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Ion Permeability Induction by the SH Cross-Linking Reagents in Rat Liver Mitochondria Is Inhibited by the Free Radical Scavenger, Butylhydroxytoluene[1]

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Abstract

The hydrophobic, potentially SH cross-linking reagent, phenylarsine oxide (PhAsO), was found to induce K^+ and Ca^{2+} effluxes from mitochondria and to accelerate the respiration rate in state 4. The hydrophobic monofunctional electrophilic agent, *N*-ethylmaleimide, does not exhibit this effect but prevents the action of PhAsO. The polar potentially SH cross-linking reagents (arsenite, diamide) induce ion fluxes only in the presence of P_i. Ion fluxes induced by the SH reagents are inhibited by butylhydroxytoluene (an inhibitor of free radical reactions), and *N,N'*-dicyclohexylcarbodiimide, not by oligomycin. It is inferred that the induction of ion fluxes in mitochondria caused by cross-linking of two juxtaposed SH groups is related to the development of free radical reactions.

Key Words: Rat liver mitochondria; SH reagents; ion transport; radical reactions; ATPase.

Introduction

Lowering the pH of the phosphate- or arsenate-containing incubation medium induces electrogenic and nonelectrogenic fluxes of cations in mitochondria (Wolkowicz and McMillin Wood, 1981; Novgorodov *et al.*, 1984).

[1] Abbreviations: PhAsO, phenylarsine oxide; NEM, *N*-ethylmaleimide; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid; RR, ruthenium red; CCCP, carbonyl cyanide-*m*-chlorophenylhydrazone; BHT, butylhydroxytoluene; DCCD, *N,N'*-dicyclohexylcarbodiimide; DTNB, 5,5'-dithio-bis-2-nitrobenzoic acid; diamide, diazenedicarboxylic acid-bis-dimethylamide; mersalyl, *O*-[3-hydroxymercuri)-2-methoxypropyl) carbamoylphenoxyacetic acid; DTE, dithioerythritol.

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Earlier we have demonstrated that under these conditions, the ion fluxes are controlled by the state of the ATP synthetase complex, and by the development of free radical reactions (Novgorodov *et al.*, 1984). As shown by Rugolo *et al.* (1981) under similar experimental conditions, the ion fluxes are controlled by the state of SH groups.

The ion fluxes are increased by the oxidation of SH groups by diamide and inhibited by DTE-induced reduction of SH groups. Therefore, ion fluxes in mitochondria induced by lowering the pH are controlled by three factors: (1) the state of the ATP complex; (2) the state of SH groups; (3) the level of free radical reactions.

In the present work we investigated the interrelationship of the state of SH groups, the state of the ATPase complex, and the level of free radical reactions in controlling ion fluxes in mitochondria. In addition, some essential characteristics of functionally significant SH groups and their environment were found. The ion fluxes were varied by SH reagents of different chemical composition. The control of these fluxes by the ATPase complex and by the level of free radical reactions was monitored by their sensitivity to oligomycin, to DCCD (inhibitors of ATP synthetase), and to BHT, a scavenger of free radicals (Hicks and Gebicki, 1981).

Methods

Rat liver mitochondria were isolated by differential centrifugation in a medium containing 250 mM sucrose, 5 mM HEPES, and 250 μM EDTA, pH 7.4 (Johnson and Lardy, 1967). The final washing was performed in the same medium in the absence of EDTA. Protein in the mitochondrial suspension was determined by the biuret method with bovine serum albumin as a standard (Gornal *et al.*, 1949). K^+ ions of the incubation medium were measured with a glass ion-selective electrode. Mitochondrial respiration was monitored with a Clark-type electrode. Ca^{2+} was measured spectrophotometrically on an Aminco DW-2a apparatus using Arsenazo III at 675 nm vs 685 nm. Mitochondria (1 mg protein/ml) were incubated in a medium containing 250 mM sucrose, 10 mM HEPES, 10 mM succinate, 400 μM KCl, 15 μM $CaCl_2$, and 2 μM rotenone. Tris-buffer was used to bring the pH of the medium to 7.4. 5 mM P_i-Tris (pH 7.4) or 40 μM Arsenazo III were added to the incubation medium where indicated.

