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THE ROLE OF SULFHYDRYL GROUPS IN OXIDATIVE PHOSPHORYLATION AND ION TRANSPORT BY RAT LIVER MITOCHONDRIA

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SUMMARY

The influence of the sulfhydryl reagent 5,5'-dithio-bis-(2-nitrobenzoic acid) (DNTB) on metabolism of liver mitochondria was studied under different experimental conditions.

1. DTNB prevented the stimulation of respiration produced by ADP and inorganic phosphate in the presence of glutamate or succinate but had little effect on 2,4-dinitrophenol-stimulated respiration.

2. The formation of ATP from ADP and phosphate was depressed by DTNB. The inhibition could be reversed by dithiothreitol.

3. The increase in mitochondrial oxygen uptake produced by calcium and phosphate was depressed by DTNB.

4. The uptake of calcium by mitochondria in the presence of ATP and glumate was only partially inhibited by DTNB. In contrast, the stimulatory effect of inorganic phosphate on calcium transport was completely prevented.

5. In the presence of glutamate and no added ATP the uptake of calcium was associated with an entrance of phosphate. DTNB almost abolished the uptake of phosphate and inhibited calcium uptake by about 60%. The decrease in calcium uptake produced by DTNB was equal, mole for mole, to the decrease in phosphate uptake.

6. DTNB completely prevented the extrusion of calcium exhibited by calcium-loaded mitochondria incubated in the presence of inorganic phosphate and a low concentration of magnesium.

7. *N*-Ethylmaleimide had effects similar to DTNB but in addition severely inhibited 2,4-dinitrophenol-stimulated respiration with glutamate as substrate.

8. It was concluded that a reactive site involving a sulfhydryl group is intimately involved in either the entrance of inorganic phosphate into the mitochondrion or in the formation of a phosphorylated intermediate essential for oxidative phosphorylation and ion transport.

Abbreviations: DTNB, 5,5'-dithio-bis-(2-nitrobenzoic acid); HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid; PCMB, *p*-chloromercuribenzoate.

INTRODUCTION

It is known that several substrate dehydrogenases[1] as well as some reactions in the respiratory chain itself[2–4] are dependent on the presence of free sulfhydryl groups.

Some studies have been concerned with the possibility that sulfhydryl groups are involved specifically in oxidative phosphorylation. FLUHARTY AND SANADI[5,6] found that in rat liver mitochondria, oxidative phosphorylation was uncoupled by cadmium ions, arsenite *plus* BAL or γ-(*p*-arsenophenyl)-*n*-butyrate. The authors postulated that a dithiol site in mitochondria was localized between the respiratory chain and the oligomycin-sensitive terminal coupling reaction. COOPER AND LEHNINGER[7] showed that sulfhydryl reagents inhibited the ATP–P_i exchange reaction in submitochondrial preparations.

Recent studies have provided strong evidence for the involvement of sulfhydryl groups in oxidative phosphorylation. FONYO AND BESSMAN[8] demonstrated that *p*-hydroxymercuribenzoate inhibited the respiratory response of liver mitochondria to ADP + P_i, Ca^{2+} + P_i and to arsenate. VAN BUSKIRK AND FRISELL[9] found that oxidative phosphorylation associated with succinate oxidation was inhibited by formaldehyde. Finally, TYLER[10] showed that mersalyl and formaldehyde depressed the respiratory response to ADP, stimulation of oxygen uptake by calcium in the presence of phosphate as well as phosphate-induced swelling in liver mitochondria. He suggested that the SH reagents interfered with entrance of phosphate ions into the mitochondria. FONYO[11] also proposed that mercurials inhibit phosphate transport in mitochondria.

The experiments to be presented in this paper confirm and extend the important observations discussed above.

METHODS

Preparation of mitochondria. Liver mitochondria were prepared by the method of HOGEBOOM[12] as described by MYERS AND SLATER[13]. The final suspension in 0.25 M sucrose was diluted to contain about 10 mg protein per ml. Protein concentrations were determined with biuret according to CLELAND AND SLATER[14].

