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# Aldehyde Dehydrogenase in Blood: A Sensitive Assay and Inhibition by Disulfiram

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TOTTMAR, O. AND E. HELLSTRÖM. *Aldehyde dehydrogenase in blood: A sensitive assay and inhibition by disulfiram.* PHARMACOL BIOCHEM BEHAV **18**: Suppl. 1, 103–107, 1983.—The characteristics of human blood aldehyde dehydrogenase with indole-3-acetaldehyde as the substrate were investigated. Blood volumes of less than 25 μl could be assayed. The $K_m$-value was below 10 μM for indole-3-acetaldehyde and 100 μM for $NAD^+$. The ALDH-activity appeared to be located exclusively in the intracellular fraction of the erythrocytes. Acetaldehyde or ethanol at concentrations up to 1 and 40 mM respectively did not affect the activity. Disulfiram caused a pronounced inhibition of the enzyme both in vitro and in vivo. The blood ALDH-activity in disulfiram-treated patients was not fully restored until 6 weeks after discontinuation of the treatment. The inhibition observed in vitro was reversed completely by 2-mercaptoethanol but only partially by glutathione. No restoration of activity in blood from disulfiram-treated patients was obtained with these two reagents. The inhibition found in vitro and in vivo was more pronounced when the assays were performed with indole-3-acetaldehyde than with acetaldehyde. The results suggest that different isozymes of ALDH are involved in the assay with these two substrates.

Aldehyde dehydrogenase  Human blood  Indole-3-acetaldehyde  Disulfiram  Antabuse®  Acetaldehyde

RECENT studies suggest that the "biological" ethanol sensitivity found in Orientals is due to the same basic mechanism as the disulfiram (antabuse)-induced ethanol sensitivity; namely, a decreased ability to metabolize acetaldehyde [1]. This in turn is caused by inhibition of aldehyde dehydrogenase (ALDH) in disulfiram-treated subjects [12], and by the lack of one of the liver ALDH isozymes in ethanol-sensitive Orientals [1].

A good correlation between ethanol sensitivity and blood ALDH-activity has been found in Japanese, and it was suggested that blood ALDH might serve as a marker for ethanol sensitivity in Orientals [9]. Furthermore, blood ALDH could also prove to be a valuable marker for screening of alcoholism, since recent studies have shown that alcoholics have a decreased ALDH-activity in liver [11] as well as in blood [6].

The purpose of the present study was to find a sensitive method for assay of ALDH in whole blood, and to study the general characteristics of this enzyme, and its suitability as a marker for the biochemical and, possible also, pharmacological effects of disulfiram.

## METHOD

### *Chemicals*

Indole-3-acetaldehyde bisulphite and indole-3-acetic acid were obtained from Sigma Biochemical Co., St. Louis, MO. Free indole-3-acetaldehyde was prepared from its bisulphite compound as described by Deitrich [3]. Disulfiram (Fluka AG, Buchs, Switzerland) was recrystallized twice from 99.5% ethanol. Sephadex G-25 columns and Percoll® were obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. 1,2-Dichloroethane was supplied by Fisher Scientific Co., New Jersey.

### *Fractionation of Blood*

White blood cells (lymphocytes and granulocytes) were isolated from defibrinated human blood on a discontinuous gradient of Percoll® according to standard methods outlined by the manufacturer (In: Percoll®-Methodology and Application, Pharmacia Fine Chemicals). Platelets were isolated as described by Chang *et al.* [2], and red cell membranes according to Hanahan and Ekholm [5].

### *Enzyme Assays*

The incubation mixture was prepared in 10 ml screw-top tubes and contained 40 mM sodium pyrophosphate buffer (pH 8.8), 0.5 mM $NAD^+$ and 0.5 ml of the blood sample (diluted 10 or 20 times with ice-cold water) in a total volume of 2.6 ml. The mixture was preincubated in a water bath at 37°C for 5 min with shaking before the reaction was started by adding 25 μM of indole-3-acetaldehyde. The reaction was stopped after 15 min by adding 0.4 ml of 10% $ZnSO_4$ and 0.2 ml of 1 N NaOH. Tissue blanks were treated with $ZnSO_4$ and NaOH before the addition of indole-3-acetaldehyde. The amount of indole-3-acetic acid formed was determined by a slight modification of the procedure of Deitrich [3]. Five ml of 1,2-dichloroethane were added to the mixture and the