Results

As was shown in several papers (Sanadi *et al.*, 1981, 1982; Rasheed *et al.*, 1984), the cross-linking of two juxtaposed SH groups in mitochondria by

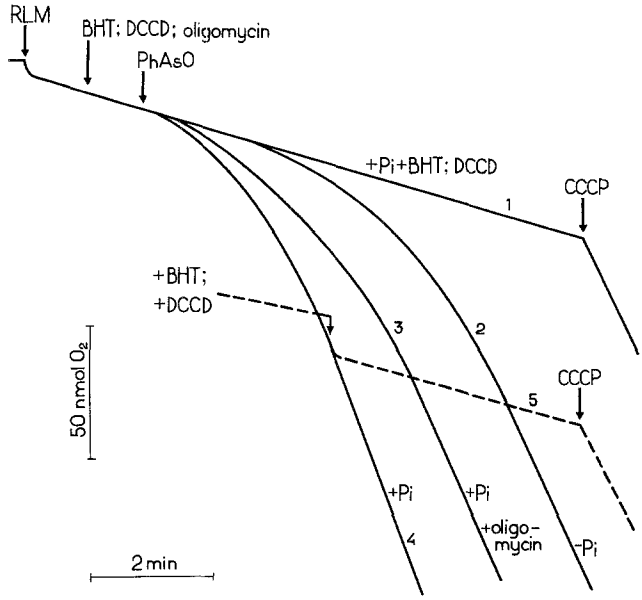

Fig. 1. Effect of the ATP synthetase inhibitors and BHT on the mitochondrial respiration rate increase by PhAsO. For experimental conditions, see Methods. (1, 3–5) In an incubation medium with 5 mM P_i; (2) in the absence of P_i. Arrows indicate the additions of 50 μM BHT, 50 μM DCCD, 2 μg oligomycin, 10 μM PhAsO, and 1.5 μM CCCP.

the hydrophobic bifunctional agent PhAsO leads to the appearance of electrogenic ion fluxes and to the deenergization of both mitochondria and submitochondrial particles. Monofunctional SH reagents do not exert such an effect.

Simultaneously with an increase in the respiration rate at state 4 (Fig. 1), the addition of 10 μM PhAsO to the incubation medium (containing 5 mM P_i, pH 7.4) induces K^+ efflux from mitochondria (Figs. 2–3) and ruthenium-red (RR)-insensitive Ca^{2+} efflux (Fig. 4).

The increase in the respiratory rate in state 4 after the addition of PhAsO points to the induction in the mitochondria of electrogenic ion fluxes which cause the dissipation of the electrochemical proton gradient and the disappearance of the transmembrane potential (not shown). Under our experimental conditions mitochondrial uncoupling may be caused by the appearance of electrophoretic proton "leaks" or by the activation of the ruthenium red-insensitive (Figs. 4 and 7) electroneutral Ca^{2+}/H^+ antiport process (Lehninger *et al.*, 1978; Roos *et al.*, 1980; Siliprandi *et al.*, 1979).

In the light of the experiments of Rahseed *et al.* (1984), where the induction of nonspecific permeability as a result of the modification of SH groups by Cd^{2+} was shown, the first variant seems to be more reasonable.

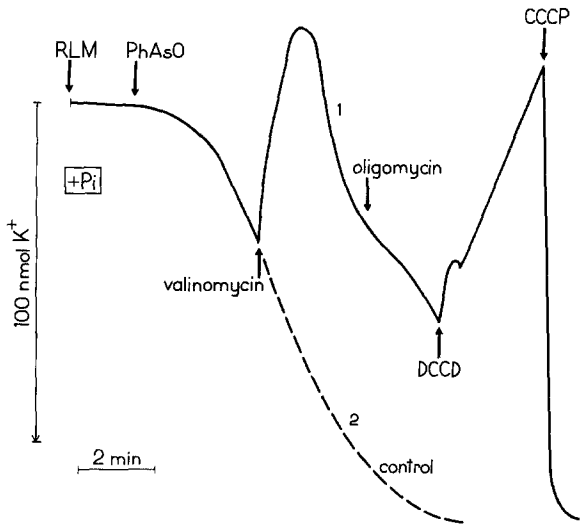

Fig. 2. Induction of K^+ efflux from mitochondria by PhAsO in the presence of 5 mM P_i. For experimental conditions, see Fig. 1. (1) Addition of 0.2 μM valinomycin to the medium after the onset of K^+ efflux; (2) control.

