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Studies on Oxidative Phosphorylation. XVI. Sulfhydryl Involvement in the Energy-Transfer Pathway*

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ABSTRACT: The effect of organic mercurials was tested on the following energy-linked reactions in bovine heart submitochondrial particles: (a) reduction of nicotinamide–adenine dinucleotide by succinate coupled to the aerobic oxidation of ascorbate–*N,N,N',N'*–tetramethyl-*p*-phenylenediamine, (b) nicotinamide nucleotide transhydrogenase reaction (reduced nicotinamide–adenine dinucleotide → nicotinamide–adenine dinucleotide phosphate) driven by the energy from the oxidation of ascorbate–tetramethyl-*p*-phenylenediamine, and (c) energy-driven intravesicular acidification measured by the bromothymol blue color change. Reaction a was inhibited about 90% by 10 μM or less of mersalyl or mercuriphenylsulfonate. The energy-linked transhydrogenase reaction (reaction b) was also highly sensitive to mercurials under conditions in which the energy-independent reverse reaction (reduced nicotinamide–adenine dinucleotide → nicotinamide–adenine dinucleotide phosphate) showed minimal sensitivity. The bromothymol blue response coupled to the aerobic oxidation of reduced nicotinamide–adenine dinucleotide or succinate was inhibited by low concentrations of mersalyl which did not affect the respiratory activity. When ascorbate–toluylene blue or adenosine triphosphate was used to initiate the bromothymol blue response, the inhibitory effect of mersalyl was enhanced by the presence of bovine serum albumin in the reaction medium. The results indicate that the mercurial acts by inhibiting the generation of nonphosphorylated high-energy intermediates and point to the involvement of sulfhydryl groups in the early reactions of oxidative phosphorylation.

There are several reports in the literature which point to the involvement of SH groups in the energy-transfer pathway of oxidative phosphorylation. Organic mercurials and other thiol binding agents have been shown to uncouple oxidative phosphorylation (Fluharty and Sanadi, 1960; Fletcher and Sanadi, 1962) and inhibit respiratory stimulation by ADP (Fonyo and Bessman, 1966) in whole mitochondria. In submitochondrial particles prepared from rat liver, *p*-hydroxymercuribenzoate inhibits phosphorylation coupled to the aerobic oxidation of ferrocytochrome *c* (Cooper and Lehninger, 1956), abolishes [^{32}Pi]ATP exchange (Cooper and Lehninger, 1957), activates latent ATPase, and inhibits dinitrophenol-stimulated ATPase (Kielley, 1963). These particulate preparations show low P/O and poor respiratory control (Bronk and Kielley, 1958) presumably due to labilization of some of the terminal steps of the energy-transfer system (Lee and Ernster, 1966).

We have examined the effect of thiol binding agents on several energy-linked reactions in submitochondrial particles in an attempt to localize the site of sulfhydryl involvement on the energy-transfer pathway. The following reactions were studied: (a) reduction of NAD by succinate driven by the aerobic oxidation of ascorbate–tetramethyl-*p*-phenylenediamine described by Packer (1963) and Vallin and Low (1964), (b) energy-linked nicotinamide nucleotide transhydrogenase reaction driven by the oxidation of ascorbate–tetramethyl-*p*-phenylenediamine (Danielson and Ernster, 1963), and (c) energy-dependent production of proton gradient measured by the absorbance change of bromothymol blue (Chance and Mela, 1967). The results presented in this communication indicate that these three reactions

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involve at least one mercurial-sensitive SH group on the energy-transfer pathway. The site of action of the mercurial is between the respiratory chain and the site of oligomycin inhibition. Some of these results have been included in a preliminary form in a previous communication (Sanadi *et al.*, 1968).

Materials and Methods

Submitochondrial Particles. Two types of submitochondrial particles were used in this study. The ammonia–EDTA-treated particles were prepared as described previously (Lam *et al.*, 1967). These particles show poor phosphorylating ability which is stimulated by low levels of oligomycin. Particles with better phosphorylating activity were prepared from bovine heart mitochondria by sonic treatment in the presence of ATP and $MgCl_2$ (these particles were referred to originally as ETPH and will be referred to hereafter as sonic particles) as described by Linnane and Ziegler (1958). These particulate preparations which were kept frozen at −70° were thawed and diluted to 5 mg of protein/ml with 0.25 M sucrose containing 5 mM Tris-SO_4 buffer (pH 7.4) before use.

Assay Systems. The reduction of NAD driven by coupling to cytochrome oxidase was carried out in a reaction system containing 50 mM Tris-SO_4 buffer (pH 7.8), 0.2–0.5 μg of oligomycin, 1 μg of antimycin A, 1 mg of particle protein, 2 mg of bovine serum albumin, 6.7 mM succinate, and either 1 mM EDTA or 0.5 mM dithiothreitol in a 3.0-ml reaction volume. After incubation at 37° for 3 min, the reaction was initiated by the addition of 20 μmoles of ascorbate, 2 μmoles of tetramethyl-*p*-phenylenediamine, and 3 μmoles of NAD in 0.25 ml.

The energy-dependent nitocintamide nucleotide transhydrogenase reaction was measured in a system containing 50 mM Tris-SO_4 buffer (pH 7.8), 0.5 μg of oligomycin, 5 μg of rotenone, 25 μg of lactate dehydrogenase, 1 mg of particle protein, 2 mg of bovine serum albumin, 10 mM lactate, 50 μM NAD, 1 mM NADP, and either 1 mM EDTA or 0.5 mM dithiothreitol in a 3.0-ml reaction volume. After incubation at 37° for 3 min, the reduction of NADP was initiated by the addition of 20 μmoles of ascorbate and 2 μmoles of tetramethyl-*p*-phenylenediamine in 0.15 ml.

