaldehyde and maleylaldehyde dehydrogenases; $NADP^+$ for benzaldehyde dehydrogenase.

enzyme (enzyme 1) exhibiting a high affinity for the substrate. The apparent K_m was 0.005 mM. When the differences between the total rates at acetaldehyde concentrations from 1 to 20 mM and the velocity of enzyme 1 were plotted as shown in the Fig. 1B, a double-reciprocal plot was obtained (see also ref. [18]). From this plot an apparent K_m of 2.3 mM was calculated for the enzyme 2. This value agrees with the apparent K_m calculated by others [18, 21] for the same mitochondrial isoenzyme (1.2–2 mM).

The plots obtained from experiments with hepatoma mitochondria were linear at the acetaldehyde concentrations between 0.05 and 0.25 mM, whereas at the concentrations of 1 and 5 mM a clear inhibition of the enzymatic activity occurred (Fig. 2). From the linear portion of the plot in Fig. 2 a K_m of 0.01 mM was calculated. These observations could indicate that, in contrast to liver mitochondria, where at least a high-K_m and a low-K_m enzyme are present, hepatoma mitochondria exhibit only a low-K_m enzyme (see below).

The double-reciprocal plots for the oxidation of acetaldehyde by liver and hepatoma microsomes (Fig. 3) and supernatants (Fig. 4) were linear. The apparent K_m was 1 mM for the enzyme of liver microsomes and 0.55 mM for that of hepatoma microsomes. The soluble fraction yielded K_m values for acetaldehyde of 0.5 and 0.81 mM for liver and hepatoma respectively.

As for acetaldehyde dehydrogenase, there were a mitochondrial, a microsomal and a cytosolic liver isoenzyme with low affinity for benzaldehyde in the presence of $NADP^+$ as coenzyme (K_m, 1.8, 1.3 and 1.6 respectively). Only a microsomal and a cytosolic high-K_m isoenzyme was found in the hepatoma (K_m, 1.3 and 0.84 mM). Both liver and hepatoma mitochondria exhibited a benzaldehyde dehydrogenase isoenzyme whose K_m was lower than that of the other subfractions (0.1 and 0.08 mM respectively). High substrate concentrations inhibited this isoenzyme in the hepatoma, although to a lower extent (14% with 5 mM substrate) than they did with the same acetaldehyde dehydrogenase isoenzyme (40%).

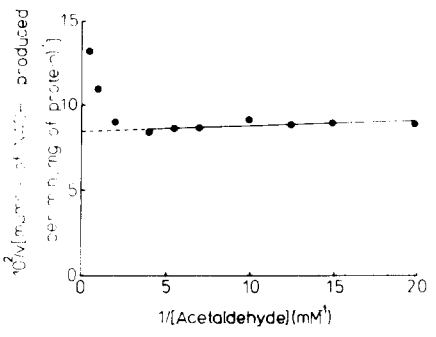

Fig. 2. Double-reciprocal plot for the oxidation of acetaldehyde by hepatoma mitochondria. Conditions as described in the legend of Fig. 1, except that the acetaldehyde concentrations varied from 0.05 to 6 mM.

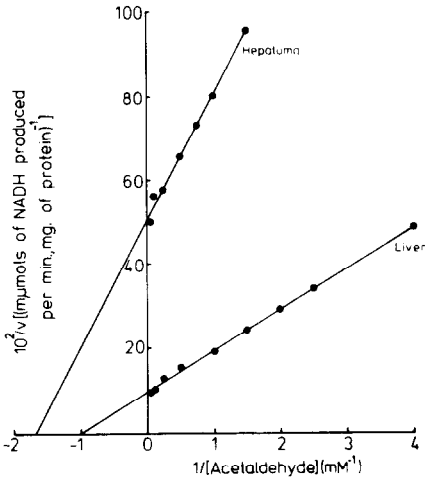

Fig. 3. Double-reciprocal plot for the oxidation of acetaldehyde by liver and hepatoma microsomes. The enzymatic activity was measured in the presence of NAD^+. The acetaldehyde concentrations varied from 0.25 to 10 mM for liver and from 0.66 to 10 mM for hepatoma.

