Triorganotins Inhibit the Mitochondrial Inner Membrane Anion Channel*

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The inner membrane of liver and heart mitochondria possesses an anion uniport pathway, known as the inner membrane anion channel (IMAC). IMAC is inhibited by matrix Mg$^{2+}$, matrix H$^+$, N,N'-dicyclohexylcarbodiimide, mercurials and amphiphilic amines such as propranolol. Most of these agents react with a number of different mitochondrial proteins and, therefore, more selective inhibitors have been sought. In this paper, we report the discovery of a new class of inhibitors, triorganotin compounds, which block IMAC completely. When the matrix pH is raised and/or tributyltin (TBT) inhibits malonate uniport via IMAC 95% at 0.9 nmol/mg. The only other mitochondrial protein reported to react with triorganotins, the F,F$_{1},$ATPase, is inhibited by about 0.75 nmol/mg. The potency of inhibition of IMAC increases with hydrophobicity in the sequence trimethyltin $<$ triethyltin $<$ tripropyltin $<$ triphenyltin; which suggests that the binding site is accessible from the lipid bilayer. It has long been established that triorganotins are anionophores able to catalyze Cl$^-$/OH$^-$ exchange; however, TBT is able to inhibit Cl$^-$ and NO$_3^-$ transport via IMAC at doses below those required to catalyze rapid rates of Cl$^-$/OH$^-$ exchange. Consistent with previous reports, the data indicate that about 0.8 nmol of TBT per mg of mitochondrial protein is tightly bound and not available to mediate Cl$^-$/OH$^-$ exchange. We have also shown that the mercurials, p-chloromercuribenzenesulfonate and mersalyl, which only partially inhibit Cl$^-$ and NO$_3^-$ transport can increase the IC$_{50}$ for TBT 10-fold. This effect appears to result from a reaction at a previously unidentified mercurial reactive site. The inhibitory dose is also increased by raising the pH and inhibition by TBT can be reversed by S$^{2-}$ and dithiols but not by monothiols.

The inner membrane of mitochondria isolated from liver and heart normally has a very low electrophoteric permeability to anions; however, when the matrix pH is raised and/or matrix Mg$^{2+}$ is depleted it becomes highly permeable to a variety of different anions (1). This transport has been ascribed to a pathway which is referred to as the inner membrane anion channel or IMAC$^1$ (2). In addition to being reversibly inhibited by matrix Mg$^{2+}$ and matrix protons (1, 3), transport via IMAC is inhibited by cationic amphiphilic drugs such as propranolol (4), and irreversibly inhibited by the alkylating agent N,N'-dicyclohexylcarbodiimide (5). More recently, it has been shown that IMAC is also inhibited by mercurials such as mersalyl and p-chloromercuribenzenesulfonate (6). Mercurials inhibit many anion carriers in mitochondria; however, the maximum extent of inhibition of IMAC is dependent on the species of the anion which is being transported and the mercurial used. The physiological function of IMAC is uncertain; however it has been proposed that it may be involved in mitochondrial volume homeostasis and nonshivering thermogenesis (2). The molecular identity of IMAC has not yet been established; however, a number of channels in the inner membrane have been identified using the patch-clamp technique (7-9). To determine whether any of these channels or transport observed in reconstituted systems reflects the activity of IMAC, potent and specific inhibitors are required. Those inhibitors identified to date have the drawback that they interact with a number of membrane proteins.

After finding that mercurials inhibit IMAC, we began looking for other electrophilic, which might be more selective. Triorganotin compounds have been used in mitochondrial research for a number of years. They inhibit the F,F$_{1}$,ATPase (10-12), have been reported to stimulate K$^+$ uniport (13), 14, and like mercurials with nonionic R-groups they are able to catalyze Cl$^-$/OH$^-$ exchange across lipid membranes (10, 15, 16). No other effects on mitochondria have been reported; however, other proteins are reported to interact with organo- tin compounds including, the Na$^+$K$^+$ATPase (10), Ca$^{2+}$-ATPases (10), hemoglobin from rat and cat (17), and hexokinases (18). Some receptor-mediated functions involving storage and release of catecholamines are also affected including uptake of $\gamma$-aminobutyric acid (19), norepinephrine (20), and serotonin (20). They are also reported to block pressure-coupled release of lysosome from polymorphonuclear leukocytes (21), and release of serotonin during platelet aggregation (22). Triorganotins are reported to be particularly toxic to certain marine organisms and, consequently, have been used in marine antifouling paints. This use has led to an increasing contamination of the marine environment (23, 24).

