Biochemical Pharmacology, Vol. 18, pp 2385–2391. Pergamon Press. 1969. Printed in Great Britain

EFFECTS OF A DISULFIDE (ELLMAN'S REAGENT) AND THIOLS ON OXIDATIVE PHOSPHORYLATION AND ION TRANSPORT BY RAT LIVER MITOCHONDRIA*

NIELS HAUGAARD, NAM HEA LEE, RICHARD KOSTRZEWA† and ELLA S. HAUGAARD

Department of Pharmacology, University of Pennsylvania,School of Medicine
Philadelphia, Pa. 19104, U.S.A.

(*Received* 4 *February* 1969; *accepted* 22 *April* 1969)

Abstract—The disulfide, 5,5′-dithio-*bis*-2-nitrobenzoic acid (DTNB), inhibits oxidative phosphorylation and uptake of calcium and phosphate ions by rat liver mitochondria. Half-maximal inhibition of oxidative phosphorylation is obtained with an amount of inhibitor of approximately 5 nmoles/mg of protein. Complete inhibition of oxidative phosphorylation and uptake of phosphate as well as abolition of phosphate-linked calcium uptake results from preincubation of mitochondria with DTNB followed by removal of excess inhibitor by washing. DTNB-treated mitochondria can be restored to normal function by additional incubation with dithiothreitol (DTT) followed by removal of excess DTT by washing. Oxidative phosphorylation during state 3 respiration can be completely stopped by DTNB and restored by DTT. It is concluded that one or more highly reactive -SH groups are essential for oxidative phosphorylation and ion transport in mitochondria.

IT HAS been demonstrated recently that organic mercurials are capable of inhibiting oxidative phosphorylation and phosphate entrance into liver mitochondria[1, 3] indicating that free sulfhydryl groups play an important role in mitochondrial metabolism. The findings were confirmed and extended by experiments which showed that Ellman's reagent,[4] 5,5′-dithio-*bis*-2-nitrobenzoic acid or DTNB, has an action on mitochondria similar to that of mercurials.[5] DTNB is a disulfide and reacts with sulfhydryl groups to form mixed disulfides according to the equation $R_1SH + R_2SSR_2 \rightleftarrows R_1SSR_2 + R_2S^- + H^+$. The anion R_2S^- has a yellow color and the reaction has been used for the determination of tissue sulfhydryl groups.[4] Results from experiments with submitochondrial particles have also provided evidence that sulfhydryl groups are involved in one or more reactions of oxidative phosphorylation.[6]

In an earlier publication from this laboratory[5] we reported that DTNB inhibited all effects of inorganic phosphate on liver mitochondria that we studied: (1) Phosphorylation of ADP and the associated increase in the rate of oxygen uptake, (2) stimulation of mitochondrial respiration by addition of calcium and phosphate ions, (3) entrance of phosphate into mitochondria, (4) calcium uptake associated with influx of inorganic phosphate, and (5) extrusion of calcium from mitochondria produced by inorganic phosphate in the presence of a low concentration of magnesium ions.

* This work was supported by grants from the National Institutes of Health, United States Public Health Service (HE-01813 and NB-03321) and the American Heart Association.

† Predoctoral trainee supported by USPHS Training Grant 2 TO1 GM 00474.

The present experiments are an extension of the earlier studies and are concerned with the quantitative aspects of the inhibitory action of DTNB and the reversibility of its effect.

METHODS

Preparation of mitochondria. Rat liver mitochondria were prepared according to the method of Hogeboom as described by Myers and Slater.[7] Protein concentrations were determined with biuret according to Cleland and Slater.[8] The final suspension in 0·25 M sucrose was diluted to contain about 10 mg protein/ml.

Incubation. For studies of ion uptake mitochondria were incubated at 25° in small beakers open to the air and agitated with magnetic stirrers. Samples were removed at time intervals and filtered through 0·45μ millipore filters. In experiments in which oxygen uptake was determined the reaction mixtures were incubated at 26° in the chamber of a Gilford oxygraph with a Clark oxygen electrode.

All media contained the amphoteric buffer HEPES.[9] Substrate, $MgCl_2$, adenine nucleotides or phosphate were added as indicated. The osmolarity was adjusted to 0·250 with KCl and pH was 7·4.

