

## DIFFERENCES IN THE KINETIC PROPERTIES AND SENSITIVITY TO INHIBITORS OF HUMAN PLACENTAL, ERYTHROCYTE, AND MAJOR HEPATIC ALDEHYDE DEHYDROGENASE ISOENZYMES

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**Abstract**—(i) The characteristics of the major human hepatic isoenzymes of aldehyde dehydrogenase (ALDH), ALDH I and ALDH II, were compared with the ALDH activities found in human placenta and erythrocytes. (ii) In human liver biopsies, the  $K_m$  of ALDH I was approximately 7  $\mu\text{mol/L}$  whereas it was 32  $\mu\text{mol/L}$  for ALDH II. The  $V_{\text{max}}$  for ALDH I was 2–3 times greater than the ALDH II  $V_{\text{max}}$ . Human liver ALDH I and II also differed in their sensitivity in inhibitors. Namely, ALDH I was less sensitive to disulfiram than the ALDH II isoenzyme. (iii) ALDH activity in human placenta and erythrocytes was much lower than in liver tissue. Kinetic data showed that placental ALDH isoenzyme had a high  $K_m$  (in the millimolar range) and increased its activity raising the pH from 7.4 to 8.8, more than the hepatic ALDH I and ALDH II isoenzymes did. Erythrocyte ALDH activity presented a dual component; the smaller one was characterized by a low  $K_m$  (micromolar range), whereas most of the ALDH activity showed a high  $K_m$  (millimolar range). (iv) Placental ALDH was resistant to nitrefazole inhibition and was inhibited by disulfiram in a manner similar to the hepatic ALDH I isoenzyme; erythrocyte ALDH was more sensitive to the inhibitory action of disulfiram and nitrefazole. (v) It is concluded that erythrocyte and placental ALDH isoenzymes are different from the hepatic ALDH I and ALDH II forms. It is also suggested that placental and erythrocyte ALDH isoenzymes are different high- $K_m$  isoenzymes.

Acetaldehyde is a toxic metabolite generated by ethanol oxidated by either the alcohol dehydrogenase in cytosol, the microsomal ethanol metabolizing system in the endoplasmic reticulum or the catalase in the peroxisomes. In addition, acetaldehyde can be oxidized to acetic acid by aldehyde dehydrogenase (EC 1.2.1.3).

Aldehyde dehydrogenase is widely distributed in mammals. The highest activities are found in the liver in the form of isoenzymes [1–4]. Four different isoenzymes, ALDH I, ALDH II, ALDH III and ALDH IV, have been visualized in human liver [5–7]. ALDH I and ALDH II isoenzymes represent low- $K_m$  aldehyde dehydrogenase and play the most important role in acetaldehyde metabolism [8, 9], whereas ALDH III and ALDH IV are high  $K_m$  aldehyde dehydrogenases. ALDH IV has been identified as a glutamic  $\gamma$ -semialdehyde dehydrogenase (EC 1.5.1.12) [10].

Erythrocytes are also capable of acetaldehyde metabolism [11, 12], however it is unclear whether they play a substantial role in the *in vivo* removal of acetaldehyde from blood. Electrophoresis of erythrocyte ALDH shows a single activity band with mobility similar to human liver ALDH II isoenzyme [8]. In the placenta, ALDH activity is also present although in small amounts [13, 14]. Furthermore, placental ALDH isoenzyme presents a

high  $K_m$  in the millimolar range [14, 15] and has also been reported to be a glutamic  $\gamma$ -semialdehyde dehydrogenase [16].

In this study we have compared the aldehyde dehydrogenase isoenzymes present in human erythrocytes or placenta with human hepatic ALDH I and ALDH II isoenzymes in an attempt to ascertain whether ALDH in erythrocytes or placenta might play an important role in acetaldehyde oxidation. That is specially relevant during pregnancy provided that acetaldehyde has been reported to induce teratogenic effects in mice [17]. To that effect, we have investigated both the kinetic variables displayed by these isoenzymes as well as their sensitivity to different ALDH inhibitors. On the basis of that, we report here that both erythrocyte and placental ALDH are different from ALDH I and ALDH II, the two major liver isoenzymes. Furthermore, the capacity of placental and erythrocyte ALDH to metabolize acetaldehyde after an ethanol dosage seems rather limited.