In this paper, we present evidence that triorganotin compounds completely inhibit IMAC at about the same dose required to block the F,F$_{1}$,ATPase. This does not appear to result from interaction at the putative mercurial binding sites (6); however, inhibition of IMAC is shown to be modulated by mercurials. A preliminary report of some of these data has been presented (25).

EXPERIMENTAL PROCEDURES

Assay of Anion Transport—Anion transport was assayed by following swelling, which accompanies net salt transport, using the light
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C1 was obtained from Aldrich. TMT C1, TET C1, TPT C1, and TOT C1 were dissolved in ethanol except triphenyltin which was dissolved in thioglycolate, and nitrate media for light scattering studies contained the K' thioglycolate, EDTA (0.1 mM), EGTA (0.1 mM), and TES (5 mM) plus rotenone (2 μg/mg). The assay medium is about 1400 nmol/ml. To determine rates of solute transport, we use a Brinkmann probe colorimeter (Model PC790) with a 1-cm probe (2-cm light path). With this probe, for optimum sensitivity we normally use mitochondria at a concentration between 0.1 and 0.2 mg/ml.

Assay of the F,F,F,ATPase Using the Light Scattering Technique—To assay the ATPase activity using the light scattering technique we exploited the fact that this proton pump can drive the electrophoretic uptake of K' upon addition of the ionophore valinomycin. In the presence of a permeant acid, such as acetate this uptake is accompanied by swelling. Use of this assay was validated in three ways; (1) in the absence of ATP no swelling is observed, (2) swelling is blocked by oligomycin (0.9 nmol/mg), (3) swelling is blocked by carboxyt relaxolyside (0.25 mmol/mg). Since it is well established that triorganotins block the ATPase (10), we have assumed that the inhibition observed in our assay reflects inhibition of the ATPase and not inhibition of the adenine nucleotide translocator. The assay medium contained the K' salts of malonate (27 mM) or NO3 (40 mM), acetate (15 mM), EGTA (0.5 mM), TES (5 mM), ADP (400 nmol), and rotenone (2 μg/mg), and valinomycin (0.5 mmol/mg). The assay media were maintained at pH 7.4 and 25 °C.

Measurement of State 3 Respiratory Rates—The rates of state 3 respiration were measured with an oxygen electrode. Mitochondria (0.5 mg/ml) were suspended in an assay medium containing the K' salts of succinate (10 mM), TES (5 mM), EGTA (0.5 mM), and phosphate (5 mM) along with succrose (210 mM), MgSO₄ (3 mM), and rotenone (1 μg/mg). ADP (400 nmol) was added after 0.5 min to initiate state 3.

Treatment of Mitochondria with Triorganotins—Inhibition by TBT was the same whether it was added directly to the assay medium or to a pretreatment medium. Consequently, TBT was usually added directly to the assay medium after addition of the mitochondria. However, in some experiments where pretreatment was desired, the normal mitochondrial stock suspension (50 mg of protein/ml) was diluted 1:1 in 0.25 M sucrose containing K' salts of TES (12 mM) and EGTA (0.5 mM) adjusted to pH 6.7 (at 25 °C) and maintained at 0 °C. The desired dose of TBT was then added and at least 1 min was allowed to elapse after mixing before mitochondria were transferred to the assay medium.

Pretreatment of Mitochondria with Mersalyl and p-CMS—This was carried out as described above for triorganotins.

Triorganotins—Mitochondria (50 mg/ml) were treated with the desired amount of NEM as described above for triorganotins, except that the suspension was kept on ice for 10 min after addition of NEM and then unless indicated otherwise thioglycollate (10 mM) was added to terminate the reaction.

Assay Media for Anion Transport—The potassium chloride, malonate, and nitrate media for light scattering studies contained the K' salts of Cl− (55 mM) or malonate (36.7 mM) or nitrate (55 mM) and EDTA (0.1 mM), EGTA (0.1 mM), and TES (5 mM) plus rotenone (2 μg/mg). The pH was adjusted to 7.4 unless indicated otherwise and the suspension was maintained at 25 °C.

Drugs and Reagents—Most drugs were obtained from Sigma. TBT Cl was obtained from Aldrich. TMT Cl, TET Cl, TPT Cl, and TOT Cl were obtained from Organometallics. All organotin compounds were dissolved in ethanol except triphenyltin which was dissolved in dimethylsulfoxide. The ionophores and rotenone were dissolved in ethanold. Thioglycollate, mersalyl, and p-CMS were dissolved in water. Rat liver mitochondria were prepared as described previously (27). Beef heart mitochondria were prepared according to the procedure of Brierley et al. (28).

