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PARTIAL PURIFICATION AND PROPERTIES OF A PHENOBARBITAL-INDUCED ALDEHYDE DEHYDROGENASE OF RAT LIVER

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Summary

Properties of the phenobarbital induced cytoplasmic aldehyde dehydrogenase (EC 1.2.1.3) have been studied in rat liver. 7—12-Fold higher levels were seen in the cytoplasmic activities after phenobarbital treatment in reactor compared to non-reactor animals with high concentrations of acetaldehyde (18 mM) and propionaldehyde (9 mM). No difference was found with 0.12 mM acetaldehyde, 2 mM glycolaldehyde, 6 mM formaldehyde or 0.5 mM betaine aldehyde. The reactor group also had slightly higher activity in the mitochondrial fraction with the high acetaldehyde and propionaldehyde concentrations. In the microsomal fraction, the activities showed no differences at any substrate concentration. An induced aldehyde dehydrogenase was purified 70-fold by chromatographic techniques. It had different molecular and enzymic properties than the main high-K_m enzyme normally present in rat liver cytoplasm. The pI of the induced enzyme was about 7.0 as measured by isoelectric focusing. It was active with several aliphatic and aromatic aldehydes but not with formaldehyde, glycolaldehyde or D-glyceraldehyde. The K_m-values for propionaldehyde and acetaldehyde were in the millimolar range. Millimolar concentrations of aromatic aldehydes caused a strong substrate inhibition. The enzyme was inhibited by submicromolar concentrations of disulfiram. Estrone, deoxycorticosterone, progesterone and diethylstilbestrol also affected the enzyme activity.

Introduction

Administration of phenobarbital to rats has been reported to result in a 10-fold increase in the NAD-linked aldehyde dehydrogenase (EC 1.2.1.3) activity in the cytoplasmic fraction of the liver. This response is controlled by the genotype of the animal and is due to the presence of a single autosomal codominant gene [1—3]. A similar effect of phenobarbital has been described also in mice [2,4]. This induction of a cytoplasmic dehydrogenase is apparent-

ly different from the known induction of microsomal and some mitochondrial enzymes by phenobarbital [2,3].

Recent studies on rat liver aldehyde dehydrogenase have revealed the presence of several enzyme types with different properties and different subcellular distribution [5—11]. In this report we present data on the subcellular distribution of aldehyde dehydrogenase activity after treatment with phenobarbital in rats with different responses to induction. The induced aldehyde dehydrogenase was partially purified and characterized in order to clarify its relation to the enzymes normally present in the cytoplasmic fraction of rat liver.

Materials and Methods

Animals

Male albino rats of the Wistar/Af/Han/Mol/(Han 67) strain, purchased as specific pathogen-free from Møllegaards Avels-laboratoriet (A/S) (Ejby, Denmark), were used. The animals were given a standard laboratory diet and water ad libitum. In order to select between the reactor and nonreactor animals the rats were given phenobarbital 80 mg/kg intraperitoneally as a single daily dose for 4 consecutive days. Liver biopsy samples weighing about 2 g were taken and analyzed for aldehyde dehydrogenase activity as described previously [11]. The animals were then divided into two groups representing a low responder (r) and a high responder (R) group in the ease of induction of cytoplasmic aldehyde dehydrogenase. After about one month from the operation the animals (six r and five R rats) were subjected to a second 4 days phenobarbital treatment and killed. Liver pieces weighing about 3 grams were taken for subcellular distribution studies. For the purification of the induced aldehyde dehydrogenase three male rats were used which had been previously shown to have a high response to induction of aldehyde dehydrogenase with phenobarbital. They were treated with phenobarbital for 4 days as described above and the whole livers were excised and used as starting material for the purification.

