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Mechanism of the Inhibition of Aldehyde Dehydrogenase *in Vivo* by Disulfiram and Diethyldithiocarbamate

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SUMMARY

Aldehyde dehydrogenase in rat liver mitochondria and supernatant fluid has been assayed by following the rate of NADH production from NAD and aldehyde.

Although it is known that disulfiram is a much more potent inhibitor *in vitro* of aldehyde dehydrogenase than diethyldithiocarbamate, the administration of either compound to rats brings about a decrease in enzyme activity in the livers of such animals. Very similar dose-response curves are obtained if it is assumed that diethyldithiocarbamate undergoes reoxidation to disulfiram *in vivo*.

The decrease and return of activity of the supernatant and mitochondrial enzymes after disulfiram administration are identical in time course. The return of activity can be blocked by cycloheximide. These results indicate that disulfiram is an irreversible inhibitor of aldehyde dehydrogenase *in vivo*.

INTRODUCTION

Disulfiram has long been known to bring about a toxic reaction to the ingestion of ethanol (1). Classically this action has been attributed to the inhibition of aldehyde dehydrogenase and subsequent accumulation of acetaldehyde (2). Recently, the inhibition of dopamine β-hydroxylase by diethyldithiocarbamate (3) and the catecholamine-releasing activity of acetaldehyde (4) have been implicated in this phenomenon.

While the action of disulfiram on a number of enzymes (5, 6), including aldehyde dehydrogenase (7), has been investigated, it was shown only recently that disulfiram, administered to rabbits, would indeed bring about a decrease in liver aldehyde dehydrogenase activity as measured *in vitro* (8). It has also been claimed that diethyldithiocarbamate is the active compound *in vivo* (9) in spite of the known ineffectiveness of this substance as an aldehyde dehydrogenase inhibitor (7).

Since preliminary experiments in rabbits (8) had indicated that the decrease in enzyme activity brought about by disulfiram was long-lasting, it seemed possible that the recovery of activity might depend upon synthesis of new enzyme protein. The present study was carried out to determine the utility of disulfiram as a tool for examination of the synthesis of the enzyme *in vivo*, and to clarify further the mechanism of action of disulfiram and diethyldithiocarbamate.

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METHODS

Disulfiram was obtained through the courtesy of Ayerst, Ltd., Quebec, and from International Chemical and Nuclear Company, or it was prepared from diethyldithiocarbamate by oxidation with I_2. In the latter case it was recrystallized twice from ethanol. Propionaldehyde was redistilled under nitrogen and assayed enzymatically (10). Indoleacetaldehyde was prepared from its bisulfite compound and assayed as previously described (11). Leucine-^{14}C (210 mCi/mmole) was obtained from Miles Laboratories. ^{35}S-Labeled disulfiram (40.6 mCi/mmole) was obtained from Amersham/Searle.

Male Sprague-Dawley rats weighing about 200 g were used throughout. They were fasted for 16 hr, then anesthetized with ether, and exsanguinated from the abdominal aorta. The level of glutathione in the blood was determined by the method of Beutler *et al.* (12). The livers were removed, weighed, and homogenized with a loosely fitting Teflon pestle in sufficient 0.25 M sucrose to make a 20% homogenate. This was centrifuged at 600 × *g* for 10 min, and the supernatant liquid was decanted and saved. The pellet was homogenized in 0.25 M sucrose with a tightly fitting Teflon pestle, and the homogenate was centrifuged at 600 × *g*. The final combined supernatant fraction (homogenate) was approximately a 10% homogenate. It was then subjected to differential centrifugation to isolate the mitochondrial and supernatant fractions as previously described (11). The mitochondria were made up to a volume equal to the original wet weight of the liver. An aliquot was removed and diluted with an equal volume of cold water. This preparation was sonically treated with the microprobe for the Branson model 125 Sonifier at a setting of 2 for a total of 3 min. During the three 1-min sonication periods, the mitochondrial suspension was immersed in an ice–salt water mixture at −7°. At least 1 min elapsed between each sonication period, during which time the suspension was placed in an ice bath at 0°. The temperature did not rise above 4°. The sonicated mitochondrial preparation was centrifuged at 110,000 × *g* for 30 min. The mitochondrial supernatant was used directly for determination of aldehyde dehydrogenase. Of the total amount of protein in the liver mitochondria of disulfiram-treated rats, 55.8 ± 1.48% (mean ± standard error) did not sediment during the centrifugation following sonication. The value for control animals is 58.3 ± 2.4%. The recovery of aldehyde dehydrogenase as determined by measurement of indoleacetic acid production from indoleacetaldehyde was essentially quatitative.