RESULTS

Triorganotins Inhibit Uniport of Malonate—Since triorganotins catalyze Cl−/OH− exchange across membranes, we began our study looking at the electrophoretic transport of malonate. Trace a of Fig. 1 is a typical light scattering trace obtained in potassium malonate. In the presence of nigericin, addition of A23187 induces a small increase in matrix volume due to the net exchange of matrix Mg2+ for 2K+ (1). Loss of matrix Mg2+ activates IMAC permitting net influx of potassium malonate and swelling upon addition of the K' ionophore valinomycin. Addition of TBT to the medium inhibits transport (traces b-d). The extent of inhibition was the same when the mitochondria were pretreated with TBT and inhibition was also observed when TBT was added after swelling had begun (data not shown).

The data contained in Fig. 2 (closed circles) show the initial rates of malonate transport, determined from light scattering kinetics, plotted versus the dose of TBT. The rate declines almost linearly with dose of TBT and inhibition is essentially

FIG. 1. TBT inhibits the uniport of malonate. Light scattering kinetics of mitochondria (0.09 mg/ml) suspended in K+ malonate assay medium are shown. Nigericin (0.5 nmol/mg) and rotenone (2 μg/mg) were added at zero time. A23187 (A23, 10 nmol/mg) and valinomycin (vd, 0.5 nmol/mg) were added as indicated. The following doses of TBT (nmol/mg) were added at 0.1 min: Trace a, 0; b, 0.3; c, 0.6; d, 0.8; e, 1.1. Rates of malonate uniport calculated from these traces, as described under "Experimental Procedures" are a, 221; b, 148; c, 78; d, 39; e, 12 nmol malonate/min·mg. The assay medium is described under "Experimental Procedures."

FIG. 2. TBT inhibits malonate uniport over the same dose range as the F,F,F,ATPase. Percent rate is plotted versus the dose of TBT for malonate uniport (○), ATP + valinomycin induced swelling (▲) and state 3 respiration (△). Rates of malonate uniport were determined from light scattering traces from the experiment shown in Fig. 1. ATPase-dependent uptake of K0Ac was also assayed in a similar medium using light scattering kinetics using the same stock of mitochondria. Rates of state 3 respiration were determined with an oxygen electrode in an isotonic sucrose medium using mitochondria from the same stock suspension incubated at 0.6 mg/ml. For further details see "Experimental Procedures."
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Complete at 1 nmol/mg. Note that Sone and Hagihara (29) have shown that 0.9 nmol TBT/mg reacts stoichiometrically with mitochondria. For comparison, we also show the dose-response curve for inhibition of the F,F,AATPase under similar conditions (closed triangles). In this experiment, acetate and 0.2 mM ATP were included in the medium and the rate of salt transport induced by the addition of valinomycin was determined from light scattering kinetics (see “Experimental Procedures”). Fig. 2 also contains a dose-response curve for inhibition of state 3 respiration in an isotonic sucrose medium (open triangles). In this experiment, when the dose is expressed in nmol of TBT/mg protein, inhibition is observed over the same range as in the swelling experiment, consistent with the conclusion that TBT reacts stoichiometrically with mitochondria (29). Furthermore, inhibition is maximal at about 0.75 nmol of TBT per mg protein, a value which is in close agreement with the data of Sone and Hagihara (29). We have also examined the effect of TBT on the activity of the phosphate carrier and the dicarboxylate carrier over this dose range and find no inhibition. Similar results were obtained in beef heart mitochondria.

In view of the findings that $N,N'$-dicyclohexylcarbodiimide (5, 30) and TBT inhibit both IMAC and the F,F,AATPase, we also investigated the effect of the classical F,F,AATPase inhibitor oligomycin on IMAC and found that it could also inhibit. To compare dose-response curves, we pretreated the mitochondria with oligomycin since at low doses it reacts slowly with the F,F,AATPase. Maximum inhibition of the ATPase was observed with 0.9 nmol/mg; however, inhibition of IMAC required much higher doses exhibiting an IC$_{50}$ of 39.8 nmol/mg (data not shown).

We have also examined inhibition of IMAC by other triorganotins. The data contained in Table I show that the potency decreases substantially as the size of the R-group and partition coefficient decrease. The most potent inhibitors are TBT and TOT and the least potent is TMT. These results suggest that the reactive site may be located in the lipid phase of the membrane.

