Erythrocyte Aldehyde Dehydrogenase: Assay of a Potential Biochemical Marker of Alcohol Abuse


Erythrocyte aldehyde dehydrogenase (ALDH; EC 1.2.1.3) may be a new biochemical indicator of alcohol abuse. An improved assay for it is described and characterized. We found that expression of erythrocyte ALDH activity in terms of hemoglobin was valid, and preferable to expression in terms of erythrocyte volume. A normal reference interval was determined from results for 375 healthy subjects (236 men, 139 women). We compared these data with results for 109 men admitted to our alcohol detoxification program. The mean erythrocyte ALDH of the alcohol abusers was 30% lower than our mean value for men (p < 0.001). Values did not change between the time the patient presented for admission and >48 h later (when blood-ethanol concentration was zero). Other variables that affect erythrocyte ALDH activities—changes in pH, temperature, other assay conditions, and drug treatments such as disulfiram and nitrate antigens—are discussed.

Additional Keyphrases: abused drugs • alcoholism • ethanol • reference interval • variation, source of • kinetic enzyme assay

The erythrocyte contains a NAD^+ dependent aldehyde dehydrogenase (ALDH; aldehyde:NAD^+ oxidoreductase, EC 1.2.1.3), which catalyzes oxidation of aldehydes to their respective acids (1). Aside from playing a possible role in the extrapatic metabolism of ethanol-derived acetaldehyde (2) and serving as an accessible model of liver ALDH (3–5), this enzyme may be a biochemical marker for diagnosis of alcohol abuse (6, 7).

Several groups have reported decreased erythrocyte ALDH activities in alcoholics (3, 6–9), but none have established normal values for erythrocyte ALDH activities with control groups exceeding 15 subjects of the same sex. Also, there is little agreement with respect to the best method of expression of ALDH activity, and a variety of aldehyde substrates are used in its determination. Here we characterize our improved assay of erythrocyte ALDH and report values for erythrocyte ALDH activity in healthy men and women in and men seeking admission to our alcohol detoxification program.

Materials and Methods

Apparatus. Instruments used included: Model 2400 recording spectrophotometer (Gilford Instrument Co., Oberlin, OH 44074), PHM 72 pH meter with a microsample combined electrode (cat. no. GK2821C; Radiometer America, West Lake, OH 44145), YSI 43TC thermometer (Yellow Springs Instrument Co., Inc., Yellow Springs, OH 45387), FJ thermoregulated water bath (Haake, Saddle Brook, NJ 07662).

Reagents. The following reagents were purchased from Sigma Chemical Co., St. Louis, MO 63178: NAD^+, grade III, cat. no. N 7004; propionaldehyde, cat. no. P6889 (redistilled under nitrogen); CM-Sephadex, cat. no. CM-50-120. Reagent-grade potassium and sodium dihydrogen phosphate, EDTA, disodium salt, trisodium citrate, citric acid, dextrose, mercaptoethanol, and Fisher Diagnostics' Cyanmethemoglobin Reagent (cat. no. 2365-22) were purchased from Fisher Chemical Co. Buffers were made with de-ionized water (resistance >18 MΩ/cm) and the pH was adjusted at ambient temperatures. Buffer A contained 20 mmol of potassium phosphate (pH 6.0), 1 mmol of EDTA, and 1.42 mmol of mercaptoethanol per liter. The "CPD mixture" contained 89.5 mmol of trisodium citrate, 15.6 mmol of citric acid, 142 mmol of dextrose, and 16.1 mmol of sodium dihydrogen phosphate (pH 5.5) per liter. The CPD-saline mixture was composed of one part CPD and 7.14 parts of isotonic saline (NaCl, 154 mmol/L). The ALDH assay buffer was potassium phosphate (0.1 mol/L, pH 7.4).

Procedures. Blood, sampled by venous puncture, was collected in heparinized (green top) or EDTA (lavender top) Vacutainer® Tubes (Becton Dickinson, Rutherford, NJ). Within 30 min the plasma was separated from the erythrocytes by centrifugation, and the cells were washed three times with isotonic saline solution buffered to pH 7.4 with potassium phosphate buffer (0.5 mmol/L final concentration). After the last wash with the buffered saline, the tubes were centrifuged at 1200 × g for 7 min, the cells were then separated from the saline and lysed by diluting to five times the cell volume with cold (5 °C) de-ionized water. The lysate was mixed for 15 s, then centrifuged (20 min, 27 000 × g) in a refrigerated Sorvall centrifuge. After the supernate was decanted, 40 μL of this enzyme-containing supernate was added to 5 mL of the cyanmethemoglobin reagent; 20 min later, the absorbance of this solution was measured at 540 nm for hemoglobin determination.

