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Phenylarsine Oxide Induces the Cyclosporin A-Sensitive Membrane Permeability Transition in Rat Liver Mitochondria

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Abstract

This paper reports an investigation on the effects of the hydrophobic, bifunctional SH group reagent phenylarsine oxide (PhAsO) on mitochondrial membrane permeability. We show that PhAsO is a potent inducer of the mitochondrial permeability transition in a process which is sensitive to both the oxygen radical scavanger BHT and to cyclosporin A. The PhAsO-induced permeability transition is stimulated by Ca^{2+} but takes place also in the presence of EGTA in a process that maintains its sensitivity to BHT and cyclosporin A. Our findings suggest that, at variance from other known inducers of the permeability transition, PhAsO reacts directly with functional SH groups that are inaccessible to hydrophilic reagents in the absence of Ca^{2+}.

Key Words: Mitochondria; permeability transition; cyclosporin A; phenylarsine oxide.

Introduction

The mitochondrial inner membrane possesses a low permeability to charged and uncharged low-molecular-weight solutes. This property is linked to the requirement of a large proton electrochemical gradient to catalyze ATP synthesis. However, it has been known since the early fifties that a number of agents or conditions can induce a Ca^{2+}-dependent increased permeability for solutes. The permeability increase may be mediated by a latent transport mechanism which makes the inner membrane permeable to

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ions and uncharged solutes (for a recent, complete review cf. Gunter and Pfeiffer, 1990). Following this review, we will call the activation of the latent transport mechanism the *permeability transition.* Although a number of agents or conditions have been shown to either enhance or inhibit the transition, the most stringent requirement appears to be the presence of Ca^{2+}.

Gunter and Pfeiffer (1990) have collected an impressive list of substances involved in the Ca^{2+}-dependent permeability transition. This list ranges from heavy metals to fatty acids and lysophospholipids, from quinones to sulfhydryl reagents, from phosphate to uncouplers, from hydrogen peroxides to nitrofurantoin, and many others. This suggests that distinct but overlapping mechanisms may induce the permeability transition, but the biochemical basis for this diversity is obscure. An important advance came with the recent discovery that the immunosuppressant peptide cyclosporin A inhibits the Ca^{2+}-dependent permeability transition independently of the inducing agent, suggesting that all inducers converge on a final common pathway which may be a cyclosporin A-sensitive proteinaceous pore (Fournier *et al.*, 1987; Crompton *et al.*, 1988; Broekemeier *et al.*, 1989).

PhAsO[4] is a hydrophobic bifunctional SH group reagent that has received considerable attention as an inducer of mitochondrial ion fluxes (Sanadi *et al.*, 1981; Diwan *et al.*, 1983, 1986; Novgorodov *et al.*, 1987). PhAsO causes a release of both K^+ and Ca^{2+}, and uncouples mitochondrial respiration in a process that is inhibited by scavangers of oxygen free radicals (Novgorodov *et al.*, 1987). In this paper, we show that PhAsO is an inducer of the mitochondrial permeability transition. Accordingly, the PhAsO effects are potentiated by Ca^{2+} and inhibited by cyclosporin A and by scavangers of oxygen radicals. Since PhAsO induces the cyclosporin A-sensitive transition even in the presence of EGTA, we suggest that with this inducer the Ca^{2+} requirement is not absolute.

Methods

Rat liver mitochondria were prepared by standard centrifugation procedures, and mitochondrial protein was assayed with the biuret reaction using bovine serum albumin as a standard. The kinetics of matrix volume changes following solute fluxes were followed as absorbance changes of the mitochondrial suspension at 540 nm. Membrane potential was determined with a $TPMP^+$-selective electrode as described by Zoratti *et al.* (1986), and

[4]Abbrevations used: PhAsO, phenylarsine oxide; BHT, buthylhydroxytoluene; $TPMP^+$, tryphenylmethylphosphonium ion; Mops, morpholinepropane sulfonic acid.