### MATERIALS AND METHODS

Fresh human liver biopsies (approximately 0.5–2 g of tissue) taken for diagnosis during abdominal surgery (cholecystectomy) from five male and four female patients who were later proven to be histopathologically unaffected were used. Immediately after excising biopsy samples were placed in ice-cold 50 mmol/L sodium phosphate buffer at pH 7.4.

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Blood samples for investigation of erythrocyte ALDH were obtained from the same liver biopsy donors. Normal human term placentae obtained after parturition from healthy pregnant women were collected at the Maternity Clinic of the Hospital "La Paz", Madrid. The placentae were kept in ice-cold 30 mmol/L sodium phosphate buffer at pH 7.5 immediately after parturition and processed within 1 hr. Subjects were informed on the nature and purpose of the study before they gave their voluntary consent. The experimental protocol was reviewed and approved by the Institutional Clinical Study Committee.

**Determination of aldehyde dehydrogenase activity.** Liver and placenta biopsies were homogenized in 20 mmol/L sodium phosphate buffer pH 7.4 (ratio 1:1, w/v) with a Teflon pestle placed in ice and sonication in a MSE sonifier (set at 12  $\mu$  for 1 min) to disrupt the integrity of subcellular organelles. This method is effective in disrupting mitochondria and releasing enzymes from the mitochondrial matrix to the medium. The sonified homogenates were centrifuged at 56,000 *g* for 20 min, at 4°. The resulting supernatants were centrifuged at 143,000 *g* for 60 min, and the final supernatants, which contained the overall non-membrane bound ALDH, were used for enzymatic assays. ALDH activity was assayed on the same day using the following spectrophotometric method described by Blair and Bodley [18] with few modifications [19]. Aldehyde dehydrogenase assay was performed at 37° in cuvettes containing 100 mmol/L glycine-NaOH, 130 mmol/L KCl, 1 mmol/L pyrazole, 1 mmol/L NAD<sup>+</sup> and 20  $\mu$ L of sample, at pH 7.4 or 8.8. The reaction was started by adding acetaldehyde 0.05, 0.3 or 20 mmol/L (final concentration) to measure the different ALDH isoenzymes. Correction for small unspecific NADH formation during the assay was appropriately corrected. The precision of the ALDH activity assay was typically about 8%.

When aldehyde dehydrogenase was assayed in erythrocytes, blood was collected with heparin, and a previous partial purification of ALDH was required. That was done according to Inoue *et al.* [20]. In brief, blood was centrifuged at 1000 *g* for 10 min and plasma was discarded. Erythrocytes were washed three times by saline and successive centrifugations. Hemolysis of washed erythrocytes was performed by 1:5 dilution of cells in 1 mmol/L EDTA, 0.1% mercaptoethanol solution and further sonication. EDTA and mercaptoethanol were added to prevent reduction of ALDH activity during the purification procedure by heavy metal ions or sulfhydryl reagents [20]. Hemolysate was centrifuged at 56,000 *g* for 20 min at 4°, and 1 mL of supernatant was passed through a column containing 20 mL of Sephadex C-50, at 5°. The column had been previously equilibrated in 20 mmol/L sodium phosphate buffer pH 6.0. After addition of sample, the column was washed with 20 mmol/L sodium phosphate buffer, pH 6.0 and eluate aliquots were collected. The eluate immediately following the void volume contained all ALDH activity, in keeping with a previous report [20]. Aldehyde dehydrogenase activity in the erythrocyte eluates was assayed at 37° in cuvettes containing 100 mmol/L sodium phosphate buffer,