The supernatant fraction containing the cytosol and microsomes was centrifuged at 122,000 × *g* for 1 hr. An aliquot from the supernatant of this centrifugation was placed on 2 × 10 cm columns of Sephadex G-25 (medium) equilibrated with 0.25 M sucrose. The elution of the protein from the column was followed visually by observing the red band as it moved down the column. The recovery of protein was 94.2% for animals treated with disulfiram and 99.4% in experiments with control animals. The recovery of aldehyde dehydrogenase as measured by indoleacetic acid production (see above) was complete.

Disulfiram suspended in 5% acacia by sonic irradiation was administered by stomach tube to animals fasted for 24 hr. The animals were fasted for an additional 16 hr following administration of the drug. Control animals received only 5% acacia and were similarly fasted. Cycloheximide was administered intraperitoneally in 0.9% NaCl. Cycloheximide was given at a dose of 1 mg/kg twice daily for each of two injections, and then at 0.5 mg/kg twice daily thereafter until termination of the experiment. The last dose was given 90 min before death. Injections of cycloheximide were initiated 24 hr after disulfiram or 5% acacia was administered. The dose used was selected as the maximum tolerated dose for the period of time necessary.

To measure incorporation of ^{14}C-leucine into liver proteins, 2.5 or 5 μCi of ^{14}C-leucine were injected intraperitoneally into rats. Thirty minutes later the livers were removed, homogenized in 0.25 M sucrose to make a 10% homogenate, and centrifuged at 600 × *g* for 10 min. Aliquots were taken from the 600 × *g* supernatant fraction for protein

determination, and an equal volume of 0.6 N perchloric acid was added to another aliquot. After centrifugation the pellet was washed twice with 0.33 N perchloric acid. The pellet was dissolved in 1 ml of "NCS" solubilizer (Amersham/Searle), and a sample was taken for counting by liquid scintillation.

The data were calculated on the basis of both wet weight and milligrams of protein. Statistical calculations were performed with an Olivetti Programma 101 computer and programs supplied with that instrument.

Mitochondrial aldehyde dehydrogenase was determined at pH 9.6 in 0.01 M sodium pyrophosphate buffer containing 0.33 mM NAD and 0.33 mM aldehyde by following the rate of NADH formation in a Gilford 2000 spectrophotometer at 340 mμ and 25°. Blank cuvettes contained no aldehyde. NADH oxidase and alcohol dehydrogenase were absent from this preparation.

Supernatant aldehyde dehydrogenase was determined similarly except that 33.3 μM pyrazole was added to inhibit alcohol dehydrogenase completely. Alcohol dehydrogenase was measured by following NADH oxidation in the presence of propionaldehyde.

Attempts were made to restore enzyme activity in the liver *in vitro* after disulfiram administration *in vivo*. Samples of liver supernatant and mitochondrial aldehyde dehydrogenase from both disulfiram-treated and control animals were incubated at 0° or 37° for 30 min with and also without 0.01 M dithiothreitol in 0.01 M sodium phosphate buffer, pH 7.4. Aliquots were removed, and enzyme activity was determined and compared.

Disulfiram-^{35}S was administered in the same manner as unlabeled material. Tissues containing ^{35}S label were homogenized in 0.25 M sucrose, and samples were taken for counting by standard liquid scintillation techniques.

RESULTS

Time course of inhibition by disulfiram. The administration of disulfiram resulted in a measurable decline in enzyme activity in 2 hr but did not reach its maximum effect until 16–40 hr (Fig. 1). This is consistent with our previous studies in rabbits and is typical of clinical experience with this drug (13). Activity returned in both the supernatant and mitochondrial fractions, with a half-time of about 24 hr. Data calculated using activity per gram of liver showed similar results, except that the mitochondrial activity never returned to more than 70% of control values even at 184 hr. Attempts to restore activity of the inhibited enzyme by addition of dithiothreitol *in vitro* were unsuccessful.

One of the likely sources of reducing equivalents for disulfiram is the glutathione (GSH) of the red blood cell. It seemed possible that with the relatively large doses of disulfiram administered, the blood GSH would decline. This proved not to be the case, however, as the blood GSH level 6 hr after disulfiram administration was unchanged from control values. Apparently the mechanism for generating reduced glutathione in the blood is sufficiently fast to keep pace with the relatively slow absorption of disulfiram.