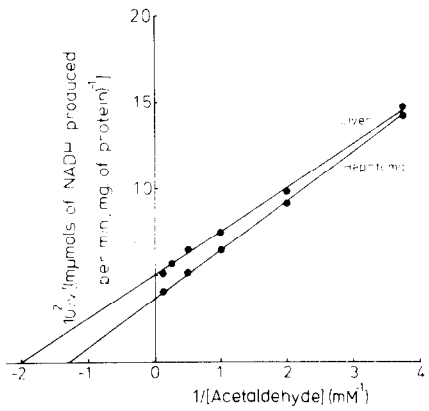

Fig. 4. Double-reciprocal plot for the oxidation of acetaldehyde by liver and hepatoma supernatants. The enzymatic activity was measured in the presence of NAD^+ and of acetaldehyde concentrations varying from 0.25 to 6 mM.

K_m values for NAD^+ and $NADP^+$ for all subcellular fractions in the presence of acetaldehyde as substrate were of the same order of magnitude, 0.03–0.065 mM, for both liver and hepatoma.

Aldehyde dehydrogenase of outer mitochondrial membrane

The above experiments seem to indicate the absence of the high-K_m enzyme in the hepatoma mitochondria. This enzyme is located in the outer membrane or in the inter-membrane space of liver mitochondria [22]. It may be studied by measuring the oxidation of acetaldehyde by intact, non-respiring mitochondria in the pre-

sence of external NAD^+ [22]. The production of NADH by external acetaldehyde dehydrogenase is followed by the non-phosphorylating oxidation linked to respiration in the presence of added cyt. *c* and NADH-cyt. *c* reductase (rotenone-insensitive) [40]. The experiment illustrated in Fig. 5 was performed with mitochondria isolated at 133,000 *g* from tissues subjected to a mild homogenization. This procedure permits the isolation of functionally and structurally intact mitochondria [29, 31]. It appears that the oxidation of acetaldehyde by highly coupled mitochondria is low. This could depend on the inhibitory effect of aldehydes on the electron transport chain in a site between NADH and cyt. *b* [4, 41]. Rotenone-inhibited liver mitochondria exhibited, after addition of NAD^+ and cyt. *c*, an oxygen uptake whose rate was proportional to the acetaldehyde concentration. In contrast, the addition of NAD^+ and cyt. *c* did not initiate oxygen consumption in rotenone-inhibited hepatoma mitochondria in the range of acetaldehyde concentrations used.

In order to investigate whether this behaviour depended on the presence of a defective NADH-cyt. *c* reductase activity in the hepatoma outer mitochondrial membrane (see ref. [42]), 1 mM NADH was substituted for acetaldehyde and NAD^+ in the reaction medium for the polarographic assays. The addition of NADH and cyt. *c* to rotenone-inhibited hepatoma mitochondria initiated an oxygen consumption of 10 natoms per min per mg of protein. The respiration of liver mitochondria in the same conditions amounted to 24.8 natoms per min per mg of protein. This can be explained on the basis of the levels of NADH-cyt. *c* reductase activity (rotenone-insensitive). The NADH oxidized per min per mg of protein was 254 ± 18 nmol for liver mitochondria and 106 ± 12 nmol for hepatoma mitochondria. This is a 2.4 times decrease of activity of hepatoma mitochondria in relation to that of liver mitochondria.