**Sodium Sulfide and Dithiols Reverse Inhibition by TBT**—The interaction of triorganotins with proteins is not well understood and there is no standard reagent for reversing the binding. Aldridge and Cremer (31) concluded that triorganotins have a low affinity for both monothiols and dithiols; however others have reported that the dithiols 2,3-dimercaptopropanol and dithioheptane are able to antagonize some effects of triorganotins (20, 32). It has also been reported that sodium sulfide can reverse the effects of triorganotins (20, 21). We have tested these agents and find that monothiols such as cysteine and thioglycolate have no effect on the action of TBT when added before or after TBT. In contrast, we find that both 2,3-dimercaptopropanol and dithioheptane can reverse the inhibition. However, even with 3 mM dithioheptane the reversal is slow and incomplete. The data contained in Fig. 3 show that sodium sulfide is also able to reverse inhibition of IMAC by TBT. The reaction is more rapid than with dithiols; however reversal is still incomplete at about 80%. The reason for this is unknown; however, sulfide alone has no effect on IMAC.

**Uniport of NO$\mathbf{;}$ and Cl$^{-}$ is Inhibited by TBT**—Transport of NO$\mathbf{;}$ and Cl$^{-}$ via IMAC is rapid (1) and can be completely blocked by $N,N'$-dicyclohexylcarbodiimide (33). In contrast, transport of these anions is only partially inhibited by mercurials, in fact although p-chloromercuribenzene sulfonate is able to block malonate transport by about 80% it has a negligible effect on NO$\mathbf{;}$ and Cl$^{-}$ transport (6). We were, therefore, interested to determine whether inhibition by triorganotins would also be incomplete with these anions. The data contained in Fig. 4 show that this is not the case. TBT almost completely inhibits NO$\mathbf{;}$ transport at about 1.4 nmol/mg (closed circles). The residual rate of transport is equal to that observed in the absence of A23187 suggesting that it represents NO$\mathbf{;}$ transport through the lipid bilayer (2) and that TBT completely blocks NO$\mathbf{;}$ transport through IMAC.

We also examined the effect of TBT on NO$\mathbf{;}$ transport in mitochondria treated with DCCD to block IMAC and found no effect providing further evidence that TBT does not affect the permeability of the lipid bilayer per se (data not shown). These data also confirm previous findings that triorganotins do not catalyze NO$\mathbf{;}$/OH$\mathbf{;}$ exchange (10).

It is interesting to note that the doses required for inhibition in NO$\mathbf{;}$ are slightly higher than those in malonate (see Table I). Rose and Aldridge (34) have shown that different anions affect the partition coefficient of TET, therefore, the observation may simply reflect a change in the concentration of TBT at the binding site.

In view of the potency of inhibition by TBT, we also examined the effect of triorganotins on NO$\mathbf{;}$ and Cl$^{-}$ transport in an isotonic sucrose medium (29). Furthermore, inhibition is maximal at about 0.75 nmol of TBT per mg protein; however, inhibition of IMAC required much higher doses exhibiting an IC$_{50}$ of 39.8 nmol/mg (data not shown).

We have also examined inhibition of IMAC by other triorganotins. The data contained in Table I show that the potency decreases substantially as the size of the R-group and partition coefficient decrease. The most potent inhibitors are TBT and TOT and the least potent is TMT. These results suggest that the reactive site may be located in the lipid phase of the membrane.

**Table I**

Inhibition of IMAC and mediation of Cl$^{-}$/OH$\mathbf{;}$ exchange by triorganotins

<table>
<thead>
<tr>
<th>Partition coefficient$^a$</th>
<th>Dose giving 95% inhibition</th>
<th>Dose giving 50% inhibition</th>
<th>Hill slope</th>
<th>Estimate of concentration in membrane giving 95% inhibition$^b$</th>
<th>Efficiency of Cl$^{-}$/OH$\mathbf{;}$ exchange</th>
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<tr>
<td></td>
<td>NO$\mathbf{;}$ Malonate</td>
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| TMT                      | 0.5                      | 6200 (0.7 mM)            | 4900 (0.5 mM) | 2300 (2.6 mM) | 2.2 250 350 3.7  
| TET                      | 3.7                      | 110 (12.5 μM)            | 68 (7.5 μM)   | 31 (3.6 μM) | 2.2 28.6 46 111 |
| TPT                      | 52                       | 8.0                      | 3.5         | 1.3       | 2.2 14.0 37 639 |
| TBT                      | 1300                     | 1.2                      | 0.9         | 0.4       | 2.5 11.5 46 2190 |
| TOT                      | 12000                    | 2.4                      | 1.5         | 0.7       | 2.5 870 380 216 |

$^a$ Partition coefficient in n-octanol from Ref. 38.