tubes were vigorously shaken for 30 sec to remove unreacted aldehyde. After centrifugation at 1000 × g for 10 min, 2 ml of the aqueous layer was added to a mixture of 10 ml 1,2-dichlorethane and 0.2 ml of 6 N HCl. After shaking for 10 min, a 8-ml aliquot was transferred to 4 ml of 0.5 M $K^+$-phosphate buffer (pH 7.4), and shaken for 10 min. The fluorescence of the buffer layer was determined with a Shimadzu RF-510 spectrofluorimeter at excitation and emission wavelengths of 284 and 368 nm, respectively. Standards of indole-3-acetic acid and tissue- and standards blanks were carried through the same procedure. All samples were made in duplicate. The recovery of indole-3-acetic acid in the presence of blood was 74.9±1.6% (mean±SD, N=9), and no significant differences in recovery were found between samples containing 10, 20 and 30 nmol indole-3-acetic acid. The activities are expressed as nmol indole-3-acetic acid formed/hr/ml blood or mg Hb. Hemoglobin content was determined by the cyanmethemoglobin method [10].

The determination of ALDH-activity in blood with acetaldehyde as the substrate was performed under similar conditions as described for the assay with indole-3-acetaldehyde, except that an incubation period of 60 min was used [6]. The activity was calculated as the rate of $NAD^+$-dependent acetaldehyde disappearance. Acetaldehyde was measured as described below.

*Determination of Indole-3-Acetaldehyde and Acetaldehyde*

The samples were mixed with 0.5 ml of 16% (w/v) ice-cold perchloric acid and centrifuged at 1000 × g for 10 min. The supernatant was neutralized with 1 ml of 0.85 M $K_2CO_3$ and the precipitate was removed by centrifugation as above. The aldehyde concentration was determined fluorimetrically with the use of a partially purified preparation of rat liver ALDH [16].

*In Vivo Experiments with Disulfiram*

*Human Studies.* Fifteen healthy non-alcoholic volunteers (8 males and 7 females, aged 18–56) participated in the study. Disulfiram (Antabuse®, Dumex) was given orally during 1 week at daily doses of 100, 200 and 400 mg. Blood samples were obtained from an antecubital vein and collected in heparinized tubes.

In another study, 2 male alcoholic patients (aged 44 and 45) were given disulfiram orally during 1 month at a daily dose of 200 mg. Blood samples were collected at periodic intervals up to 10 weeks after the last dose of disulfiram.

*Animal Studies.* Disulfiram (300 mg/kg) was suspended in 5% (w/v) gum arabicum by sonication and given as a single dose intraperitoneally to female NMRI mice (Anticimex, Sweden). At different times after the treatment, the mice were anesthetized with chloroform and decapitated. A mixture of arterial and venous cervical blood was collected into heparinized tubes. The livers were removed and homogenized in a medium (pH 7.2) containing 0.25 M sucrose, 0.5 mM EDTA and 5 mM Tris-HCl. The ALDH-activity in liver homogenates was determined with acetaldehyde (25 μM) as the substrate [15].

*Inhibition Studies In Vitro*

Disulfiram, dissolved in methanol, was added to a reaction mixture containing 40 mM sodium pyrophosphate buffer (pH 8.8), 0.5 mM $NAD^+$ and 0.5 ml of the hemolysate in a total volume of 2.65 ml. The final concentration of disulfiram was 1 or 5 μM. After preincubation for 5 min with disulfiram at 37°C, indole-3-acetaldehyde was added to a final concentration of 25 μM and the incubation was continued for 15 min. In some experiments, disulfiram-pretreated samples were incubated with 2-mercaptoethanol (50 or 100 mM) or glutathione (5 mM) for 5 min before addition of indole-3-acetaldehyde. The presence of 2-mercaptoethanol, glutathione or methanol did not affect the control activity. Gel filtration of disulfiram-treated blood samples were performed with Sephadex G-25 columns equilibrated with 50 mM sodium pyrophosphate buffer (pH 8.8).

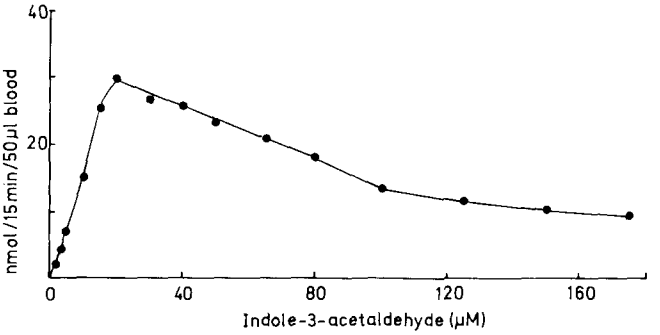


FIG. 1. Aldehyde dehydrogenase activity in human blood at different concentrations of indole-3-acetaldehyde.