Reagents

NAD^+, D-glucuronolactone, 8-hydroxyquinoline 5-sulphonic acid and diethyl stilbestrol were purchased from Sigma Chemical Company, St. Louis, Mo. Estrone, deoxycorticosterone and progesterone were products of Ikapharm, Ramat-Gan, Israel. Phenylacetaldehyde, pyrazole and 2,2-diethoxy-ethyltrimethylammonium iodide were obtained from Aldrich-Europe, Beerse, Belgium. Benzaldehyde and anisaldehyde were from BDH Chemicals Ltd., Poole, Dorset, U.K. and all other aldehydes and disulfiram were obtained from Fluka AG, Buchs, Switzerland. Chloral hydrate, 2-mercaptoethanol and all the remaining chemicals used were products of E. Merck, Darmstadt, Germany. The ampholines 8143 and 8155 were purchased from LKB, Bromma, Sweden. CM-cellulose (CM 22) was a product of Whatman Biochemicals Ltd., Maidstone, Kent, U.K., and was prepared for use as directed by the manufacturer. Sephadex G-200 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Betaine aldehyde was synthesized from 2,2-diethoxy-ethyltrimethylammonium iodide according to the principle of Bergel et al. [12].

Subcellular fractionation

The subcellular fractionation was performed as described previously [11], with the difference that the pellet obtained after centrifugation at 12 500 × *g* was not discarded but designated as lysosomal fraction. The mitochondrial, lysosomal and microsomal pellets were suspended in 0.16 M KCl. Before activity measurements all fractions were treated with 1% Triton X-100 (w/v, final concentration).

Purification of the induced aldehyde dehydrogenase

For the purification of the induced aldehyde dehydrogenase the livers of three reactor (R) rats were used as starting material. After decapitation and excision of the livers, a 10% (w/v) homogenate was made with a Potter-Elvehjem type homogenizer in ice-cold 0.25 M sucrose solution which contained 10 mM sodium phosphate, pH 7.0, 5 mM 2-mercaptoethanol and 2 mM 8-hydroxyquinoline sulphonic acid. All the following steps were carried out at +4°C. The cytoplasmic fraction was isolated by centrifuging the homogenate, first for 20 min at 12 000 × *g* and then for 90 min at 76 000 × *g*.

The clear cytoplasmic fraction was then dialyzed twice, against 20 vol. of 8 mM sodium phosphate, pH 6.0, plus 5 mM 2-mercaptoethanol and 2 mM 8-hydroxyquinoline sulphonic acid. The dialyzed sample was then applied to a CM-cellulose column (3.0 × 40 cm) equilibrated with the dialysis buffer. After the application of the sample the column was eluted with 2 vol. of the starting buffer and then a linear sodium phosphate gradient from 8 to 100 mM was started. The eluted fractions (13 ml) were analyzed for their protein concentration and aldehyde dehydrogenase activity. The fractions containing the highest activity were collected and designated as CM-fraction I (eluted with the starting buffer) and CM-fraction II (eluted with the phosphate gradient). A part of the CM-fraction II was concentrated to about one tenth in volume in an Amicon cell with a PM 10 membrane. The concentrated enzyme was then passed through a Sephadex G-200 column (4.0 × 50 cm) equilibrated with 10 mM sodium phosphate pH 6.0 plus 5 mM 2-mercaptoethanol and 2 mM 8-hydroxyquinoline sulphonic acid.

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 5—8 and 7—9 were used with a 1% (w/v) concentration of ampholyte. 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 and aldehyde dehydrogenase activity of each fraction were measured.

Assay methods

Aldehyde dehydrogenase activity was measured at 25°C by following the NADH production either spectrophotometrically at 340 nm with a Gilford model 2000 attachment to the Beckman DU monochromator or fluorometrically with a Farrand A-4 ratio fluorometer. The standard assay mixture contained 70 mM sodium pyrophosphate, 1.33 mM NAD^+, 1.67 mM pyrazole and 9 mM propionaldehyde, pH 8.0, unless stated otherwise. In the activity local-

ization analyses of column chromatography samples of the eluted fractions were incubated for 30 min at 37° C with the assay mixture. 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.

Protein was determined in homogenates by the biuret method [13] and in purified preparations and column eluates by the method of Lowry et al. [14]. Dry bovine serum albumin (Armour) was used as standard.

Results

Subcellular distribution of aldehyde dehydrogenase activity. Activities were measured as described in Materials and Methods, with 9 mM propionaldehyde, 18 mM and 0.12 mM acetaldehyde, 2 mM glycolaldehyde, 6 mM formaldehyde and 0.5 mM betaine aldehyde as substrates.