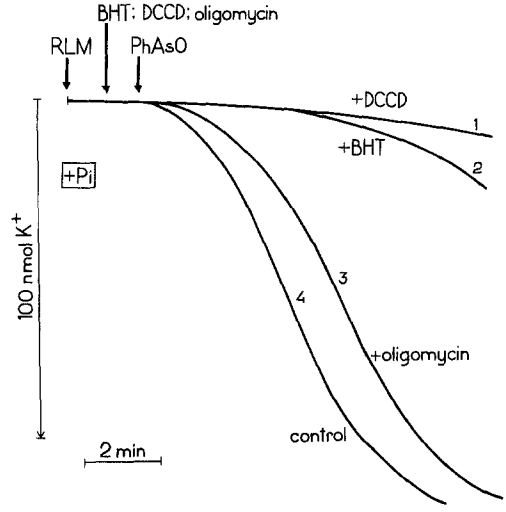

Fig. 3. Effect of the ATP synthetase inhibitors and BHT on 10 μM PhAsO-induced K^+ efflux from mitochondria. For experimental conditions, see Figs. 1 and 2. The incubation medium contained 5 μM P_i. (4) Control.

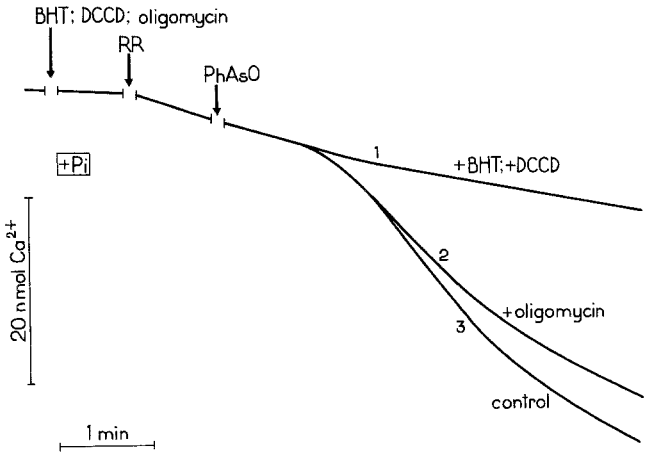

Fig. 4. Effect of the ATP synthetase inhibitors and BHT on RR-insensitive Ca^{2+} efflux from mitochondria induced by PhAsO. For experimental conditions, see Fig. 1. The incubation medium contained 5 mM P_i and 40 μM Arsenazo III. BHT, DCCD, and oligomycin were added 1 min after the introduction of mitochondria when Ca^{2+} accumulation from the incubation medium had been completed. Electrogenic pumping of Ca^{2+} was inhibited by 3 nmol RR. (3) Control.

As seen from Fig. 2, valinomycin reverses the K^+ efflux at the initial steps, suggesting the nonelectrogenic character of this process. BHT (a scavenger of free radicals) added to the incubation medium prevents a PhAsO-induced increase in the respiration rate (Fig. 1), the K^+ efflux (Fig. 3, curve 2), and the Ca^{2+} efflux (Fig. 4, curve 1). DCCD blocks the action of PhAsO on ion fluxes (Figs. 1–4), but oligomycin, a more specific ATP-synthetase inhibitor, does not affect the ion transport induction. This demonstrates that the PhAsO-induced ion fluxes do not depend on the state of the ATPase complex. BHT and DCCD *per se* do not induce a respiratory rate increase as K^+ and Ca^{2+} fluxes (not shown).

Under these conditions, BHT- or DCCD-induced inhibition of respiration is completely reversed by the uncoupler CCCP (Fig. 1, curves 1 and 4). This indicates that the inhibition of mitochondrial respiration by these agents is due to the suppression of electrogenic ion fluxes and not to the direct inhibition of the respiratory chain.