Buffer A was degassed and 20 mL was added to 300 mg of Sephadex CM-50. After the Sephadex beads were wetted, a stirring bar was added and the suspension was stirred for 5 min. Then 1.35 mL of the suspension was pipetted (while being stirred) into a 0.7 cm (i.d.) Bio-Rad disposable column. This procedure produced a 1- mL bed volume. The buffer was drained to the top of the bed and 1 mL of the enzyme-containing supernate was added to the column. After the supernate had entered the bed, the column was washed with buffer A until a total of 1.6 mL of eluent was collected. This was centrifuged (5 min, 27 000 × g) and the resulting supernate was kept stopped on ice until it was assayed. The erythrocyte ALDH was assayed by adding 0.2 mL of the supernate to 2.2 mL of the assay buffer, which contained 1 mmol of NAD^+ and 20 mmol of propionaldehyde per liter.
The reactions were performed in Teflon-stoppered cuvets to prevent evaporation of the aldehyde substrate. The rate at which the absorbance at 340 nm increased was recorded at 30 °C in the spectrophotometer. The rate of change of absorbance at 340 nm was converted to its molar equivalent by using the molar absorptivity for NADH: $6.2 \times 10^3$ L·mol$^{-1}$·cm$^{-1}$. The resulting activity (corrected for dilution on the Sephadex column) in units of micromoles of aldehyde oxidized per minute per milliliter of erythrocyte lysate was divided by the value for milligrams of hemoglobin (Hgb) per milliliter of erythrocyte lysate solution to yield the normalized activity (U/g of Hgb).

Data analysis. Groups of data points are summarized by the mean (¡) and standard deviation (SD). The coefficient of variation is based on the group SD and ¡; or, in the case of several groups of different subjects (Table 1), the CV was calculated from SDs that are square roots of analysis of variance estimates of variance components. The percentage change in ALDH activity was linearly related to pH, temperature, and volume of Sephadex CM-50 for each individual set of points. The combined or pooled linear regression line was determined and plotted over mean responses (see Figure 1). The corresponding linear correlation was calculated by using Fisher's z-transformation procedure. Mean ALDH of two groups was compared by use of Student's t-test. For multiple comparisons among three or more mean ALDH values for dependent data sets, we used the least significant difference (10) procedure after a significant two-way analysis of variance F-test.

Results

Variation in ALDH activity. When a blood sample was divided into eight equal aliquots, each of which was concurrently assayed for erythrocyte ALDH, the CV for specific activity was 2.97% (Table 1). For 11 groups of 20 healthy people plus one group of 16 at a local industrial firm, the CV for ALDH activities in different people was 23.0%; for mean activities of the 11 groups of 20 people, it was 9.85%. The mean ALDH activity for 10 determinations for the same individual during two years showed a CV of 18.8%.

Hemoglobin normalization. When erythrocytes from 11 subjects were hemolyzed while increasing the NaCl concentrations, the release of hemoglobin and ALDH was decreased (Figure 1). However, these decreases were complementary, there being no observed changes in the specific activity of ALDH (Figure 1).

Effects of changing pH, temperature, centrifuging time, and Sephadex CM-50 of ALDH activity. The effects of changing the assay conditions, and changing the bed volume of Sephadex CM-50 are shown in Figure 2. A change of 0.1 pH unit caused 8–9% changes in specific activity; temperature differences of 1 °C caused differences of 6–7%. Changing the bed volume of Sephadex CM-50 caused no changes in specific activity, but the magnitude of the error bars indicates the potential for large variance at this step.

To determine the effects of varying the centrifugation time after the erythrocyte lysis, we centrifuged lysates from 13 subjects for 10, 20, or 30 min, then assayed the supernates for erythrocyte ALDH activity. The lack of significant differences between the ALDH activities of the three groups (paired t-test) meant that the 20-min centrifugation step could be shortened to 10 min without affecting erythrocyte ALDH activities.