Pretreatment of mitochondria with DTNB and dithiothreitol (DTT). In some experiments mitochondria were allowed to react with DTNB and subsequently washed with sucrose solution. DTNB-treated mitochondria were also exposed to DTT and the excess DTT removed by washing. The procedure was as follows: To 6 ml of mitochondrial suspension in 0·25 M sucrose (*ca.* 20 mg protein/ml) was added 0·35 ml 5 mM DTNB in 0·1 M HEPES (pH 7·4)-0·05M KCl. The final concentration of DTNB was 0·5 mM. After 2 min at 25° the mixture was centrifuged at 12,000 *g* for 10 min in a refrigerated centrifuge. The mitochondria were resuspended in 6 ml 0·25 M sucrose and divided into two 3-ml portions. To one portion 0·35 ml 10 mM DTT (in 0·1 M HEPES-0·05 M KCl) was added (final concentration of DTT, 2 mM). The HEPES-KCl solution without DTT was added to the other portion. After 3 min at 25° the mixtures were centrifuged at 12,000 *g* for 10 min. The mitochondria were resuspended in 0·25 sucrose, recentrifuged and again suspended in 3 ml 0·25 M sucrose. Additional mitochondria were carried through all stages of the procedure except for addition of DTNB or DTT in order to obtain washed control preparations.

Aliquots of the mitochondrial suspensions treated as above were then used for experiments on oxidative phosphorylation and ion uptake as indicated in the tables.

Analytical determinations. For calcium analysis the solutions obtained after millipore filtration were diluted with 0·1 N HCl-0·01 M $SrCl_2$ and calcium determined by atomic absorption spectroscopy.

Inorganic phosphate was measured by the procedure of Fiske and SubbaRow[10] and ATP by a specific enzymatic method.[11] Perchloric acid (30 μl 9·4 M) was added to the collecting tubes for the millipore filtrate in experiments in which ATP or phosphate was determined. Aliquots of the filtrate were neutralized with K_2CO_3-triethanolamine buffer and the supernatant fluid after removal of K-perchlorate was used for analysis.

Chemicals. HEPES was obtained from Calbiochem Co., 5,5′-ditho-*bis*-2-nitrobenzoic acid (DTNB) from Aldrich Chemical Co., and dithiothreitol (DTT) from Nutritional Biochemicals.

EXPERIMENTAL RESULTS

Effects of DTNB on the respiratory response to ADP and phosphate. Oxygen consumption of mitochondrial suspensions was determined in the presence of glutamate and inorganic phosphate before and after additionn of ADP. DTNB was added to give a wide range of concentrations. The results of these experiments are recorded in Fig. 1.

DTNB had almost no effect on respiration in the absence of phosphate acceptor, but caused a progressive inhibition of the rate of oxygen uptake in the presence of ADP (state 3 respiration). Half-maximal inhibition was obtained at a concentration of 10–12 μM. This corresponds to only about 5 nmoles inhibitor per mg mitochondrial protein. Complete inhibition of the respiratory response to ADP was observed at 40 μM DTNB (18 nmoles/mg protein).

Reversibility of DTNB effect on respiration. The inhibition by DTNB of the effect of ADP on mitochondrial respiration could be completely reversed by addition of

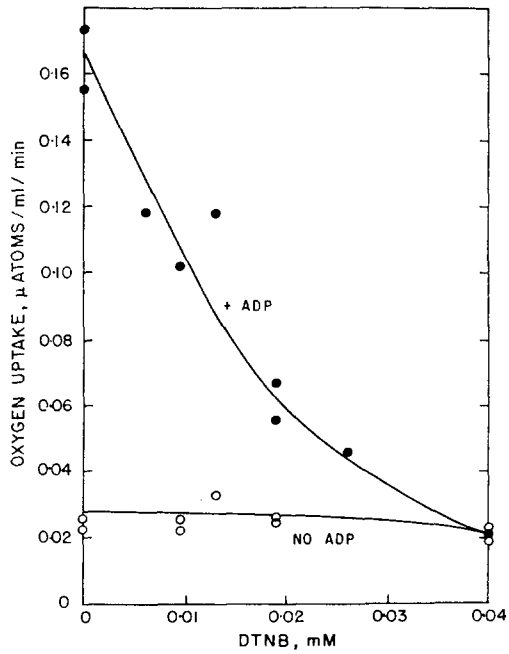

FIG. 1. Effects of different concentrations of DTNB on oxygen uptake of rat liver mitochondria in the presence and absence of ADP. Composition of reaction system: 40 mM HEPES (pH 7·4), 10 mM $MgCl_2$, 10 mM Na-glutamate, 3 mM potassium phosphate, KCl to 250 mosM. ADP when added, 1 mM. 2·22 mg protein/ml. Temp. = 26°.

the sulfhydryl compound, dithiothreitol (DDT). Such experiments are reported in Fig. 2.