## RESULTS

*Characteristics of Human Blood ALDH with Indole-3-Acetaldehyde as the Substrate*

The experiments were performed with water-hemolyzed blood samples as detailed in the Method. The ALDH-activity at different concentrations of indole-3-acetaldehyde is shown in Fig. 1. Highest activity was observed at 20–25 μM. A marked substrate inhibition was found at higher concentrations. An apparent $K_m$-value of 10 μM for indole-3-acetaldehyde was calculated from a Lineweaver-Burk plot of the data in the low concentration range in Fig. 1. However, the true $K_m$-value is probably much lower since a substantial amount of the substrate was bound to blood proteins (see below).

No activity was observed in the absence of $NAD^+$ (Fig. 2). The $K_m$-value for $NAD^+$ was 0.1 mM, as measured at a fixed substrate concentration of 25 μM. Maximal activity was found with 0.5 mM $NAD^+$.

A pH-optimum of 8.8 was obtained in sodium pyrophosphate buffer. No clear pH-optimum was observed in $K^+$-phosphate buffer.

The formation of indole-3-acetic acid was linear with time for 25 min when 25 μl blood was used in the assay. In assays with larger blood volumes, the activity declined after 15 min, and this was evidently caused mainly by depletion of the substrate (see Fig. 2), and also, to some extent to the instability of the enzyme [8].

About 50% of the amount of indole-3-acetaldehyde added to tissue blanks could not be recovered by perchloric acid extraction (Fig. 2). A $NAD^+$-independent disappearance of indole-3-acetaldehyde occurred during the incubation before perchloric acid treatment. The rate of this disappearance was

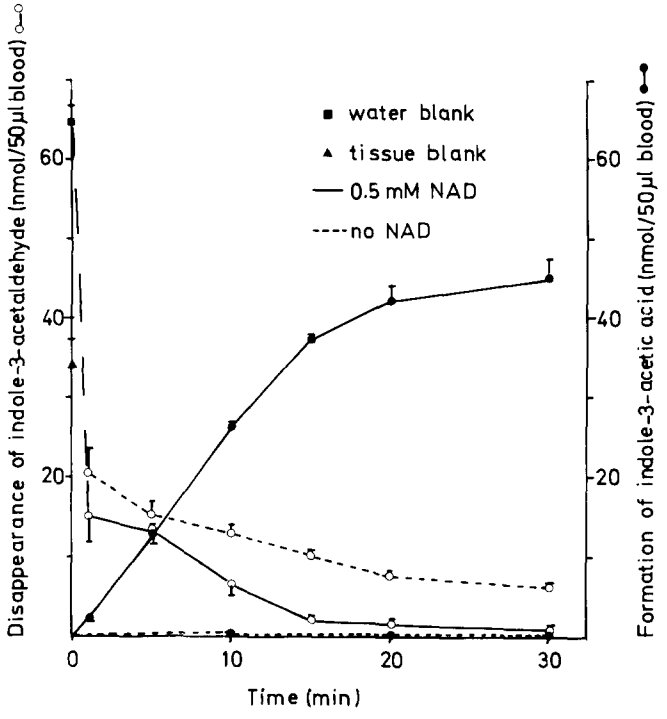


FIG. 2. Disappearance of indole-3-acetaldehyde and formation of indole-3-acetic acid in human blood in the presence and absence of $NAD^+$. The initial concentration of indole-3-acetaldehyde in the reaction mixture was 25 $\mu$M. The values are the means±SD from 3 determinations.

very rapid during the first 2 minutes of incubation, and it continued at a slower rate during the following incubation period. About 2 and 10% of added indole-3-acetaldehyde was recovered after 30 min in the presence and absence of $NAD^+$, respectively. Approximately 70% was recovered as indole-3-acetic acid. Thus 25–30% of the substrate was apparently tightly bound to the blood proteins.

A much less pronounced binding was found when the bisulphite form of indole-3-acetaldehyde was used as substrate (about 90% recovery from tissue blanks). The time-dependent disappearance of the aldehyde in the absence of $NAD^+$ was also found in these experiments. The rate of formation of indole-3-acetic acid was similar to that observed with the free aldehyde.