1 mmol/L NAD<sup>+</sup>, pH 7.4 and 200  $\mu$ L of eluate. The reaction was started by the addition of 0.05 or 20 mmol/L acetaldehyde (final concentration) to measure ALDH activity of low and high  $K_m$  values, respectively. For  $K_m$  and  $V_{max}$  measurements of ALDH, acetaldehyde was added at final concentrations ranging from 0.005 to 40 mmol/L. In some experiments, ALDH inhibitors such as disulfiram and nitrefazole, dissolved in dimethylsulfoxide, were added to the incubation media. Identical volumes of dimethylsulfoxide were added to the control (no inhibitor) cuvettes in these experiments. In all the assays, measurements were made with a Beckman DU-8B (Kinetics II) spectrophotometer at 340 nm, and 1 unit of enzymatic activity was considered to correspond to 1  $\mu$ mol of substrate transformed per min. Kinetic parameters were obtained by determining ALDH activity at high (0.1–40 mmol/L) and low (0.005–0.05 mmol/L) acetaldehyde concentration ranges. Proteins were determined by the method of Lowry *et al.* [21]. Results are expressed as mean  $\pm$  SE and statistical comparison among the groups was performed by analysis of variance and further post-hoc *t*-tests.

## RESULTS

We have previously reported [19] that soluble microsome-free extracts from human liver only contained the two major aldehyde dehydrogenase isoenzymes, called ALDH I (or E<sub>2</sub>) and ALDH II (or E<sub>1</sub>) [5, 6]. The activity of hepatic ALDH isoenzyme ALDH I (E<sub>2</sub>) is greater than ALDH II (E<sub>1</sub>) (Table 1). In turn, ALDH activity in erythrocytes or in the placenta is much lower than ALDH I or ALDH II when expressed either per gram of tissue or as total activity (Table 1). No differences were found between male and female subjects when comparing ALDH activity from erythrocytes or liver extracts.

The kinetic parameters of ALDH from different tissues are presented in Table 2. In liver, at either pH 7.4 or 8.8, ALDH I isoenzyme shows a very low  $K_m$  value for acetaldehyde (6–9  $\mu$ mol/L) and a relatively high  $V_{max}$ . The  $K_m$  of hepatic ALDH II isoenzyme is substantially higher than for ALDH I but still remains in the micromolar range (32–34  $\mu$ mol/L). The  $V_{max}$  of hepatic ALDH II represented, at both pH 7.4 or 8.8, just 30–35% of the  $V_{max}$  for hepatic ALDH I. Two different components were obtained when the double-reciprocal plot for the ALDH activity was analysed in erythrocytes (data not shown). Therefore,  $K_m$  values for acetaldehyde were determined at high and low substrate concentration ranges in the erythrocytes extracts. This is in keeping with another report [20] in which such a dual component was detected even after purification to homogeneity. Thus, the high affinity component presented a  $K_m$  in the micromolar range, but the low affinity component, the majority of the total erythrocyte ALDH activity, showed a  $K_m$  in the millimolar range. Therefore, the  $K_m$  displayed by the erythrocyte low affinity component was much greater compared with the  $K_m$  values obtained for hepatic ALDH I or ALDH II isoenzymes (Table 2). Furthermore, the millimolar range for the placental ALDH  $K_m$  was much larger than the  $K_m$  for the

Table 1. Aldehyde dehydrogenase activities in human liver, erythrocytes and placenta

	Aldehyde dehydrogenase activity		
	mUnits/g tissue	μUnits/mg protein	Units/whole tissue
Liver			
ALDH I	651 ± 71	677 ± 79	1230
ALDH II	236 ± 58*	248 ± 62*	446
Erythrocytes	33 ± 6†	111 ± 18	71
Placenta	7 ± 1†	217 ± 44	4

Results are mean ± SE of 9, 11 and 25 observations in liver, erythrocytes and placenta, respectively. Aldehyde dehydrogenase activity was assayed in tissue extracts at pH 7.4, 37° and optimal acetaldehyde concentrations (0.05 mmol/L for hepatic ALDH I, 0.3 mmol/L for hepatic ALDH II, and 20 mmol/L in erythrocyte and placenta extracts). Erythrocyte aldehyde dehydrogenase activity accounted for by 0.13 mUnits/mg hemoglobin.

