Pharmacology Biochemistry & Behavior, Vol. 18, Suppl. 1, pp. 167–170, 1983.

Oxidation of Acetaldehyde and Presence of Aldehyde Dehydrogenase in Rat Erythrocytes[1]

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WEINER, H., N. TRUESDALE-MAHONEY AND A. J. PELLETIER. *Oxidation of acetaldehyde and presence of aldehyde dehydrogenase in rat erythrocytes.* PHARMACOL BIOCHEM BEHAV **18:** Suppl. 1, 167–170, 1983.—Rat liver erythrocytes were found to oxidize acetaldehyde at 7 nmoles/min/ml blood at 37°C. This is less than 1% the rate that occurs in liver. An aldehyde dehydrogenase was isolated from erythrocytes, but was not purified. The enzyme had a K_m of 170 μM toward acetaldehyde at pH 7.4. The enzyme, which could oxidize both aliphatic and aromatic aldehydes, was more active at pH 9 than at 7. Disulfiram proved to be both an *in vivo* and *in vitro* inhibitor of the enzyme. Due to the low total capacity of the erythrocytes to metabolize acetaldehyde, it is doubtful they perform any important role in ethanol metabolism.

Aldehyde dehydrogenase Erythrocytes Acetaldehyde Ethanol Disulfiram Rat

ALDEHYDE dehydrogenase (ALDH), the enzyme responsible for catalyzing the oxidation of aldehydes to acids, is found in virtually every organ in the body. The best studied enzymes are those obtained from liver and brain [15]. It is in those two organs that the metabolism of acetaldehyde to acetate is mainly studied [14]. In rats, 95% of the acetaldehyde which is produced during the metabolism of ethanol is oxidized in liver [2]. The organs responsible for the remaining metabolism is not known with certainty.

Human erythrocytes [7] and blood [11] have been shown capable of oxidizing acetaldehyde. This observation led to a search for human erythrocyte ALDH. Recently, it was reported that such an enzyme exists and has properties similar to those of liver cytosol ALDH [3–6]. It has been reported that an inverse correlation exists between the level of ALDH activity in the erythrocytes and a person's heart rate after being administered a dose of ethanol [3]. Knowing that human erythrocytes oxidize acetaldehyde opens the question as to the importance of this system in maintaining the low blood acetaldehyde levels found after a person or laboratory animal consumes large quantities of ethanol [14]. Inasmuch as rodents are the major laboratory animals used in alcohol-related studies, we wanted to determine if ALDH was also present in rat erythrocytes. In this communication, we report on the ability of rat erythrocytes to metabolize acetaldehyde, and on the identification of an ALDH in these cells.

METHOD

Reagents

Aldehydes were from Eastman Organic Chemicals. NAD^+, $NADP^+$, disulfiram, dithiothreitol and glucose-6-phosphate were from Sigma Chemical Co. Other chemicals were of analytical grade and were used without purification. Deionized, distilled water was used to prepare the sodium salts used as buffers.

Preparation of Aldehyde Dehydrogenase

Blood was collected from both male and female adult Wistar rats obtained from the Department of Biochemistry's breeding facility. Animals were decapitated and blood collected in heparinized beakers. Blood was centrifuged at 4,000×g for 10 min at 4°C to pellet the cells. The packed cells were rinsed three times with normal saline. Cells were suspended in 1 or 2 volumes of cold 30 mM phosphate, pH 6, containing 0.1% dithiothreitol and 1 mM EDTA. The cells were lysed by allowing them to freeze and thaw three times. The hemolysate was submitted to centrifugation at 14,000×g for 30 min at 4°C. To the resulting supernatant was added enough ammonium sulfate to make the solution 30% with respect to saturation. The solution was stirred at 4°C for 4 hours and then centrifuged to remove debris. Ammonium sulfate was added to the supernatant to bring its concentra-

[1]This paper is based on a poster presented at the first congress of the International Society for Biomedical Research on Alcoholism, July 6–10, 1982. This work was supported in part by Grant PCM 7816051 from the National Science Foundation and Grant AAO1395 from the National Institute on Alcohol Abuse and Alcoholism. This is Journal Paper No. 9290 from the Agricultural Experiment Station, Purdue University.

[2]H. W. is a recipient of Research Scientist Development Award K02AA00028 from the National Institute on Alcohol Abuse and Alcoholism and is the author to whom requests for reprints should be addressed.

