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EFFECT OF DISULFIRAM (TETRAETHYLTHIURAM DISULFIDE) ON MITOCHONDRIAL OXIDATIONS

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Abstract—Disulfiram inhibits the oxidation of NAD-linked substrates by mitochondria but does not affect their succinoxidase activity. The oxidation of β-hydroxybutyrate was totally blocked by 0·27 mM disulfiram. Disulfiram did not change the P/O ratios. Uncoupled mitochondria were less sensitive to disulfiram than phosphorylating mitochondria. Disulfiram inhibited the dinitrophenol-activated ATPase of mitochondria by about 60 per cent whilst having little effect on Mg^{2+}-activated ATPase. The ATP–P_i exchange reaction was approximately 50 per cent inhibited by disulfiram. The inhibitory effect of disulfiram on mitochondria is irreversible. 2-Mercaptoethanol protected the mitochondria if present before the addition of disulfiram. The mechanism of action of disulfiram and the implications of the results for the understanding of the disulfiram-alcohol reaction and the mechanism of oxidative phosphorylation are discussed.

DISULFIRAM has long been known as a vulcanizing accelerator, antiparasitic substance and metal chelator, and was first employed as an antialcoholic drug in 1948.[1] The combined effects of ethanol and disulfiram in the animal organism have been studied intensively and from many aspects, but the mechanism of the so-called disulfiram–alcohol reaction has remained obscure. Disulfiram is a chemically active compound and reacts readily with sulfhydryl groups to form mixed disulfides. There are also a number of enzymes which are inhibited by disulfiram, e.g. glyceraldehyde-3-phosphate dehydrogenase, aldehyde dehydrogenase and xanthine oxidase[2, 3].

Disulfiram slows down the oxidation of ethanol[4], causes an accumulation of acetaldehyde[1] and inhibits the endogenous respiration of the liver.[5, 6] In a search for the cause of the decreased cellular respiration it was found that disulfiram had effects on the mitochondrial respiratory chain. This discovery may have a dual outcome: (1) explaining the mechanism of the disulfiram–alcohol intoxication and (2) enabling disulfiram to be used as a tool in studies on the mitochondrial electron transport and energy-conserving reactions.

EXPERIMENTAL

Rat liver mitochondria were isolated in 0·25 M sucrose containing 1 mM EDTA and 5 mM tris-HCl, pH 7·5. Submitochondrial particles were prepared by dilution of the mitochondrial suspension 1 : 1 with water and sonication with a Branson Sonifier, Model No. 125 (Branson Instruments, Inc, Danbury, Conn, USA) at 20 kHz in ten bursts of 5 sec at 10 sec intervals Mitochondria were centrifuged down at 9000 *g*, and no attempts were made to eliminate the soluble enzymes liberated from the particles.

The oxygen consumption was measured polarographically using a Clark type electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, USA) connected through a suitable resistor to a Speedomax H potentiometric recorder (Leeds & Northrup Co., Philadelphia, Pa., USA). ATPase was measured according to Siekevitz *et al.*[7] The ATP–phosphate exchange reaction was determined by the method of Löw *et al.*[8] slightly modified. Disulfiram was obtained from A/S Dumex, Copenhagen, Denmark. Sodium diethyl dithiocarbamate was purchased from Th. Schuchardt, G.m.b.H., Göorlitz, Germany, oxidized glutathione and oxidized lipoic acid from the Sigma Chemical Co., St. Louis, Mo., USA.