Effects of quick freeze on ALDH activity. If alcoholic

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\text{Table 1. Variation in Erythrocyte ALDH Activity for Different Sampling Conditions}\\
\text{Source of samples} & \text{Degrees of freedom} & \text{Mean} & \text{SD} & \text{CV, %} \\
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\text{Between different people on same day} & 205 & 0.1547 & 0.03563 & 23.03 \\
\text{In same person over a two-year period} & 9 & 0.1594 & 0.03001 & 18.83 \\
\text{Between groups of 20 people over a 17-day period} & 10 & 0.1547 & 0.01524 & 9.85 \\
\text{Between aliquots of the same sample} & 7 & 0.1841 & 0.00546 & 2.97 \\
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Fig. 1. Effects of increasing sodium chloride concentrations (from 0 to 30 g/L) during lysis on lysate hemoglobin concentration (--), lysate ALDH activity (---), and ALDH activity normalized to hemoglobin concentration (....)

Fig. 2. Effects of altered pH (A), temperature (B), and volume of Sephadex CM-50 (C) on percent change in erythrocyte ALDH activity (% ALDH) from normal assay conditions (pH 7.4, 30 °C, 1.35 mL of Sephadex)

Other assay conditions as described in the text. Pooled linear regression equations are (1) % ALDH = -620 + 83.6 A ($r = .982$), (2) % ALDH = -177 + 6.05 B ($r = .984$), and (3) % ALDH = -15.1 + 12.2 C ($r = .73$)
patients abdinate from drinking ethanolic beverages, erythrocyte ALDH activities, which are decreased in alcoholics, increase toward normal values within several weeks (6, 11). If the mechanism of the ethanol-induced inhibition of ALDH were irreversible, this return to normal within two weeks could not occur. Because the lifetime of the human erythrocyte is 120 days, not enough new erythrocyte ALDH would be synthesized within two weeks to account for the return of the enzyme activity. To account for the decreased but reversible inhibition, we hypothesized that a significant proportion of erythrocyte ALDH, although primarily a cytosolic enzyme, might be bound to the erythrocyte membrane as a consequence of alcohol abuse; if so, during the lysate centrifugation, most of the ALDH activity would be removed with the membrane pellet. We tested this hypothesis by comparing ALDH activities from standard cold-water lysis with activities from erythrocytes that were quick frozen by immersing washed cells into a solid CO₂-acetone bath for 1.5 min, then thawed at ambient temperature before addition of the cold water. We found no linear correlation between the ALDH activity after cold-water lysis, and the change in activity after the quick freeze (Figure 3).

**Effects of ethanol and ethanol withdrawal on erythrocyte ALDH.** Blood samples obtained for ethanol analysis were also assayed for erythrocyte ALDH activities in 22 alcohol detoxification patients. After 48 h, follow-up blood samples were analyzed for blood ethanol concentration and erythrocyte ALDH activities. The initial mean (± SD) blood ethanol concentration 2.85 ± 1.31 g/L had decreased to zero in all follow-up samples. Erythrocyte ALDH activities were the same in both sets of samples.

**Reference values for human erythrocyte ALDH.** Erythrocyte ALDH activities were measured in 87 women (ages 38 ± 12 years, mean ± SD) and 142 men (ages 40 ± 11 years) from a local industrial firm. The respective activities measured within 3 h of sampling were 0.149 ± 0.039 and 0.157 ± 0.036 U/g of Hgb (Figure 4A), in agreement with published values for normal erythrocyte ALDH activity (4, 5, 7).

In another study, two days passed between the time of initial sampling, the separation of the erythrocytes from the plasma and resuspension in CPD-saline (see Reagenta), and the assay; samples were stored at 5°C until assayed. Erythrocyte ALDH activities (mean ± SD) for 52 women and 94 men were 0.148 ± 0.036 and 0.150 ± 0.039 U/g of Hgb, respectively—not different from the other healthy group (Figure 4B).

**Human erythrocyte ALDH in alcohol abusers.** The mean (± SD) erythrocyte ALDH activity for 109 men seeking admission to our alcohol detoxification program was 0.111 ± 0.051 U/g of Hgb (Figure 4C). This was significantly (p

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**Fig. 3.** Effect of freeze–thaw lysis on erythrocyte ALDH activity from 83 subjects.
Change in ALDH activity after freeze–thaw lysis as compared with routine lysis (x-axis) vs erythrocyte ALDH activity after routine lysis of cells (y-axis).

**Fig. 4.** Distribution of erythrocyte ALDH activities in healthy employees of two local industrial firms
A, 87 women, 142 men; enzyme activity assayed within 3 h after sampling; B, 52 women, 94 men; enzyme activity assayed 48 h after sampling; C, 109 men seeking alcohol detoxification (mean blood alcohol 2.16, SD 1.40 g/L).
<0.001) less than the mean erythrocyte ALDH activity of the healthy men in Figure 4A. The blood ethanol concentration of the detoxification group was 2.16 (SD 1.40) g/L. There was no significant correlation between blood alcohol concentrations and erythrocyte ALDH activity.