Incubation. In experiments in which calcium uptake was determined, the mitochondria were incubated in small beakers surrounded by circulating water at 25°. The total volume of the reaction mixture was 6–8 ml, and 1 ml-samples were taken for filtration through 0.45-μ millipore filters. The beakers were open to the air and the contents agitated with magnetic stirrers.

All media contained the amphoteric buffer *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) (ref. 15) adjusted to pH 7.4. ATP, substrate, $MgCl_2$ and sodium phosphate (pH 7.4) were present as indicated. KCl was added to give a osmolarity of 0.250.

When oxygen uptake was measured, the reaction mixture was incubated at 26° in the chamber of a Gilford oxygraph with a Clark oxygen electrode.

Determination of calcium. The solutions obtained after millipore filtration were diluted about one-tenth with 0.1 M HCl–0.01 M $SrCl_2$ and the calcium concentration determined by atomic absorption spectroscopy. Since a large proportion of calcium and magnesium ions are combined with components of the reaction mixture during

incubation, these metals are referred to as Ca and Mg rather than Ca^{2+} and Mg^{2+}.

Other analytical determinations. Inorganic phosphate was determined by the method of FISKE AND SUBBAROW[16] and ATP by a specific enzymatic method[17]. When adenine nucleotide or P_i was determined, 30 μl 9.4 M perchloric acid were added to each tube used for collection of the millipore filtrate. An aliquot of the filtrate was neutralized with K_2CO_3–ethanolamine buffer and the supernatant fluid after removal of potassium perchlorate used for analysis.

Chemicals. HEPES was obtained from Calbiochem, oligomycin from Sigma Chemical Company, 5,5'-dithio-bis-(2-nitrobenzoic acid) (DTNB) from Aldrich Chemical Company, and *N*-ethylmaleimide from Nutritional Biochemicals.

EXPERIMENTAL RESULTS

Effects of DTNB on mitochondrial respiration. Table I describes results of experiments designed to study the effect of DTNB on the oxygen uptake of rat liver mitochondria under different experimental conditions.

It is seen that the respiratory response to ADP and P_i was severely inhibited by the SH reagent when either glutamate or succinate was added as substrate. DTNB did not greatly influence respiration in the absence of phosphate acceptor or when stimulated by 2,4-dinitrophenol. In experiments in which the concentration of DTNB

TABLE I

EFFECTS OF DTNB ON THE RESPIRATORY RESPONSES OF RAT LIVER MITOCHONDRIA TO ADP AND CALCIUM IONS

Composition of reaction system: 40 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 10 mM succinate or glutamate. KCl to 250 mosM. When added: potassium phosphate 3 mM, ADP 0.18 mM, $CaCl_2$ 0.35 mM, 2,4-dinitrophenol 0.011 mM. Protein concentrations: Expt. 1, 1.05 mg/ml; Expt. 2, 1.56 mg/ml. $T = 26°$.

Expt.	*Substrate*	*DTNB (mM)*	*Additions*	*Oxygen uptake (µatoms/mg protein per min)*
1	Succinate	0	P_i	0.011
		0	P_i + ADP	0.045
		0	P_i + ADP + 2,4-dinitrophenol	0.060
		0.04	P_i	0.010
		0.04	P_i + ADP	0.017
		0.04	Pï + ADP + 2-4-dinitrophenol	0.077
	Glutamate	0	P_i	0.009
		0	P_i + ADP	0.064
		0	P_i + ADP + 2,4-dinitrophenol	0.057
		0.04	P_i	0.011
		0.04	P_i + ADP	0.014
		0.04	P_i + ADP + 2,4-dinitrophenol	0.048
2	Glutamate	0	none	0.008
		0	$CaCl_2$	0.043
		0	P_i	0.011
		0	$CaCl_2$ + P_i	0.055
		0.07	none	0.010
		0.07	$CaCl_2$	0.020
		0.07	P_i	0.010
		0.07	$CaCl_2$ + P_i	0.019

was varied, it was observed that the concentration of the inhibitor necessary to produce 50% depression of the respiratory response to ADP was about 0.012 mM.