*Stability, Activity in Different Blood Fractions and in Other Species*

Human blood samples could be stored at −20 or −70°C for at least 5 weeks, or at +5°C for 1 week with no loss of activity.

More than 90% of the total ALDH-activity in whole blood was recovered in a membrane-free hemolysate of blood. No activity could be detected in plasma, blood platelets, white blood cells or in the red cell membranes isolated from human blood.

Similar ALDH-activity as that found in human blood was also present in blood from monkey, rabbit, guinea-pig and mouse, but no activity was detected in blood from Sprague-Dawley rats.

TABLE 1

EFFECT OF ORAL ADMINISTRATION OF DISULFIRAM ON ALDEHYDE DEHYDROGENASE ACTIVITY IN HUMAN BLOOD*

| Treatment | Dose (mg/day) | ALDH-activity (nmol/h/mg Hb) | Percent inhibition | No. of subjects |
|---|---|---|---|---|
| Control | — | 25.0 ± 2.7 | — | 15 |
| Disulfiram | 100 | 9.4 ± 3.7 | 62 ± 15 | 7 |
| Disulfiram | 200 | 4.1 ± 3.1 | 84 ± 10 | 7 |
| Disulfiram | 400 | 0.5 | 98 | 1 |

*Disulfiram was administered for 1 week.

*Effects of Ethanol and Acetaldehyde on ALDH-Activity*

The presence of ethanol up to a concentration of 40 mM in the assay mixture did not affect the formation of indole-3-acetic acid. Similarly, acetaldehyde at concentrations below 1 mM had no effect on the activity in assays with 25 $\mu$M indole-3-acetaldehyde. A 25% lower activity was observed in the presence of 5 mM acetaldehyde.

For a comparison, the ALDH-activity was also measured with acetaldehyde as the substrate. Concentrations higher than 1 mM were needed to obtain maxiumum activity. The activity observed at 25 $\mu$M of acetaldehyde was 35 times lower than that observed with a similar concentration of indole-3-acetaldehyde.

*Inhibition by Disulfiram In Vitro and In Vivo*

Almost complete inhibition of the ALDH-activity was found after a preincubation period of 1–5 min of the assay mixture in the presence of 5 $\mu$M disulfiram (Table 1). The activity was fully restored within 5 min after addition of 2-mercaptoethanol (50–100 mM). Only partial restoration of activity was obtained with glutathione (5 mM) or after gel filtration of the disulfiram-treated sample.

Administration of disulfiram orally during 1 week to humans caused a pronounced inhibition of blood ALDH (Table 2). Almost complete inhibition was observed in one subject given daily doses of 400 mg. In contrast to the results obtained in vitro, the inhibition of ALDH in blood from disulfiram-treated subjects could not be reversed by treatment of blood samples with 2-mercaptoethanol, glutathione or gel filtration (Table 1).

The recovery of ALDH-activity after discontinuation of disulfiram treatment was followed in two patients who had been given disulfiram at a daily dose of 200 mg during 4 weeks (Fig. 3). One day after the last dose, the activity of blood ALDH was less than 10% of the control activity in healthy subjects. The activity increased slowly during the following weeks, and control activity was not observed until after 6 weeks.

The blood ALDH-activity in subjects given the lowest dose of disulfiram was also measured with acetaldehyde (25 $\mu$M) as substrate. The inhibition was significantly lower than that observed in assays with 25 $\mu$M of indole-3-acetaldehyde (45±8 as compared to 61±8%, N=5; $p<0.02$, Student's $t$-test). The inhibition by disulfiram in vitro was also much less pronounced in assays with acetaldehyde than that observed with indole-3-acetaldehyde. A residual activity of

TABLE 2

INHIBITION OF BLOOD ALDEHYDE DEHYDROGENASE BY DISULFIRAM IN VITRO AND IN VIVO, AND EFFECTS OF 2-MERCAPTOETHANOL, GLUTATHIONE AND GEL-FILTRATION ON THE INHIBITED ENZYME

| Experiment | | In vivo* % inhibition | In vitro† % inhibition |
|---|---|---|---|
| 1. 2-mercaptoethanol | 0 mM | 97 | 93 |
| 2-mercaptoethanol | 50 mM‡ | 96 | 0 |
| 2-mercaptoethanol | 100 mM‡ | 97 | 0 |
| 2. Glutathione | 0 mM | 98 | 93 |
| Glutathione | 5 mM‡ | 98 | 67 |
| 3. Before Sephadex G-25 | | 83 | 82 |
| After Sephadex G-25 | | 87 | 73 |

*Disulfiram was administered orally for 1 week (200 mg/day).

