On the Mechanism of Oxidative Phosphorylation. VI. Localization of the Dithiol in Oxidative Phosphorylation with Respect to the Oligomycin Inhibition Site*

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Oxidative phosphorylation in rat liver mitochondria is uncoupled by γ-(*p*-arsenophenyl)-*n*-butyrate. The uncoupling activity is enhanced by several monothiol compounds but reversed by 2,3-dimercaptopropanol. The arsenical also stimulates the latent ATPase activity, and the stimulation is greater in the presence of 2-mercaptoethanol, 2-mercaptoethylamine, and thiophenol but unchanged by thioglycollate, cysteine, and glutathione. In the presence of a high concentration of arsenophenylbutyrate, the monothiols have only a small and varied effect but 2,3-dimercaptoethanol effectively reverses the ATPase stimulation. The data provide further support for the postulated participation of a dithiol group in oxidative phosphorylation. Arsenophenylbutyrate restores respiration which has been inhibited by oligomycin in initially tightly coupled mitochondria. Furthermore, oligomycin inhibits the ATPase evoked by the arsenical. Similar results have been obtained with cadmium ion, and with arsenite in combination with 2,3-dimercaptopropanol. It is concluded that the postulated dithiol site must be localized between the electron transport chain and the oligomycin-sensitive terminal coupling reaction.

Recent work from this laboratory established that cadmium ion (Cd^{++}) and arsenite in the presence of 2,3-dimercaptopropanol uncouple oxidative phosphorylation and stimulate the latent ATPase activity of mitochondria (Jacobs *et al.*, 1956; Fluharty and Sanadi, 1960, 1961; Fletcher and Sanadi, 1962; Fletcher *et al.*, 1962; Fluharty and Sanadi, 1962b). The effects appeared closely parallel to those of 2,4-dinitrophenol. Arsenite and Cd^{++} were considered to produce these effects by binding a dithiol function (*i.e.*, two juxtaposed —SH groups), although the requirement for dimercaptopropanol to potentiate the effects of arsenite had no previous analogy. The interpretation with respect to Cd^{++} is also open to some criticism on the basis of its possible binding by nucleotide phosphate groups and chelation with nitrogen and sulfur (Vallee *et al.*, 1961). However, since then, myosin ATPase (Fluharty and Sanadi, 1962a) and acetyl CoA carboxylase (Hatch and Stumpf, 1961) have been observed to exhibit a similar dithiol requirement to evoke the sensitivity to arsenite. In order to confirm the interpretation regarding dithiol involvement, we present in this paper data on the uncoupling and ATPase-stimulating effects of γ-(*p*-arsenophenyl)-*n*-butyrate, a reagent whose specificity to dithiol groupings has been established by Reiss (1958). The site of action of such dithiol-specific reagents in the oxidative phosphorylation reactions has been localized to some extent by the use of oligomycin, a reagent introduced by Lardy and co-workers (1958).

Experimental Procedure

The preparation of rat liver mitochondria and the assays for oxidative phosphorylation and ATPase activity have been described in earlier communications (Jacobs *et al.*, 1956; Fluharty and Sanadi, 1960, 1961). In these experiments the uncoupling agent and thiol compounds were incubated routinely for 10 minutes in an ice bath with the mitochondria in 0.3 M sucrose and then mixed with the other reaction components. This preliminary incubation was essential for optimal effects with arsenophenylbutyrate and also for the Cd^{++}-stimulated ATPase. An exception was 2,4-dinitrophenol, which was included in the reaction medium. Arsenophenylbutyrate solutions were prepared daily in 0.3 M sucrose by neutralization of the free acid with potassium hydroxide. Other experimental details have been given in previous communications (Jacobs *et al.*, 1956; Fluharty and Sanadi, 1960, 1961; Fletcher and Sanadi, 1962).

Oligomycin was kindly supplied by Dr. H. A. Lardy, and arsenophenylbutyric acid was the generous gift of Dr. L. Hellermann.