$^b$ Assumes 0.8 nmol/mg is tightly bound.
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Fig. 3. Reversal of TBT inhibition of malonate uniport by sulfide. Light scattering kinetics of mitochondria (0.09 mg/ml) suspended in potassium malonate assay medium are shown. Rotenone (2 μg/mg) was added at zero time, nigericin (0.5 nmol/mg) at 0.2 min, A23187 (433, 10 nmol/mg) at 0.3 min, and valinomycin (ca., 0.5 nmol/mg) at 1.5 min. Trace a, control; trace b, sodium sulfide (S²⁻, 0.1 mM) was added at 0.4 min; trace c, TBT (1.7 nmol/mg) was added at 0.1 min; trace d, both TBT and sodium sulfide were added as above. For further details see “Experimental Procedures.”

Fig. 4. The effect of TBT on NO₃⁻ and Cl⁻ transport. The rate of transport determined from light scattering kinetics is plotted versus the dose of TBT. NO₃⁻ transport (a) was measured in mitochondria (0.11 mg/ml) treated with A23187 (10 nmol/mg) and nigericin (0.5 nmol/mg) to activate IMAC and valinomycin (0.5 nmol/mg) to catalyze K⁺/H⁺ antiport. Cl⁻ uniport (b) was assayed in the same way except that nigericin was omitted. Cl⁻/OH⁻ exchange (c) was assayed in the same medium except that nigericin was omitted and nigericin (1 nmol/mg) was added to mediate K⁺/H⁺ antiport, thereby permitting net KCl influx. The assay media contained rotenone (2 μg/mg) and are described under “Experimental Procedures.”

examined the effect of TBT on Cl⁻ transport (see Fig. 4). Two experiments were carried out. In the first, to look at the effect on IMAC, we added A23187 to activate IMAC and valinomycin to mediate K⁺ uniport but omitted nigericin in order to minimize net KCl transport resulting from K⁺/H⁺ antiport and TBT-mediated Cl⁻/OH⁻ exchange. In the second experiment, we omitted A23187 and valinomycin to minimize the activity of IMAC and added nigericin to facilitate net KCl influx dependent on TBT-mediated Cl⁻/OH⁻ exchange. Cl⁻ uniport via IMAC is found to be maximally inhibited by 1.4 nmol TBT/mg. The residual rate of transport which does not increase with dose of TBT, can be explained by transport of KCl mediated by the combined activities of the endogenous K⁺/H⁺ antiporter and TBT-mediated Cl⁻/OH⁻ exchange, with the activity of the antiporter being limiting. Other experiments were carried out which confirmed that the antiporter is not inhibited by TBT over this dose range (data not shown).

In the absence of A23187 and valinomycin, TBT-mediated Cl⁻ transport is observed; however, consistent with the notion that mitochondria contain a number of tight-binding sites for TBT, rates are low below a dose of 0.7–0.8 nmol/mg. This interpretation is supported by the finding that thresholds are also observed with TOT and TPT but not with TET and TMT (data not shown). In all cases, after this threshold, the rate of transport increases linearly with the concentration of the triorganotin. The slopes of these plots provide a means of comparing the ability of each of these compounds to catalyze Cl⁻/OH⁻ exchange. The results, expressed as nmol/min·mg·M are presented in Table I. These values do not fall in the same sequence reported by Selwyn’s group for mitochondria (10); however, they fit quite well with the values obtained in chloroplasts (see Ref. 10). Some of this difference can be attributed to the higher mitochondrial concentration used by Selwyn’s group and the lack of correction for the tight binding of TBT, TPT, and TO (10, 35).