There was about a 12-fold increase in the cytoplasmic aldehyde dehydrogenase activity in the reactor group compared with the non-reactor animals when 9 mM propionaldehyde was the substrate (Table I). The mitochondrial activity was also significantly higher ($P < 0.01$) in the reactor animals. The difference in the activities when measured in the homogenates was about 2.5-fold between the groups. When 18 mM acetaldehyde was used as substrate, the reactor group had 7-fold higher activity in the cytoplasmic fraction and about 2-fold higher activity in the homogenate. Also with this substrate the mitochondrial activities were slightly though significantly ($P < 0.025$) higher in the reactor group.

When the acetaldehyde concentration was lowered to 0.12 mM the activities in cytoplasmic and mitochondrial fractions were similar in both groups. With 2 mM glycolaldehyde as substrate there was no difference between the groups in any fractions. The activity with 18 mM D-glucuronolactone as substrate was about 3 times higher in the cytoplasmic fractions of reactor (R) rats. The activity with formaldehyde could not be measured accurately in the cytoplasmic fraction due to the additional formaldehyde dehydrogenase activity and endogenous reduced glutathione. In the mitochondrial fractions activities with 6 mM formaldehyde were 0.25 ± 0.07 and 0.28 ± 0.04 units/g liver wet weight for the reactor and non-reactor groups, respectively. Neither could any significant difference be found in the cytoplasmic betaine aldehyde dehydrogenase activity (in the reactor group 1.08 ± 0.07 and in the nonreactor group 0.95 ± 0.19 units/g liver wet weight).

There were no differences between the groups in the aldehyde dehydrogenase activities of the microsomal, lysosomal or nuclear fractions with any aldehyde substrate tested.

Purification of the induced aldehyde dehydrogenase. The degree of induction of aldehyde dehydrogenase activity in the reactor rats used for purification was similar to that of the reactor group in the subcellular distribution studies. In the CM-cellulose chromatography one enzyme fraction could be eluted with the starting buffer (Fig. 1). This unbound fraction was of the same relative magnitude as that found in similar experiments with non-induced liver material. The CM-fraction I was active also with glycolaldehyde and had all of the betaine aldehyde dehydrogenase activity of the cytoplasmic fraction. A linear

TABLE I

SUBCELLULAR DISTRIBUTION OF ALDEHYDE DEHYDROGENASE ACTIVITY IN REACTOR (R) AND NON-REACTOR (r) RATS AFTER TREATMENT WITH PHENOBARBITAL

Values are means ± S.D. $N = 6$ in r group and 5 in R group.

Substrate		Homogenate	Nuclear fraction	Activity (units/g liver wet weight)			
				Mitochondrial fraction	Lysosomal fraction	Microsomal fraction	Cytoplasmic fraction
Propionaldehyde	r	2.70 ± 0.23	0.84 ± 0.19	0.99 ± 0.07	0.25 ± 0.04	1.06 ± 0.10	0.25 ± 0.05
9 mM	R	6.29 ± 0.68***	0.96 ± 0.15	1.17 ± 0.11**	0.31 ± 0.10	1.00 ± 0.12	3.01 ± 0.82***
Acetaldehyde	r	2.21 ± 0.22	0.64 ± 0.15	0.71 ± 0.05	0.21 ± 0.03	0.92 ± 0.09	0.21 ± 0.03
18 mM	R	4.63 ± 1.01***	0.74 ± 0.13	0.86 ± 0.06*	0.25 ± 0.07	0.87 ± 0.10	1.40 ± 0.40***
Acetaldehyde	r	0.35 ± 0.06	0.18 ± 0.05	0.20 ± 0.02			0.04 ± 0.02
0.12 mM	R	0.30 ± 0.08	0.17 ± 0.03	0.21 ± 0.04			0.02 ± 0.02
Glycolaldehyde	r	1.08 ± 0.13	0.45 ± 0.12	0.49 ± 0.05			0.17 ± 0.02
2 mM	R	1.07 ± 0.24	0.44 ± 0.09	0.48 ± 0.08			0.20 ± 0.04

* $P < 0.025$
** $P < 0.01$
*** $P < 0.001$
} for difference between r and R groups.