Results

Arsenophenylbutyrate in the concentration range of 20 to 50 μM uncoupled phosphorylation associated with the oxidation of succinate (Fig. 1). A moderate inhibition of oxidation also occurred, but it was not sufficient to mask the uncoupling. Under our experimental conditions, complete uncoupling was infrequent, and the concentration of arsenical necessary to produce the same degree of inhibition of oxidation or uncoupling varied somewhat from one mitochondrial preparation to another. In the presence of an excess of 2-mercaptoethylamine, thioglycollate, or cysteine, the oxidation inhibition produced by arsenophenylbutyrate was reduced but the uncoupling activity was enhanced considerably. Although the protection against inhibition of oxidation in the presence of 2-mercaptoethanol appeared to be less than that with the other thiols, the stimulation of uncoupling activity was greatest. These thiol compounds, in the absence of arsenophenylbutyrate, altered the oxygen uptake and P/O values by less than 10%, which was within the range of experimental error in these measurements. In contrast to the potentiating effect of the monothiols on the uncoupling produced by arsenophenylbutyrate, 2,3-dimercaptopropanol restored coupled phosphorylation (Table I). It is important to note that when the arsenical and dimercaptopropanol were in nearly equimolar concentration, the uncoupling effect persisted. When the dithiol concentration was approximately 4-fold higher, the uncoupling was mostly reversed. This reversal was no different when both mono and dithiol compounds were present simultaneously.

The stimulation of the latent ATPase activity of mitochondria by arsenophenylbutyrate is seen in Figure

* Paper V in the series was Fletcher, Fluharty and Sanadi (1962).

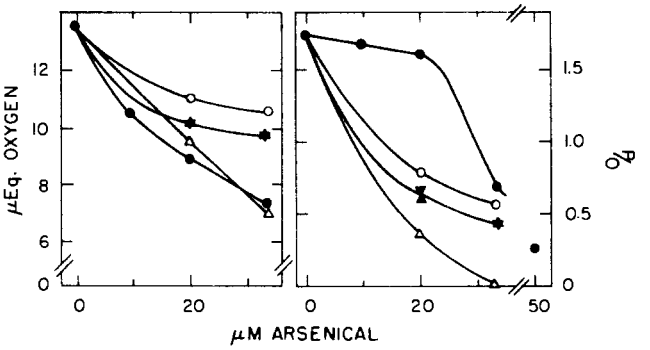

FIG. 1.—Effect of arsenophenylbutyrate on succinate oxidation and coupled phosphorylation. The mitochondria were incubated with the arsenical and a 5-fold molar excess of thiol compound in 0.3 M sucrose for 10 minutes in an ice bath. One milliliter of the incubation mixture was then added to the remainder of the reaction mixture in Warburg flasks, giving a final volume of 3 ml. The concentrations shown in the figure refer to those present in the final reaction medium, which also contained 10 mM phosphate, *p*H 7.4 (as potassium salts), 2 mM ATP, 6.7 mM $MgCl_2$, 10 mM glucose, 0.2 M sucrose, 6.7 mM succinate, 0.5 mg of hexokinase, and approximately 8 mg of mitochondrial protein. The reaction was carried out at 30° for 18 minutes. The curves on the left show oxygen uptake and on the right P/O ratios. ●, Arsenophenylbutyrate alone; ○, plus cysteine; △, plus 2-mercaptoethanol; ▲, 2-mercaptoethylamine; ▼ plus thioglycollate.

TABLE I

REVERSAL OF ARSENOPHENYLBUTYRATE UNCOUPLING BY 2,3-DIMERCAPTOPROPANOL

The arsenical was allowed to incubate with the mitochondria at 4° in 0.3 M sucrose for 10 minutes. The thiol reagent was then added to the same medium, and the incubation was continued for an additional 5 minutes. Finally, the mixture was added to the remainder of the reaction components. Concentrations again refer to the final reaction system.

Arsenical (mM)	Dimercaptopropanol (mM)	Mercaptoethanol (mM)	O_2	P/O
—	—	—	10.2	1.75
0.033	—	—	9.4	0.53
0.033	—	0.30	10.6	0.39
0.033	0.04	—	10.4	0.54
0.033	0.12	—	13.1	1.47
0.033	0.20	—	13.3	1.45
—	0.20	—	12.9	1.72

2. The extent of ATPase stimulation with the optimal arsenical concentration was similar to that obtained with 2,4-dinitrophenol under similar conditions. The ATPase activity was unchanged when an excess of cysteine, thioglycollate, or glutathione was present together with the arsenophenylbutyrate. On the other hand, 2-mercaptoethanol, thiophenol, and 2-mercaptoethylamine enhanced the stimulation of ATPase produced by the arsenical. The order of effectiveness of the thiols was consistently reproducible. Concentrations of thiophenol greater than 5 μM could not be tested with confidence since a precipitate, presumably of the thioarsenite, was often produced.