TABLE 1

METABOLISM OF ACETALDEHYDE IN RAT ERYTHROCYTES BEFORE AND AFTER DISULFIRAM TREATMENT

	Activity					
	Untreated		Treated*			
Rat	nmole† / ml-min	nmole‡ / mg-protein-min	nmole† / ml-min	nmole‡ / mg-protein-min	Inhibiton (%) ml^{-1}	Inhibiton (%) $mg\text{-}protein^{-1}$
1	6.4	0.025	1.2	0.005	81	78
2	2.0	0.008	1.1	0.005	45	32
3	13.4	0.055	1.2	0.009	91	84
4	5.1	0.018	1.2	0.004	77	78
5	5.1	0.020	4.3§	0.016§	16§	20§
Mean ± SD	6.4 ± 4.2	0.025 ± 0.018	1.2 ± 0.05	0.006 ± 0.002	74	68
P			<0.05	<0.1		

*Rats (1–4) were given 200 mg/kg disulfiram by gavage 16 hrs prior to blood removal. Rat 5 was given only a 100 mg/kg dose

†Activity based on a 1 ml of whole blood.

‡Activity based on erythrocyte protein concentration.

§Excluded from averages since drug dose was different.

tion to 70% of saturation. After 12 hr of stirring at 4°C, this solution was centrifuged and the pellet redissolved in 30 mM phosphate buffer, pH 6.8, containing 0.1% dithiothreitol and 1 mM EDTA. The solution was dialyzed extensively against the same buffer at 4°C. The dialysate was subjected to ion exchange chromatography on carboxymethyl-Sephadex at 4°C, using the 30 mM phosphate buffer, pH 6.0, to elute the enzyme. Approximately 1 ml of the dialysate was placed on a 0.9×20 cm column and 0.75 ml fractions collected. The enzyme eluted immediately after the void volume. No attempts were made to purify the enzyme further.

Enzyme Assays

Assays were performed by measuring the rate of NADH formation by monitoring the fluorescence increase with an Aminco Spectromicrofluorometer. Routinely, assays for aldehyde dehydrogenase were performed in a 0.1 M phosphate pH 9 at 25°C containing 0.5 mM NAD^+. Other assays were performed in the buffer at the pHs indicated. Glucose-6-phosphate dehydrogenase was assayed at pH 7.4 or 9.0 in 0.1 M phosphate at 25°C in the presence of 25 μM $NADP^+$ and 1 mM glucose-6-phosphate.

Acetaldehyde Metabolism in Intact Rat Erythrocytes

Whole blood was collected from rats by heart puncture and then centrifuged. The cells were suspended in normal saline containing 10 mM phosphate at pH 7. The cells were centrifuged and washed three times as outlined above. To 1.5 ml of saline-phosphate was added 0.5 ml of the cell suspension. This was incubated at 37°C in a 4.5 ml sealed septum vial. Acetaldehyde was added so the final concentration was approximately 200 μM. The mixture was incubated for 10 min at 37°C, then perchloric acid was added (final concentration 0.3 M). The vials were cooled to 4°C and centrifuged. Supernatant (0.4 ml) was removed and injected into another vial. Ethanol was added as the internal standard. The vials were heated to 40°C and 0.5 ml of the vapor removed and injected into a Hewlett-Packard gas chromatograph equipped with a flame ionizer detector. A duplicate sample was analyzed without incubation to serve as the zero-time point. The acetaldehyde concentration was calculated by the difference between the two samples. Protein concentration was determined with a kit from BioRad Laboratory using albumin as a standard. Duplication runs of each sample were averaged. Gas chromatography conditions employed were a six-foot column of Carbopak C (80/100) on 0.2% CW 1500. Temperatures were: oven, 40°C; injector, 120°C; and detector, 150°C. The nitrogen flow rate was 10 ml/min.

Inhibitors

For *in vivo* studies, disulfiram was suspended in 1% water soluble carboxymethyl cellulose (200 mg/ml) [1]. One ml/kg was administered by gavage 12–16 hours prior to drawing blood. For *in vitro* studies, disulfiram was dissolved in ethanol. Control assays were performed to verify that the added ethanol did not inhibit the enzyme. Chloral hydrate was dissolved in buffer.

RESULTS

Oxidation of Acetaldehyde in Intact Rat Erythrocytes

The ability of intact erythrocytes to oxidize acetaldehyde was determined with cells obtained from four rats. To insure that the loss in acetaldehyde was not due to its binding to hemoglobin, a zero time point assay was performed with each sample. The difference between acetaldehyde concentrations at t=0 and at t=20 min was used as the rate of oxidation. It was found that intact rat erythrocytes, like those obtained from human, could oxidize acetaldehyde. The rate was 8.07±3.12 (SD) nmoles/min/ml blood.

