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COMPARISON OF THE EFFECTS OF DISULFIRAM AND DIMERCAPTOPROPANOL ARSENITE ON MITOCHONDRIAL STRUCTURE AND FUNCTION

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Abstract—The effects of disulfiram and arsenite + BAL* on the respiratory chain-linked $NADH_2$ dehydrogenase and oxidase activities, reduction of mitochondrial flavoprotein and cytochrome *b*, effects on mitochondrial volume and mitochondrial ATPases were examined.

Disulfiram inhibited $NADH_2$ oxidase activity without affecting the dehydrogenase moiety of the enzyme. $NADH_2$ oxidase was inhibited by BAL + arsenite. Disulfiram inhibited the reduction of cytochrome *b* by $NADH_2$ without affecting reduction by succinate. BAL + arsenite inhibited the reduction of cytochrome *b* by $NADH_2$ and succinate. Large-amplitude swelling of the mitochondria was induced by disulfiram, whereas rapid small-amplitude swelling was observed in the presence of BAL + arsenite. Both disulfiram and BAL + arsenite inhibited the DNP-activated ATPase of mitochondria in a closely similar manner. The role of structural alterations of mitochondria on the effects of disulfiram are discussed.

THE EXISTENCE of functional —SH groups in the flavoprotein region of the mitochondrial respiratory chain is well documented[1–4] and the role of —SH groups in $NADH_2$-ubiquinone reductase has been subjected to extensive studies. Recently, the observation of the effects of disulfiram on mitochondrial hydrogen transfer and some partial reactions of oxidative phosphorylation again raised the question of the role of —SH groups in the first phosphorylation site.[5] The present investigation was undertaken as an extension of these studies, the principal objects being (1) to determine the mode of action of the above mentioned drug on isolated mitochondria and on a purified preparation of the respiratory chain (sonicated particles), with special reference to dithiol-disulphide interaction, and (2) to evaluate the significance of structural changes of mitochondria for the inhibition of energy-linked reactions.

EXPERIMENTAL

Female albino rats of the Wistar strain were used. Age of the animals was five months. The animals were fed standard diet, without starving before sacrifice. Liver mitochondria were prepared essentially as described by Schneider.[6] The isolation of mitochondria was accomplished in 0·25 M sucrose, containing 1 mM EDTA and 5 mM Tris chloride, pH 7·4. EDTA was omitted from the final suspension. Submitochondrial particles were prepared according to Kielley and Bronk.[7] For assess-

* Abbreviations: BAL, 2,3-dimercaptopropanol; DNP, 2,4-dinitrophenol; $NADH_2$, reduced nicotinamide-adenine dinucleotide; TETD, tetraethylthiuram disulfide (disulfiram).

ment of the reduction of the respiratory carriers, an Aminco–Chance dual wavelength spectrophotometer was used (American Instrument Co., Silver Spring, Md., USA). Reduction of flavoprotein and cytochrome *b* was indicated by changes in the difference of absorbances at 465 and 510 nm and at 434 and 490 nm, respectively.[8, 9] $NADH_2$ oxidase activity was measured by observing the rate of decrease of absorbance of $NADH_2$ at 340 nm. $NADH_2$ dehydrogenase activity was assayed by using ferricyanide as electron acceptor,[1] the rate of decrease of absorbance at 420 nm being registered with a Beckman DK-1 recording spectrophotometer. Volume changes of mitochondria were assessed by recording the absorbance changes of the suspension at 520 nm. For continuous monitoring of the ATPases, hydrogen ion production related to ATP hydrolysis[10] was measured with a Beckman combination electrode and Radiometer TTT 1c pH meter connected to a potentiometric recorder. Protein was determined by the biuret method after solubilizing the particles with 0·67% sodium desoxycholate. Corrections for turbidity or colour of the solution were made by destroying the biuret colour with cyanide,[11]

RESULTS

Mitochondrial respiratory carriers. Sonicated particles were used in order that external $NADH_2$ might be used. The reduction rates of the carriers after addition of substrates are extremely rapid, so that it was found most satisfactory to record the steady state reduction levels rather that reaction velocities. Disulfiram caused a slight increase of the reduction of flavoprotein, indicating that the point of interference is on the oxygen side of flavoprotein. BAL + arsenite left the reduction level of flavoprotein almost unaffected (Table 1). Rotenone caused a slight decrease of the reduction level of flavoprotein.