†The reaction mixture was preincubated at 37°C for 5 min in the presence of 5 $\mu$M (exp 1 and 2) or 1 $\mu$M (exp 3) of disulfiram.

‡Blood samples from disulfiram-treated subjects and disulfiram-treated blood samples from control subjects were incubated with 2-mercaptoethanol or glutathione for 5 min before addition of 25 $\mu$M indole-3-acetaldehyde.

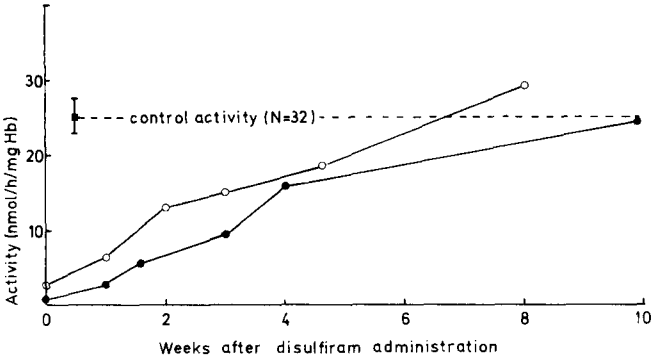


FIG. 3. Return of blood aldehyde dehydrogenase activity in two alcoholic patients pretreated with disulfiram (200 mg/day) for 1 month.

40% of the control activity was found even after a preincubation + assay period of 70 min in the presence of 40 $\mu$M disulfiram.

*Comparison of the Inhibition of Blood and Liver ALDH in Disulfiram-Treated Mice*

Groups of mice were treated with a single dose of disulfiram (300 mg/kg) and the ALDH-activity in blood and liver was measured 1–21 days after treatment (Fig. 4). The liver and blood ALDH-activity was measured with 25 $\mu$M of indole-3-acetaldehyde and acetaldehyde, respectively. At this concentration of acetaldehyde the main part of the liver activity is caused by a mitochondrial low-$K_m$ ALDH (unpublished results). The inhibition of the blood and liver ALDH was 81 and 27%, respectively, 24 hr after disulfiram administration. No inhibition of liver ALDH was found after 4 days, whereas the blood enzyme was inhibited by 68, 49

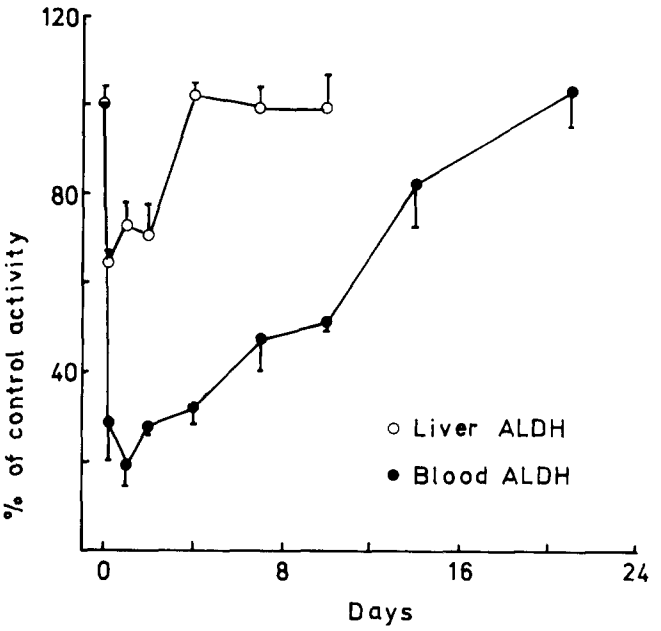


FIG. 4. Time-course of inhibition and recovery of aldehyde dehydrogenase activity in mouse liver and blood after disulfiram administration. Disulfiram was given intraperitoneally in a dose of 300 mg/kg. The control activity was 6.8±0.3 nmol NADH formed/min/mg of protein (N=6) and 33.6±5.5 nmol indole-3-acetic acid/h/mg Hb (N=8) in liver and blood respectively. Each point represents the mean±SD from 3 animals.

and 18% after 4, 10 and 14 days, respectively. Control activity in blood was found after 21 days.