\* Denotes a significant difference between hepatic ALDH I and ALDH II, at P < 0.05.

† Denotes a significant difference with hepatic ALDH I, at P < 0.05.

Table 2. Kinetic parameters of aldehyde dehydrogenase in human liver, erythrocytes and placenta

	<i>K<sub>m</sub></i> (μmol/L)	<i>V<sub>max</sub></i> (mUnit/g)
Assayed at pH 7.4		
Hepatic ALDH I	6.4 ± 4.8	661 ± 21
Erythrocyte low- <i>K<sub>m</sub></i> isoenzyme	7.4 ± 2.1	8.6 ± 2.1
Hepatic ALDH II	34.2 ± 18.9	342 ± 72
Erythrocyte high- <i>K<sub>m</sub></i> isoenzyme	1185 ± 317*	33.8 ± 5.1*
Assayed at pH 8.8		
Hepatic ALDH I	9.1 ± 3.2	853 ± 88
Hepatic ALDH II	32.5 ± 5.3	267 ± 103
Placental high- <i>K<sub>m</sub></i> isoenzyme	3670 ± 410*	30.7 ± 3.6*

Results are mean ± SE of six observations in liver and erythrocytes and 18 in placenta. Aldehyde dehydrogenase activities were assayed at 37° at different acetaldehyde concentrations (ranging from 5 μmol/L to 40 mmol/L) and at pH 7.4 or pH 8.8. Hepatic low *K<sub>m</sub>* ALDH isoenzyme corresponds to ALDH I and hepatic high *K<sub>m</sub>* ALDH isoenzyme corresponds to ALDH II.

\* Denotes a significant difference between values from the liver extracts and the erythrocyte or placenta extracts, at P < 0.05.

hepatic ALDH I and ALDH II isoenzymes (Table 2).

The effect of pH on ALDH activity was next investigated in liver as well as in placental ALDH (Table 3). Under all circumstances, the optimum pH for activity was in the vicinity of pH 8.8 (data not shown). When the pH was raised from 7.4 to 8.8, ALDH activity increased dramatically (Table 3). However, whereas the increase in activity in hepatic ALDH I and ALDH II isoenzymes ranged between 67 and 109%, placental ALDH activity was modified to a greater extent (208%) by the rise in pH.

All this initial information supports the hypothesis that erythrocyte and placental ALDH isoenzymes are different from the major ALDH isoenzymes found in liver, ALDH I and ALDH II. To gain further insight into this question, the effect of the

Table 3. Effect of pH on human liver and placenta aldehyde dehydrogenase activities

	pH		% Increase
	7.4	8.8	
	ALDH activity (mUnits/g tissue)		
Hepatic ALDH I	651 ± 71	1087 ± 138*	67
Hepatic ALDH II	236 ± 58	493 ± 84*	109
Placenta	7.4 ± 1.5	22.8 ± 2.2*	208

Results are mean ± SE of nine observations in liver and 25 observations in placenta. Aldehyde dehydrogenase activity was assayed in tissue extracts at 37°, pH 7.4 or 8.8 and 20 mmol/L acetaldehyde when measured in placenta, or 0.05 and 0.3 mmol/L acetaldehyde when measured in liver extracts. Hepatic aldehyde dehydrogenase activity measured at 0.05 mmol/L acetaldehyde corresponds to the hepatic ALDH I isoenzyme, whereas the activity assayed at 0.3 mmol/L is the result of both ALDH I and ALDH II isoenzymes.