We have shown that the hydrophobic monofunctional SH reagent NEM causes no induction of ion fluxes, and moreover completely prevents the PhAsO effect. NEM prevents the effect of PhAsO both on the electrogenic fluxes (Fig. 5) and also on the K^+ (Fig. 6) and the RR-insensitive Ca^{2+} (Fig. 7) effluxes. NEM is known to inhibit the phosphate carrier (Fonyo and Vignais, 1980). Therefore experiments on preventing PhAsO induction of ion

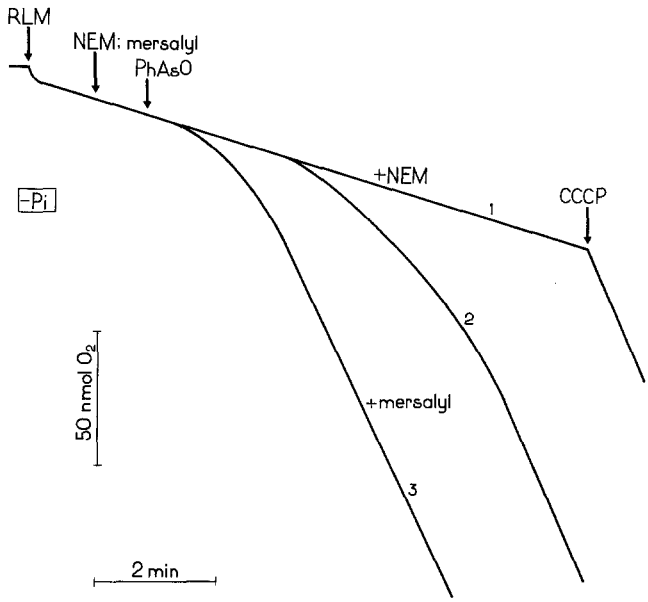

Fig. 5. Effect of NEM and mersalyl on the PhAsO-induced increase in the mitochondrial respiration rate in the absence of P_i. For experimental conditions, see Methods. The medium was supplemented with 10 μM PhAsO, 100 μM NEM, 20 μM mersalyl, and 1.5 μM CCCP. (2) Control.

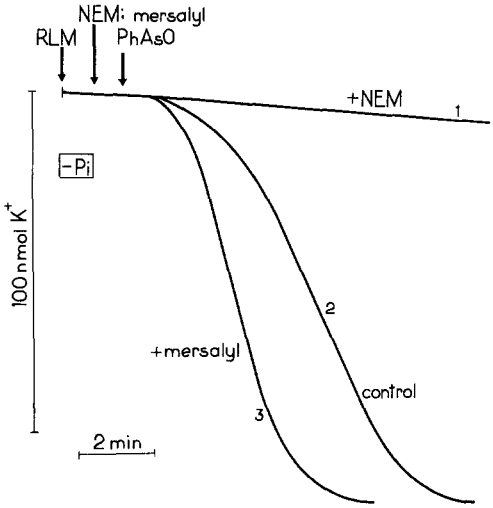

Fig. 6. Effect of NEM and mersalyl on K^+ efflux from mitochondria induced by PhAsO in the absence of P_i. For experimental conditions, see Fig. 5.

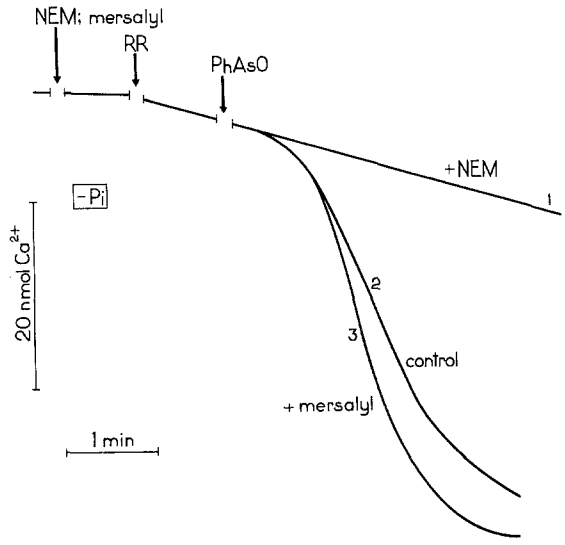

Fig. 7. Effect of NEM and mersalyl on RR-insensitive efflux of Ca^{2+} induced by PhAsO in the absence of P_i. For experimental conditions, see Fig. 5. 40 μM Arsenazo III was supplemented additionally. NEM and mersalyl were added 1 min after the introduction of mitochondria when Ca^{2+} accumulation from the medium had been complemented. Electrogenic pumping of Ca^{2+} was inhibited by 3 nmol RR.

fluxes by NEM were carried out in the absence of P_i. Moreover, the hydrophilic membrane-impermeable monofunctional SH reagent mersalyl, which also inhibits the P_i-carrier, does not prevent the PhAsO effect (Figs. 5–7).