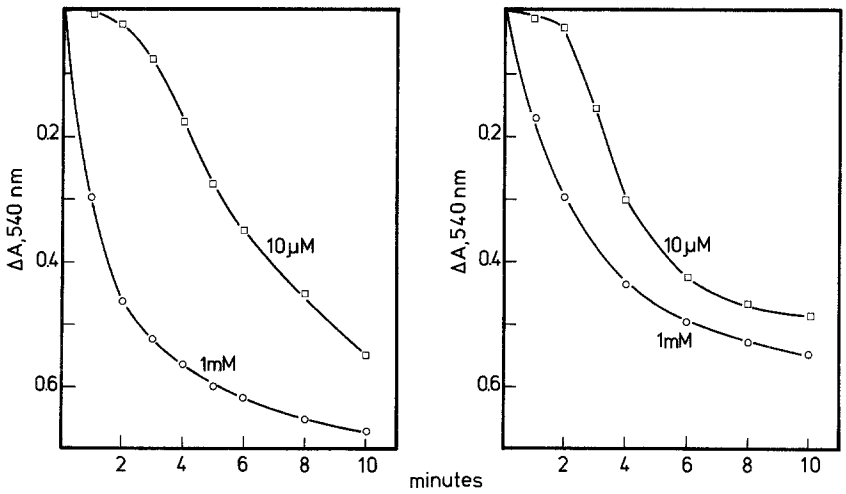

Fig. 1. PhAsO-induced permeability to sucrose in the presence of Ca^{2+}. Mitochondria (0.5 mg/ml) were incubated in a medium containing 200 mM sucrose, 10 mM Tris-Mops, 0.1 mg/ml, BSA, 20 μM $CaCl_2$, 2 μM rotenone, and either 10 μM (□) or 1 mM (○) PhAsO. In the figure on the right the medium was supplemented with 5 mM Tris-succinate.

K^+ fluxes with a K^+-selective electrode as described (Bernardi and Azzone, 1983). The incubation conditions are specified in the figure legends, and all chemicals were of the highest purity commercial available. Cyclosporin A was a generous gift of Sandoz Pharma AG (Basel).

Results

Properties of the PhAsO-induced Permeability Transition in Ca^{2+}-Supplemented Mitochondria

Since PhAsO can induce increased ion permeability prior to the generalized permeability increase (Novgorodov *et al.*, 1987), we have first tested the effect of PhAsO in inducing the membrane permeability transition in sucrose media in the presence of Ca^{2+}. Figure 1 shows that PhAsO caused a major absorbance decrease both in the presence or absence of respiration (Fig. 1b and a, respectively). The absorbance decrease is indicative of increased permeability to sucrose leading to matrix swelling. At 10 μM PhAsO the permeability increase occurred after a lag phase which disappeared at higher PhAsO concentrations. The permeability transition was (i) largely

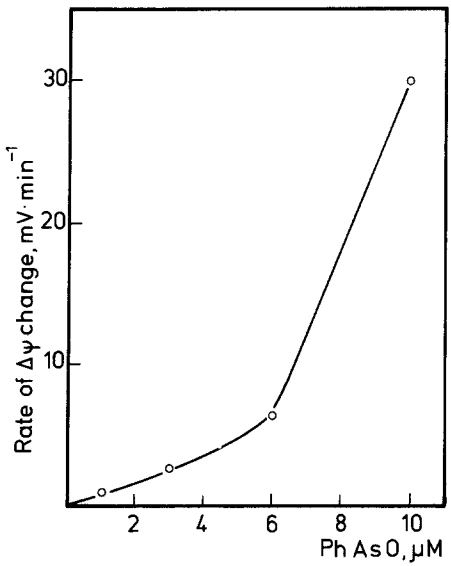

Fig. 2. PhAsO-induced decline of membrane potential in the presence of Ca^{2+}. Mitochondria (1 mg/ml) were incubated in a medium containing 180 mM sucrose, 20 mM choline chloride, 10 mM Tris-Mops, 5 mM Tris-succinate, 1 mM Tris-phosphate, 20 μM $CaCl_2$, 2 μM rotenone, 0.1 mg/ml BSA, 5 μM $TPMP^+$, and the indicated concentrations of PhAsO. Values on the ordinate refer to the rate of decline of membrane potential following addition of PhAsO to mitochondria that had reached a steady-state $TPMP^+$ distribution.

independent of whether mitochondria were respiring or not, although the lag phase was longer in nonrespiring mitochondria, and (ii) most likely due to dithiol oxidation, since it could not be inhibited by 10 mM β-mercaptoethanol (not shown).