\* Denotes a significant difference between pH 7.4 and 8.8, at P < 0.05.

known ALDH inhibitors, disulfiram [22] and nitrefazole [23], was investigated. Results comparing the effect of inhibitors on ALDH isoenzymes in liver and placenta are presented in Table 4. In keeping with previous observations [9, 24], low concentrations of disulfiram (10 μmol/L) caused a greater inhibition of hepatic ALDH II as compared to that observed in ALDH I (Table 4). Addition of 10 μmol/L disulfiram caused an inhibition of placental ALDH similar to the one observed in hepatic ALDH I, the isoenzyme which is resistant to disulfiram (Table 4). Under these conditions, 100 μmol/L nitrefazole did not modify hepatic ALDH II or placental ALDH activities, although it did cause a 30% inhibition in ALDH I activity. Thus, we conclude that placental ALDH isoenzyme is different from the hepatic ALDH I or ALDH II forms, on the basis of its sensitivity to disulfiram and nitrefazole, in addition to previous commented differences.

Table 4. Effect of disulfiram and nitrefazole on hepatic and placental aldehyde dehydrogenase activities

Additions	Hepatic ALDH I	Hepatic ALDH II (mUnits/g tissue)	Placenta
None	1069 ± 107	464 ± 129	16 ± 2
Disulfiram 10 µmol/L	961 ± 134 (11%)	270 ± 106* (58%)	13 ± 2 (18%)
Disulfiram 100 µmol/L	54 ± 28* (95%)	14 ± 11* (97%)	1.7 ± 4.4* (89%)
Nitrefazole 100 µmol/L	730 ± 101* (32%)	508 ± 143 (-)	15 ± 2 (3%)

Results are mean ± SE of four observations in liver and 21 in placenta. Data in brackets indicate percentage of inhibition caused by the inhibitors. Aldehyde dehydrogenase activity was assayed in tissue extracts at 37°, pH 8.8 and different acetaldehyde concentrations (20 mmol/L in placental extracts and as explained in legend to Table 3 in liver extracts).

\* Denotes a significant difference with the control group (no additions), at  $P < 0.05$ .

Table 5. Effects of disulfiram and nitrefazole on hepatic and erythrocyte aldehyde dehydrogenase activities

Additions	Liver		Erythrocyte	
	ALDH I	ALDH II (mUnits/g tissue)	Low $K_m$	High $K_m$
None	576 ± 147	311 ± 128	7.3 ± 1.3	38.2 ± 5.3
Disulfiram 10 µmol/L	558 ± 160 (4%)	111 ± 91* (64%)	1.0 ± 0.59* (87%)	5.1 ± 2.1* (87%)
Disulfiram 100 µmol/L	0* (100%)	19 ± 19* (94%)	0* (100%)	0* (100%)
Nitrefazole 100 µmol/L	426 ± 123 (26%)	32 ± 25* (90%)	4.7 ± 1.1 (35%)	24 ± 4* (37%)

Results are mean ± SE of four observations in liver and eight in erythrocytes. Data in brackets indicate percentage of inhibition caused by disulfiram or nitrefazole. Aldehyde dehydrogenase activity was assayed in tissue extracts at 37°, pH 7.4, and different acetaldehyde concentrations (20 mmol/L when erythrocyte extracts were used and 0.05 and 0.3 mmol/L in liver extracts). Low- $K_m$  and high- $K_m$  hepatic isoenzyme activities were determined as in Table 3.

\* Denotes a significant difference with the control group (no additions), at  $P < 0.05$ .

Additional experiments were performed to investigate the effects of disulfiram or nitrefazole on hepatic and erythrocyte ALDH isoenzymes (Table 5). Unlike placental ALDH, both the low and high  $K_m$  components of erythrocyte ALDH showed a high sensitivity to disulfiram; thus, 10 µmol/L disulfiram caused a greater inhibition in erythrocyte ALDH activity than in hepatic ALDH II isoenzyme, the disulfiram-sensitive hepatic isoenzyme (Table 5). Furthermore, 100 µmol/L nitrefazole caused a 35–37% inhibition of erythrocyte ALDH activity that fell between the inhibition observed in ALDH II (90%) and ALDH I (26%). These results allow us to conclude that erythrocyte ALDH isoenzyme is also different from the hepatic ALDH I and ALDH II isoenzymes.