The reaction systems for the assay of the energy-independent transhydrogenase reactions were similar to the one given above and have been described in the appropriate legends. The rate of reduction of the nicotinamide nucleotide, in all these cases, was followed at 340 mμ with the Gilford automatic recording attachment to the Beckman DU monochromator. When sonic particles were used the reaction system contained 0.25 M sucrose.

The energy-linked production of proton gradient was determined in a reaction system containing 20 mM Tris-Cl buffer (pH 7.4), 0.25 M sucrose, 6.7 μM bromothymol blue, 0.5 mg of particle protein, and 1 mM EDTA or 0.5 mM dithiothreitol in 3 ml. The reaction was started by stirring in the electron donor in 0.05 ml and the change in the color of the membrane-bound dye was determined by the decrease in absorbance at 618–700 mμ in the Aminco-Chance dual-wavelength spectrophotometer (Chance and Mela, 1967). Oxygen uptake was measured with a Clark or vibrating platinum microelectrode.

Chemicals. The *N,N,N′,N′*-tetramethyl-*p*-phenylenediamine supplied by Eastman Organic Chemicals was purified as follows. The yellow powder was dissolved in boiling ethanol, decolorized by activated charcoal, and filtered. HCl (1 or 2 drops) was added to the blue filtrate, chilled, and the hydrochloride was precipitated by the addition of diethyl ether. The white precipitate was filtered under suction, washed with ether, and dried under vacuum. The powder when stored in amber bottles did not change color for several months. Most of the other chemicals including rabbit muscle lactate dehydrogenase were products of Sigma Chemical Co. The uncoupling agent, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone, was a generous gift from Dr. P. G. Heytler. Toluylene blue ($E_0' = +0.115$) was obtained from British Drug Houses. Solutions of ascorbate, toluylene blue, tetramethyl-*p*-phenylenediamine, carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone, mersalyl, and *p*-chloromercuriphenylsulfonate[1] were prepared fresh daily. All solutions were adjusted to pH 7.4 with KOH or HCl before use. Water, double distilled in an all-glass apparatus, was used in these experiments.

Results

Energy-Linked Reduction. In this reaction, the energy generated at the third site of phosphorylation during the aerobic oxidation of ascorbate–tetramethyl-*p*-phenylenediamine is utilized for the reduction of NAD by succinate by reversed electron transfer. Reversed electron flow from ferrocytochrome *c* to NAD and reoxidation of NADH are prevented by the addition of antimycin A. When the reaction was carried out in the absence of bovine serum albumin, dithiothreitol, or EDTA, an absorbance increase equivalent to 10–12 mμmoles of NADH min^{-1} mg^{-1} of protein was observed. This absorbance increase like the energy-dependent NAD reduction showed a requirement for oxygen and essentially stopped when the system became anaerobic, but differed from it in the following respects. It was observed at wavelengths other than 340 mμ, was not dependent upon the concentration of particle protein or the presence of NAD or tetramethyl-*p*-phenylenediamine in the reaction system, and was observed when the particles were mixed with ascorbate in the presence of buffer. The absorbance increase at 340 mμ did not change on the subsequent addition of lactate dehydrogenase and pyruvate and was completely inhibited by dithiothreitol or EDTA both of which stimulated the energy-dependent reaction. In most of these properties it resembled the ascorbate-induced swelling observed in isolated liver mitochondria by Hunter (1961). In routine experiments

[1] Hereafter referred to as MPS.

the reduction of NAD was confirmed by the addition of lactate dehydrogenase and pyruvate when the reaction system became anaerobic.

Requirement for Thiol Compound or EDTA and Bovine Serum Albumin. With either sonic or ammonia particles no significant reduction of NAD was observed unless dithiothreitol or EDTA was added to the reaction system. The activity obtained with EDTA was always less than that with dithiothreitol. Although bovine serum albumin by itself did not stimulate the reaction, its addition along with dithiothreitol or EDTA increased the rate of reduction of NAD (Table I). To obtain maximum activity these reagents had to be added before incubation. Only one-fifth of the activity was observed when dithiothreitol was added after the reaction was initiated. Also, pretreatment of the particles with dithiothreitol, EDTA, or both did not eliminate their requirement in the reaction system. For example, ammonia particles sedimented after treatment with 1.4 mM dithiothreitol or 14 mM EDTA still showed no appreciable activity unless dithiothreitol or EDTA along with bovine serum albumin was added to the assay medium. Stimulation of the reaction by bovine serum albumin, EDTA, and other metal-chelating agents has been observed earlier (Vallin and Low, 1964).

TABLE I: Effect of Bovine Serum Albumin, Dithiothreitol, and EDTA on Energy-Linked NAD Reduction in Submitochondrial Particles.[a]

Addition	NAD Reduced (mμmoles min^{-1} mg^{-1} of protein)	
	Ammonia Particles	Sonic Particles
None	10.6	0.0
Bovine serum albumin	12.0	0.0
EDTA	9.7	16.8
Bovine serum albumin + EDTA	48.0	33.8
Dithiothreitol	53.5	29.0
Bovine serum albumin + dithiothreitol	82.0	48.4

[a] The reaction system and conditions of assay were as described in Materials and Methods except that the reagents shown in the table were omitted. As indicated, bovine serum albumin (2 mg), dithiothreitol (0.5 mM), and EDTA (1 mM) were added to the reaction system before incubation. Addition of EDTA together with dithiothreitol does not stimulate the reaction any more than dithiothreitol alone.