Next we have measured the membrane permeability to (i) H^+ ions by following the decline of membrane potential with a $TPMP^+$-selective electrode and (ii) to H^+ and K^+ ions by following K^+ distribution with a K^+-selective electrode. Figure 2A shows that addition of PhAsO to Ca^{2+}-supplemented mitochondria respiring in a sucrose medium initiated a process of depolarization the rate of which increased with the increase of the PhAsO concentration. Fig. 3 shows the dose dependence of PhAsO-induced net K^+ efflux from respiring mitochondria. Under these conditions K^+ efflux is a complex function of several parameters, namely: (i) the proton permeability, (ii) the K^+ permeability, and (iii) the flux ratio between proton leaks and proton pumping. Since PhAsO induced net K^+ efflux, we conclude that it induced an increase of permeability for both H^+ and K^+ to an extent capable of causing a release of matrix K^+, in accord with earlier observations of Diwan *et al.* (1986) and of Novgorodov *et al.* (1987).

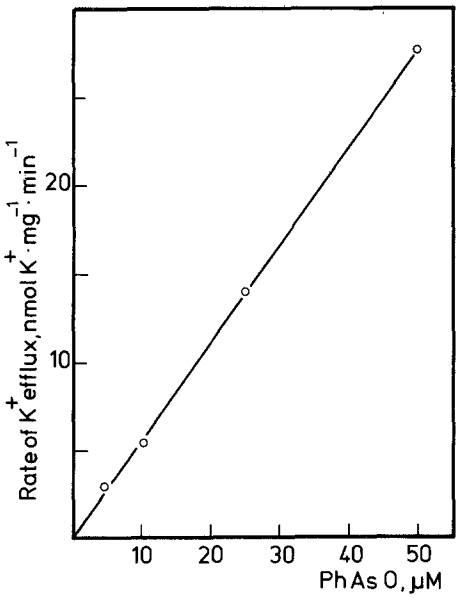

Fig. 3. PhAsO-induced K^+ efflux in the presence of Ca^{2+}. Experimental conditions were as in Fig. 2, except that $TPMP^+$ was omitted. Values on the ordinate refer to the rate of K^+ efflux induced by the addition of the indicated concentrations of PhAsO.

Figure 4 shows that the PhAsO-induced increase of K^+ permeability was inhibited by both BHT and cyclosporin A. The effects of BHT on membrane potential and K^+ efflux occurred in the same range of concentrations previously reported to inhibit the membrane permeability transition caused by other agents (Novgorodov *et al.*, 1987; Carbonera and Azzone, 1988; Crompton *et al.*, 1988). Since cyclosporin A inhibited the permeability transition in the same range of concentrations reported by Broekemeier *et al.* (1989) for a number of inducers, this experiment demonstrates for the first time that PhAsO is indeed one more member of the growing family of inducers of the permeability transition.

Properties of the PhAsO-Induced Permeability Transition in Ca^{2+}-Depleted Mitochondria

A relevant question is whether the effect of PhAsO is simply that of enhancing a Ca^{2+}-induced process or rather the effect of PhAsO can take place also in Ca^{2+}-depleted mitochondria. To answer this question, we have tested the ability of PhAsO to induce the permeability transition in EGTA-supplemented mitochondria. The first experiment was carried out by adding