Effect of Mercurials on Inhibition by TBT—To determine whether triorganotins inhibit anion uniport by reacting at the same site as mercurials, we investigated the effect of TBT on mitochondria treated with mersalyl and p-CMS. We began the study by looking at NO₃⁻ transport; (a) because TBT does not catalyze NO₃⁻ transport and (b) because mercurials do not completely block NO₃⁻ transport through IMAC, and therefore, if the mercurial were able to displace TBT or protect the TBT-binding site, transport should be observed. Preliminary experiments showed that addition of p-CMS to TBT-inhibited mitochondria did indeed reactivitate NO₃⁻ transport. To examine the dose-response of this effect, we pretreated the mitochondria with various doses of p-CMS and then assayed swelling in media to which 3.3 nmol of TBT/mg was added. The data contained in Fig. 5 show that in the absence of p-CMS, this dose of TBT blocks NO₃⁻ transport by 96%, and that this inhibition is reversed as the dose of p-CMS is increased from 7 to 12 nmol/mg. Note that the rates reach those observed in the absence of TBT. For comparison, we also show the rates of malonate transport in mitochondria from the same pretreatment mixes. Note that in the absence of TBT, p-CMS inhibits malonate transport. In the presence of TBT, p-CMS partially reverses the inhibition, consistent with our earlier conclusion that p-CMS does not completely block malonate transport (6). Note that these assays were
carried out at pH 8.4 in order to avoid the stimulatory effect of p-CMS observed at lower pH values, and to maximize the percentage inhibition of malonate transport (6). Similar results were also obtained at pH 7.4 with mersalyl.

These data suggest that TBT and mercurials may inhibit IMAC by reacting at the same site. To further investigate this possibility, we examined the dose-response curve for inhibition by TBT in mitochondria pretreated with p-CMS (20 nmol/mg) (Fig. 6A). To our surprise, we found that TBT was still able to inhibit transport; however, the dose required was 10-fold higher. Although this finding suggests that p-CMS and TBT must bind at different sites, it does not rule out the possibility that they may bind competitively (see below).

To try to determine whether the binding of p-CMS to its inhibitory site was responsible for the shift in the dose-response curve to TBT, we examined the effect of thioglycolate, which as we have previously shown reverses the inhibition induced by mercurials (6). As shown in Fig. 6A, addition of thioglycolate to the assay medium does not reverse the shift in the dose-response curve, in fact even higher doses of TBT are required for inhibition. Note that in the absence of p-CMS thioglycolate has no effect on the dose-response curve. Also, when thioglycolate was added to the pretreatment before p-CMS, p-CMS had no effect (not shown).

Since the effect of p-CMS was not reversed by thioglycolate, we also investigated its effect on TBT inhibition of other anions and found that the inhibitory doses for malonate and 1,2,3-benzenetricarboxylate were also increased. Since in the absence of p-CMS a large proportion of TBT appears to be bound, the actual increase in $K_i$ may be larger than reflected by the increase in $IC_{50}$. This conclusion is supported by the finding that when TET and TOT were used, p-CMS increased the $IC_{50}$ 11-fold and 24-fold for malonate and NO$_3^-$, respectively (data not shown).

These data suggest that a different mercurial-binding site must be involved. A second candidate is the mercurial binding site which is responsible for the shift in the $IC_{50}$ values for H$^+$, Mg$^{2+}$, and propranolol (6, 36, 37). Like the effect on TBT inhibition, these effects are not reversed by thioglycolate. Since these effects are also induced by N-ethylmaleimide (37), we investigated the effect of NEM on the dose-response to TBT. As shown by the data contained in Fig. 6B, no effect was observed. However, since it is possible that mercurials and NEM could bind to the same site but induce different effects, we also investigated the effect of p-CMS on the TBT-dose-response curve in NEM-treated mitochondria. As shown by the data contained in Fig. 6B, NEM was unable to protect against the effect of p-CMS. Thus, this effect of p-CMS cannot be attributed to reaction at either of the mercurial binding sites previously identified (6).

To investigate whether p-CMS and TBT binding might be competitive, we have examined the dose-response to p-CMS at two different doses of TBT, 2.4 and 8.4 nmol/mg. The data contained in Fig. 7 show that reversal of inhibition occurs over the same dose range at both TBT concentrations. This suggests that they are not competitive. One could also argue that p-CMS reverses inhibition by unmasking TBT binding sites in other proteins thereby lowering the free concentration of TBT. This, however, appears to be unlikely because inhibition by TET (300 nmol/mg) and TMT (4.1 mmol/mg) is also reversed over the same p-CMS dose range (see Fig. 7) and, it is unlikely that the free concentrations of these agents could change, since they inhibit at relatively high concentrations. This conclusion is also supported by the finding that inhibition of the F,F,$\Delta$ATPase by TBT, assayed as described in Fig. 2 is totally unaffected by treatment of the mitochondria with p-CMS (data not shown).