TABLE 1. EFFECT OF DISULFIRAM OR BAL + ARSENITE ON THE STEADY STATE REDUCTION OF FLAVOPROTEIN AND CYTOCHROME *b* DURING OXIDATION OF $NADH_2$ OR SUCCINATE BY SUBMITOCHONDRIAL PARTICLES

Additions	Per cent reduction* of flavoprotein and cytochrome *b* aerobically					
	$NADH_2$		Succinate		$NADH_2$ + succinate	
	Flavin	Cyto. *b*	Flavin	Cyto. *b*	Flavin	Cyto. *b*
—	56	33	26	15	72	30
TETD	74	13	—	18	95	23
BAL + $NaAsO_2$	54	10	—	—	72	10
Rotenone	42	18	—	—	64	28

* The reduction observed anaerobically in the presence of an excess of $NADH_2$ and succinate is designated as 100 per cent.

Components: 230 μmole KCl, 69 μmole of potassium phosphate, pH 7·4, 23 μmole of $MgCl_2$, 2 mg of particle protein, volume 2·5 ml, 50 μl of 40 mM $NADH_2$, 25 μl of 0·1 M sodium succinate, 10 μl of 12·5 mM TETD in ethanol, 10 μl of 2·5 mM rotenone in ethanol, 25 μl of 50 mM $NaAsO_2$ and 25 μl of 25 mM BAL were added as indicated.

The effects of disulfiram and rotenone on the reduction of cytochrome *b* by $NADH_2$ and succinate were closely similar. Both decreased the amount of cytochrome *b* reducible by succinate + $NADH_2$, indicating an inhibition of hydrogen transfer

between flavin and cytochrome *b* (Table 1). BAL + arsenite had a different pattern of action, almost totally abolishing the reduction caused by succinate. Inhibition of succinate dehydrogenase was suggested, and this was also verified by polarographic determination of succinoxidase activity.

Mitochondrial $NADH_2$ dehydrogenase and $NADH_2$ oxidase activities. Disulfiram inhibited the $NADH_2$ oxidase activity of submitochondrial particles, without affecting the $NADH_2$ dehydrogenase activity (Fig. 1). BAL or arsenite alone had negligible

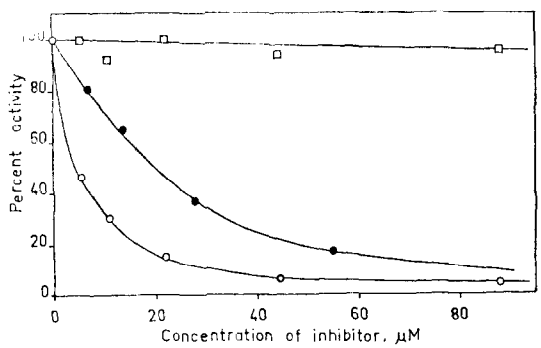

FIG. 1. Effect of disulfiram and BAL + arsenite on the $NADH_2$ dehydrogenase and oxidase activities of submitochondrial particles. Components: 270 μmole KCl, 81 μmole potassium phosphate, pH 7·4, 27 μmole $MgCl_2$, volume 2·8 ml. □ = effect of TETD on the dehydrogenase activity in the presence of 1 μmole $NADH_2$ and 2·5 μmole $K_3(Fe(CN)_6)$, 0·43 mg of particle protein. ○ = effect of TETD on the oxidase activity in the presence of 0·5 μmole $NADH_2$, 0·43 mg of particle protein. ● = effect of BAL arsenite on the oxidase activity in the presence of 0·5μ mole $NADH_2$, BAL as indicated, 2·5 μmole $NaAsO_2$, 0·29 mg of particle protein.

effects on $NADH_2$ oxidase activity, but when added together, had an inhibitory effect (Fig. 1). The effects on the dehydrogenase moiety of the enzyme could not be tested because of incompatibility of BAL and ferricyanide.