Enzymes other than ALDH could be responsible for the metabolism of acetaldehyde. To show that ALDH was performing the oxidation, disulfiram, an irreversible inhibitor of ALDH, was employed. Disulfiram was administered to a different group of four rats whose individual rates of acetaldehyde oxidation in erythrocytes were measured previously. Acetaldehyde oxidation rate was then remeasured in erythrocytes from a new blood sample and this rate was compared

TABLE 2

IN VITRO INHIBITION OF ALDH BY DISULFIRAM*

Disulfiram (mM)	Velocity (nmole NADH/min)
0	0.064
0.1	0.064
0.2	0.050
0.3	0.025
0.4	ND†
0.5	ND

*Partially purified ALDH was incubated with disulfiram and assayed with 0.3 mg NAD^+ and 400 μM acetaldehyde in 1 ml phosphate pH 7.4 at 25°C.

†No activity detected.

TABLE 3

K_m OF VARIOUS SUBSTRATES AT DIFFERENT pH, USING PARTIALLY PURIFIED RAT ERYTHROCYTE ALDH*

	K_m (μM)				
Substrate	pH 6.0	7.4	8.6	9.0	9.5
Propionaldehyde	290	280	180	190	170
Acetaldehyde		170		30	
p-Nitrobenzaldehyde				120	

*Post carboxymethyl sephadex obtained enzyme was used in the 1 ml assays which were performed at 25°C in the presence of 0.3 mg NAD^+ and variable aldehyde concentrations.

to the pre-drug treatment data. These results are presented in Table 1. By comparing the average group activity, expressed either per ml blood or per mg erythrocyte protein, it was observed that disulfiram caused an 81 and 76% inhibition, respectively. Results obtained for individual animals are also presented in the table. Though large individual variations in the percent inhibitor were observed, between 32 and 91% (mean 71±21 (SD)), the rate of acetaldehyde oxidation was inhibited by the drug treatment. A half dose of disulfiram (100 mg/kg) produced approximately 20% inhibition in one rat. Thus, based on inhibitor studies, it appears that an ALDH was present in rat erythrocytes just as it is in human.

Identification of Rat Erythrocyte ALDH

It was not possible to assay for ALDH activity in crude hemolysates. Blank reactions were so great that it was impossible to measure a change in fluorescence or optical density due to NADH formation. Even after extensive dialysis, the crude homogenate contained a large quantity of endogenous substrates which produced a large blank reaction when NAD^+ was added to an assay mixture without added aldehydes.

A simple ammonium sulfate fractionation step followed by chromatography on CM-sephadex allowed the recovery of ALDH activity. The protein which precipitated between 30 and 70% saturated ammonium sulfate was dialyzed and subjected to chromatography on carboxymethyl Sephadex. The ALDH activity eluted immediately after the void volume. Acrylamide gel electrophoresis revealed that 9 protein bands were present after the chromatography step (data not shown). Glucose-6-phosphate dehydrogenase proved to be one of these proteins. Inasmuch as this enzyme is only active with $NADP^+$ as a cofactor, its presence did not hinder the further characterization of the ALDH activity.

Isolating a dehydrogenase which oxidizes acetaldehyde in the presence of NAD^+ does not prove that the enzyme is a true ALDH. Knowing that disulfiram inhibited acetaldehyde oxidation in intact erythrocytes [4] and that chloral hydrate is an inhibitor of rat brain and liver ALDH [1] presented a way to test whether or not the activity isolated was that of an ALDH. Disulfiram proved to be an effective inhibitor of the catalytic activity (Table 2). Complete inhibition was obtained when the chromatography fractions were incubated with 0.4 mM disulfiram at pH 7.4. Chloral hydrate was found to be a competitive inhibitor with a K_i of approximately 10 mM at pH 9 when propionaldehyde was used as a substrate. These findings strongly support the notion that the enzyme activity being measured was due to the action of a rat erythrocyte ALDH.

Properties of Rat Erythrocyte ALDH

The post ammonium sulfate fractions prove to be relatively stable. The material could be frozen in the presence or absence of 20% glycerol with less than 15% of the catalytic activity being lost per month. The sample could be lyophilized without any undue loss in catalytic activity.