Administration of diethyldithiocarbamate. Since it is known that disulfiram is quickly and completely reduced to diethyldithiocarbamate in the intact animal (14), the latter compound was also tested as an inhibitor of aldehyde dehydrogenase activity (Fig. 1). After subcutaneous injection of this compound, activity fell more rapidly than after its oral administration. Dose-response curves for diethyldithiocarbamate and disulfiram are given in Fig. 2. The dose of diethyldithiocarbamate is calculated as disulfiram equivalents, since 2 molecules of the carbamate are required to make 1 molecule of disulfiram. The values were determined 40 hr after administration of the inhibitors.

Effect of inhibition of protein synthesis. Cycloheximide effectively blocked the return of aldehyde dehydrogenase activity in the mitochondrial fraction ($p < 0.001$ at 88 hr, disulfiram plus cycloheximide vs. cycloheximide alone). Similar results were obtained for the supernatant enzyme ($p < 0.001$ at 88 hr) (Table 1).

Experiments were carried out to determine the effect of cycloheximide on protein synthesis in rat liver. Treatment with cycloheximide or cycloheximide plus disulfiram

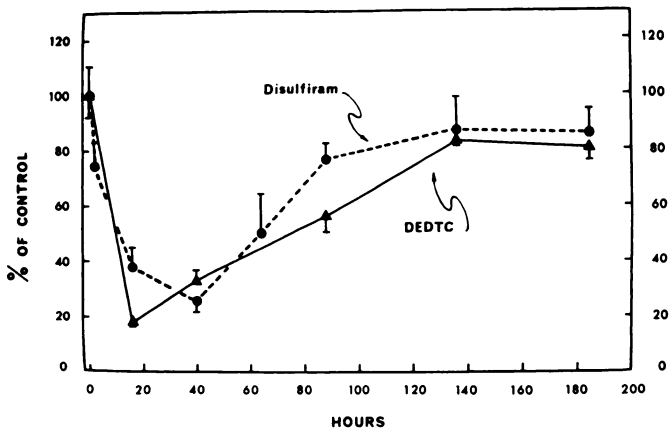

FIG. 1. *Time course of inhibition of rat liver mitochondrial aldehyde dehydrogenase activity after administration of disulfiram and diethyldithiocarbamate (DEDTC).*

Disulfiram (2.02 mmoles/kg in 5% acacia) or 5% acacia as control was administered orally at zero time to 24-hr-fasted rats. Diethyldithiocarbamate (100 mg/ml in 0.1 M sodium phosphate buffer, pH 7.4) was administered subcutaneously (2.02 mmoles/kg as disulfiram) at zero time to 24-hr-fasted rats. Controls received injections of sodium phosphate buffer alone. Aldehyde dehydrogenase in the mitochondrial and supernatant fractions of the cell was determined as described in the text. The data were calculated using the activity per milligram of protein as 100% for the zero-time controls. Brackets denote standard errors of the mean. Each point represents at least seven animals for disulfiram and four animals for diethyldithiocarbamate. For mitochondria, 100% activity is 110 ± 11.7 nmoles of NADH per milligram of protein per 5 min, and for the supernatant, 100% activity is 17.5 ± 1.6 nmoles of NADH per milligram of protein per 5 min. Only the curves for the mitochondrial enzyme are presented, for clarity, since the curves for the supernatant enzyme are almost identical.

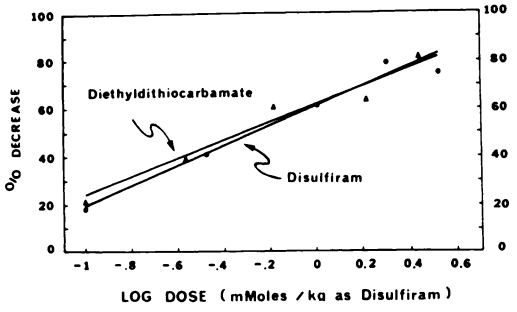

FIG. 2. *Dose-response curves for inhibition of rat liver supernatant aldehyde dehydrogenase*

The inhibitors were administered as described for Fig. 1. The dose of diethyldithiocarbamate plotted on the abscissa was calculated as disulfiram equivalents; i.e., 2 molecules of diethyldithiocarbamate are required to form 1 molecule of disulfiram. The results for inhibition of the mitochondrial enzyme are virtually identical. The values shown were determined 40 hr after administration of the inhibitors. The lines are drawn from a linear least-squares fit to the data. (●——●, disulfiram; ▲——▲, diethyldithiocarbamate.)

lowered the incorporation of ^{14}C-leucine into liver protein (Table 2).