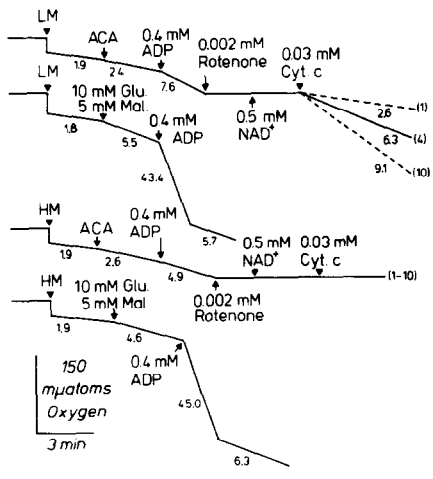

Fig. 5. Oxidation of acetaldehyde by aldehyde dehydrogenase in liver and hepatoma mitochondria. The respiration was recorded as described under Material and Methods. Numbers near the traces indicate the oxygen uptake in natoms *per* min *per* mg *of protein. Numbers in parentheses indicate the final concentration of acetaldehyde (ACA) in* mmol/l.

Inhibition of aldehyde dehydrogenase by diethyldithiocarbamate

The administration to rats of diethyldithiocarbamate or disulfiram is followed by the irreversible inhibition of aldehyde dehydrogenase [43]. Return of activity following the maximum inhibition of aldehyde dehydrogenase in liver mitochondria and supernatants was shown to be linked to the resynthesis of new enzyme molecules [43]. The effect of diethyldithiocarbamate on the acetaldehyde dehydrogenase of hepatoma homogenate and subcellular fractions were studied to investigate the ability of tumour cells to resynthesize the enzyme after its irreversible inactivation. For comparison, both adult and neonatal liver were used. The data in Fig. 6 show the results obtained with homogenates. The curves for the mitochondrial, microsomal and cytosolic enzymes were almost identical and are not presented. It appears that diethyldithiocarbamate injection to rats was followed by enzyme inhibition, reaching its maximum at 12 hr for adult liver and about 30 hr for both hepatoma and neonatal liver. The activity return was slower in hepatoma and neonatal liver, with half-times of about 130 hr, against a half-time of 30 hr for the adult liver.

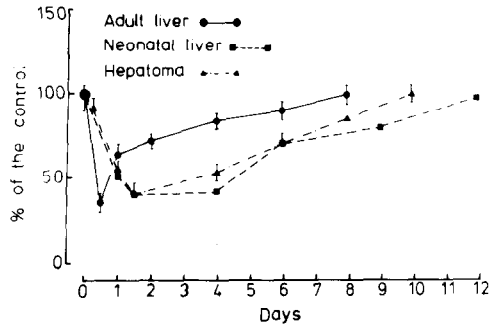

Fig. 6. Time-course of inhibition of acetaldehyde dehydrogenase activity of adult rat liver, neonatal liver and hepatoma homogenate after administration of diethyldithiocarbamate. Diethyldithiocarbamate (100 mg/ml *in* 0.1 M *sodium phosphate buffer,* pH 7.4) *was injected subcutaneously* (2 mmol/kg) *at 0-time to 24-hr fasted rats. Controls received phosphate buffer alone. The enzymatic activity was determined in the presence of* NAD^+ *and* 10 mM *substrate. The data are calculated as a percentage of the activity of the 0-time controls. Points with vertical bars represent mean values of at least 5 experiments ± standard deviation. Other points are mean values of 2 experiments. The* 100% *specific activities were* 16.2 ± 1.2 *for adult liver,* 11.3 ± 0.6 *for neonatal liver and* 4.3 ± 0.2 *for hepatoma.*

DISCUSSION

The hepatoma AH-130 exhibits a decrease of NAD^+- and $NADP^+$-dependent aldehyde-dehydrogenase activities as compared to liver with all substrates tested, except 0.025 mM benzaldehyde, in the presence of NAD^+. A decrease of NAD^+-dependent aldehyde dehydrogenase has already been described in different primary or transplanted tumours [6, 7]. A decrease of $NADP^+$-dependent aldehyde dehydrogenase has been found in ethionine-induced as well as spontaneous liver tumours, in several other tumours of rats bearing 2-acetylaminofluorene-induced hepatomas and in a number of Morris hepatomas [9]. These results contrast with the evidence that NAD^+- and $NADP^+$-dependent aldehyde dehydrogenase activity increases in some primary hepatomas [9, 10]. This discrepancy cannot be explained at present. Perhaps differences in the purity of cell population, cell age and physiological state affect the aldehyde dehydrogenase activity of different tumours.