Volume changes of mitochondria. When mitochondria were incubated in the presence of disulfiram in a medium composed of 100 mM KCl, 5 mM Tris chloride and 1 mM EDTA, pH 7·4, large-amplitude swelling of the mitochondria occurred rapidly. In the presence of 10 mM $MgCl_2$, the amplitude of the swelling was smaller but the rate of the phenomenon was unaffected (Fig. 2). Spontaneous swelling did not occur in the medium used. Arsenite caused a slow small-amplitude swelling of the mitochondria, BAL alone having no effect. However, when arsenite and BAL were added successively, a rapid small-amplitude swelling occurred. The amplitude of the light scattering change was not affected by the concentration of BAL, but the reaction rate decreased with decreasing concentration of BAL (Fig. 3).

Mitochondrial ATPases. DNP-induced ATPase: In the presence of disulfiram a slowly increasing inhibition occurred (Fig. 4). Arsenite alone was practically without effect, as was also BAL. However, when both were added together, ATPase was inhibited in a manner closely resembling that of disulfiram (Fig. 5). Mg^{2+}-activated ATPase: When added to a suspension of intact mitochondria, disulfiram did not

affect the low rate of ATP hydrolysis observed. Actually the same results were obtained with BAL + arsenite. When mitochondria were aged by addition of desoxycholate (0·1 per cent), no effects on ATPase could be detected with either disulfiram or BAL + arsenite.

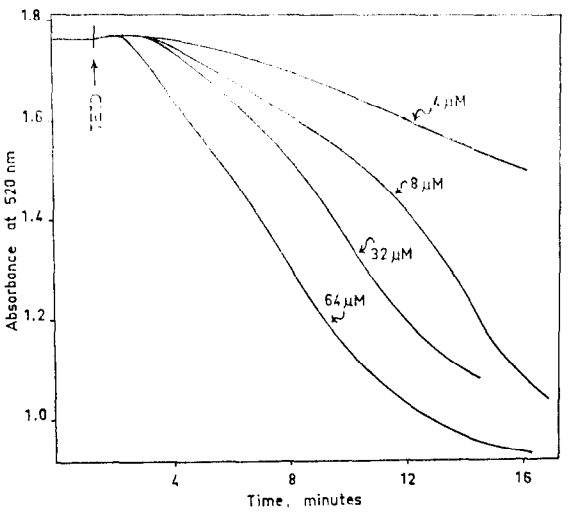

FIG. 2. Disulfiram-induced swelling of mitochondria. Components: 200 μmole KCl, 100 μmole Tris chloride pH 7·4, 20 μmole $MgCl_2$, 2 μmole $EDTA_2$, 0·57 mg of particle protein, volume 2 ml.

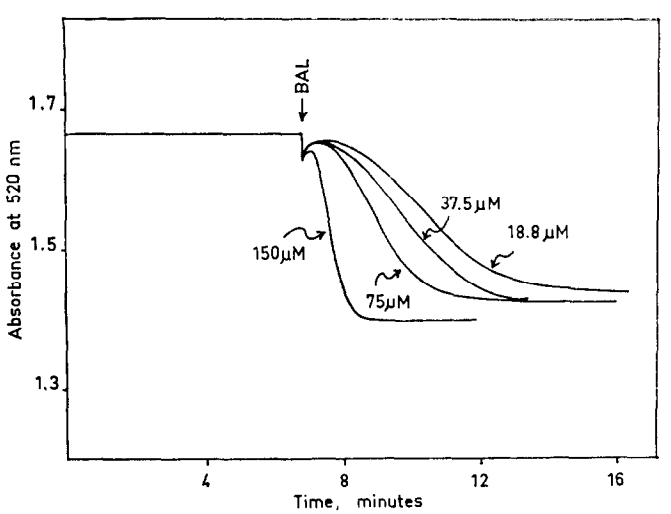

FIG. 3. BAL-induced swelling of mitochondria. Components: 200 μmole KCl, 100 μmole Tris chloride, pH 7·4, 20 μmole $MgCl_2$, 2 μmole EDTA, 0·57 mg of particle protein, 2·5 μmole $NaAsO_2$ and BAL as indicated, volume 2 ml.