## DISCUSSION

Several studies have shown that blood can metabolize acetaldehyde [4, 7, 14, 18]. Recently Inoue [8] isolated an ALDH from human blood which resembles a cytosolic ALDH present in human liver. Both these enzymes have a low affinity for acetaldehyde and show high sensitivity to disulfiram. It is possible, however, that blood contains several forms of ALDH. Starch electrophoresis of an intracellular fraction of human blood cells indicated the presence of four ALDH isozymes [13]. The precise localization of these enzymes are not known.

The $NAD^+$-dependent blood ALDH-activity is generally assayed with acetaldehyde as the substrate. The activity is measured by following the acetaldehyde disappearance in assays with whole blood [7,18], or by direct spectrophotometric recording of the formation of NADH in assays with hemoglobin-free samples [9]. We found, however, neither of these methods sufficiently simple or sensitive for routine clinical use.

Indole-3-acetaldehyde has been used in several other studies as substrate in assay of ALDH in liver and brain. Using this method, Deitrich [3] found ALDH-activity in most tissues of the rat. However, no activity was detected in rat blood, which is consistent with our results. Deitrich [3] observed higher activity with the free form of indole-3-acetaldehyde than with the bisulphite form of the aldehyde.

We found similar ALDH-activity in assays with these two substrate forms. This might be explained by the fact that we used a higher pH (8.8 compared to 7.4) at which a larger amount of the aldehyde exists in its free form. The use of the bisulphite form in the assays has several advantages—it is commercially available, it is more stable, and less binding of the aldehyde to blood proteins occurs during the assay.

Our results indicate that different forms of ALDH are involved in the assays with acetaldehyde and indole-3-acetaldehyde. The $K_m$-value for indole-3-acetaldehyde was apparently below 10 $\mu$M, whereas a $K_m$-value of 0.2–0.7 mM has been found for acetaldehyde in assays on blood hemolysates [6,7]. Acetaldehyde at concentrations up to 1 mM did not affect the activity with indole-3-acetaldehyde. Furthermore, the inhibition by disulfiram both in vivo and in vitro was markedly lower in assays with acetaldehyde than with indole-3-acetaldehyde.

It is interesting to note that two $K_m$-values for acetaldehyde (17 and 830 $\mu$M) were obtained in kinetic studies on purified human blood ALDH [8]. The enzyme preparation showed, however, only a single protein band in gel electrophoresis [8].

Disulfiram was found to be a potent inhibitor of blood ALDH-activity, which is consistent with previous studies on whole blood and purified blood ALDH [7,8]. The complete reversal of the inhibition in vitro by high concentrations of 2-mercaptoethanol was also observed by Inoue [8], and recently in experiments with a cytosolic ALDH from human liver [17]. Physiological concentrations of glutathione caused only partial restoration of the blood ALDH-activity and the liver enzyme [17]. No restoration of ALDH-activity by 2-mercaptoethanol or glutathione was observed in experiments with blood from disulfiram-treated subjects. This indicates that the mechanism of inhibition by disulfiram in vivo is different to that observed in short-term experiments in vitro.

The inhibition of blood ALDH in disulfiram-treated subjects was very pronounced, even at the lowest dose (100 mg). In a previous study, where the activity was measured with acetaldehyde [6], a much lower inhibition (50%) was found in patients given daily doses of 400 mg during 2 weeks. These results indicate that assays with acetaldehyde include a disulfiram-resistent activity which accounts for 50% of the acetaldehyde-oxidizing capacity in blood.

The ALDH-activity with indole-3-acetaldehyde seems to be confined solely to the intracellular fraction of erythrocytes. The observed long-lasting inhibition of blood ALDH was therefore expected since erythrocytes lack the ability to synthetize proteins and have a life-span of 120 days.

The results obtained show that the present assay of blood ALDH is a very sensitive test for disulfiram inhibition. However, it is difficult to evaluate its value as a marker for ethanol sensitivity in disulfiram-treated patients. The experiments on mice showed that disulfiram causes a much more long-lasting inhibition of blood ALDH than of liver ALDH. Therefore, even a pronounced inhibition of the blood enzyme does not necessarily mean that the liver has a decreased capacity to oxidize acetaldehyde. Whether blood ALDH, as measured with the present method, can be used as an index of the liver ALDH-activity in untreated subjects or in alcoholics remains to be investigated.

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