Rat erythrocyte ALDH, like most other mammalian ALDH's, proved to be capable of oxidizing both aromatic and aliphatic aldehydes. Though only a limited number of substrates were tested, it appears that the maximum velocity increases with increasing pH. Using propionaldehyde as a substrate, it was found that the velocity was 180% at pH 9.5 and just 10% at pH 6 when compared to the physiological pH of 7.4. In Table 3 are listed the K_m values for aldehydes as a function of pH. Acetaldehyde had a K_m of 170 μM when assayed at pH 7.4. At pH=9, the K_m for acetaldehyde decreased to 30 μM. The latter value is virtually the same as reported for the human erythrocyte enzyme which was assayed at pH 8.6 [5].

As shown in Table 1, individual rats had different capacities to oxidize acetaldehyde in their erythrocytes. The enzyme prepared from blood of two individuals was compared. From the double reciprocal plot presented in Fig. 1, it can be seen that the K_m value was essentially the same, but that the total activity in the individual animals differed by 60%. The latter is consistent with the data present in Table 2. No attempt was made to determine if the rate of acetaldehyde oxidation in intact cells correlated with the specific activity or total activity of the enzyme isolatable from those cells.

DISCUSSION

Rat erythrocytes, like human erythrocytes, contains ALDH. The rat enzyme was not characterized with respect to antibody cross reactivity, but it appears to have properties which make it similar to other rat ALDH. It is inhibited by disulfiram and chloral hydrate, exhibits an increased velocity at elevated pH's, and oxidizes both aromatic and aliphatic aldehydes.

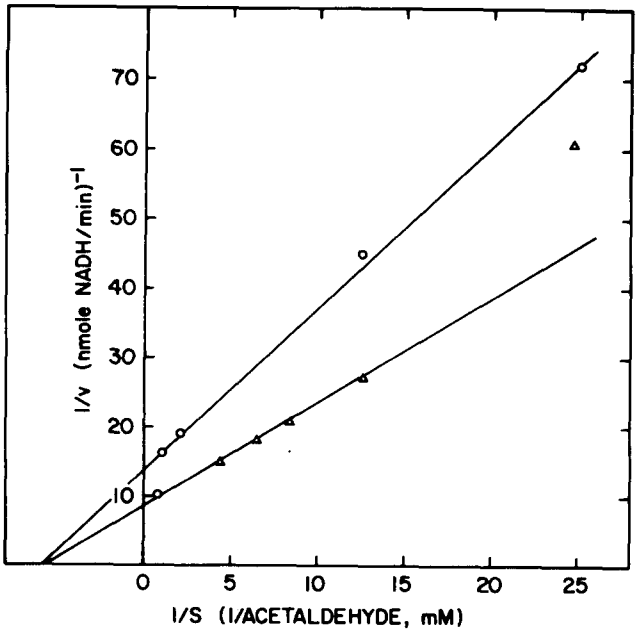

FIG. 1. Lineweaver-Burk plots for ALDH (post carboxymethyl Sephadex) obtained from two different rats when assayed at pH 7.4 at 25°C (K_m=174 μM).

The average rate of aldehyde oxidation for the 9 animals used in this overall study was 7.8±3.1 nmoles/min/ml whole blood. Thus, in rat, the total erythrocyte capacity to oxidize acetaldehyde is approximately 100 nmoles per min. This is to be compared to the total liver capacity of 30–40 μmoles per min [8,9]. It is not surprising that with this low activity and the difficulty in assaying for activity in crude homogenates that rat erythrocyte ALDH was not previously identified.

Data has been presented which show that the non-hepatic oxidation rate of acetaldehyde is approximately 2,000 nmoles per min in rats administered ethanol [2]. It can be expected, based on the data presented here, that erythrocytes would contribute no more than 5% to the total non-hepatic metabolism of acetaldehyde. What then is the physiological role of this low level of aldehyde dehydrogenase. It is possible that erythrocytes contain the enzyme for its protection against xenobiotics which may produce aldehydes. Alternatively, the enzyme may be involved in the oxidation of biogenetic aldehydes. It is known that platelets [13] and vessels [12] contain monoamine oxidase. Conceivably, erythrocyte ALDH may aid in the metabolism of the products of the monoamine oxidase reaction. Metabolic studies with isolated erythrocytes will have to be performed in order to gain greater insights into the possible physiological role of this ALDH.

Though the erythrocyte enzyme does not seem to be important in acetaldehyde metabolism, this enzyme could have a potential for practical use. Many compounds, including a number of drugs, inhibit liver ALDH [10]. The erythrocyte ALDH may be able to serve as a marker for screening for *in vivo* inhibition of liver ALDH.

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