The stimulation of respiration produced by addition of calcium ions was inhibited but not completely abolished by 0.04 mM DTNB. On the other hand, the additional sustained increase in respiration observed when both $CaCl_2$ and phosphate were added was prevented by this concentration of DTNB.

N-Ethylmaleimide had effects similar to DTNB on the respiratory responses of mitochondria to both ADP and calcium in the presence of phosphate. However, *N*-ethylmaleimide also severely inhibited 2,4-dinitrophenol-stimulated respiration with glutamate but not with succinate as substrate.

DTNB was introduced by ELLMAN[18] as a reagent for determination of sulfhydryl groups and reacts with these groups to form mixed disulfides. The fact that DTNB has an action on mitochondria similar to that of mercurials or formaldehyde[8–11] strengthens the view that SH groups are of importance in mitochondrial metabolism.

The effect of sulfhydryl inhibitors on calcium uptake by mitochondria. Experiments demonstrating an effect of DTNB on calcium uptake by liver mitochondria are presented in Fig. 1.

Uptake of calcium was determined in the presence of glutamate and ATP and in the presence and absence of inorganic phosphate. As we have reported previously[19], P_i produces a marked immediate stimulation of calcium uptake in the presence of a respiratory substrate. When DTNB was added, there was some inhibition of calcium uptake in the absence of added phosphate, but the inhibitory action of DTNB was

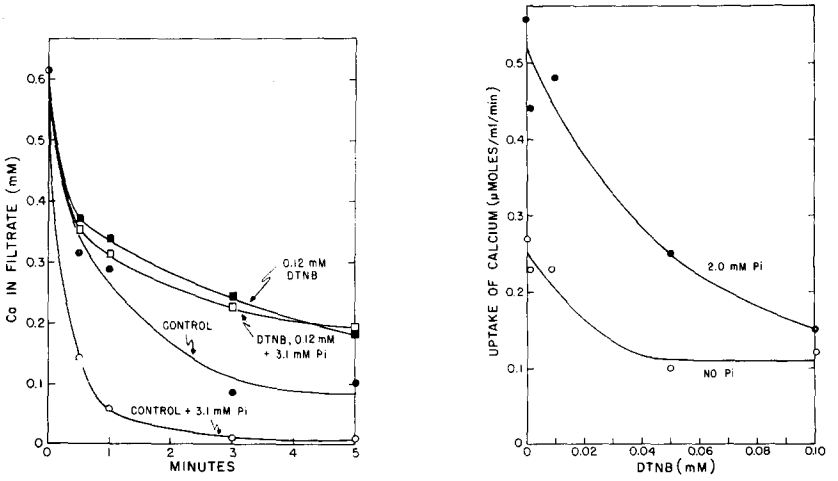

Fig. 1. Effect of DTNB on uptake of calcium by rat liver mitochondria in the presence and absence of phosphate. Composition of reaction system: 33 mM HEPES (pH 7.4), 7.1 mM $MgCl_2$, 9.2 mM glutamate, 4.6 mM ATP. Potassium phosphate when added 3.1 mM, KCl to 250 mosM. $CaCl_2$ (0.62 mM) added after 2 min of preincubation with and without DTBN (0.12 mM). 1.54 mg protein per ml. Temperature 25°.

Fig. 2. Inhibition of calcium uptake of rat liver mitochondria by DTNB. Composition of reaction system: 27 mM HEPES (pH 7.4), 6.7 mM $MgCl_2$, 6.7 mM glutamate, 3.3 mM ATP, potassium phosphate when added 2.0 mM. KCl to 250 mosM. $CaCl_2$ (0.6 mM) added after 1-min preincubation with and without DTNB at different concentrations. Incubation for 1 min after $CaCl_2$ addition. 1.02 mg protein/ml. Temperature 25°.

much greater with added phosphate. In effect, DTNB abolished the effect of phosphate on calcium uptake. *N*-Ethylmaleimide had a similar action. Fig. 2 depicts results of experiments in which calcium uptake by mitochondria was measured at different concentrations of DTNB. The inhibitor caused some decrease in calcium uptake in the absence of added phosphate, but had a greater effect on phosphate-stimulated calcium influx. At about 0.1 mM concentration the effect of P_i on calcium uptake was virtually abolished.