Other thiol compounds, 2,3-dimercaptopropanol, and β-mercaptoethanol also stimulated the reaction to the same extent as dithiothreitol but only at higher concentrations (Figure 1). The activity obtained with cysteine or glutathione was consistently about one-half of that with dithiothreitol. Dithioerythritol stimulated the reaction in the same manner as dithiothreitol.

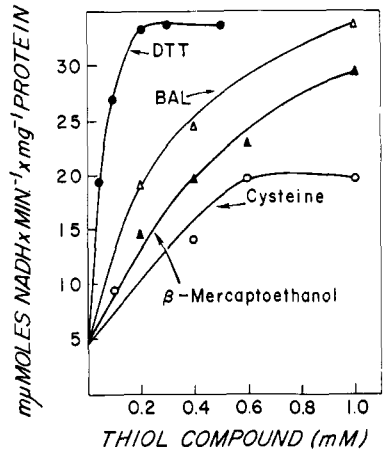

FIGURE 1: Effect of thiol compounds on energy-linked NAD reduction. The reaction was carried out with ammonia particles as given in Materials and Methods except that dithiothreitol was omitted and the thiol compounds were added as indicated in the figure. (●) Dithiothreitol, (△) 2,3-dimercaptopropanol, (▲) β-mercaptoethanol, and (○) cysteine.

Disulfide compounds like oxidized glutathione, cystine, cystamine, or cystine dimethyl ester neither stimulated the reaction when added in the place of dithiothreitol nor had any inhibitory effect when added to the reaction system along with dithiothreitol.

Effect of Oligomycin. Oligomycin stimulated the reaction over 15-fold with ammonia particles and about 3-fold with sonic particles. Very little activity (6.8 mμmoles of NADH min^{-1} mg^{-1} of protein) was detectable with ammonia particles unless oligomycin was added to the reaction system. The activity increased with increasing concentration of oligomycin to give an S-shaped curve and maximal stimulation (87.0 mμmoles of NADH min^{-1} mg^{-1} of protein) was observed with 0.2 μg of oligomycin mg^{-1} of protein. Concentrations of oligomycin as high as 10 μg mg^{-1} of protein did not inhibit the reaction to any significant extent. The stimulatory effect of oligomycin would indicate that the reaction is driven by nonphosphorylated high-energy intermediates.

Relation to Cytochrome Oxidase Activity. The reduction of NAD was dependent upon aerobic oxidation, stopped when the system became anaerobic, and was inhibited by terminal respiratory inhibitors like sulfide and cyanide. As seen in Figure 2, the energy-linked NAD reduction increased rapidly with increasing concentration of tetramethyl-*p*-phenylenediamine and was saturated with approximately 0.15 mM tetramethyl*p*-phenylenediamine. Oxygen uptake, however, was still not saturated at concentrations of tetramethyl-*p*-phenylenediamine well above the 0.15 mM level. Similar results have been obtained in the bromothymol blue color change when it is driven by the oxidation of ascorbate–toluylene blue. The toluylene blue saturates the bromothymol blue response at 15–20 μM while oxygen consumption is saturated at 70–75 μM. These and related data will be published in a later communication. It would appear from

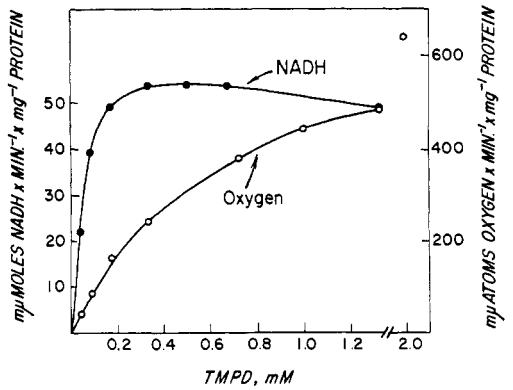

FIGURE 2: Effect of tetramethyl-*p*-phenylenediamine concentration on NAD reduction and oxygen uptake. The reaction system for NAD reduction has been described in Materials and Methods and contained 0.5 mM dithiothreitol. The concentration of tetramethyl-*p*-phenylenediamine was varied as indicated. The oxygen uptake was determined in the same reaction medium.

these results that the increased rate of oxygen consumption does not always indicate an increase in the supply of utilizable energy. It is also possible that the reactions which utilize the energy counted become saturated at lower levels and an excess of energy supply becomes wasteful. A third factor may be uncoupling by excess dye. A careful comparison of the saturation level for different energy-linked reactions may be helpful in choosing between these possibilities.

Effect of pH and Salt Concentration. The rate of NAD reduction was optimal at pH 7.8. The activity was 8.6% at pH 6.4, 52% at pH 6.8, 60% at pH 8.3, and 44% at pH 8.8 relative to the maximum activity. A threefold increase in the molarity of the buffer (Tris-SO_4) decreased the activity to 42% of the original. The reaction was inhibited 56% by 10 mM $CaCl_2$ while the same concentrations of KCl, Na_2SO_4, Na_2AsO_4, and Na_2HPO_4 did not show any inhibition. The inhibition by Ca^{2+} could be due, at least partly, to uncoupling.

Activity of Different Submitochondrial Particles. Ordinarily, ammonia particles showed activity ranging from 40 to 90 (mμmoles of NAD reduced min^{-1} mg^{-1} of protein). This activity did not change when the reaction was carried out in 0.25 M sucrose. Under comparable conditions sonic particles showed an activity of about 40 which was increased 30–50% when the reaction medium contained 0.25 M sucrose. The activity of urea-depleted submitochondrial particles (Andreoli *et al.*, 1965) was only 15.