RESULTS

When the effect of disulfiram on mitochondrial respiration with different substrates was studied, it was found that the oxidation of β-hydroxybutyric acid could be totally inhibited by 0·14 mM disulfiram (Figs. 1 and 2). With pyruvate and or malate

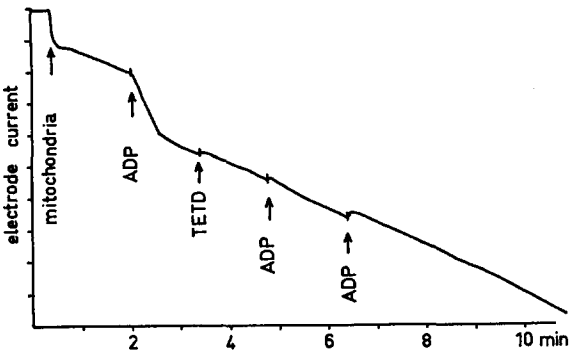

FIG. 1. Effect of disulfiram on the oxidation of β-hydroxybutyrate by mitochondria.
Components: 30 μmoles of potassium phosphate pH 7·4, 100 μmoles of KCl, 10 μmoles of $MgCl_2$ 5 μmoles of β-hydroxybutyrate, 125 μmoles of sucrose, initial volume 1·5 ml. Mitochondria from 250 mg of liver in 250 μl of 0·25 M sucrose, 0·5 μmoles of ADP in 25 μl and 0·25 μmoles of disulfiram (TETD) in 10 μl of ethanol were added as indicated.

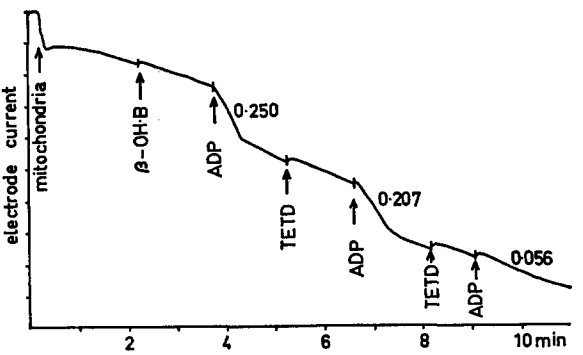

FIG. 2. Effect of increasing concentrations of disulfiram on the oxidation of β-hydroxybutyrate by mitochondria.
Components: 30 μmoles of potassium phosphate, pH 7·4, 100 μmoles of KCl, 10 μmoles of $MgCl_2$ and 125 μmoles of sucrose, initial volume 1·5 ml. Mitochondria from 250 mg of liver in 250 μl of 0·25 M sucrose, 5 μmoles of β-hydroxybutyrate (β-OHB) in 25 μl, 0·5 μmoles of ADP in 25 μl and 0·062 μmoles of disulfiram (TETD) in 10 μl of ethanol were added as indicated. Figures in the graph indicate the rate of disappearance of oxygen in μatoms/min after the addition of ADP.

as substrates a 26 per cent inhibition was caused by 0·27 mM disulfiram. The succinoxidase activity of mitochondria was not affected by disulfiram. Disulfiram did not change the P/O ratios, which were 3·0–3·1 with the NAD-linked substrates.

To decrease the possible membrane effects of the strongly lipophilic disulfiram, experiments were made with submitochondrial particles. It was found that the respiratory chain-linked $NADH_2$ oxidase activity was inhibited 86 per cent by 0·25 mM disulfiram. This inhibition did not change with further increase of the concentration of the

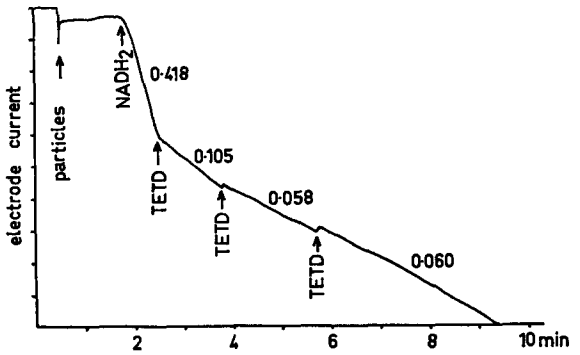

FIG. 3. Effect of disulfiram on the oxidation of $NADH_2$ by submitochondrial particles. Components: 30 μmoles of potassium phosphate pH 7·4, 100 μmoles of KCl, 10 μmoles of $MgCl_2$, 125 μmoles of sucrose, initial volume 1·5 ml. The particles in 400 μl of 0·125 M sucrose, 2 μmoles of $NADH_2$ in 100 μl, and 0·25 μmoles of disulfiram in 10 μl of ethanol were added as indicated. The figures in the graph indicate the oxygen consumption rate in μatoms/min.