The monofunctional hydrophilic SH reagents DTNB and iodoacetic acid exhibit the same effect as mersalyl. Addition of mersalyl, NEM, DTNB, and iodoacetic acid *per se* does not cause a respiratory rate increase or the induction of K^+ and Ca^{2+} ion fluxes (not shown). But, as seen from Figs. 5–7, mersalyl potentiates the effect of PhAsO on induction of ion fluxes.

Such hydrophilic potentially cross-linking SH reagents as membrane-impermeable diamide (Siliprandi *et al.*, 1978; Vignais *et al.*, 1975) and arsenite, which readily penetrate mitochondria via the phosphate carrier (Harris and Achenjang, 1977), do not induce electrogenic ion fluxes in the absence of P_i (not shown). As seen in Fig. 9, introduction of 50 μM arsenite to a P_i-containing medium exerts the same effect as diamine (Fig. 8), causing a rapid increase in the respiration rate in state 4. The diamide- and arsenite-induced increase in the respiration rate is inhibited by BHT and DCCD both at the initial step and after the onset of deenergization (CCCP reversed inhibition); oligomycin has no effect (Figs. 8 and 9).

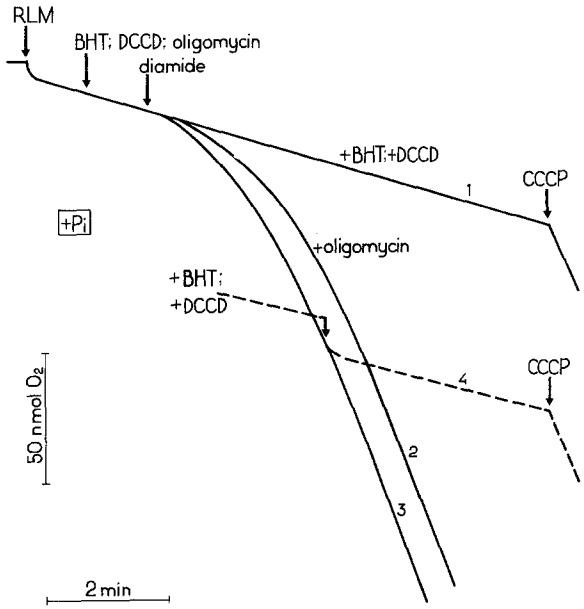

Fig. 8. Effect of the ATP synthetase inhibitors and BHT on the 100 μM diamide-induced in the mitochondrial respiration rate in the presence of P_i. For experimental conditions, see Fig. 1.

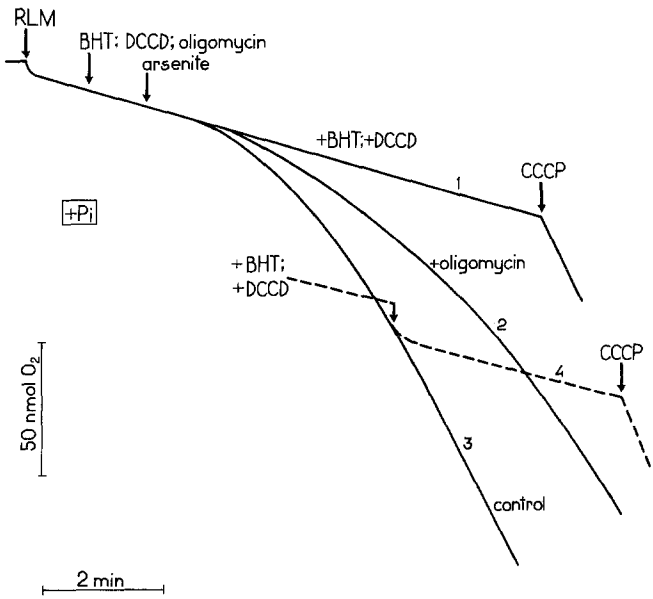

Fig. 9. Effect of the ATP synthetase inhibitors and BHT on the 100 μM arsenite-induced increase in the mitochondrial respiration rate in the presence of P_i. For experimental conditions, see Fig. 1.