Effect of Bovine Serum Albumin, Dithiothreitol, and EDTA on Oxidation Reactions. Since the energy-dependent NAD reduction was stimulated by bovine serum albumin, EDTA, and dithiothreitol (Table I), the effect of these compounds on oxygen uptake with the substrates related to the assay was tested. The NADH and cytochrome oxidase activities (320 and 410 mμatoms of oxygen min^{-1} mg^{-1} of protein, respectively) were unaffected by the addition of bovine serum albumin, EDTA, or dithiothreitol at the concentration used in the assay. The oxidation of succinate (60 mμatoms of oxygen min^{-1} mg^{-1} of protein), on the other hand, was stimulated twofold by EDTA and fourfold by dithiothreitol. Addition of bovine serum albumin did not show any further stimulatory effect. Increased supply of reducing power from succinate may not be the primary reason for the stimulation of the energy-linked NAD reduction by bovine serum albumin, EDTA, or dithiothreitol because the succinate oxidase activity even without the addition of these compounds (60 mμatoms of O_2 min^{-1} mg^{-1} of protein) was high enough to support the energy-linked reaction (40–80 mμmoles of NADH min^{-1} mg^{-1} of protein) in the presence of dithiothreitol or EDTA. However, this possibility cannot be excluded rigorously since an increase in the steady-state concentration of the reduced electron carrier could conceivably stimulate reversed electron flow appreciably. It would appear from these results that the site of dithiothreitol and EDTA effect may be the succinate dehydrogenase limb of the respiratory chain before its junction with the cytochrome chain.

Effect of Mercurials. The energy-linked reduction of NAD was sensitive to organic mercurials. The reaction was inhibited 85% by 5 μM MPS (Figure 3). The inhibitory effect of both MPS and mersalyl was completely reversed by dithiothreitol (0.1 mM), 2,3-dimercaptopropanol (0.2 mM), β-mercaptoethanol (0.5 mM), or cysteine (0.5 mM).[1]

Under the conditions of the experiment the cytochrome oxidase activity of the particles was practically insensitive to mercurials (Figure 3). This is in agreement with the observation of Slater (1949) who reported that even prolonged incubation of submitochondrial particles with mercurial did not significantly inhibit the cytochrome oxidase activity. Cooperstein (1963) has identified cytochrome oxidase as an enzyme involving intact disulfide groups. The oxidation of NADH was partially sensitive to mercurial (29% inhibition by 10 μM MPS Figure 3). However, higher concentrations of the mercurial did not inhibit the reaction to any great extent (46% at 200 μM, point not shown in Figure 3). Tyler *et al.* (1965) have reported that NADH oxidase activity becomes highly sensitive to mercurials when the particles are preconditioned by exposure to a cycle of NADH oxidation.

The sensitivity of succinate oxidation to mercurials depended upon the experimental conditions. The oxidase activity was inhibited less than 20% when 10 μM mercurial was added to the particles after the addition of succinate and 70% when added before its addition (Figure 3). This is consistent with the finding that the inhibitory action of sulfhydryl binding agents on succinate dehydrogenase activity is at the level of the formation of the enzyme–substrate complex (Hopkins *et al.*, 1938; Slater, 1949).

The above results indicate that the inhibitory action of organic mercurials on the energy-linked reduction of NAD may not be due to the inhibition of electron transport activity at the succinate or NADH dehydrogenase

[1] The reaction was inhibited 17, 58, and 79%, respectively, by 5, 50, and 200 μM *N*-ethylmaleimide, a sulfhydryl-alkylating agent.

TABLE II: Effect of Bovine Serum Albumin, Dithiothreitol, EDTA, and Oligomycin on the Energy-Linked Transhydrogenase Reaction.[a]

Addition	NADP Reduced (mμmoles min^{-1} mg^{-1} of protein)			
	Ammonia Particles		Sonic Particles	
	No Oligomycin	Oligomycin	No Oligomycin	Oligomycin
None	19.2	19.2	19.6	19.6
Bovine serum albumin	19.2	23.2	19.6	19.6
EDTA	19.2	29.0	19.6	19.6
Bovine serum albumin + EDTA	23.0	43.3	19.6	24.2
Dithiothreitol	21.7	38.7	24.2	24.2
Bovine serum albumin + dithiothreitol	43.2	43.3	38.6	38.6

[a] The reaction system and conditions of assay were the same as described in Materials and Methods. Additions of bovine serum albumin (2 mg), EDTA (1 mM), dithiothreitol (0.5 mM), and oligomycin (0.5 μg) were made as indicated, before the incubation.

level, but to the inhibition of the generation or utilization of the high-energy intermediates.

Energy-Linked Transhydrogenase Activity. The results presented above indicate that one or more mercurial-sensitive SH groups may be involved in the energy-transfer pathway. However, the evidence was rendered inconclusive because of the partial mercurial sensitivity of the electron transport system especially at the succinate dehydrogenase level. Therefore, the effect of mercurials on the energy-dependent nicotinamide nucleotide transhydrogenase reaction (Danielson and Ernster, 1963) driven by the mercurial-insensitive cytochrome oxidase system (site III) was examined. As in the previous set of experiments, the energy was provided by the oxidation of ascorbate–tetramethyl-*p*-phenylenediamine. The lactate–lactate dehydrogenase system was used to keep the NAD in the reduced state in view of the relative insensitivity of lactate dehydrogenase to mercurials (Schwert and Winer, 1963).