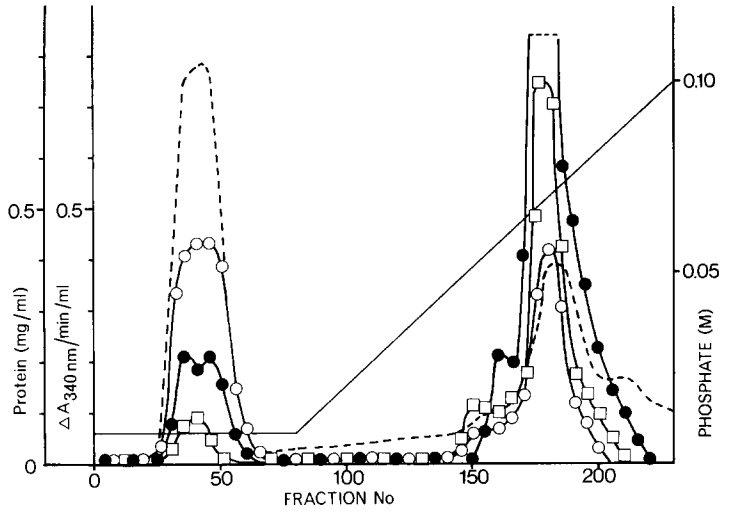


Fig. 1. CM-cellulose chromatography of rat liver cytoplasmic fraction after phenobarbital treatment. The experimental procedure and the assay methods are described in the text. The fractions were analyzed for aldehyde dehydrogenase activity with 2.0 mM propionaldehyde (●), 1.0 mM glycolaldehyde (○) and 18 mM D-glucuronolactone (□) as substrates and for protein concentration (- - -).

phosphate concentration gradient eluted the induced enzyme activity at about 70 mM phosphate concentration. In comparison with a similar experiment using a non-induced liver cytoplasmic fraction there was at least a 10-fold increase in the activity with propionaldehyde. This CM-fraction II was also active with D-glucuronolactone as substrate.

Sephadex G-200 chromatography of the CM-fraction II (Fig. 2) did not separate the propionaldehyde and D-glucuronolactone dehydrogenase activities. By these two steps about 70-fold purification was achieved (Table II). At-

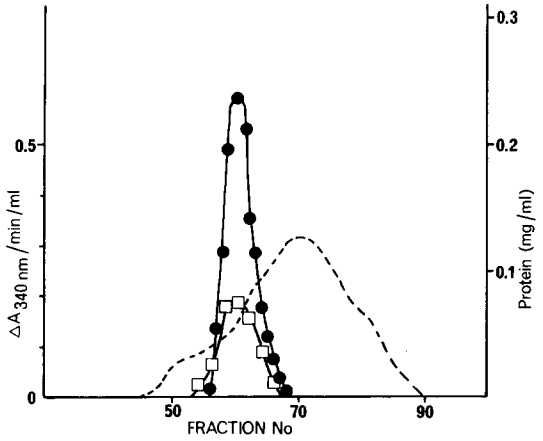


Fig. 2. Sephadex G-200 gel filtration of the CM-fraction II. The experimental procedure and the assay methods are described in the text. Fractions were analyzed for aldehyde dehydrogenase activity with 2.0 mM propionaldehyde (●) and 18 mM D-glucuronolactone (□) as substrates and for protein concentration (- - -).