Discussion

Recently strong evidence was obtained for the presence in mitochondria of two different types of thiol groups which may be involved in ion transport regulation (Sanadi *et al.*, 1981; Rasheed *et al.*, 1984). The addition of Cd^{2+} or PhAsO, the thiol reagents with high preference to juxtaposed dithiol, to respiring rat liver mitochondria results first in the activation of membrane potential-dependent, valinomycin type K^+ uptake, followed by the uncoupling and discharge of ion gradients. The first phase of PhAsO and Cd^{2+} action (activation of K^+ uptake) can be mimicked by relatively membrane-impermeable monofunctional SH reagents, mersalyl and *p*-hydroxymercuribenzoate. This agrees with the results of other authors (Brierley *et al.*, 1967; Southard and Green, 1974; Diwan *et al.*, 1983).

Sanadi *et al.* (1981) and Rasheed *et al.* (1984) conclude that the *p*-hydroxymercuribenzoate and mersalyl-sensitive site is distinct from the other uncoupling site since these inhibitors do not cause the uncoupling and discharge of ion gradients. Parallel with the uncoupling, caused by the hydrophobic site modification, a nonspecific increase in membrane permeability takes place (Rasheed *et al.*, 1984).

Diwan and Lehrer (1978) showed that the apparent K_m for SH reagent sensitive K^+ uptake is in the 5–10 mM range. In the present work only the ion fluxes caused by the uncoupling site modification were investigated. To avoid K^+ uptake activation by SH reagents, the experiments were performed in a low-potassium medium (400 μM K^+). Under the conditions used, monofunctional SH reagents *per se* caused no induction of potassium fluxes. The uncoupling site seems to be in a hydrophobic environment and to be readily accessible to Cd^{2+} and PhAsO. The uncoupling action of PhAsO and Cd^{2+} is abolished by low levels of 2,3-dimercaptopropanol but is potentiated by the excess of 2-mercaptoethanol, which shows the involvement of dithiol type groups in the response (Sanadi *et al.*, 1981).

When S–X–S or S–S bonds are formed, two types of changes occur in the system. Chemical modification of the protein, simultaneous disappearance of the S–H bonds (substituted by S–X–S or S–S) in two juxtaposed SH groups, takes place; on the other hand, cross-linkage stabilizes the spatial arrangement of the two juxtaposed sulfurs versus each other and causes their immobilization.

It is still an open question which of these effects controls the ion transport induction system and uncoupling of mitochondria. Experiments with the hydrophobic monofunctional agent (NEM), which prevents the action of the hydrophobic bifunctional agent PhAsO (Figs. 5–7), demonstrate unambiguously that the immobilization effect is a functionally significant one: a covalent modification of SH groups *per se* is not sufficient for ion transport induction. Significantly, the polar monofunctional electrophilic SH

reagents (mersalyl, DTNB, and iodoacetic acid which, in contrast to the former reagents, penetrate the mitochondrial matrix; Gaudemer and Latruffe, 1975) do not prevent the PhAsO effect—an indication that the SH groups, which are the targets of a PhAsO attack, are localized in the hydrophobic environment. Moreover, as seen from Figs. 5–7, mersalyl not only fails to suppress the PhAsO action, but potentiates it. DTNB shows the same effect. The potentiating action of hydrophilic monofunctional SH reagents may be connected with the screening of hydrophilic SH groups, and this leads to increase in PhAsO concentration in the mitochondrial membrane hydrophobic zone. A comparion of the action of the hydrophobic and hydrophilic potentially SH cross-linking reagents shows that the hydrophobic PhAsO is capable of inducing ion transport in mitochondria both in the presence and in the absence of P_i in the incubation medium. The polar compounds, diamide and arsenite, induce the effect only in the presence of P_i.

The high polarity of the molecules prevents their transport directly through the membrane to the mitochondrial matrix, and their accumulation in the membrane. According to some data, in a medium lacking P_i the mitochondrial membrane is impermeable to diamide (Fonyo and Vignais, 1980; Vignais *et al.*, 1975); conversely, arsenite is transported across the membrane via the phosphate carrier (Harris and Achenjang, 1977). The fact that arsenite in the absence of P_i does not induce ion transport (Sanadi *et al.*, 1981) shows that the specific character of the polar potentially cross-linking agents is due to their inability, in a P_i-free medium, to reach the SH groups localized in a hydrophobic environment. This interpretation of the experimental results presupposes that P_i alters the properties of the system in such a way that the SH groups, controlling ion transport, become accessible to the polar reagents.