It was necessary to show that cycloheximide was not effective simply by prolonging the presence of inhibitor in the body. Table 3 presents the results of an experiment in which the amount of ^{35}S label was determined in control and cycloheximide-treated rats after administration of labeled disulfiram. Clearly, cycloheximide did not slow the rate of loss of ^{35}S.

DISCUSSION

As was expected from our previous results (8) and from clinical impressions (13), the decline in aldehyde dehydrogenase activity produced by disulfiram is long-lasting. The fact that the decrease and half-time for recovery of activity followed the same time course for both the supernatant and mitochondrial enzymes indicates that the mechanisms responsible for inhibition and recovery are equally operative for both enzymes. It is

TABLE 1

Effect of cycloheximide on recovery of aldehyde dehydrogenase in rat liver mitochondria and supernatant after disulfiram treatment

The conditions were the same as for Fig. 1, except that 1 mg/kg of cycloheximide was given for two doses daily, starting 24 hr after disulfiram, or 5% acacia was given, followed by 0.5 mg/kg of cycloheximide twice daily. The values are those obtained at 88 hr.

Treatment	Percentage of zero-time control values	
	Supernatant	Mitochondria
	mean ± *SE* (*n*)	
Disulfiram	101 ± 10.5 (5)	76.4 ± 5.8 (5)
Cycloheximide[a]	93.7 ± 8.2 (5)	98.5 ± 12.2 (5)
Disulfiram plus cycloheximide[b]	31.7 ± 4 (6)	23.6 ± 2 (6)
	$p < 0.001$[b]	$p < 0.001$[b]

[a] The entire time course both with cycloheximide and with disulfiram plus cycloheximide was followed. Only at 88 hr and beyond, however, did the disulfiram-treated animals recover sufficiently to demonstrate the inhibition of recovery by cycloheximide. The values are still significant at 112 hr ($p < 0.01$). Beyond that time cycloheximide alone depresses aldehyde dehydrogenase activity, so that there is no difference between the values for cycloheximide alone and cycloheximide plus disulfiram.

[b] Disulfiram plus cycloheximide vs. either cycloheximide alone or disulfiram alone.

probable, although not proven, that disulfiram inhibits aldehyde dehydrogenase by sulfhydryl group oxidation (7) *in vitro*. The results of Hellerman, Coffey, and Neims (6) proving this to be the mechanism of disulfiram inhibition of D-amino acid oxidase make this an attractive hypothesis.

Direct comparisons between the decline and restoration of aldehyde dehydrogenase following disulfiram or diethyldithiocarbamate are difficult, since disulfiram is insoluble in water and was given orally, while diethyldithiocarbamate is highly ionized and water-soluble and was given subcutaneously. The time course of effect is somewhat different for the two compounds. As expected, the rates of decline and restoration of activity with diethyldithiocarbamate were somewhat more rapid. The recovery phase thus began earlier, and significant recovery was seen at 40 hr. However, at 184 hr about the same amount of enzyme was present in both the supernatant and mitochondria, as was also the case after disulfiram administration.

The fall in aldehyde dehydrogenase activity after diethyldithiocarbamate administration may have resulted from the conversion of this compound to disulfiram *in vivo*. It is known that diethyldithiocarbamate is 100 times less effective than disulfiram as an inhibitor of aldehyde dehydrogenase *in vitro* (7). The slight inhibition observed with diethyldithiocarbamate *in vitro* may have been due to 1% contamination with disulfiram.

TABLE 2

Protein synthesis in rat liver

Animals were treated as outlined for Fig. 1 and Table 1. The livers were taken 88 hr after treatment with disulfiram or 5% acacia (64 hr after initiation of cycloheximide treatment). Values are calculated as incorporation of ^{14}C-leucine per gram of liver, wet weight.