The effect of DTNB on calcium and phosphate uptake by liver mitochondria. When calcium enters mitochondria suspended in a medium containing inorganic phosphate, there is also an entrance of phosphate into the mitochondria. This can best be studied with a reaction mixture not containing adenine nucleotides. Such an experiment is reported in Fig. 3.

In the absence of inhibitor there was a large uptake of both calcium and phosphate, about 2 molecules of phosphate being taken up for every 3 of calcium. As the concentration of DTNB was increased, there occurred a progressive inhibition of both calcium and phosphate uptake by the mitochondria. At about 0.03 mM DTNB the phosphate uptake was completely abolished and the inhibition of calcium uptake had reached a maximum of 60%. It should be noted that the decrease in phosphate and calcium uptake produced by DTNB was equal in quantity (0.21 μmole/ml for each ion). These observations indicate that there are 2 mechanisms for calcium uptake under these experimental conditions. One requires inorganic phosphate and consists of the entrance of both calcium and phosphate in equal amounts. This reaction is inhibited

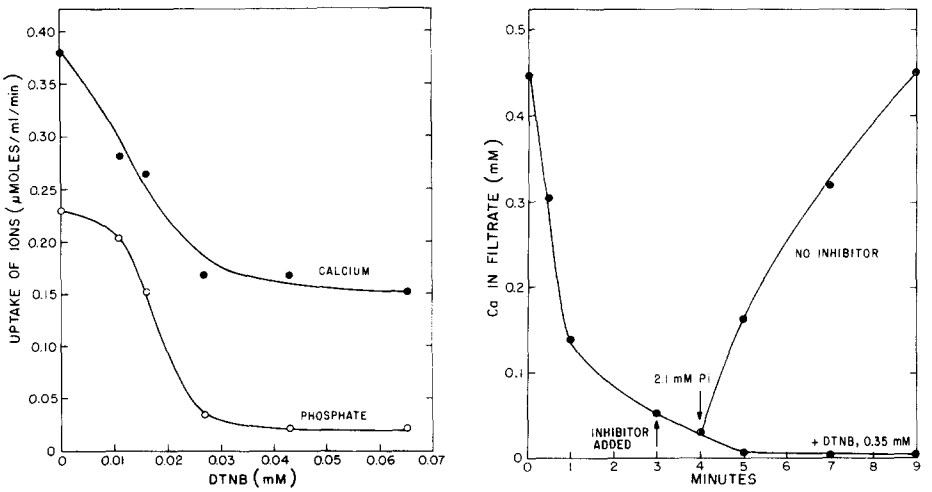

Fig. 3. Effect of DTNB on phosphate and calcium uptake by rat liver mitochondria. Composition of reaction system: 31 mM HEPES (pH 7.4), 7.5 mM $MgCl_2$, 7.5 mM glutamate, 0.54 mM potassium phosphate, KCl to 250 mosM. $CaCl_2$ (0.54 mM) added after 1-min preincubation with different concentrations of DTNB. Incubation for 1 min after $CaCl_2$ addition. 1.85 mg protein/ml. Temperature 25°.

Fig. 4. Effect of DTNB on the phosphate-induced release of mitochondrial calcium in a low magnesium system. Composition of reaction system: 35 mM HEPES (pH 7.4), 0.35 mM $MgCl_2$, 8.7 mM glutamate, 8.7 mM ATP, 0.44 mM $CaCl_2$. DTNB (0.35 mM) added after 3 min of incubation. Potassium phosphate (2.1 mM) added after 4 min of incubation. KCl to 250 mosM. 1.58 mg protein/ml. Temperature 25°.

by a sulfhydryl reagent. Another type of calcium uptake is not associated with phosphate influx and is not inhibited by DTNB.