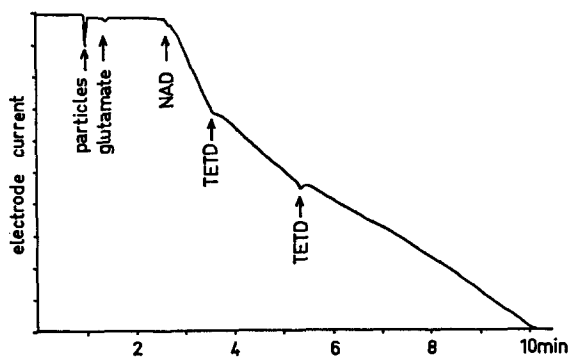

FIG. 4. Effect of disulfiram on the oxidation of glutamate by submitochondrial particles. Components: 30 μmoles of potassium phosphate pH 7·4, 100 μmoles of KCl, 10 μmoles of $MgCl_2$ and 125 μmoles of sucrose, initial volume 1·5 ml. The particles in 400 μl of 0·125 M sucrose, 20 μmoles of potassium glutamate in 100 μl, 2 μmoles of NAD in 100 μl and 0·25 μmoles of disulfiram in 10 μl of ethanol were added as indicated.

inhibitor (Fig. 3). Analogous effects were obtained when glutamate and NAD were used as substrate (Fig. 4). A total block in the oxidation of β-hydroxybutyrate in the presence of NAD was caused by disulfiram after a lag-time of about 30 sec (Fig. 5). No effect could be detected on the oxidation of succinate (Fig. 6).

The amount of disulfiram sufficient to cause total inhibition was found to be roughly proportional to the quantity of mitochondria in the incubation mixture, when β-hydroxybutyrate was used as substrate (Table 1). This phenomenon is reminiscent of that seen in rotenone inhibition[9].

Washing the mitochondria twice with the suspension medium after preincubation with disulfiram at 0° did not restore the activity.

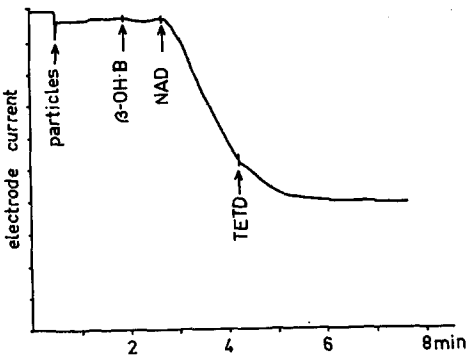

FIG. 5. Effect of disulfiram on the oxidation of β-hydroxybutyrate by submitochondrial particles. The conditions used were as described in Fig. 4. Five μmoles of hydroxybutyrate in 25 μl, 2 μmoles of NAD in 100 μl and 0·25 μmoles of disulfiram were added as indicated.

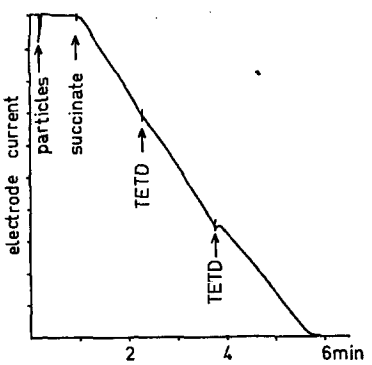

FIG. 6. Effect of disulfiram on the succinoxidase activity of submitochondrial particles. The conditions used were as described in Fig. 4. Five μmoles of succinate in 25 μl and 0·25 μmoles of disulfiram were added as indicated.

TABLE 1. DEPENDENCY OF THE DISULFIRAM INHIBITION OF MITOCHONDRIAL β-HYDRO XYBUTYRATE OXIDATION ON THE AMOUNT OF MITOCHONDRIA

mg of liver equivalent to the amount of mitochondria used	μmoles of disulfiram	Inhibition percentage
500	0·25	44
250	0·25	63
100	0·125	67

Components: 30 μmoles of potassium phosphate pH 7·4, 100 μmoles of KCl, 10 μmoles of $MgCl_2$, 250 μmoles of sucrose, 2·5 μmoles of hydroxybutyrate, volume 2 ml.