The influence of four monothiol compounds and of 2,3-dimercaptopropanol on the ATPase activity evoked by a saturating concentration of arsenophenylbutyrate is seen in Figure 3. While the monothiols had only a small and variable effect on the arsenical stimulation of ATPase, the dithiol antagonized it rather strongly. A 3-fold molar excess of dimercaptopropanol over the

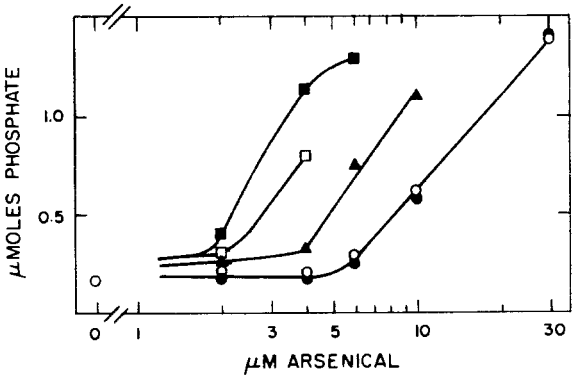

FIG. 2.—Effect of arsenophenylbutyrate on mitochondrial ATPase. The arsenical and mitochondria were incubated as in Fig. 1. The thiols were in 10-fold molar excess over the arsenical where indicated. In this case 0.30 ml of the mixture was added to the remaining reaction components to make a total volume of 1 ml. The reaction was carried out at 30° for 10 minutes and then stopped by the addition of 0.5 ml of 15% trichloroacetic acid solution. After removal of precipitated protein, inorganic phosphate was determined on an aliquot. The final reaction mixture contained 2 mM ATP, *p*H 7.4, 70 mM KCl, and 1 mg mitochondrial protein plus other components as indicated. The concentrations in the final assay mixture are shown in the figure. ○, Arsenophenylbutyrate; ●, plus cysteine; ■, plus 2-mercaptoethanol; ▲, plus 2-mercaptoethylamine; □, plus thiophenol. The results with the arsenical plus thioglycollate or plus glutathione were identical, within experimental error, with those obtained with arsenical plus cysteine.

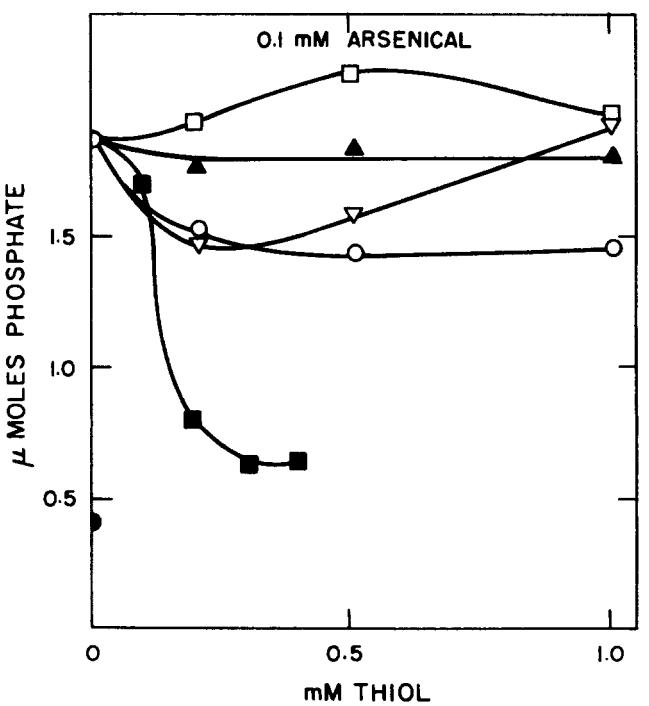

FIG. 3.—The effect of thiols on arsenical stimulation of the mitochondrial latent ATPase. The mitochondria were incubated with 0.1 μmole of arsenophenylbutyrate in 0.3 ml of 0.3 M sucrose for 10 minutes in an ice bath. The thiol compounds were then added in 0.1 ml sucrose solution and the incubation was continued for 5 minutes. The reaction components were subsequently added in 0.6 ml. The other experimental conditions are shown under Figure 2. All incubations except ● contained arsenophenylbutyrate. Other additions are represented as follows: ○, 2-mercaptoethanol; □, cysteine; ▲, 2-mercaptoethylamine; ▽, thioglycollate; ■, 2,3-dimercaptopropanol.