Effect of DTNB on extrusion of calcium by rat liver mitochondria. The addition of inorganic phosphate to mitochondria loaded with calcium in the presence of a low concentration of magnesium causes extrusion of calcium and mitochondrial swelling[19]. DTNB was found to inhibit completely the phosphate-induced extrusion of calcium as shown in Fig. 4.

Mitochondria were allowed to take up calcium in the presence of ATP and glutamate. The addition of P_i caused an extrusion of all the calcium which had been taken up. When DTNB was added 1 min before the phosphate, no extrusion of calcium occurred. Separate experiments showed that mitochondrial swelling was also inhibited.

Inhibition by DTNB of oxidative phosphorylation and its reversal by dithiothreitol. The finding that DTNB inhibits ADP-stimulated respiration indicates that ATP formation itself is inhibited by the SH reagent. This was tested directly in experiments in which liver mitochondria were incubated in the presence of glutamate, ADP and phosphate, and the ATP formed was measured by an enzymatic method. As the DTNB concentration was raised, there was a progressive increase in the degree of inhibition of ATP synthesis. The maximum effect of DTNB was obtained at 0.020 mM DTNB and 50% inhibition at about 0.012 mM (8 nmoles/mg protein). Addition of *N*-ethylmaleimide to liver mitochondria incubated with succinate as substrate depressed formation of ATP from AMP. Inhibition was complete at 0.030 mM *N*-ethylmaleimide.

Reversal of the effect of DTNB on oxidative phosphorylation is shown by the experiment reported in Fig. 5.

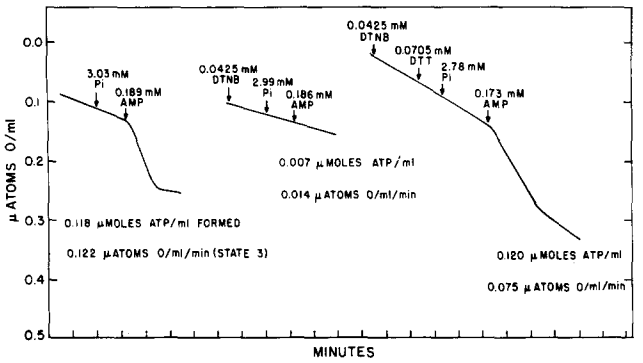

Fig. 5. Reversal of the effect of DTNB on ATP production by dithiothreitol (DTT). Composition of reaction system: 31 mM HEPES (pH 7.4), 7.7 mM $MgCl_2$, 7.7 mM glutamate, KCl to 250 mosM. Potassium phosphate, AMP, DTNB and dithiothreitol as indicated on the figure. 1.45 mg protein per ml. Temperature 26°.

In the presence of glutamate and P_i, 0.043 mM DTNB abolished the stimulation of respiration produced by AMP. The formation of ATP was almost entirely suppressed. The addition of dithiothreitol, 2 min after DTNB, effectively reversed the action of the inhibitor.

DISCUSSION

The experiments reported here have confirmed the observations of FONYO AND BESSMAN[8] and TYLER[10] that addition of a sulfhydryl reagent to liver mitochondria

inhibits the respiratory response to ADP or calcium in the presence of inorganic phosphate. The experiments provide further evidence that it is the response of mitochondria to inorganic phosphate that is specifically inhibited by sulfhydryl reagents. Oxidative phosphorylation, stimulation of respiration by calcium and phosphate, calcium uptake associated with influx of phosphate ions and phosphate-induced mitochondrial swelling and calcium extrusion are all processes that can be inhibited by low concentrations of sulfhydryl reagents. It appears that there is a common step in all these reactions that includes an enzyme or compound containing one or more sulfhydryl groups. As suggested by FONYO[11], the entrance of phosphate into the mitochondrion may be the reaction specifically inhibited by SH reagents. However, it is also possible that removal of SH groups may prevent esterification of phosphate ions entering the mitochondrion and in this way further influx of phosphate ions may be inhibited. Whatever the detailed mechanism of action involved, the sulfhydryl reagents make phosphate unavailable for metabolic reactions and abolish the usual effects of phosphate on mitochondrial function.

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