The results obtained show that ion transport induction by any of the compounds studied (PhAsO, diamide, arsenite) is suppressed by adding BHT, a scavenger of free radicals, to the medium (Figs. 1, 8 and 9). This indicates a certain relationship between the cross-linking of two juxtaposed SH groups, free radical processes in mitochondria, and ion transport induction.

There is some correlation between the development of lipid peroxidation (determined from the accumulation of malonic dialdehyde) and the values of SH reagent-dependent ion fluxes (not shown). But to resolve the question whether a free radical reaction is involved in the SH reagent action or not, additional experiments should be carried out to elucidate the concrete nature of the free radical process involved in ion transport induction and the mechanism of free radical formation.

The fact that oligomycin, in contrast to DCCD, fails to inhibit the PhAsO action indicates that the DCCD action is not connected with the

modification of the ATPase complex but is directed to some other systems (Azzi *et al.*, 1984; Warhurst *et al.*, 1982; Martin *et al.*, 1984).

There is evidence that DCCD may directly suppress some specific systems of K^+ transport (Martin *et al.*, 1984; Jung *et al.*, 1980). In the case of Mg^{2+} depletion, DCCD blocks the electroneutral K/H^+ antiporter in rat liver mitochondria (Martin *et al.*, 1984; Garlid *et al.*, 1986), which was identified as an 82,000-dalton protein.

Moreover, the specific inhibition of energy-dependent K^+ influx in mitochondria by DCCD has been demonstrated (Gauthier and Diwan, 1979; Jung *et al.*, 1980; Diwan, 1982).

Our results demonstrate a new action of DCCD—the suppression of nonspecific PhAsO-induced ion permeability.

According to the experiments of Broekemeier *et al.* (1985), it may be suggested that the ruthenium red-insensitive Ca^{2+} efflux and the apparent electroneutral (according to he valinomycin test) K^+ efflux are connected with the heterogenic nature of the mitochondrial population and the gradual transition of a part of the mitochondrial population to the state of nonspecific ion permeability under the action of PhAsO.

Further analysis showed that the ion transport induction system studied in this work is qualitatively similar to that caused by lowering the pH of the incubation medium (Novgorodov *et al.*, 1984). In either case a parallel increase in the electrogenic fluxes, the K^+ efflux, and the RR-insensitive Ca^{2+} efflux takes place; at the initial steps, the K^+ efflux is reversed by valinomycin. Moreover, both in the case of ion flux induction by the SH reagents and in the case of induction by lowering the pH of the incubation medium, BHT, a scavenger of free radicals, and DCCD suppress the ion fluxes. The important difference is that the ion transport at lower pH of the incubation medium is inhibited by oligomycin (Novgorodov *et al.*, 1982, 1984), whereas the effect of the SH reagents is not suppressed by this inhibitor.

The involvement of disulfide bond formation in the induction of ion fluxes under similar conditions was also suggested by others (Zoccarato *et al.*, 1981; Rugolo *et al.*, 1981; Siliprandi *et al.*, 1978).

The qualitative similarity of these two ion transport induction systems (induced by lowering the pH and by the potentially SH cross-linking reagents) enables us to suggest that lowering the pH also leads to S–S bond formation, the ATP synthetase complex controlling the cross-linkage. Oligomycin, changing the ATP synthetase state, prevents the formation of S–S bonds. If this proposal proves to be correct, the ATP synthetase complex, as well as certain SH groups of the protein and BHT-inhibited free radical reactions, are involved in the system of ion transport induction at low pH. This model suggests that under conditions where an S–S bond is formed due to a direct effect of the cross-linking reagent, the regulatory component (ATP synthetase

complex) controlling the spontaneous formation of this bond must be lost. Just such an effect was observed in the experiments (Figs. 1, 3, 4, 8 and 9).