The respiration of mitochondria was not affected by diethyldithiocarbamate, oxidized glutathione or oxidized lipoic acid.

When attempts were made to protect the enzymes with a substance containing a sulfhydryl group, it was found that the effect of disulfiram was abolished by addition of 2-mercaptoethanol to the incubation mixture before the addition of disulfiram (Table 2). The inhibitory effect on the oxidation of β-hydroxybutyrate could not be reversed by addition of 2-mercaptoethanol afterwards even in fourfold the concentration necessary for the protective effect.

TABLE 2. EFFECT OF 2-MERCAPTOETHANOL ON THE DISULFIRAM INHIBITION OF THE OXIDATION OF β-HYDROXYBUTYRATE BY MITOCHONDRIA

Mercaptoethanol (mM)	Disulfiram	O_2 consumption μatoms/min	Inhibition percentage
—	+	0·041	68
0·1	+	0·046	64
0·2	+	0·061	52
0·4	+	0·056	56
0·8	+	0·066	48
1·6	+	0·071	44
3·2	+	0·102	19
6·4	+	0·096	24
6·4	—	0·111	12
—	—	0·126	0

Components: 30 μmoles of potassium phosphate, pH 7·4, 100 μmoles of KCl, 10 μmoles of $MgCl_2$, 250 μmoles of sucrose, 10 μmoles of β-hydroxybutyrate, 0·25 μmoles of disulfiram when indicated, mitochondria from 200 mg of liver (4·1 mg of protein). Oxygen consumption was measured after the addition of 0·5 μmoles of ADP. Total volume 2·0 ml.

TABLE 3. EFFECT OF DISULFIRAM ON THE OXIDATION OF β-HYDROXYBUTYRATE BY UNCOUPLED MITOCHONDRIA

	Utilization of oxygen μatoms/min		
	Control	0·25 μmoles of disulfiram	Inhibition percentage
ADP	0·328	0·121	63
DNP	0·204	0·160	22

Components: 30 μmoles of potassium phosphate pH 7·4, 100 μmoles of KCl, 10 μmoles of $MgCl_2$, 5 μmoles of hydroxybutyrate, 188 μmoles of sucrose, mitochondria from 250 mg of liver, volume 1·8 ml. Stimulation of respiration was achieved by 0·5 μmoles of ADP or 0·125 μmoles of dinitrophenol (DNP).

If the mitochondria were uncoupled with 2,4-dinitrophenol, they were less sensitive to disulfiram, and concentrations of disulfiram twice as high were needed to produce the same effect as on phosphorylating mitochondria (Table 3).

The dinitrophenol-activated ATPase was inhibited by about 60 per cent by disulfiram whereas the Mg^{2+}-activated ATPase was hardly at all affected (Table 4). The latent ATPase of intact mitochondria was not activated by disulfiram. The ATP-P_i exchange reaction was inhibited 50 per cent by disulfiram (Table 5).

TABLE 4. EFFECT OF DISULFIRAM ON MITOCHONDRIAL ATPASES

	Pi released, μmoles		
	DNP	Mg^{++}	None
Control	3·51	3·11	0·55
Disulfiram	0·99	2·48	0·50

Components: 10 μmoles of ATP, 50 μmoles of tris-HCl, pH 7·4, mitochondria from 50 mg of liver, volume 2 ml. Dinitrophenol 0·25 μmoles, $MgCl_2$ 5 μmoles and disulfiram 0·5 μmoles as indicated. Incubation time 15 min at 30°. For the determination of the Mg^{2+}-induced ATPase ageing of the mitochondria was brought about with 0·1% sodium desoxycholate.