Effect of Bovine Serum Albumin, EDTA, Dithiothreitol, and Oligomycin. The transhydrogenase reaction did not show a dependence upon oligomycin for activity under our assay conditions. The stimulation obtained with bovine serum albumin, EDTA, or dithiothreitol was also less pronounced than in the energy-linked reduction of NAD by succinate (Tables I and II). However, bovine serum albumin and dithiothreitol together produced consistent stimulation. The lesser dependence of the transhydrogenase reaction upon oligomycin may be a reflection of the lower requirement of energy for this reaction as contrasted with the reduction of NAD (Danielson and Ernster, 1963). It may be mentioned in this context that the transhydrogenase activity measured with lactate and lactate dehydrogenase was less than the activity obtained when the alcohol–alcohol dehydrogenase system was used to generate NADH (112 mμmoles of NADPH formed min^{-1} mg^{-1} of ammonia particle protein). It is possible that the high amounts of lactate (10 mM) used in the reaction system may affect the transhydrogenase activity.

Effect of Mercurials. The energy-linked transhydrogenase reaction was inhibited over 90% by 10 μM MPS (Figure 4A) or mersalyl. The inhibition was reversed by dithiothreitol (0.1 mM) and other thiol compounds (data are not presented). The effect of mer-

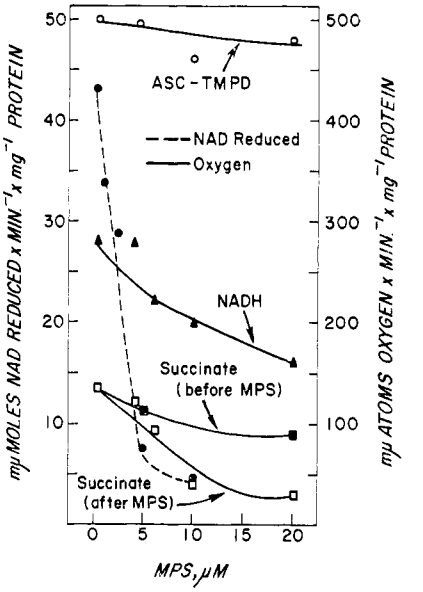

FIGURE 3: Effect of MPS on energy-linked NAD reduction and oxidase activities. The energy-linked NAD reduction was measured as given in Materials and Methods and contained 0.5 mg of ammonia particle protein, 1 mM EDTA, 2 mg of bovine serum albumin, and 0.5 μg of oligomycin. The reaction system for the assay of oxidase activity contained 50 mM Tris-SO_4 buffer (pH 7.8), 0.25 μg of oligomycin, 1 mg of bovine serum albumin, 1 mM EDTA, and 0.5 mg of ammonia particles protein in a total reaction volume of 1.5 ml. After incubation at 23° for 3 min, the reaction was started by the addition of 1.5 μmoles of NADH in 0.15 ml, 10 μmoles of succinate in 0.1 ml or 10 μmoles of ascorbate, and 1 μmole of tetramethyl-*p*-phenylenediamine in 0.13 ml. The mercurial was added before the substrate in all experiments except the one indicated in the figure. Oxygen uptake was measured polarographically using a Clark electrode.

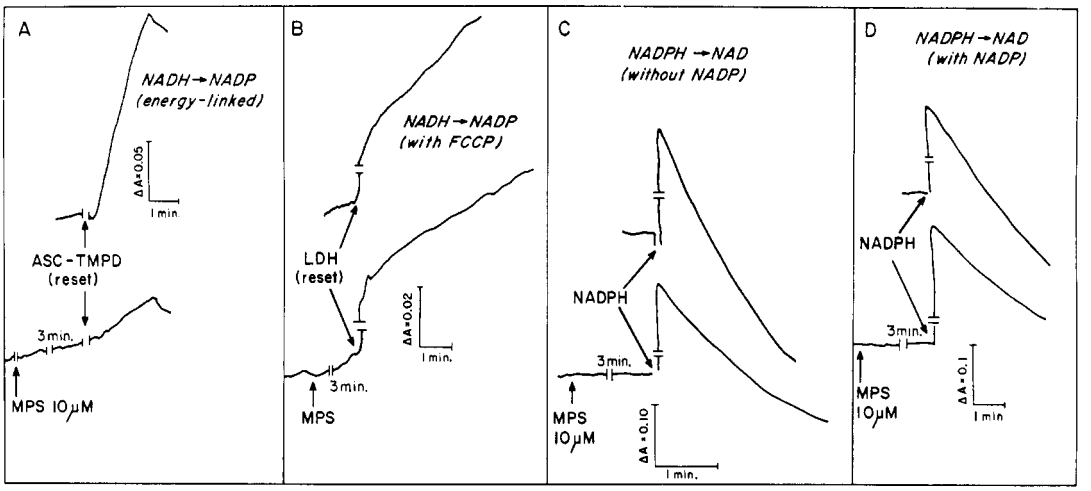

FIGURE 4: Effect of MPS on transhydrogenase activity. The energy-dependent transhydrogenase reaction (NADH → NADP) represented by A in the figure was assayed as described in Materials and Methods and contained bovine serum albumin, EDTA, and oligomycin. The same reaction without energy involvement (B) was carried out in the presence of 1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. The reaction was initiated by the addition of lactate dehydrogenase which produces an immediate increase in absorbance due to reduction of NAD. The reaction system for the reverse reaction (NADPH → NAD) was essentially the same as for the energy-linked reaction except that pyruvate (3 mM) was used instead of lactate to oxidize the NADH formed and the reaction was started by the addition of 0.5 μmole of NADH in 0.05 ml in the place of ascorbate-tetramethyl-*p*-phenylenediamine. The medium for C did not contain NADP while that for D contained 1 mM NADP. Wherever indicated mercurial (10 μM) was added 3 min before the start of the reaction.