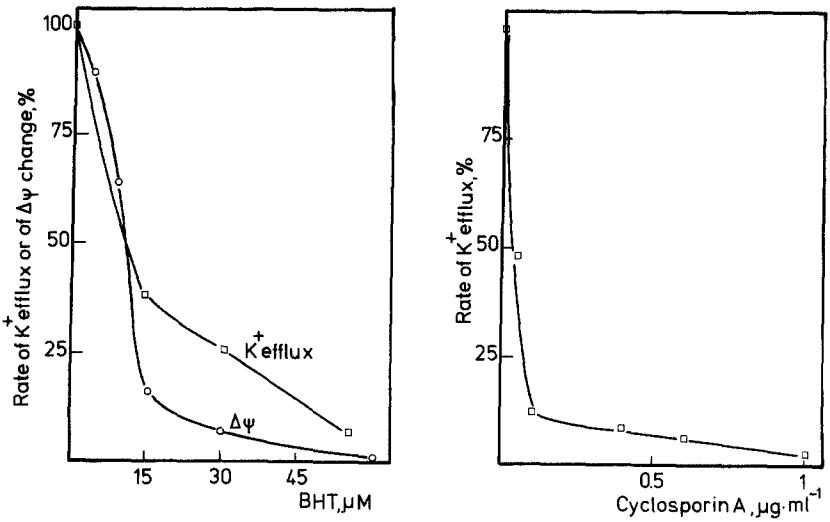

Fig. 4. Effect of BHT and cyclosporin A on the PhAsO-induced permeability transition in the presence of Ca^{2+}. Experimental conditions were as in Fig. 2, except that $TPMP^+$ was omitted in the K^+ efflux measurements and PhAsO was 10 μM. (Left) Values on the ordinate refer to the effect of the indicated concentrations of BHT on the rate of decline of membrane potential (○) or on the rate of K^+ efflux (□). (Right) The effect of cyclosporin A on the rate of K^+ efflux is shown.

PhAsO to respiring mitochondria, and measuring the rate of K^+ efflux in a K^+-free sucrose medium. Intact mitochondria do not release matrix K^+ not only because the membrane has a low passive permeability for both K^+ and H^+ but also because the proton pumping activity maintains a high membrane potential. Figure 5 shows that PhAsO was able to induce a dose-dependent process of K^+ efflux in the range 0–250 μM. The K^+ efflux indicates that even in the absence of Ca^{2+}, PhAsO was able to induce an increased permeability for both K^+ and H^+, a process that reflects the permeability transition (see Fig. 6). At 50 μM PhAsO the rate of K^+ efflux of EGTA-treated mitochondria was about half of that observed in Ca^{2+}-supplemented mitochondria (compare Figs 3 and 5). In their study on the effects of PhAsO on mitochondrial K^+ fluxes, Diwan *et al.* (1986) could not detect a permeability change below 50 μM PhAsO. This minor discrepancy is most likely due to the fact that in the study of Diwan *et al.* (1986) the concentration of mitochondria was 5 to 8 times higher than that employed here.

Figure 6 shows that the process of K^+ efflux induced by 500 μM PhAsO in EGTA-treated mitochondria was inhibited by both cyclosporin A and BHT. It is significant that the concentrations of cyclosporin A required for the inhibition were close to those required to abolish the permeabilty transition

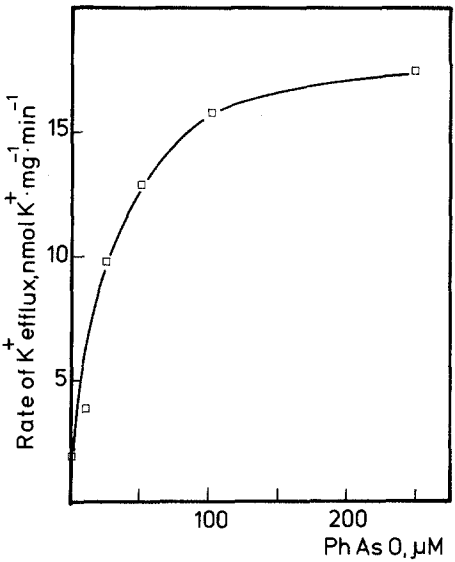

Fig. 5. PhAsO-induced K^+ efflux in the presence of EGTA. Mitochondria (1 mg/ml) were incubated in a medium containing 180 mM sucrose, 20 mM choline chloride, 10 mM Tris-Mops, 5 mM Tris-succinate, 1 mM Tris-phosphate, 2.5 mM EGTA, 2 μM rotenone, 0.1 mg/ml BSA, and 25 μM KCl. Values on the ordinate refer to the rate of K^+ efflux following the addition of the indicated concentrations of PhAsO.

in Ca^{2+}-treated mitochondria. The main difference, however, was that the cyclosporin A inhibition was not complete, i.e., about 25% of the PhAsO-induced K^+ efflux was insensitive to cyclosporin A. On the other hand, the concentration of BHT required for 50% inhibition was about 4 times higher.

Diwan *et al.* (1986) have reported that addition of PhAsO resulted in a stimulation of the rate of K^+ influx into respiring mitochondria. In Fig. 7 the correlation between the PhAsO-induced permeability transition and the rate of K^+ diffusion was examined directly by measuring the rate of penetration of KSCN in respiratory-inhibited mitochondria. Since SCN^- is a permeant anion, the rate of KSCN penetration is limited by the electrophoretic K^+ permeability. Therefore in this system the rate of absorbance decrease following the addition of PhAsO is a quantitative assay of the induced increase of K^+ permeability. Figure 7 shows that the rate of absorbance decrease was negligible in untreated mitochondria and increased markedly parallel to the increase of the PhAsO concentration in the medium in the range 0–500 μM. Figure 7 also shows that the permeability increase due to PhAsO was largely abolished by the addition of 30 μM BHT or 1 μg/ml of cyclosporin A.