Our results indicate that the decrease of aldehyde dehydrogenase-specific activity in hepatoma AH-130 concerns all the subcellular fractions examined except the cytosol. The decrease is greater at high substrate levels. In fact, the liver microsomal aldehyde dehydrogenase activities are 3–12 times higher and the mitochondrial activities 1.3–3 times higher in the presence of 10 mM than of 0.025 mM substrate. In contrast, the hepatoma microsomal enzyme undergoes a slight increase at high substrate level, while the mitochondrial activity is inhibited. $NADP^+$-dependent benzaldehyde dehydrogenase has a very low activity in all hepatoma subfractions at 10 mM substrate and it is undetectable at 0.025 mM substrate. The determination of K_m for substrate indicates the existence of a high-affinity isoenzyme in the mitochondria. However, the low activity of this isoenzyme, both in normal liver and hepatoma, suggests that it has probably no physiological importance, as is also true for the low-affinity $NADP^+$-dependent benzaldehyde dehydrogenase isoenzymes.

The contribution of various intracellular compartments to the aldehyde dehydrogenase activity has been shown by the study of the intracellular distribution pattern of acetaldehyde, maleylaldehyde and benzaldehyde dehydrogenases. The distribution of liver acetaldehyde dehydrogenase observed in this paper was generally consistent with the observations of Tottmar *et al.* [18] and Koivula and Koivusalo [21]. These authors described two mitochondrial and one microsomal enzymes. However, they found a lower cytosolic activity. Probably a portion of the cytosolic dehydrogenase, found in this paper, derived from mitochondria during tissue fractionation. The marker enzyme analysis indicated that a fraction of inter-membrane mitochondrial compartment was collected with the cytosol. In the hepatoma, at high substrates levels the acetaldehyde and maleylaldehyde dehydrogenases are present primarily in the soluble fraction, only 13–19% of the activity being linked to the intracellular organelles. In the liver cells this is about 72 and 53% for acetaldehyde dehydrogenase and maleylaldehyde dehydrogenase respectively. The decrease of the hepatoma isoenzymes bound to membranes is even more marked for $NADP^+$-dependent benzaldehyde dehydrogenase. The low K_m isoenzyme of hepatoma AH-130 mitochondria is inhibited by aldehyde concentrations higher than 0.5 mM. Therefore the enzyme activities of hepatoma homogenate and mitochondria appear to be artificially generated in the presence of high substrate levels. When a low concentration of the substrate is used the enzyme activity is nearer to the physiological conditions. At a low substrate level 80% of the aldehyde dehydrogenase is linked to liver mitochondria and only 55% to hepatoma mitochondria. Thus, as expected, the subcellular distribution of aldehyde dehydrogenase was influenced by the differences in the affinity for the substrate of the isoenzymes in the various cellular compartments. However, an increase in the relative amounts of the cytosolic enzyme in the hepatoma was observed at all substrate concentrations. The altered subcellular distribution and the decreased activity of the hepatoma aldehyde dehydrogenase do not appear to be influenced by differences between liver and hepatoma concerning the enzyme affinity for the substrate. The apparent K_m values for the microsomal and cytosolic enzymes were, in fact, of the same order for the two tissues. Neither were differences between the two tissues recorded for the low-K_m mitochondrial enzyme for substrate concentrations lower than 0.5 mM.