arsenical neutralized the stimulation almost completely and reverted the ATPase activity to the level in untreated mitochondria. The stimulation was unaffected, however, when the dithiol and the arsenical were present in nearly equimolar concentrations. The thiol compounds by themselves did not alter the latent ATPase activity.

It is seen in Table II that Cd^{++}, arsenophenylbutyrate, and arsenite in combination with dimercaptopropanol produced significant release of the respiratory inhibition caused by oligomycin in the "tightly coupled" mitochondria. The ATPase activity stimulated by these dithiol reagents was strongly inhibited by oligomycin (Table III). These effects of the dithiol reagents are very similar to the effects of 2,4-dinitrophenol (Lardy *et al.*, 1958).

TABLE II

RELEASE OF OLIGOMYCIN RESPIRATORY INHIBITION

Experimental conditions were as described in Figure 1. The oligomycin was included with reaction components and was not present in the prior incubation of the uncoupling agent with mitochondria. Levels of oligomycin and uncoupling agents were chosen so as to give optimal release of respiratory inhibition in spite of the partial oxidation inhibition by uncoupling agent alone.

Uncoupling Agent	Concentration (μM)	Oligomycin (μg/ml)	O_2 (μeq)	P/O
AsO_2-dimercaptopropanol	—	—	11.1	2.1
	—	1.7	3.0	0.2
	170	—	12.6	0.3
	170	1.7	13.0	0.0
Arsenophenylbutyrate	—	—	10.0	1.8
	—	0.33	4.5	0.4
	33	—	8.5	0.4
	33	0.33	6.8	0.3
Cd^{++}	—	—	8.7	
	—	0.67	1.0	
	3.3	—	7.6	
	3.3	0.67	7.6	

The stimulation of the latent ATPase by Cd^{++} shown in Table III was maximal between 6 and 10 μM under the assay conditions given and declined with higher concentrations. It was found that if the preincubation of the Cd^{++} with the mitochondria were omitted, and instead the mitochondria were added to the reaction medium containing Cd^{++}, much higher concentrations of the latter were required for maximal activation. The decreased effectiveness under these conditions could result from partial removal of Cd^{++} by chelation with ATP.

DISCUSSION

The uncoupling and ATPase-stimulating action of arsenite in combination with a dithiol compound and of Cd^{++} have been attributed to the binding of a dithiol grouping functional in oxidative phosphorylation (Fluharty and Sanadi, 1960, 1961). The requirement of a dithiol compound in potentiating the effects of arsenite was ascribed to the presence of a hypothetical nonpolar region near the active site which was rendered accessible to the arsenite after combination with dimercaptopropanol to form a lipophilic cyclic dithioarsenite. If this explanation has any validity, organic arsenicals (compounds with nonpolar substitutions on arsenite) may be expected to be active by themselves. The data in Figures 1 to 3 clearly show that arsenophenylbutyrate is indeed a strong uncoupling agent

TABLE III

EFFECT OF OLIGOMYCIN ON ATPASE INDUCED BY UNCOUPLING AGENTS

Experimental details are as described for Figure 2. All uncoupling agents, except 2,4-dinitrophenol, were incubated with the mitochondria prior to the addition to the remaining reaction components.