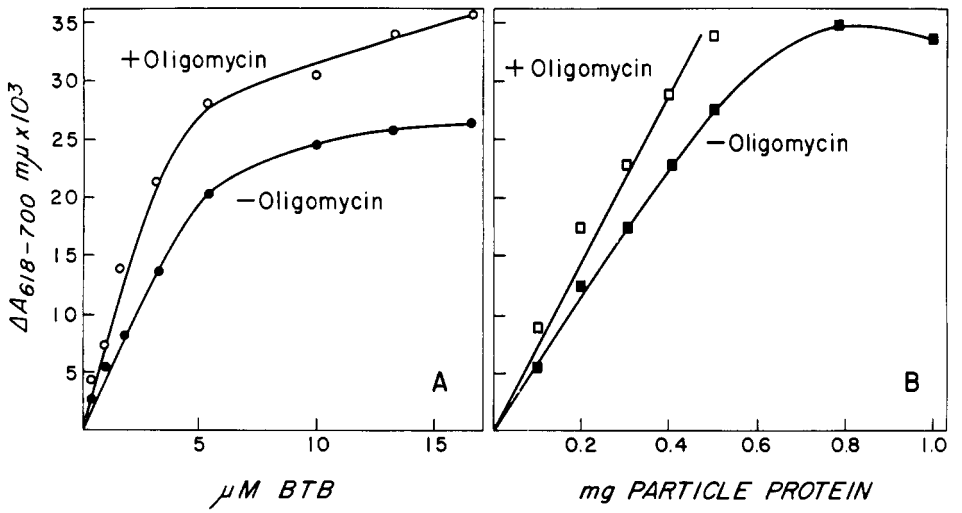

FIGURE 5: Effect of bromothymol blue and protein concentration on the extent of absorbance decrease. The reaction system contained 20 mM Tris-Cl buffer (pH 7.4), 0.25 M sucrose, and 0.5 mg of ammonia particle protein (A) and 13.3 μM bromothymol blue (B) in 2.97 ml. The reaction was started by stirring in 0.3 μmole of NADH in 0.03 ml. The bromothymol blue concentration was varied in A and protein concentration in B. (● and ■) Ammonia particle alone; (○ and □) same in the presence of 0.5 μg of oligomycin.

curials on the nonenergy-dependent transhydrogenase reaction was tested under three different conditions. The reaction in the forward direction (NADH → NADP) was measured under the same conditions as those used for the measurement of the energy-dependent reaction but with the addition of 1 μM carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone to inhibit energy production. The rate of formation of NADPH (4.8 mμmoles min^{-1} mg^{-1} of ammonia particle protein) was inhibited 21% by 10 μM MPS (Figure 4B). The reaction in the reverse direction (NADPH → NAD) was measured by following the rate of disappearance of added NADPH in a system containing pyruvate and lactate dehydrogenase to trap the resulting NADH. Under these conditions the activity was higher (96.8 mμmoles of NADPH min^{-1} mg^{-1} of ammonia particle protein) than that of the energy-linked forward reaction and was inhibited 45–50% by 10 μM MPS (Figure 4C). When NADP (1 mM) was also added to the reaction system to simulate the conditions used for the measurement of the energy-dependent reaction, the activity decreased (38.7 mμmoles of NADPH min^{-1} mg^{-1} of protein) and was inhibited 25–30% by 10 μM MPS (Figure 4D). It may be mentioned that the mercurial effectively inhibited the energy-linked reaction (over 80%) even when added after the reaction

had been initiated with ascorbate–tetramethyl-*p*-phenylenediamine. Under similar conditions the reverse reaction (NADPH → NAD) showed no significant inhibition (less than 20%).

The greater sensitivity of the energy-linked transhydrogenase reaction to mercurials indicated the possibility of SH involvement in the generation of energy at the third phosphorylation site. However, the presence of functional SH in the transhydrogenase enzyme itself cannot be completely ruled out in view of the inhibitory effect (although low) of mercurial on the reverse reaction.

In all of the above experiments, the concentration of mercurial necessary to produce a given level of inhibition depends upon the particle protein concentration and the time of its exposure to the inhibitor. These factors have been controlled as much as possible in order to make the comparison of the responses in different reactions reasonably meaningful.

Energy-Linked Bromothymol Blue Response. It was shown by Chance and Mela (1967) that bromothymol blue supplemented submitochondrial particles exhibited a decrease in absorbance at 618–700 mμ on the initiation of respiratory activity by the addition of NADH or succinate or on the addition of ATP. The energy dependence of this response was established by them. Since cytochrome oxidase is insensitive to mercurials (Figure 3), bromothymol blue response linked to the generation of energy exclusively at this region of the respiratory chain appeared to be a suitable system to study SH involvement. It was observed that bromothymol blue response similar in nature to that produced by NADH or succinate was produced on the aerobic oxidation of ascorbate in the presence of the red ox dye toluylene blue ($E_0' = +0.115$; Clark, 1960). This response was unaffected by respiratory poisons like rotenone and antimycin A but was inhibited by Na_2S which is a terminal respiratory poison or by the uncoupling agent, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (Sanadi *et al.*, 1968), indicating clearly energy requirement as well as association with cytochrome oxidase activity. No response was produced when menadione or cytochrome *c* was substituted for toluylene blue. The response with tetramethyl-*p*-phenylenediamine was about one-half that obtained with toluylene blue.