Liver mitochondria were incubated in the oxygraph in the presence of glutamate. Curve A shows the typical response to the addition of first inorganic phosphate and then ADP. There was a large increase in respiration after addition of ADP followed by a precise cut-off (state 3 → state 4) when the added ADP had become phosphorylated.

In the experiments depicted in curve B DTNB was first added. Subsequent addition of Pi and ADP now had no effect on respiration. When DTT was added 2 min later, the rate of oxygen uptake, after a slight lag period, again increased and eventually a cut-off occurred as in the control experiment. The reversal of the DTNB effect on respiration, although dramatic, was not complete in that the rate of oxygen uptake during state 3 after DTT was only 70 per cent of that found in the control experiment.

The experiment depicted in curve C shows that DTNB can stop the respiratory response to ADP when added during state 3 respiration. Subsequent addition of DTT again causes a reversal of the inhibition. It is interesting that there is a short lag period before the effects of either DTNB or DTT are observed. The extra oxygen uptake observed after addition of the fixed amount of ADP was about the same in all three experiments recorded in Fig. 2.

Effect of pretreatment of mitochondria with DTNB. The experiments reported in Table 1 demonstrate that the inhibitor does not have to be present in solution during the incubation of the mitochondria in order to exert its effect.

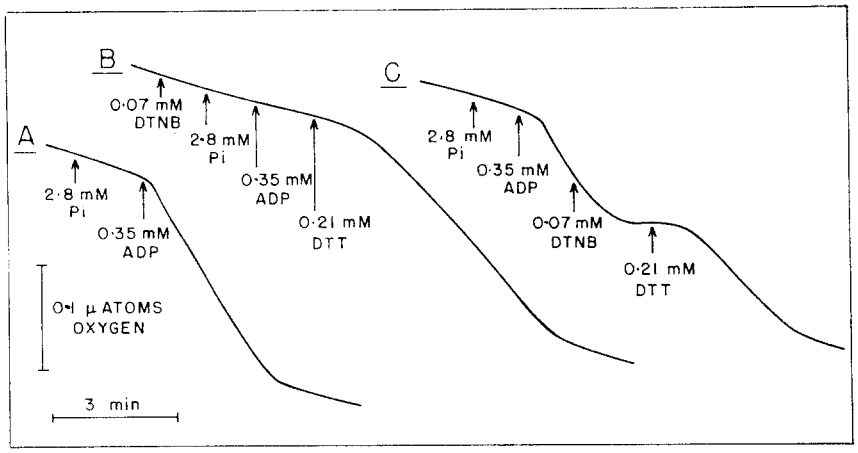

FIG. 2. Effects of DTNB and DTT on respiration of rat liver mitochondria. Composition of reaction system: 37 mM HEPES (pH 7·4), 9·3 mM $MgCl_2$, 9·3 mM Na-glutamate, KCl to 250 mosM. Other additions as indicated. 1·02 mg protein/ml. Temp. = 26°

In this experiment DTNB was added to a mitochondrial suspension. After 2 min at room temperature the suspension was centrifuged The sedimented mitochondria were then washed with a sucrose solution and resuspended as described in the Methods section. Control mitochondria were treated in exactly the same way except for the omission of DTNB.

The results reported in Table 1 show that pretreatment of the mitochondria with DTNB caused almost complete abolition of the respiratory response to AMP and abolished ATP formation. When dithiothreitol (DTT) was added to the DTNB-treated mitochondria, the respiratory response to the addition of AMP again appeared and the formation of ATP was restored. Addition of cysteine caused a partial reversal of the inhibitory effect of DTNB on oxidative phosphorylation. Finally, exposure of DTNB-treated mitochondria to DTT and subsequent washing of the mitochondria resulted in a preparation which responded to AMP in the same way as the control

TABLE 1. EFFECT OF PRETREATMENT OF RAT LIVER MITOCHONDRIA WITH DTNB ON OXIDATIVE PHOSPHORYLATION—REVERSAL OF INHIBITORY EFFECTS WITH SULFHYDRYL REAGENTS

Treatment of Mitochondria*	Addition to incubation medium	Oxygen uptake (μatoms $\times 10^3$/min/mg protein)		ATP synthesis (μmoles/ml)	P/O ratio†
		Without AMP	With AMP		
Sucrose-washed	—	10·7	55·2	0·282	2·06
DTNB-treated	—	11·9	14·2	0	—
DTNB-treated	0·14 mM DTT	11·9	45·0	0·269	1·86
DTNB-treated	1·3 mM cysteine	14·2	23·7	0·116	1·04
DTNB + DTT-treated	—	11·7	48·8	0·275	1·95