Uncoupling Agent (μM)	Oligomycin (μg/ml)	Phosphate Released (μmoles)
None	None	1.04
AsO_2-Dimercaptopropanol (100)	None	2.87
AsO_2-Dimercaptopropanol (100)	1.0	0.85
AsO_2-Dimercaptopropanol (100)	2.0	0.55
Dinitrophenol (120)	None	2.89
Dinitrophenol (120)	2.0	0.34
None	None	0.46
Arsenophenylbutyrate (50)	None	1.21
Arsenophenylbutyrate (50)	1.0	0.00
Arsenophenylbutyrate (100)	None	1.15
Arsenophenylbutyrate (100)	1.0	0.17
None	1.0	0.00
None	None	0.26
Cd^{++} (6)	None	2.13
Cd^{++} (6)	1.0	0.51
Cd^{++} (30)	None	1.76
Cd^{++} (30)	1.0	0.62
None	1.0	0.15

and activator of the mitochondrial ATPase. The potentiation of the uncoupling by monothiols is reminiscent of the observations of Jacoby (1958), who found that inhibition of the aldehyde dehydrogenase reaction by arsenite required the presence of 2-mercaptoethanol. In the activation of the mitochondrial ATPase by arsenophenylbutyrate the simultaneous presence of mercaptoethanol, thiophenol, or mercaptoethylamine rendered the arsenical even more effective, but cysteine, thioglycollate, and glutathione were inert (Fig. 2). The stimulating thiols all have distinct solubility in nonpolar solvents around neutral *p*H, while the inert thiols are essentially insoluble in lipid solvents. Although their effects on arsenical stimulation of ATPase may be fortuitous, the data are consistent with the hypothesis concerning the presence of a nonpolar region around the arsenite binding site in ATPase activation, since it can be argued that mercaptoethanol, thiophenol, and the mercaptoethylamine increase further the lipid solubility of arsenophenylbutyrate. It is pertinent to note in this regard that Hemker and Hülsman (1961) have considered lipid solubility as a factor in explaining the relative uncoupling activity of several dinitrophenols. Since the presence of the monothiols is not essential for the uncoupling and ATPase-stimulating effects of the arsenical, it would seem unlikely that they are acting by reducing a disulfide group on an enzyme.

The uncoupling of oxidative phosphorylation and ATPase stimulation produced by arsenophenylbutyrate were not reversed when 2,3-dimercaptopropanol was also present in equimolar concentration (Table I and Fig. 3). Higher concentrations of the dithiol, however, consistently reversed the arsenical effects. These results are quite similar to the previously reported

observations with arsenite (Fluharty and Sanadi, 1960, 1961) and indicate that the enzyme site and the external dithiol have nearly the same affinity for the arsenophenylbutyrate. The postulated involvement of a dithiol in oxidative phosphorylation rests largely on these relative affinities of the enzyme and dimercaptopropanol for arsenicals.

The dithiol reagents and 2,4-dinitrophenol (Lardy *et al.*, 1958) have identical influence on the effects of oligomycin in oxidative phosphorylation and related reactions. All of them release the inhibition of respiration produced by oligomycin in tightly coupled mitochondria (Table II). Also, the antibiotic inhibits the ATPase evoked by the reagents (Table III). Since oligomycin has been shown to inhibit a terminal coupling reaction in oxidative phosphorylation (Lardy *et al.*, 1958), it would appear that the dithiol reagents, like dinitrophenol, interact with a site located between the electron transport event and the oligomycin-sensitive step. The tentative mechanism proposed earlier (Fluharty and Sanadi, 1960) for the role of dithiols in oxidative phosphorylation is consistent with such localization.

There is a striking similarity between the effects of dithiol reagents and of 2,4-dinitrophenol. Both uncouple oxidative phosphorylation and stimulate the latent ATPase of mitochondria (Fluharty and Sanadi, 1961). Their sites of action in the mitochondrial reactions appear to be identical or close together. They also activate the myosin ATPase (Fluharty and Sanadi, 1962a; Blum, 1962; Chappell and Perry, 1955; Greville and Needham, 1955). The close resemblances in these many respects may be more than conicidence and raise the question whether these compounds react with the same functional site. This interesting possibility gains further support from the work of Cooper (1960) and Kielley (1961), who have presented evidence that an —SH group may be concerned with the ATPase-stimulating action of dinitrophenol in phosphorylating mitochondrial particles. A third key system where dithiols may play an important role is photophosphorylation. Newton (1962) has produced evidence based on disulfide interchange reactions for the involvement of dithiols in photophosphorylation in preparations from *Rhodospirillum rubrum*. It may well be that the dithiol function is central in the energy-trapping reactions in mitochondria and in photosynthetic organelles as well as in the mechanochemical response of muscle to ATP.

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