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References

Azzi, A., Casey, R. P., and Nalecz, M. J. (1984). *Biochim. Biophys. Acta* **768**, 209–226.
Brierley, G. P., Settlemire, C. T., and Knight, V. A. (1967). *Biochem. Biophys. Res. Commun.* **28**, 420–425.
Broekemeier, K. M., Schmid, P. G., Schmid, H. H. O., and Pfeiffer, D. R. (1985). *J. Biol. Chem.* **260**, 105–113.
Diwan, J. J., De Lucia, A., and Rose, P. E. (1983). *J. Bioenerg. Biomembr.* **15**, 277–288.
Diwan, J. J. (1982). *J. Bioenerg. Biomembr.* **14**, 15–22.
Diwan, J. J., and Lehrer, P. H. (1978). *Membr. Biochem.* **1**, 43–60.
Fonyo, A. and Vignais, P. V. (1980). *J. Bioenerg. Biomembr.* **12**, 137–149.
Garlid, K. D., Di Resta, D. J., Beavis, A. D., and Martin, W. H. (1986). *J. Biol. Chem.* **261**, 1529–1535.
Gaudemer, Y., and Latruffe, N. (1975). *FEBS Lett.* **54**, 30–34.
Gauthier, L. M., and Diwan, J. J. (1979). *Biochem. Biophys. Res. Commun.* **87**, 1072–1079.
Gornal, A. G., Bardawill, C. C., and David, M. M. (1949). *J. Biol. Chem.* **177**, 751–766.
Harris, E. J., and Achenjang, F. M. (1977). *Biochem. J.* **168**, 129–132.
Hicks, M., and Gebicki, J. M. (1981). *Arch. Biochem. Biophys.* **210**, 56–63.
Johnson, D., and Lardy, H. (1967). *Methods Enzymol.* **10**, 94–96.
Jung, D. W., Shi, G.-Y., and Brierley, G. P. (1980). *J. Biol. Chem.* **255**, 408–412.
Lehninger, A. L., Vercesi, A., and Bababunmi, E. A. (1978). *Proc. Natl. Acad. Sci USA* **79**, 1690–1694.
Martin, W. H., Beavis, A. D., and Garlid, K. D. (1984). *J. Biol. Chem.* **259**, 2062–2065.
Novgorodov, S. A., Dragunova, S. F., and Yaguzhinsky, L. S. (1982). *Biofizika (USSR)* **27**, 244–248.
Novgorodov, S. A., Marshansky, V. N., and Yaguzhinsky, L. S. (1984). *Biochimiya (USSR)* **49**, 185–192.
Rasheed, B. K., Diwan, J. J., and Sanadi, D. R. (1984). *Eur. J. Biochem.* **144**, 643–647.
Roos, I., Crompton, M., and Carafoli, E. (1980). *Eur. J. Biochem.* **110**, 319–325.
Rugolo, M., Siliprandi, D., Siliprandi, N., and Toninello, A. (1981). *Biochem. J.* **200**, 481–486.
Sanadi, D. R. (1982). *Biochim. Biophys. Acta* **683**, 39–56.
Sanadi, D. R., Hughes, J. B., and Saroj, J. (1981). *J. Bioenerg. Biomembr.* **13**, 425–431.
Siliprandi, N., Siliprandi, D., Bindoli, A., and Toninello, A. (1978). In *Functions of Glutathione in Liver and Kidney* (Sies, H., and Wendel, A., eds.), Springer-Verlag, Berlin, pp. 139–147.
Siliprandi, N., Rugolo, M., Siliprandi, D., Toninello, A., and Zoccarato, F. (1979). In *International Symposium on Function and Molecular Aspects of Biomembrane Transport* (Quaglieriello, E., Palmieri, F., Papa, S., and Klingenberg, M., eds.), Elsevier-North Holland, Amsterdam, pp. 147–155.
Southard, J. H., and Green, D. E. (1974). *Biochem. Biophys. Res. Commun.* **61**, 1310–1316.
Vignais, P. M., Chalert, J., and Vignais, P. V. (1975). In *Biomembranes: Structure and Function*, Vol. 34, Budapest, pp. 307–313.
Warhurst, J. W., Dawson, A. P., and Selwyn, M. J. (1982). *FEBS Lett.* **149**, 249–252.
Wolkowicz, P. E., and McMillin Wood, J. (1981). *Arch. Biochem. Biophys.* **209**, 408–422.
Zoccarato, F., Rugolo, M., Siliprandi, D., and Siliprandi, N. (1981). *Eur. J. Biochem.* **114**, 195–199.