Effect of pH on Inhibition by TBT—Not only is the activity of IMAC inhibited by protons (1), but also the inhibition by Mg$^{2+}$ (3) and propranolol (4) is modulated by pH. Consequently, it is of interest whether inhibition by triorganotins is also modulated by pH. Since TBT appears to inhibit IMAC in a dose range where it binds tightly to sites in the mitochondria, even quite a large increase in $K_i$ may produce only a small increase in the $IC_{50}$. Therefore, to assess better the effect of pH on inhibition by triorganotins, we looked at TMT. Since TMT has a very high $IC_{50}$ and low partition...
coefficient, the free concentration should be proportional to the total added. Fig. 8 shows data obtained in potassium malonate at pH 7.4 and 8.4. The IC₅₀ values for TMT rise from 0.26 mM at pH 7.4 to 1.02 mM at pH 8.4. Furthermore, when TBT is used, one finds that the dose required to block malonate transport at pH 8.4 is about twice that required at pH 7.4.

**DISCUSSION**

In this paper, we have presented evidence that triorganotin compounds inhibit the inner membrane anion channel of rat liver and bovine heart mitochondria. Most of these studies have been carried out with TBT which is able to block transport through IMAC completely at doses of about 1–1.4 nmol of TBT per mg of mitochondrial protein at pH 7.4. Since our experiments were carried out at mitochondrial concentrations of 0.1 mg/ml, this is approximately 0.1 μM; however, the free aqueous concentration will be considerably less than this if, as reported, 0.9 nmol of TBT/mg binds stoichiometrically to mitochondria (29) and TBT has a partition coefficient of about 1300 (38). Other inhibitors of IMAC which have been described are much less potent than this. For example, inhibition by mercurials requires 15–20 nmol/mg (6), and the IC₅₀ for the most potent cationic amphiphile, amiodarone, is about 6 nmol/mg (4), and the IC₅₀ for Mg²⁺ is about 38 μM (3). Thus, TBT is the most potent inhibitor of IMAC identified to date.

All the triorganotins examined were found to inhibit IMAC; however, their potencies differ considerably increasing in the sequence TMT < TET < TPT < TOT < TBT. With the exception of TOT, this sequence follows the sequence of their partition coefficients (see Table I), therefore we believe that they probably react at a site in the lipid bilayer. A similar conclusion has been drawn for inhibition of mitochondrial oxidative phosphorylation, chloroplast phosphorylation, the Na⁺,K⁺ATPase and the Ca⁺⁺ATPase from sarcoplasmic reticulum (10). The difference in partition coefficients, however, does not seem sufficient to explain completely the difference in potencies. Using the partition coefficients from Wulf and Byington (38) and assuming that 0.8 nmol/mg is tightly bound, we have estimated the free concentration of each triorganotin in the membrane at doses which give 95% inhibition. The results shown in Table I suggest that TBT, TPT, and TET may have similar affinities for IMAC; however the affinities of TMT and TOT are about 10-fold lower. One reason for the greater potency of TBT over other agents such as mercurials is that triorganotins appear to react with far fewer proteins. Mercurials bind tightly to SH groups and consequently, they react with many proteins in the inner membrane including those involved in transport (39). In contrast, to our knowledge, there is only one other protein in mitochondria which reacts with triorganotins, the F,F₄ATPase (29). Inhibition of oxidative phosphorylation by triorganotins was first demonstrated in 1955 by Aldridge and Cremer (31) and since then has been the subject of a number of studies (see Selwyn (10) for a review). Sone and Hagihara (29) carried out dose-response studies and found that inhibition increased linearly with the dose of TBT and that stoichiometric amounts (0.9 nmol/mg) are required to inhibit. The partition coefficient of TBT is not high enough to explain this tight binding, consequently this tightly bound TBT is most likely bound to the ATPase and other proteins. This conclusion is supported by our finding that no appreciable Cl⁻/OH⁻ exchange can be observed at doses of TBT lower than 0.8 nmol/mg. Thus, up to this dose the free concentrations in the membrane and aqueous phase must both be very low. Since we find that inhibition of IMAC also increases linearly over this same dose range, at least at pH 7.4, IMAC must be one of the proteins responsible for the tight binding of TBT. This conclusion is supported by our finding that washing of mitochondria in bovine serum albumin-containing media does not reverse inhibition of IMAC.