Our data indicate that the high-K_m acetaldehyde dehydrogenase of the outer mitochondrial membrane is absent in hepatomas. This is shown by the inability of rotenone-inhibited hepatoma mitochondria to oxidize acetaldehyde in the presence of added NAD^+ and cytochrome *c*. The NADH-cyt. *c* reductase activity (rotenone-insensitive) of these mitochondria, even though 2.4 times lower than that of liver mitochondria, can still permit NADH oxidation. It is possible that the high-K_m mitochondrial isoenzyme is loosely bound to the hepatoma outer membrane and it is easily released during the isolation procedures. There is, however, no proof that this hypothesis is correct.

It is not clear if the alterations of distribution and activity of hepatoma aldehyde dehydrogenase are related to alterations of the subcellular membranes and/or genetic expression of tumour cells. Carcinogen-induced gene de-repression was shown by Lindahl [13] to contribute at least partially to the generation of hepatoma-specific aldehyde dehydrogenase. And yet there is so far no evidence for the existence of a defective gene expression in the synthesis of aldehyde dehydrogenase in the hepatoma AH-130: on the basis of substrate and coenzyme specificity a tumour-specific aldehyde dehydrogenase was not identified. The only alteration dealing with the aldehyde dehydrogenase production in hepatoma cells consists of a decreased ability to synthesize new enzyme molecules after diethyldithiocarbamate inhibition. A similar behaviour was also found in neonatal liver and could indicate the existence of a lower aldehyde dehydrogenase turnover in the growing tissues, a phenomenon which has been observed for different proteins in these tissues [44].

It has been observed that necrosis does occur in monkey kidney cells treated with up to 10 mM aldehyde [45]. Necrosis occurs at the 2–3 mM level in Ehrlich ascites tumour cells [23, 24]. Conroy *et al.* [25] observed that the *in vitro* treatment of murine tumour cells with 0.54–1.28 mM aldehydes resulted in the inhibition of tumour development following their injection to mice. During the aldehyde treatment of tumour-bearing animals [23, 24] the aldehyde concentration presumably reaches relatively high levels in the blood and tissues. The aldehyde dehydrogenase kinetics and distribution in liver cells should favour the aldehyde catabolism when the aldehyde molecules attain high levels. In fact, the enzyme activity is high in all the subcellular compartments in the presence of high aldehyde concentrations. This could explain the relatively short half-lives of aldehyde *in vivo* [25]. According to our results, high substrate levels do not greatly stimulate the acetaldehyde dehydrogenase activity of tumour homogenate. Consequently, aldehydes should easily reach concentrations that inhibit the protein synthesis as well as mitochondrial oxidations and cause ultrastructural alterations in tumour cells [2–4, 46]. This behaviour should be favoured by the lack of enzyme activity in the subcellular sites where these important metabolic processes occur, i.e. ergastoplasmic membranes and mitochondria. Even so, the *in vivo* toxicity for normal tissues of pharmacological aldehyde doses has not yet been established. Consequently, it is not known whether the high aldehyde concentrations used in our experiments are harmless for normal liver cells. Moreover, the differences of sensitivity to aldehydes between normal liver and hepatoma could depend on factors other than the aldehyde dehydrogenase decrease in tumour cells. For instance, a different sensitivity to aldehyde-induced protein cross-links. In this respect it should be interesting to evaluate the aldehyde toxicity for those tumours which show a high aldehyde dehydrogenase activity [10].

The results described in this paper refer to a transplantable, rapidly growing hepatoma which was serially transferred intraperitoneally. Thus the alterations described could depend on the selection of particular cell subpopulations. In fact, growth intraperitoneally has been shown to be selective [47]. Researches on different tumours, particularly on primary hepatomas, should clarify whether the described features may be considered a generalized phenomenon. Also, our data regard the aldehyde dehydrogenase which is the most important catabolizing enzyme for aldehydes. It should be interesting to study other catabolic enzymes such as aldehyde oxidase and aldehyde reductase in order to evaluate the actual ability of tumour cells to catabolize aldehydes. Further work is needed to assess these aspects of the problem.

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