* As described in Methods Section.
† Calculated from oxygen uptake and measured ATP formation during state 3.
Composition of reaction system: 31 mM HEPES (pH 7·4), 7·7 mM $MgCl_2$, 7·7 mM Na-glutamate, 2·8 mM potassium phosphate, KCl to 250 mosM. AMP when added 0·31 mM. Temp. = 26°

sucrose-washed mitochondria. Identical results were obtained in an experiment similar to that presented in Table 1 but with ADP as a phosphate acceptor. It is evident from these experiments that oxidative phosphorylation by liver mitochondria can be aboli- by a disulfide reagent which forms a mixed disulfide with SH-groups and can be completely restored by treatment of the mitochondria with a reagent, such as DTT, which effectively regenerates SH-groups from S-S-bonds.

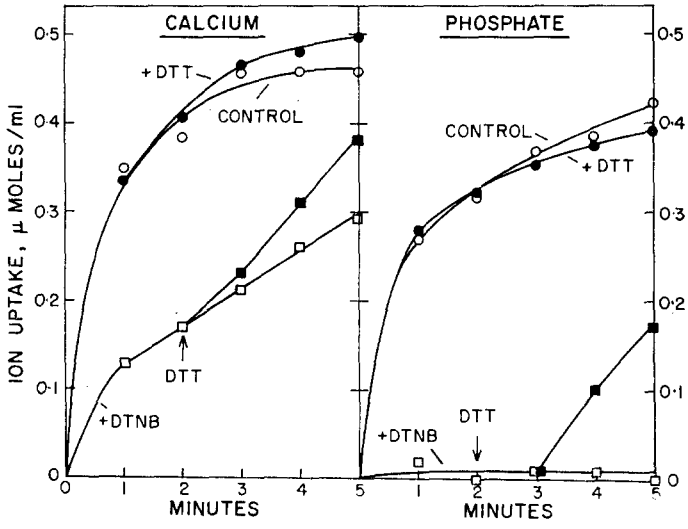

FIG. 3. Effects of DTNB and DTT on calcium and phosphate uptake by rat liver mitochondria. Composition of reaction system: 29 mM HEPES (pH 7·4), 7·1 mM $mgCl_2$, 7·1 mM Na-glutamate, 0·71 mM potassium phosphate, 0·56 mM $CaCl_2$, KCl to 250 mosM. DTNB when added, 0·13 mM. DTT when added, 0·26 mM. 2·95 mg protein/ml. Temp. = 25°.

Effects of DTNB on mitochondrial calcium and phosphate uptake and reversal by DTT. DTNB has been shown to inhibit completely the uptake of phosphate ions by rat liver mitochondria and to abolish that fraction of calcium influx associated with entrance of phosphate into the mitochondria.[5] This is well demonstrated in the experiment recorded in Fig. 3. The experiment also shows that DTT effectively overcomes the inhibitory action of DTNB on both calcium and phosphate uptake.

When added alone, DTT had a slight stimulatory effect on calcium uptake by mitochondria.

Effect of pretreatment of mitochondria with DTNB on the uptake of calcium. Mitochondria pretreated with DTNB in a way similar to that used in the experiment on oxidative phosphorylation reported in Table 1 were deficient in the ability to take up calcium ions. This is demonstrated in the experiments described in Table 2.

TABLE 2. EFFECT OF PRETREATMENT OF RAT LIVER MITOCHONDRIA WITH DTNB ON CALCIUM AND PHOSPHATE UPTAKE—REVERSAL OF INHIBITORY EFFECTS WITH DTT

Exp. No.	Treatment of mitochondria*	Addition to incubation medium	Calcium uptake μmoles/min/mg protein()	Phosphate uptake μmoles/min/mg protein
1	Sucrose-washed	—	0·250	0·195
	Sucrose-washed	0·10 mM DTNB	0·090	0·019
	Sucrose-washed	0·20 mM DTT	0·248	0·185
	Sucrose-washed	0·10 mM DTNB + 0·20 mM DTT	0·230	0·195
	DTNB-treated	—	0·095	0·020
	DTNB-treated	0·20 mM DTT	0·235	0·190
	DTNB + DTT treated	—	0·235	0·165
2	Sucrose-washed	—	0·239	0·165
	Sucrose-washed	0·10 mM DTNB	0·092	0·010
	Sucrose-washed	0·10 mM DTNB + 0·19 mM DTT	0·239	0·159
	DTNB-treated	—	0·109	0
	DTNB-treated	0·19 mM DTT	0·184	0·081
	DTNB-treated	0·39 mM DTT	0·203	0·104
	DTNB-treated	0·58 mM DTT	0·199	0·115