Treatment (*n*)	Percentage of control incorporation	*p*
	mean ± *SE*	
Control (10)	100 ± 11.5	
Disulfiram (5)	146 ± 2.8	<0.02
Cycloheximide (7)	53 ± 6.0	<0.02
Disulfiram plus cycloheximide (4)	45 ± 4.6	<0.05

The dose-response curves for disulfiram and diethyldithiocarbamate have nearly the same slopes, and while this evidence is not conclusive, it is consistent with a common mechanism of action for the two compounds. Again, because of the different routes of administration and different characteristics

TABLE 3

Effect of cycloheximide on retention of label after ^{35}S-disulfiram administration

Rats were given 100 mg/kg of ^{35}S-disulfiram (0.8 mCi/mmole) orally as described under METHODS for unlabeled disulfiram. Cycloheximide was given intraperitoneally exactly as described for Table 1 and Fig. 1. The animals were killed 88 hr after receiving disulfiram. Tissues were homogenized in 0.25 M sucrose, and aliquots were taken for scintillation counting. Plasma was counted without dilution by sucrose. The values below are in disintegrations per minute per milligram of protein; calculations in terms of disintegrations per minute per gram of tissue yielded similar results. None of the values for treated animals is significantly different from the controls.

Treatment (*n*)	^{35}S radioactivity		
	Liver	Brain	Plasma
	dpm/mg (± *SEM*)		*dpm/ml* (±*SEM*)
Control (12)	29.8 ± 2.9	35.7 ± 2.0	3201 ± 435
Cycloheximide (6)	31.7 ± 3.1	32.8 ± 2.3	4349 ± 677

of the compounds, it is unlikely that precisely the same dose-response curves would be obtained.

Since administered disulfiram cannot be found *in vivo* and is reduced to diethyldithiocarbamate in the blood upon absorption (14), there is little difference between treatment with these agents except that the disulfide-reducing capacity of the body, and of the blood in particular, will be taxed while disulfiram is being absorbed. Strömme has shown that administered ^{35}S-diethyldithiocarbamate is bound equally as well as ^{35}S-disulfiram to the proteins of both liver supernatant and plasma, 1–4 hr after intraperitoneal injection (14). The bound material was identified largely as diethyldithiocarbamate in mixed disulfide linkage and was released by treatment with GSH. As Strömme suggests (14), this result could come about only by (a) reoxidation of diethyldithiocarbamate to disulfiram, which then interacts with protein —SH groups to produce the mixed disulfide, or (b) direct combination of —SH groups of proteins with the —SH group of diethyldithiocarbamate, mediated by some oxidizing agent to form the mixed disulfide directly. Sulfhydryl groups of aldehyde dehydrogenase (7,15) itself might participate in this action *in vivo*, or one of the mixed disulfides could interact with aldehyde dehydrogenase and bring about inhibition.

The inhibition of dopamine β-hydroxylase by disulfiram offers a contrast to the effects on aldehyde dehydrogenase observed here. The inhibitor of dopamine β-hydroxylase is, in all probability, diethyldithiocarbamate, and inhibition occurs by chelation of the copper present in the enzyme (3). In studying the inhibition of this enzyme after intraperitoneal administration of disulfiram it has been found that the onset of action is rapid, 1 hr or less (16), that the duration of action is about 6 hr, and that a dose of 300 mg/kg to rats brings about a maximal effect (17). All these observations are in contrast to those made in the present work.

It is known that inhibition of aldehyde dehydrogenase *in vitro* by disulfiram is prevented but not reversed by sulfhydryl-containing compounds (7). Thus, inhibition of the enzyme *in vivo* might be irreversible and synthesis of new enzyme might be required. Treatment with dithiothreitol did not restore activity, nor did Sephadex G-25 gel filtration bring about any restoration of enzyme activity in the supernatant fraction. The experiments with cycloheximide also bear on this problem. There was complete inhibition of the recovery of aldehyde dehydrogenase activity after disulfiram and cycloheximide treatment, indicating a requirement for new protein synthesis for return of activity.

The incorporation of ^{14}C-leucine was inhibited 46% following cycloheximide treatment; yet virtually complete inhibition of recovery of enzyme activity was achieved. The reason for this observation is not clear, unless cycloheximide is somehow selective in

its inhibition of protein synthesis. It is also possible that cycloheximide inhibited the synthesis of some enzyme which rapidly turns over and which is required either for destruction of the inhibitor of aldehyde dehydrogenase or for removal of the inhibitor from the enzyme. The results of the ^{35}S experiment seem to cast doubt on this possibility, however.

Our results confirm the earlier report (8) that administration of disulfiram lowers aldehyde dehydrogenase activity in liver, and extend this finding to diethyldithiocarbamate, which is equally effective after administration *in vivo*. We also have obtained preliminary evidence that the inhibition is irreversible and return of activity is dependent upon protein synthesis.

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