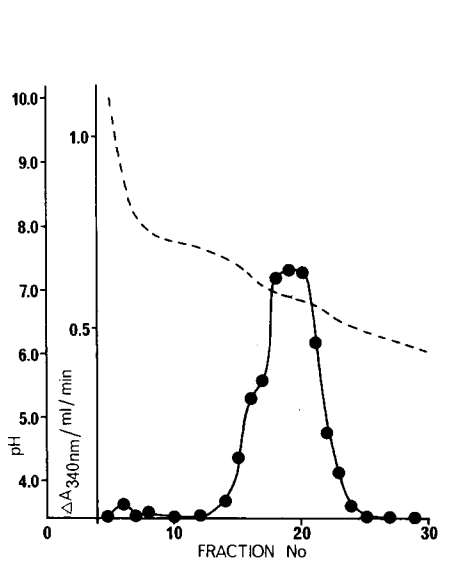
TABLE II

PURIFICATION OF THE INDUCED ALDEHYDE DEHYDROGENASE

Purification step	Total activity (units)	Total protein (mg)	Spec. act. (units/mg protein)
Cytoplasmic fraction	63.5	2990	0.0212
CM-cellulose Fr I	0.50	437	0.0011
Fr II	32.3	160	0.202
Sephadex G-200 gel filtration	12.1	9.1	1.33

tempts were also made to improve the purification by isoelectric focusing before the gel chromatography step. Because of considerable inactivation of the enzyme during the focusing better purification was not obtained by this method. The focused enzyme was used, however, for characterization purposes, after an intensive dialysis against 0.01 M sodium phosphate pH 7.4 plus 2 mM 2-mercaptoethanol to remove the ampholytes.

Thermostability of the induced aldehyde dehydrogenase activity. The cytoplasmic fraction from the phenobarbital-treated reactor rats gave different patterns of thermal inactivation with propionaldehyde and 4-carboxybenzaldehyde as substrates when exposed to 57°C at pH 7.4 (Fig. 4). The 4-carboxybenzaldehyde oxidizing activity disappeared considerably faster than the propionaldehyde oxidizing activity.



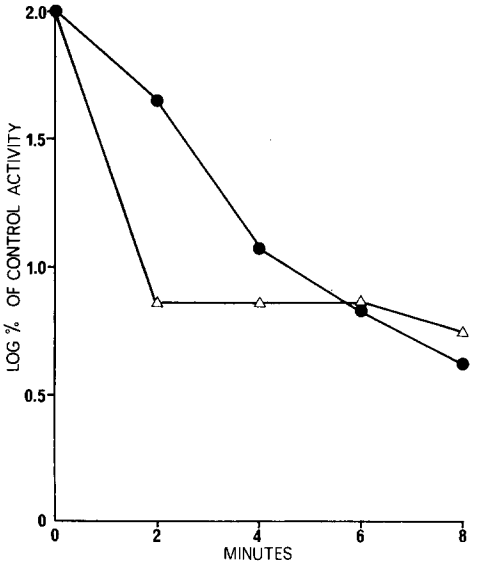


Fig. 3. Isoelectric focusing of the CM-fraction II. The experimental procedure and the assay methods are described in the text. Fractions were analyzed for aldehyde dehydrogenase activity with 2.0 mM propionaldehyde (●) and for pH (- - -).

Fig. 4. Heat denaturation of propionaldehyde (●) and *p*-carboxybenzaldehyde (△) oxidizing activities of the cytoplasmic fraction in phenobarbital treated reactor-rats. A sample was exposed to 57°C in 0.05 M sodium phosphate pH 7.4 containing 2 mM 2-mercaptoethanol. Then aliquots were withdrawn, centrifuged and assayed for aldehyde dehydrogenase activity as described in the text.

Determination of molecular weight. A calibrated Sephadex G-200 column was used. An apparent molecular weight of 150 000 was estimated for the propionaldehyde and also for the D-glucuronolactone oxidizing activities of the CM-fraction II.

Isoelectric focusing. The bound CM-fraction II revealed two peaks with aldehyde dehydrogenase activity by isoelectric focusing (Fig. 3). Most of the activity was focused at pH 7.0 as a symmetrical peak. A minor aldehyde dehydrogenase fraction with a pI of 8.5 was also found which evidently represents an enzyme fraction previously described as being present in non-induced rat liver cytoplasm [11]. Because of disturbing blank reactions due to ampholine, D-glucuronolactone dehydrogenase could not be accurately measured in the focused fractions.

Substrate specificity. The partially purified enzyme had the highest activity with propionaldehyde from the 15 aldehydes tested (Table III). The enzyme was not active with formaldehyde, glycolaldehyde, D-glyceraldehyde, betaine aldehyde or 4-carboxybenzaldehyde as substrates.