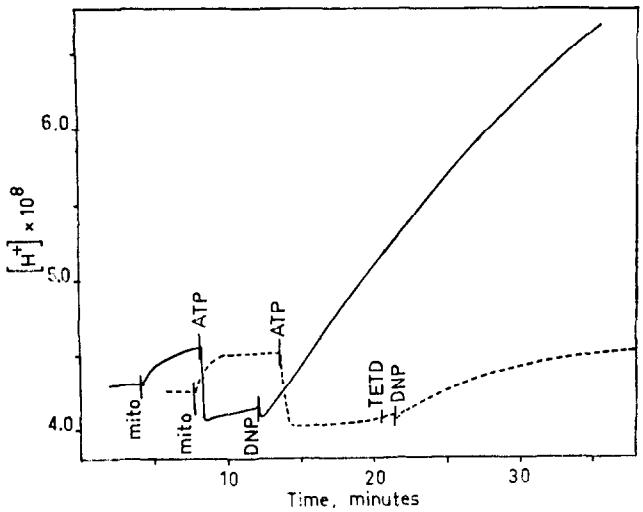

FIG. 4. Effect of disulfiram on the DNP-induced ATPase of mitochondria. Components: 200 μmole KCl, 30 μmole Tris chloride, 2·5 μmole ATP, 0·25 μmole DNP, 0·125 μmole TETD, 1·2 mg of mitochondrial protein, volume 2 ml.

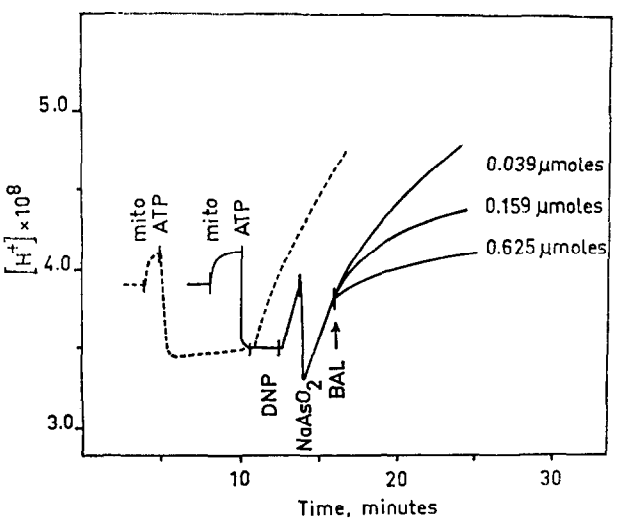

FIG. 5. Effect of BAL + arsenite on the DNP-induced ATPase of mitochondria. Components: 200 μmole KCl, 30 μmole Tris chloride, pH 7·4, 20 μmole $MgCl_2$, 2·5 μmole ATP, 0·25 μmole DNP, 1·25 μmole $NaAsO_2$, BAL as indicated, volume 2 ml.

DISCUSSION

The kinetic and spectrophotometric data presented in this paper unequivocally demonstrate that the sites of action of disulfiram and BAL + arsenite are on the ubiquinone side of the $NADH_2$-ubiquinone reductase enzyme. A more distal locus sensitive to these reagents cannot be excluded, but at least in case of disulfiram it must be proximal to the point of adjoining the succinoxidase system.