Effect of Bromothymol Blue and Protein Concentration. The experiments of Chance and Mela (1967) were performed using a bromothymol blue concentration of 3.3 μM and particle protein concentration of 0.5–1.5 mg/ml. We observed that using a fixed particle concentration of 0.5 mg of protein/3 ml, the response increased with increasing bromothymol blue concentration up to about 5 μM, and tapered off beyond this point (Figure 5A). It may be mentioned that even with high levels of bromothymol blue (13.3 μM) the extent of response was little affected by large changes in the buffering capacity of the medium. For example, in a system containing 0.5 mg of ammonia particle protein, 13.3 μM bromothymol blue, and 0.5 μg of oligomycin, the absorbance decrease on the addition of 100 μM NADH was 27.6, 34.0, and 21.6 $\times 10^{-3}$ when the respective Tris-Cl

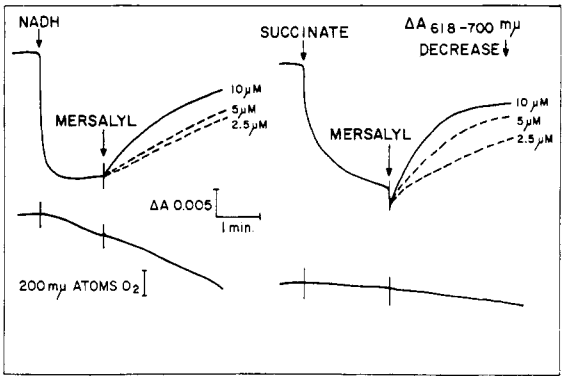

FIGURE 6: Effect of mersalyl on bromothymol blue response with NADH and succinate as substrates. The reaction system contained 20 mM Tris-Cl buffer (pH 7.4), 0.25 M sucrose, 1 mg of ammonia particle protein, 6.7 μM bromothymol blue, 1 mM EDTA, and 1 μg of oligomycin in a total reaction volume of 6 ml. The reaction was started by stirring in 2 μmoles of NADH or 12 μmoles of succinate in 0.03 ml. The tracings at the bottom indicate oxygen uptake measured simultaneously with a vibrating platinum electrode.

buffer concentration in the medium was 2, 20, and 200 mM. This is in general agreement with the observations of Chance and Mela (1967). In the case of whole mitochondria, Mitchell *et al.* (1968) observed that the absorbance change was greater when the bromothymol blue concentration was increased from 3.3 to 15 μM. It may be stated, however, that the concentration of bromothymol blue did not affect our results in the experiments reported below. These were repeated at two different bromothymol blue concentrations (13.3 μM and 6.7 mM or lower).

The effect of protein concentration on the magnitude of the absorbance change obtained during NADH oxidation is shown in Figure 5B. In the presence of 13.3 μM bromothymol blue the absorbance change increased linearly with increase in protein concentration up to about 0.5 mg of particle protein. Further increase in particle concentration caused very little change in absorbance. The same trend was shown in the presence of oligomycin. Although the protein/bromothymol blue ratio is different from that used by Chance and Mela (1967), the initial slope of the curve (Figure 5B), corresponding to an absorbance change of 0.06 $\times$ mg^{-1} of protein, is the same as the value obtained by them for submitochondrial particle A. This would indicate that the ammonia particles are capable of binding more bromothymol blue than those used by Chance and Mela (1967), but the extent of response obtained with both types of particles is similar.

Effect of EDTA, Dithiothreitol, and Oligomycin. The response of bromothymol blue when coupled to respiratory activity was stimulated by EDTA and dithiothreitol. In a typical experiment 1 mM EDTA increased the absorbance change 39% with NADH and 86% with succinate. With ascorbate–toluylene blue, practically no response was obtained unless EDTA or dithiothreitol was added to the reaction medium (Sanadi *et al.*, 1968). For maximal activity they were added prior to the addition of toluylene blue. Addition of EDTA or dithiotheitol after toluylene blue elicited only partial

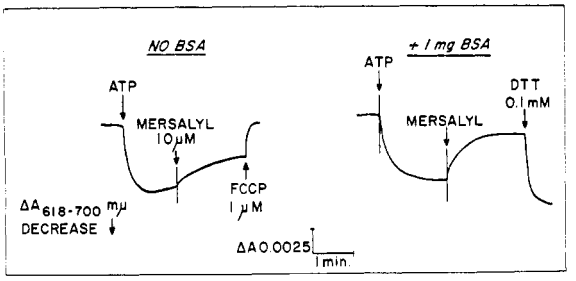

FIGURE 7: Effect of mersalyl on ATP-driven bromothymol blue response. The reaction system contained 20 mM Tris-Cl buffer (pH 7.4), 0.25 M sucrose, 6.7 μM bromothymol blue, 3.3 mM $MgCl_2$, and 0.5 mg of sonic particle in 2.95 ml. The response was initiated by adding 4 μmoles of ATP in 0.04 ml. The experiment traced on the right contained 1 mg of bovine serum albumin.

response. With respect to these properties, the bromothymol blue response resembled the energy-linked NAD reduction described earlier.

The effect of oligomycin on the bromothymol blue response with both the ammonia particles and sonic particles has been reported (Sanadi *et al.*, 1968). The stimulation by oligomycin was greater with the ammonia particles than with sonic particles. In this respect also this response resembled the reduction of NAD by reversed electron flow. The differential response shown by the two types of particles to oligomycin is consistent with the suggestion that poorly phosphorylating particles like the ammonia particles are characterized by a high rate of breakdown of the high-energy intermediates and that this energy leak is inhibited by oligomycin (Lee and Ernster, 1966).