K_m-values. The induced aldehyde dehydrogenase had an apparent K_m of $3.5 \cdot 10^{-5}$ M for NAD^+ when measured at a constant propionaldehyde concentration of 9.0 mM. A high apparent K_m, 4.0 mM, was obtained for acetaldehyde and a somewhat lower value, 0.55 mM, for propionaldehyde (measured with 1.33 mM NAD^+). The K_m values for aromatic aldehydes were around 10^{-5} M and a marked inhibition by substrate was seen at aldehyde concentrations as low as 10^{-4} M. The apparent K_m values for aldehydes are not corrected for the hydration of aldehydes.

Effect of pH and phosphate. The maximal rate of the partially purified enzyme with propionaldehyde as substrate was achieved at pH 9.2—9.5. The activity was considerably higher in sodium pyrophosphate buffer than in so-

TABLE III

SUBSTRATE SPECIFICITY OF THE INDUCED ALDEHYDE DEHYDROGENASE

The partially purified enzyme from the focusing step was used. Aldehyde dehydrogenase was assayed as described in the text with 1.33 mM NAD in 70 mM sodium pyrophosphate pH 8.0.

Substrate	Concentration (mM)	Relative velocity
Formaldehyde	3.0	0
Acetaldehyde	3.0	0.37
Propionaldehyde	3.0	1.00
N-butanal	3.0	0.96
Hexanal	0.16	0.73
4-carboxybenzaldehyde	0.16	0
Banzaldehyde	0.04	0.53
Anisaldehyde	0.04	0.23
3-nitrobenzaldehyde	0.04	0.88
4-nitrobenzaldehyde	0.04	0.30
4-chlorbenzaldehyde	0.04	0.92
Phenylacetaldehyde	0.04	0.86
Betaine aldehyde	3.0	0
DL-glyceraldehyde	3.0	0
Glycolaldehyde	3.0	0

TABLE IV

EFFECT OF SOME INHIBITORS OR ACTIVATORS ON THE INDUCED ALDEHYDE DEHYDROGENASE

The partially purified enzyme from the focusing step was used. Aldehyde dehydrogenase was assayed in 70 mM sodium pyrophosphate buffer pH 8.0 with 1.33 mM NAD and 3.0 mM propionaldehyde.

Inhibitor	Concentration (mM)	% of activity without an inhibitor/activator
Disulfiram	$0.3 \cdot 10^{-7}$	43
	$0.7 \cdot 10^{-7}$	31
	$1.7 \cdot 10^{-7}$	23
Sodium arsenite	$0.67 \cdot 10^{-3}$	87
	$0.67 \cdot 10^{-3}$ + 3.3 mM ME	82
	$6.7 \cdot 10^{-3}$	78
	$6.7 \cdot 10^{-3}$ + 3.3 mM ME	49
EDTA	$0.67 \cdot 10^{-3}$	98
	$6.7 \cdot 10^{-3}$	69
Estrone	$3.3 \cdot 10^{-6}$	185
Deoxycorticosterone	$3.3 \cdot 10^{-6}$	120
	$3.3 \cdot 10^{-5}$	24
Diethyl stilbestrol	$3\,3 \cdot 10^{-6}$	350
Progesterone	$3.3 \cdot 10^{-6}$	38

dium phosphate or sodium hydroxide/glycine buffers. There was no evidence for stimulation by phosphate when the concentration was increased from 0.01 to 0.15 M.

Effect of some inhibitors, steroids and diethylstilbestrol. The effects of some potential effectors on the induced aldehyde dehydrogenase are presented in Table IV. Disulfiram was a very potent inhibitor of the enzyme even at submicromolar concentrations. The concentration of sodium arsenite needed for effective inhibition was relatively high but the inhibition was somewhat potentiated by the addition of 2-mercaptoethanol to the incubation mixture. In addition to the list of inhibitors presented in Table IV, chloral hydrate was found to be a competitive inhibitor with the aldehyde with a K_i of 0.23 mM.