It is known that the $NADH_2$ side of the enzyme conceals masked —SH groups, which are rendered sensitive to inhibitors by preconditioning the enzyme with $NADH_2$.[2, 3] The ubiquinone side of the enzyme has been considered to have a 'slowly reacting' —SH group.[2] Lack of effect of arsenite or BAL alone represents a new aspect of the interactions between flavoprotein and ubiquinone; if BAL + arsenite are considered to be specific inhibitors of dithiol group,[12] existence of the latter in this region is plausible to assume. It may be noticed that the 'highly reactive' —SH groups between $NADH_2$ and the enzyme have been explained to be a dithiol-disulfide system, being reactive only in the reduced state.[2] Preconditioning of the enzyme with small amounts of $NADH_2$ has no effect on susceptibility to disulfiram.[13] In its effects disulfiram differs from the usual —SH inhibitors by its more all-or-none mode of action on the sites of the flavoenzyme.

From the time relationships of the effects on the light-scattering characteristics and ATPases of mitochondria some conclusions might be drawn. If the swelling is considered to reflect a structural change[14] (as may plausibly be assumed, because the swelling in the present experiments was found to be independent of added ATP or oxidizable substrates or uncoupler added before the swelling agent), the effects of disulfiram are different from those of BAL + arsenite. The latter cause swelling of limited amplitude, in contrast to the slower and more pronounced swelling caused by disulfiram. The slowly appearing swelling is in accordance with the gradually increasing inhibition of the DNP-induced ATPase. However, when the osmotic environment of the mitochondria was altered and isotonic sucrose used instead of saline medium, no changes in the lights scattering appeared. Obviously, the permeability of the mitochondria to sucrose is not significantly altered by disulfiram. It seems that the primary action of disulfiram in its effects on the swelling phenomenon is exerted on the permeability characteristics of the mitochondria, and that swelling only occurs as a result of decreasing osmotic support of a rapidly penetrating medium.

The modes of action of disulfiram and BAL + arsenite on the ATPases are closely similar; possibly a common site of action exists. When the structural integrity of mitochondria is disturbed, Mg^{2+}-activated ATPase activity usually results. In spite of the observed volume effects that suggest structural changes, the reagents used did not evoke such an activity.

The swelling of mitochondria induced by BAL and arsenite is strikingly different from that caused by disulfiram. It is possible that there are different categories of disulfide bonds or —SH groups preventing unpleating of the mitochondrial membrane or that BAL + arsenite provide ligands inhibiting the swelling phenomenon.

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REFERENCES

1. S. MINAKAMI, F. J. SCHINDLER and R. W. ESTABROOK, *J. biol. Chem.* **239**, 2042 (1964).
2. D. D. TYLER, R. A. BUTOW, J. GONZE and R. W. ESTABROOK, *Biochem. biophys. Res. Commun.* **19**, 551 (1965).
3. H. MERSMANN, J. LUTHY and T. P. SINGER, *Biochem. biophys. Res. Commun.* **25**, 43 (1966).

4. T. E. KING, R. L. HOWARD, J. KETTMAN, JR., B. M. HEGDEKAR, M. KUBOYAMA, K. S. NICKEL and E. A. POSSEHL, in *Flavins and Flavoproteins*, (Ed. E. C. SLATER), p. 477. Elsevier, Amsterdam (1966).
5. I. HASSINEN, *Biochem. Pharmac.* **15**, 1147 (1966).
6. W. C. SCHNEIDER, *J. biol. Chem.* **176**, 259 (1948).
7. W. KIELLEY and J. R. BRONK, *J. biol. Chem.* **230**, 521 (1958).
8. B. CHANCE and B. HAGIHARA, *Proc. 5th int. Symp. Biochem., Moscow* **5**, 3 (1963).
9. A. KRÖGER and M. KLINGENBERG, *Biochem. Z.* **344**, 317 (1966).
10. P. MITCHELL and J. MOYLE, *Nature, Lond.* **208**, 147 (1965).
11. L. SZARKOWSKA and M. KLINGENBERG, *Biochem. Z.* **338**, 674 (1963).
12. L. A. STOCKEN and R. H. S. TOMPSON, *Biochem. J.* **40**, 529 (1946).
13. M. HALLMAN and I. HASSINEN, unpublished.
14. A. LEHNINGER and D. NEUBERT, *Proc. nat. Acad. Sci., USA* **47**, 1929 (1961).