Effect of Mercurials. The effect of mercurials on the absorbance decrease of the dye was tested with the three electron donors. Addition of mersalyl after the response had been activated by NADH resulted in partial reversal of the response indicating inhibition either at the electron transport or at the energy-transfer level. The rate and extent of reversal depended upon the concentration of mersalyl (Figure 6). Inhibition of respiratory activity may be ruled out as the cause of reversal of bromothymol blue response because oxygen uptake was not inhibited by mersalyl under these conditions. The rate of reversal of the response was greater with succinate as the substrate. Oxygen uptake under the conditions of the experiment was low (about 40 mμatoms min^{-1} mg^{-1} of protein) but did not indicate any significant inhibition by mersalyl (Figure 6). When the bromothymol blue response was produced at the third phosphorylation site by using ascorbate–toluylene blue, the effect of mersalyl was enhanced by the presence of bovine serum albumin in the medium (Sanadi *et al.*, 1968). In agreement with the results presented in Figure 3, simultaneous measurement of oxygen uptake showed no inhibition by mersalyl (data not given). When mersalyl was added to the reaction system before the addition of ascorbate–toluylene blue, the response was completely inhibited if bovine serum albumin was present. In the absence of bovine serum albumin the response was inhibitied 25–30%.

ATP-Driven Bromothymol Blue Response. It has been shown (Chance and Mela, 1967) that the bromothymol blue response can be produced in the absence of respiratory substrates by the addition of ATP. The ATP-driven bromothymol blue response was stimulated by $MgCl_2$ and dithiothreitol. It was inhibited by EDTA in the absence of added Mg^{2+} (Table III) indicating involvement of Mg^{2+}. The response increased with increasing concentration of ATP up to 1 mM. Half-maximal response was obtained with 0.4 mM ATP. The response was relatively insensitive to changes in the buffering capacity of the medium as in the case of the substrate-driven response. For example, when the concentration of Tris-Cl in the medium was 2, 20, and 200 mM, respectively, the corresponding absorbance change obtained with sonic particles was 20.4, 11.0, and 13.0 $\times 10^{-3}$.

TABLE III: Effect of EDTA, Dithiothreitol, and Mg^{2+} on ATP-Driven Bromothymol Blue Response.[a]

Addition	ΔAbsorbance × 10^3	
	Ammonia Particles	Sonic Particles
None	5.0	5.0
EDTA (1 mM)	1.3	0.0
Dithiothreitol (0.5 mM)	5.5	7.0
$MgCl_2$ (3.3 mM)	8.0	8.0
$MgCl_2$ + EDTA	7.0	8.0
$MgCl_2$ + dithiothreitol	10.0	10.0

[a] The reaction system contained 20 mM Tris-Cl buffer (pH 7.4), 0.25 M sucrose, 0.5 mg of particle protein, and 6.7 μM bromothymol blue in 2.97 ml. The reaction was started by stirring in 3 μmoles of ATP in 0.03 ml. The indicated additions were made before the start of the reaction with ATP.

The ATP-driven bromothymol blue absorbance change was also inhibited by mersalyl and the inhibition was enhanced by the presence of bovine serum albumin in the medium (Figure 7) as in the case of the ascorbate–toluylene blue activated response. This would further confirm that the inhibition by the mercurial and its enhancement by bovine serum albumin are effects on the energy-transfer reactions rather than on electron transport.

Discussion

Since mercaptide formation is one of the most characteristic and specific reactions of SH groups (Boyer, 1959), inhibition by organic mercurials and reversal of the inhibition by thiol compounds have been used successfully and extensively in the elucidation of the role of SH groups in the activities of proteins. Employing essentially this technique, evidence for SH involvement in mitochondrial and submitochondrial energy transfer was obtained. Fluharty and Sanadi (1963) localized the thiol site between the respiratory chain and the oligo-

mycin-sensitive-terminal coupling reaction in the mitochondrion. The results of Fonyo and Bessman (1966) also pointed to the same conclusion. The data presented in this communication indicate that in submitochondrial particles, reactions which are dependent upon high-energy intermediates generated by respiratory activity are sensitive to mercurials. These reactions are not inhibited by oligomycin, and the results are, thus, consistent with the above conclusion.

In a previous report (Lam, 1968), it was shown that factor B, a highly purified protein which restores energy-linked reactions in phosphorylation-deficient submitochondrial particles (Lam *et al.*, 1967) has an SH group which is essential for its activity. Evidence was presented (Sanadi *et al.*, 1968) that it is a component of or is concerned with the formation of the nonphosphorylated high-energy intermediate (X∼C) either from aerobic oxidation or anaerobically from ATP. All of the reactions examined in this report are presumed to be driven by X∼C, and it is conceivable that these inhibiting effects are due to binding of the SH group of factor B. However, it has also been noted that energy-linked reactions driven by energy derived from oxidation are more sensitive to mercurials than the same reactions energized by ATP (Sanadi *et al.*, 1968). Thus, the possibility must be kept open that a second SH-containing component with greater affinity toward mercurials may be present in the reactions associated with the generation of high-energy intermediates from respiration. The specific roles of factor B and of the proposed second SH-containing component have to be elucidated before the exact function of the SH groups (whether they are involved in catalysis or in maintenance of a critical structure) can be determined.

A point that merits discussion is the role of bovine serum albumin in potentiating the inhibitory effect of mercurial on the bromothymol blue response. This is intriguing because bovine serum albumin by itself decreases the absorbance change by nonspecific binding of bromothymol blue (Chance and Mela, 1967). Lowering of the bromothymol blue concentration inside the particles by bovine serum albumin does not appear to be the reason for this effect because no increased inhibition by mersalyl is observed when the bromothymol blue level is adjusted to give the same absorbance change as in the bovine serum albumin supplemented assay system. It is also unlikely that bromothymol blue antagonizes the inhibitory effect of mersalyl because addition of bromothymol blue did not have any effect on the inhibition of the transhydrogenase reaction. The possibility that bovine serum albumin makes some SH group in the particle protein more reactive by conformational changes cannot be excluded. It has been shown that bovine serum albumin plays such a role in the case of denatured Taka-amylase (Isemura *et al.*, 1967).

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