Estrone, deoxycorticosterone and diethylstilbestrol caused a marked activation of the enzyme at micromolar concentrations. When the concentration of deoxycorticosterone was raised the effect became an inhibition. Progesterone had an inhibitory effect at micromolar concentrations.

Discussion

Previous reports by Deitrich et al. [2,3] had already given evidence that not all of the cytoplasmic aldehyde dehydrogenase types in rat liver may be induced by phenobarbital. They also suggested, mainly on the basis of the complex thermostability pattern of the cytoplasmic aldehyde dehydrogenase activity, that the induced enzyme may be different from the normal enzyme in untreated rats and also from the enzyme in phenobarbital-treated non-reactor rats. Several different enzyme types may also be induced or the phenobarbital treatment may change the properties of an enzyme normally present.

In the present study the degree of induction of rat liver cytoplasmic aldehyde dehydrogenase by phenobarbital in the reactor animals was in accord with the previous results [2,10]. A great difference between the reactor and

non-reactor groups was seen only in the cytoplasmic activities measured with high (millimolar) concentrations of acetaldehyde or propionaldehyde. Acetaldehyde at 0.12 mM concentration, 6 mM formaldehyde and 2 mM glycolaldehyde were not oxidized differently by any subcellular fraction of the two groups. Our results also show that the cytoplasmic betaine aldehyde dehydrogenase (EC 1.2.1.8) [15] is not induced by phenobarbital treatment. The cytoplasmic D-glucuronolactone dehydrogenase activity (EC 1.1.1.70) was 3-fold higher in the reactor group than in the non-reactor group confirming the previous results of Marselos and Hänninen [10]. It has been suggested that the cytoplasmic D-glucuronolactone and aldehyde dehydrogenase activities are due to a single enzyme [11,16,17]. Although the degree of induction was different for these two activities, we were not able to separate them with the chromatographic techniques used in this study.

In the mitochondrial fraction a small increase was seen in the reactor group in the activities measured with a high concentration of acetaldehyde or propionaldehyde, but not when measured with glycolaldehyde or with a low acetaldehyde concentration. The increase would thus be in the high-K_m mitochondrial enzyme II [11]. This effect was, however, small, compared with that in the cytoplasmic fraction and a slight contamination of the mitochondrial fraction by cytoplasm cannot be ruled out. Deitrich et al. [2] found no difference in the mitochondrial activities between phenobarbital-treated reactor and non-reactor rats but they used the relatively low aldehyde concentration of 0.33 mM. A small decrease has also been reported [18] in mitochondrial aldehyde dehydrogenase activity measured with 5 mM acetaldehyde in phenobarbital-treated non-reactor rats with non-treated rats as controls.

The induced aldehyde dehydrogenase can be separated from a non-induced cytoplasmic aldehyde dehydrogenase by CM-cellulose chromatography. This noninduced fraction, not bound to CM-cellulose at pH 6.0 obviously corresponds to the cytoplasmic DEAE-fraction II which we have previously described [11], and possibly does not represent a true cytoplasmic enzyme. The induced fraction has a similar elution pattern in the ion exchange chromatography to the cytoplasmic high K_m-enzyme (DEAE-fraction I in ref. 11, unpublished observations on CM-cellulose) in untreated rats. There are, however, several differences in the properties of these two enzyme fractions. The isoelectric point of the induced enzyme is lower at 7.0, than that of the normal enzyme, at 8.5. A small activity peak at pH 8.5 was, however, found in the isoelectric focusing of the CM-fraction II from induced livers, but in the focusing of the non-induced cytoplasmic fraction no peak was found at pH 7.0. The induced and the normal enzyme were found to have several differences in substrate specificity especially for formaldehyde, glycolaldehyde and aromatic aldehydes. The K_m-values for aldehydes also differed. Further, the induced enzyme is also more strongly inhibited by disulfiram than the normal enzyme.

The present results suggest that the increase in liver aldehyde dehydrogenase activity after treatment with phenobarbital in reactor rats is due to an enzyme which has different molecular and enzymic properties from the cytoplasmic aldehyde dehydrogenases normally detectable in untreated animals. Whether it truly represents a new enzyme or some kind of conversion of a normal enzyme fraction remains to be elucidated.

Acknowledgements

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