* As described in Methods section.
Composition of reaction system: 31 mM HEPES (pH 7·4), 7·7 mM $MgCl_2$, 7·7 mM Na-glutamate, KCl to 250 mosM. DTNB or DTT as indicated. Potassium phosphate (pH 7·4) to 0·98 mM added at 80 sec; $CaCl_2$ (to 0·58 mM) added at 90 sec. Incubation time after Ca addition 1 min., Temp.25°.

Treatment of mitochondria with DTNB followed by washing with sucrose resulted in an inhibition of calcium and phosphate uptake identical to that seen when DTNB was actually present in the incubation medium. Addition of the sulfhydryl compound DTT to the medium or exposure of DTNB-treated mitochondria to DTT followed by washing resulted in reversal of the inhibition of calcium and phosphate uptake.

DISCUSSION

The results presented here provide further evidence that sulfhydryl groups in mitochondria are essential for oxidative phosphorylation and ion transport. Mitochondria which have been exposed to DTNB and subsequently washed can no longer catalyze oxidative phosphorylation or take up phosphate and calcium ions associated with

the entrance of phosphate. When DTNB is added to a mitochondrial suspension, a yellow color appears immediately, indicating that the reagent has reacted with the mitochondria to form mixed disulfide bonds. Excess reagent can be removed by washing without reversing the inhibition. When dithiothreitol (DTT) is added to washed DTNB-treated mitochondria, the supernatant solution becomes yellow, showing that the DTNB that has reacted with the mitochondria is being released. Mitochondria so treated, after further washing, have an essentially normal ability to catalyze oxidative phosphorylation and uptake of calcium and phosphate. It is particularly interesting that oxidative phosphorylation can be stopped by a disulfide during state 3 respiration (Fig. 2) and be reversed by a thiol. The lag period that occurs before the effect of each compound can be seen indicates that a relatively slow chemical reaction takes place before mitochondrial function changes. The findings that chemicals containing S-S and SH-groups have such remarkable effects on mitochondrial function lead one to speculate that disulfide and sulfhydryl compounds normally present in the cell may play a role in regulating oxidative phosphorylation and ion transport.

The exact site of the SH-groups in mitochondria that react with DTNB is not known. In recent studies Kurup and Sanadi[7] found that organic mercurials inhibit several energy-linked reactions in bovine heart submitochondrial particles and concluded that the mercurials inhibited the generation of nonphosphorylated high-energy intermediates. These observations indicate that the inhibition of ion uptake and oxidative phosphorylation by DTNB is not caused by a change in mitochondrial permeability to ions, but is a result of a deficiency in energy production. This view is in accord with our previous findings that it is the respiration-dependent calcium uptake by mitochondria that is particularly susceptible to inhibition by DTNB.[5]

REFERENCES

1. A. Fonyo and S. P. Bessman, *Biochem. biophys. Res. Commun.* **24**, 61 (1966).
2. D. D. Tyler, *Biochem. J.* **107**, 121 (1968).
3. A. Fonyo, *Abstr. 5th Meeting Fed. Eur. Biochem. Soc., Prague*, p. 5, (1968).
4. G. L. Ellman, *Archs. Biochem. Biophys.* **82**, 70 (1959).
5. N. Haugaard, N. H. Lee, R. Kostrzewa, R. S. Horn and E. Haugaard, *Biochem. biophys. Acta* **172**, 198 (1969).
6. C. K. R. Kurup and D. R. Sanadi, *Biochemistry, N.Y.* 7, 4483 (1968).
7. D. K. Myers and E. C. Slater, *Biochem. J.* **67**, 558 (1957).
8. K. W. Cleland and E. C. Slater, *Biochem. J.* **53**, 547 (1953).
9. N. E. Good, G. D. Winget, W. Winter, N. Connolly, S. Izawa and R. M. M. Singh, *Biochemistry, N.Y.* **5**, 467 (1966).
10. C. H. Fiske and Y. Subbarow, *J. biol. Chem.* **66**, 375 (1925).
11. H. V. Bergmeyer, in *Methods of Enzymatic Analysis*, p. 539. Academic Press, New York (1963).