The finding that the doses of TBT required to block IMAC and the F,F₄ATPase are very similar plus the fact that both are also blocked by DCCD led us to the discovery that the classical ATPase inhibitor oligomycin is also an inhibitor of IMAC. In view of these common properties, one could raise the question as to whether IMAC is related to the proton channel of the ATPase. This does not, however, appear to be the case. Although at pH 7.4 in normal mitochondria TBT inhibits these pathways over the same dose range, in mitochondria treated with p-CMS inhibition of NO₃⁻ uniport requires a 10-fold higher dose, whereas inhibition of the ATPase is unaffected. In addition, both oligomycin and DCCD block Fₐ at doses which have little effect on IMAC (5, 40).

Although the effects of triorganotins on biological systems have been studied for over 30 years, little is known about how they react with proteins. Like Aldridge and Cremer (31), initially we thought that triorganotins would react with proteins in a similar way to mercurials. However, Aldridge and Cremer (31) came to the conclusion that TET has little affinity for monothiols or dithiols. Our data support this conclusion, monothiols have no effect on inhibition of IMAC by triorganotins and the dithiols, dithiothreitol and 2,3-di-mercaptopropanol, only partially reverse inhibition at relatively high concentrations. In order to understand the toxicity of triorganotins, Rose and Aldridge (41) looked at the distribution of TET in the rat and found that a large part of it was bound to hemoglobin. This interaction is highly specific and hemoglobins from all other species tested except the cat do not bind TET (17, 40). Rose (17) identified histidine residues at the binding site and later it was concluded that the bound tin atom is penta-coordinated with cysteine and histidine residues as the axial ligands (42, 43). More recently, Siebenlist and Taketa (18) have identified the specific residues involved. In other proteins such as yeast hexokinase (19), it has been concluded that neither of these residues is involved. Thus, it appears that triorganotins do not react with specific amino acid residues but that a specific three-dimensional arrangement is more important for tight binding. If this is the case, it would explain why triorganotins bind to so few proteins, why the affinity could be sensitive to small changes in the conformation of the protein, and also why the size of the organo group may affect the binding constant. The stability

![Fig. 8. The effect of pH on inhibition of malonate uniport by TMT. Percent rate of malonate uniport is plotted versus the concentration of TMT at pH 7.4 (0) and 8.4 (O). The experiment was carried out as described in the legend to Fig. 2. Mitochondria from the stock suspension were transferred directly to the assay medium at the indicated pH. The composition of the assay medium is described under "Experimental Procedures."](image-url)
of the pentacoordinate structure would also explain why di-thiols and not monothiols are able to remove the tin compound. A wide variation in the affinity of triorganotins for different proteins would also explain why some effects of triorganotins are unaffected by dithiols (20, 31, 44), while others are reversed (19, 31, 45, 46). The finding that reversal of inhibition is incomplete could be explained by the existence of two triorganotin binding sites only one of which is accessible to dithiothreitol. The existence of two sites is also supported by our finding that the Hill coefficient for inhibition by all triorganotins is close to 2.

Although our initial idea that triorganotins may react at the mercurial binding site appears to be wrong, it is interesting that we have identified a dramatic effect of mercurials on the inhibition by TBT. This is particularly evident with NO3- for which the dose required to inhibit is increased 10-fold for TBT, 24-fold for TET, and 27-fold for TOT. This effect does not appear to be related to reaction of the mercurial at either of the two putative mercurial binding sites designated sites 1 and 2, previously described (6). NEM has no effect on inhibition of IMAC by TBT and has no effect on the shift in the TBT dose–response curve induced by p-CMS, consequently the effect of p-CMS cannot be attributed to reaction at mercurial binding site 1 (6, 37). Moreover, since inhibition of IMAC by mercurials is reversed by monothiols whereas the effect of p-CMS on TBT inhibition is not, the effect cannot be attributed to reaction at mercurial binding site 2. Thus, we have to resort to postulating the existence of a third mercurial reactive site to explain this effect. The existence of a third site, which does not react with NEM, could explain why the effects of NEM and mersalyl on inhibition of IMAC by propranolol reported (37) differ. Although we observe a 10-fold increase in the dose of TBT required to block NO3- transport via IMAC in p-CMS-treated mitochondria, the increase in Ka is probably significantly larger than this. For example, if it is assumed that when one adds 1.2 nmol/mg, 0.8 nmol/mg is bound, the free concentration (aqueous) will be 35 nM; however, if the same amount is tightly bound when the dose is increased 10-fold to 12 nmol/mg the free concentration (aqueous) becomes 1 μM. Thus, a 10-fold increase in dose would lead to a 28-fold increase in free concentration. This analysis is consistent with the finding that the dose of TET required to inhibit IMAC, which far exceeds the number of tight binding sites, is increased 24-fold by p-CMS.

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