Discussion

Treatment with disulfiram (250 mg per day) completely inhibits erythrocyte ALDH within seven days (4). This inhibition is irreversible; therefore, after treatment is discontinued, erythrocyte ALDH activities do not return to normal values until new erythrocytes are synthesized (12). Thus, subjects treated with disulfiram in the past four to five months should be excluded from ALDH analysis. Moreover, patients treated with oral sulfonlyurea hypoglycemic and nitrate ester anti-anginal medications have decreased activities of erythrocyte ALDH (5) and should be excluded. Other substances that may cause erythrocyte ALDH inhibition are cephalosporins (moxalactam, cefamadole, and cefoperazone), metronidazole, and pargyline (13).

Acute administration of ethanol to nonalcoholic subjects (peak blood ethanol concentration, 1 g/L at 2 h) increases the erythrocyte ALDH activity by 106% at 4 h (14). In contrast, our results indicated that mean blood ethanol concentrations of 2.85 g/L had no effect on ALDH activities in patients undergoing alcohol detoxification. Thus, ethanol may affect the activity of this enzyme differently in ethanol-“naive” and ethanol-tolerant people. This difference in response could be used to assess the degree of ethanol tolerance in patients presenting with high (>3 g/L) blood alcohol concentrations. Patients with prior history of chronic drinking episodes may be at a relatively lower risk of complications from high concentrations of blood ethanol, and would not have increased activities of erythrocyte ALDH. Patients who were not drinking chronically prior to presenting might exhibit increased erythrocyte ALDH activities and, thus, could be at higher risk of ethanol-induced complications. Therefore, measurement of this enzyme activity may have additional clinical utility in the management of ethanol intoxication.

Lin et al. (6) reported an erythrocyte ALDH activity of 66 U/L of erythrocytes for 20 nonalcoholic controls. Dividing this value by 335 mg of Hgb per liter of erythrocytes (mean corpuscular Hgb, 30.2 pg/erythrocyte, divided by mean corpuscular volume, 90.1 μm²/erythrocyte (15)) yields 0.197 U/g of Hgb, which is 30% higher than the mean value reported here. The CV for their determinations was 68% (vs 23% for ours); thus, the values presented here are possibly more accurate. However, the mean erythrocyte ALDH activity for 44 alcoholics reported by Lin et al. was 38% lower than their control mean. This agrees well with our previous results from 38 subjects (7), whose mean erythrocyte ALDH activity was 46% lower than the mean reported here for men. Thus the discrepancy between the two laboratories may be due to methodological differences.

Reports of human erythrocyte ALDH activity based on acetaldehyde as the substrate are in agreement: 10 U/g of erythrocytes (3, 9, 11), one-fifth to one-sixth of the activity determined with propionaldehyde as substrate (5). Acetaldehyde was also used as the substrate in a study of erythrocyte ALDH activities in 57 alcoholics and 57 nonalcoholics (16), but the enzyme activity was reported in units per liter of hemoglobin-free preparation. Because we could not convert these activities to units per volume of erythrocytes or per mass of Hgb, we cannot comment on these values. The higher activity obtained with propionaldehyde and the greater difficulty of working with acetaldehyde (increased volatility, hydration, and polymerization), we consider propionaldehyde the preferred substrate for assay of erythrocyte ALDH activity.

Expression of erythrocyte ALDH activity in U/g Hgb is advantageous because analysis for hemoglobin is routine in clinical chemistry laboratories. Although several anemias are associated with chronic alcohol use (17), abnormal concentrations of hemoglobin are not typically found in alcoholics (18, 19). Also, expressing activity units per erythrocyte volume may not take into account the fact that inflated cell volumes are highly characteristic of excessive alcohol use (18, 19) and thus could underestimate ALDH activities in persons with high cell volume values.

A potential source of error during the spectrophotometric ALDH assay is the high background absorbance from residual hemoglobin. The procedures outlined here were developed for rapid assay of erythrocyte ALDH. Thus, the volume of Sephadex CM-50 used to adsorb the hemoglobin was minimized to expedite the column procedure. Adding 200 μL of the centrifuged Sephadex CM-50 column eluate to 2.2 mL of the reaction mixture gave a typical background absorbance of 2–3 A at 340 nm. At such small amounts of transmitted light, stray-light artifacts may occur in some spectrophotometers. If this occurs, decrease the background absorbance by using a larger bed volume of Sephadex CM-50 or decrease the volume of column eluate from 200 to 100 μL.

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