TABLE 5. EFFECT OF DISULFIRAM ON THE ATP–PHOSPHATE EXCHANGE REACTION

Time in min	Percentage of total ^{32}P in ATP	
	control	disulfiram
5	8·6	4·8
10	13·7	5·7

Components: 10 μmoles of ATP, 10 μmoles of potassium phosphate pH 7·4 containing ^{32}P, 35 μmoles of tris-HCl pH 7·4, 5 μmoles of $MgCl_2$, 250 μmoles of sucrose and mitochondria from 50 mg of liver, volume 2 ml. Disulfiram 0·5 μmoles as indicated. Incubation temperature 30°.

DISCUSSION

Summarizing the evidence presented above, it could be demonstrated that disulfiram inhibits the oxidation of NAD-linked substrates by mitochondria, especially β-hydroxybutyrate, but does not affect the oxidation of succinate. From experiments with mitochondria and submitochondrial particles, the conclusion could be drawn that disulfiram exerts its effect on the mitochondrial $NADH_2$ dehydrogenase and β-hydroxybutyrate dehydrogenase. Disulfiram also inhibits the ATP–P_i exchange reaction and the dinitrophenol-activated ATPase.

Thus, the effects of disulfiram on the mitochondria closely resemble those of amobarbital (5-ethyl-5-isoamyl-barbituric acid), if the effect of disulfiram on β-hydroxybutyrate dehydrogenase is excluded.

The mechanism of effect of various substances on oxidative phosphorylation, especially at site I, can be divided into two categories, (1) interference with electron transport and (2) with energy conservation. Only rotenone shows great specificity for electron transport. Amobarbital, on the other hand, exerts an effect on both electron transport and the energy-linked reactions. It has been demonstrated that amobrabital inhibits the oxidation of NAD-linked substrates, DNP-induced ATPase and the ATP–P_i exchange rate. Löw *et al.*[8] concluded from their amobarbital data that the ATP–P_i exchange reaction is more closely related to the first phosphorylation site than to the others. A similar conclusion can be reached from the disulfiram inhibition experiments, and it is therefore believed that the present experiments support the conclusions of Löw *et al.*

Similar patterns of inhibition at the first phosphorylation site can be achieved with many substances not chemically related to each other. The mechanism of action of amobarbital is not exactly known.[9, 10] However, the mechanism of action of disulfiram

on the respiratory chain might be easier to understand because of the known reactivity of disulfiram and the known properties of other enzymes affected by it.

It is known that thiol groups are essential for the activity of the enzymes of the mitochondrial respiratory chain. It has been demonstrated that $NADH_2$-ubiquinone reductase contains acid labile sulfide[11] and that thiol reagents act as uncouplers of oxidative phosphorylation. In particular in view of the effects of Cd^{2+} and arsenite it has been suggested that dithiol groups play a central role in oxidative phosphorylation.[12] β-Hydroxybutyrate dehydrogenase is also inhibited by Cd^{2+} and arsenite and BAL, which is believed to indicate the presence of vicinal thiol groups in the enzyme.[13, 14] As the results of the present study show, β-hydroxybutyrate dehydrogenase is also inhibited by disulfiram. Thus, it is proposed as a working hypothesis that the mechanism of action of disulfiram is based on interference with the vicinal thiol groups of the enzymes involved.

The possibility that vicinal thiol groups may play a role in oxidative phosphorylation must be considered in the light of the present results. Whether these groups are integral to the active center of the enzyme or involved in the conformation of the enzyme molecule cannot be decided.

It is noteworthy that glutathione and lipoic acid induce the same degree of volume change in the mitochondria as disulfiram,[15] but have no effect on mitochondrial oxidations. It seems reasonable to conclude that the disulfide bridge is essential to the action of disulfiram and that possibly the terminal alkyl groups of the disulfiram molecule are needed to bind the inhibitor to the acceptor site of the enzyme.

The present results reopen the question of the implications of the effects of disulfiram on the respiratory chain for the understanding the disulfiram–alcohol reaction. The old hypothesis of acetaldehyde intoxication seems to need revision in the light of recent investigations,[16] and it is quite possible that a deficiency of NAD, caused by disulfiram, may be the fundamental cause of the reaction.

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