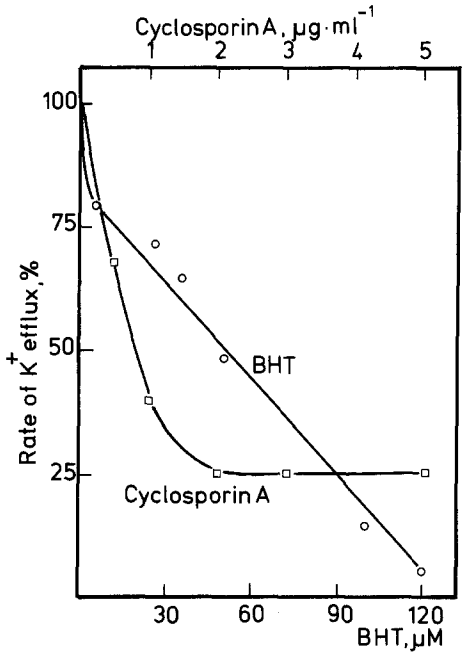

Fig. 6. Effect of BHT and cyclosporin A on the PhAsO-induced permeability transition in the presence of EGTA. Experimental conditions were as in Fig. 5. Values on the ordinate refer to the rate of K^+ efflux following addition of 500 μM PhAsO in the presence of the indicated concentrations of BHT (○) or cyclosporin A (□).

Discussion

A large number of conditions and agents lead to the permeability transition of the inner mitochondrial membrane (reviewed by Gunter and Pfeiffer, 1990). This phenomenon has two basic aspects: the induction process and the permeability increase. For almost 30 years the permeability increase has been regarded as a "damage" to the inner membrane of no physiological significance. Recently, however, mainly because of the work of the groups of Pfeiffer and Crompton, the problem of the permeability transition, pioneered by Hunter and Haworth (1979a, b, c), has been reevaluated. It now appears that the permeability transition shows features consistent with the opening of a proteinaceous pore. Indeed, the permeability transition is reversible on a time scale of seconds, and the reversal exhibits first-order kinetics and involves the synchronized closing of all pores in individual mitochondria (Crompton and Costi, 1988). Strong evidence for the involvement of a pore also comes from the finding that the transition is inhibited by cyclosporin A

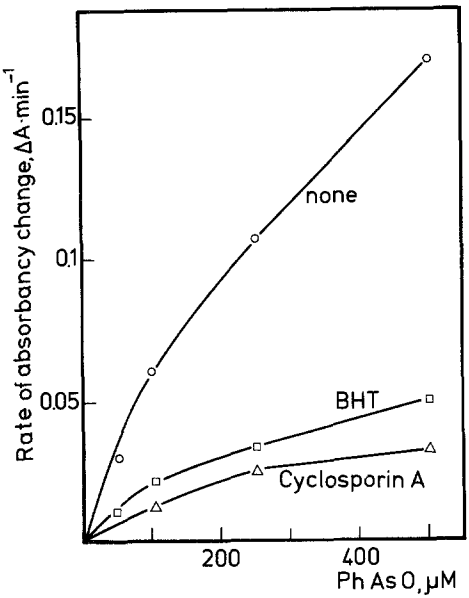

Fig. 7. Effect of BHT and cyclosporin A on passive K^+ permeability induced by PhAsO in the presence of EGTA. Mitochondria (0.5 mg/ml) were incubated in a medium containing 50 mM KSCN, 10 mM Tris-Mops, 1 mM EGTA, 2 μM rotenone, 0.1 mg/ml BSA, and the indicated concentrations of PhAsO. (○) no further additions; (□) 30 μM BHT; (△) 1 μg/ml cyclosporin A.

with a high affinity (Fournier *et al.*, 1987; Crompton *et al.*, 1988; Broekemeier *et al.*, 1989).