#### DISCUSSION

The results of the present study suggest that, as judged by its kinetic properties and sensitivity to inhibitors, the ALDH activity found in the placenta and erythrocytes corresponds to isoenzymes other than hepatic ALDH I (or  $E_2$ ) and ALDH II (or  $E_1$ ).

This conclusion is in agreement with a recent report demonstrating that human placental ALDH is a glutamic  $\gamma$ -semialdehyde dehydrogenase similar to the hepatic ALDH IV isoenzyme [10, 16]. The physiological relevance of placental and erythrocyte ALDH in acetaldehyde oxidation is questionable; erythrocyte ALDH activity is very low, which suggests that it does not contribute much to acetaldehyde removal from the blood. In addition, the placental capacity for acetaldehyde removal has been described as rather limited in the rat [14].

Regarding hepatic ALDH I and ALDH II isoenzymes, we have reported that both isoforms are obtained in supernatant resulting from homogenization, sonication and centrifugation (143,000 g), implying its presence in cytosol or in mitochondrial matrix. It is believed that ALDH I is predominantly of mitochondrial origin whereas ALDH II is of cytosolic origin [9, 25]. The fact that ALDH III (or  $E_3$ ) or ALDH IV (or  $E_4$ ) are not detected under our conditions suggests that these isoenzymes are membrane-bound (perhaps in the microsomal fraction). Both hepatic ALDH I and ALDH II, should play an important role in oxidizing

acetaldehyde. However ALDH I is quantitatively more important than ALDH II, and accounts for 75% of the overall hepatic low  $K_m$  ALDH activity. In this regard, it should be pointed out that the existence of a correlation between alcohol sensitivity and elevated blood acetaldehyde levels in conjunction with ALDH I deficiency in Japanese subjects after an acute dose of ethanol has been previously reported [26–28]. Our data also clearly demonstrate that hepatic ALDH II is more sensitive to disulfiram than hepatic ALDH I, in keeping with other data [8, 9, 24].

Both kinetic parameters and sensitivity to inhibitors clearly allow us to conclude that erythrocyte ALDH is not similar to hepatic ALDH II isoenzyme although they share similar pI values [8]. Thus, erythrocyte ALDH has a major component characterized by high  $K_m$  values (in the millimolar range), and erythrocyte ALDH is more sensitive to disulfiram and less sensitive to nitrefazole than hepatic ALDH II.

ALDH activities found in placenta and erythrocytes seem to be different isoenzymes. This conclusion is based on two considerations, one kinetic and the other related to inhibitors sensitivity. Placental ALDH is a high  $K_m$  isoenzyme whereas erythrocyte has two components, a small fraction showing low  $K_m$  values and a major component presenting high  $K_m$  values. In addition, the pattern of inhibition by disulfiram and nitrefazole differs in erythrocyte and placental extracts. This conclusion is also supported by structural data; it has been reported that purified erythrocyte ALDH has a molecular weight of 210,000 as determined by gel filtration, and SDS–polyacrylamide gel electrophoresis yields a single protein band with a molecular weight of 51,500 [20]. However, purified human placental ALDH appears to be a dimer whose molecular weight is approximately 114,000 [16].

In conclusion, data from the present study demonstrates: (i) ALDH I is quantitatively the major low  $K_m$  aldehyde dehydrogenase isoenzyme found in fresh human liver biopsies, thereby indicating a more important role in acetaldehyde metabolism than ALDH II; (ii) placental and erythrocyte ALDH isoenzymes are different from hepatic ALDH I and ALDH II, based on kinetic parameters and sensitivity to inhibitors; (iii) in turn, the placental ALDH is not the same isoenzyme as erythrocyte ALDH. Taking into account the high  $K_m$  values displayed by placental and erythrocyte ALDH and the low maximal activities observed, we conclude that both activities play only a limited role in acetaldehyde removal from blood.

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