This discovery facilitates the interpretation of the pathway(s) underlying mitochondrial swelling under a number of conditions, allowing discrimination between (i) specific pore induction, sensitive to cyclosporin A; (ii) permeability increase due to formation of lysophopholipids, sensitive to trifluoperazine (Broekemeier and Pfeiffer, 1989); (iii) unspecific permeability increase due to a detergent-like effect, insensitive to either inhibitor. By using the relatively unambiguous criterion of sensitivity to cyclosporin A, in this paper we have shown that PhAsO is a *bona fide* activator of the permeability transition.

The most puzzling part of the permeability transition concerns the induction process, which can be triggered by a number of unrelated compounds provided that Ca^{2+} ions are present in the incubation medium (see the comprehensive list compiled by Gunter and Pfeiffer, 1990). The finding that the bifunctional, hydrophobic thiol reagent PhAsO is able to induce the permeability transition even in the presence of EGTA suggests that Ca^{2+} catalyzes some early step(s) in the induction process that PhAsO is able to circumvent. The Ca^{2+}-catalyzed step must involve the formation of oxygen free radical species, since the permeability transition induced in the presence

of Ca^{2+} by phosphate, diamide, and hydroperoxides (Carbonera and Azzone, 1988) and by PhAsO (Novgorodov *et al.*, 1987) is inhibited by the oxygen free radical scavanger BHT. On the other hand, cross-linking of adjacent SH groups must be involved in the induction process. The critical SH groups appear not to be easily accessible to hydrophilic SH reagents (Novgorodov *et al.*, 1987). For example, the bifunctional, hydrophilic SH group reagent diamide is unable to induce the permeability transition in the presence of EGTA, while the transition is readily observed upon addition of Ca^{2+} (Siliprandi *et al.*, 1978). Since the Ca^{2+} requirement is not observed with hydrophobic, bifunctional SH group reagents such as Cd^{2+} (Rasheed *et al.*, 1984) or PhAsO (this paper), it appears that Ca^{2+} ions catalyze the exposure of critical SH groups regulating the pore permeability to solutes. Studies aimed at substantiating this hypothesis are under way in our laboratory.

Acknowledgments

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References

Bernardi, P., and Azzone, G. F. (1983). *Biochim. Biophys. Acta* **724**, 212–223.
Broekemeier, K. M., and Pfeiffer, O. R. (1989). *Biochem. Biophys. Res. Commun.* **163**, 561–566.
Broekemeier, K. M., Dempsey, M. E., and Pfeiffer, D. R. (1989). *J. Biol. Chem.* **264**, 7829–7830.
Carbonera, D., and Azzone, G. F. (1988). *Biochim. Biophys. Acta* **943**, 245–255.
Crompton, M., and Costi, A. (1988). *Eur. J. Biochem.* **178**, 489–501.
Crompton, M., Ellinger, H., and Costi, A. (1988). *Biochem. J.* **255**, 357–360.
Diwan, J. J., De Lucia, A., and Rose, P. E. (1983). *J. Bioenerg. Biomembr.* **15**, 277–288.
Diwan, J. J., Srivastava, J., Moore, C., and Holey, T. (1986). *J. Bioenerg. Biomembr.* **18**, 123–134.
Fournier, N., Ducet, G., and Crevat, A. (1987). *J. Bioenerg. Biomembr.* **19**, 297–303.
Gunter, T. E., and Pfeiffer, D. R. (1990). *Am. J. Physiol.* **258**, C755–C786.
Hunter, D. R., and Haworth, R. A. (1979a). *Arch. Biochem. Biophys.* **195**, 453–459.
Hunter, D. R., and Haworth, R. A. (1979b). *Arch. Biochem. Biophys.* **195**, 460–467.
Hunter, D. R., and Haworth, R. A. (1979c). *Arch. Biochem. Biophys.* **195**, 468–477.
Novgorodov, S. A., Kultayeva, E. V., Yaguzhinsky, L. S., and Lemeshko, V. V. (1987). *J. Bioenerg. Biomembr.* **19**, 191–202.
Rasheed, B. K., Diwan, J. J., and Sandai, D. R. (1984). *Eur. J. Biochem.* **144**, 643–647.
Sandai, D. R., Hughes, J. B., and Saraj, 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 Wendal, A., eds.), Springer-Verlag, Berlin, pp. 139–147.
Zoratti, M., Favaron, M., Pietrobon, D., and Azzone, G. F. (1986). *Biochemistry* **25**, 760–767.