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Organotin compounds as energy-potentiated uncouplers of rat liver mitochondria

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Triorganotins will titrate membrane potential ($\Delta\psi$) and the accompanying pH gradient (Δ pH) in estimates of protonmotive force (Δp) in isolated rat liver mitochondria in an apparent concentration-dependent manner and in minimal halide media (5 μmol dm^{-3}). Under these conditions the concentrations of organotin required to produce a drop of 80–120 mV in Δp approach or are in excess of those required to inhibit ATP-synthase activity, which are at least three-fold greater than those which inhibit ATP hydrolytic activity. The addition of exogenous chloride ion did not appreciably alter the steady-state or rate estimates of triorganotin-mediated membrane potential ($\Delta\psi$) depolarization. The evidence indicates that triorganotins possess an uncoupling effect which is independent of halide/hydroxyl exchange or direct inhibition of the ATPase complex. The activity of various triorganotins may be best understood according to their abilities to uncouple or directly inhibit oxidative phosphorylation at the enzymic level, in the absence of halide/hydroxyl exchange.

Keywords: Organotin, membrane potential, pH gradient, mitochondria, protonmotive force, ATP, oxidative phosphorylation

INTRODUCTION

The major effects of triorganotins on mitochondria have been attributed to three basic modes of action. The first is a direct inhibition of the ATPase complex which manifests itself in the loss of ATPase and ATP-synthase activities.[1–3] This oligomycin-like effect appears to be the result of an interaction with the F_0 component of the F_1F_0 ATPase,[2,4] causing the inhibition of proton (H^+) conduction through the system.[5–7] The second mode is to induce a chloride/hydroxide exchange activity across the inner mitochondrial membrane which causes an equilibration of respiration-generated pH differences.[8,9] The third mode of action is to cause gross swelling of the mitochondria in either potassium chloride (KCl) or potassium isethionate media accompanied by the loss of most energetic functions.[1,10]

Studies on the nature of the triorganotin binding sites in mitochondria have yielded complex binding curves which have been interpreted as indicative of two classes of binding site. There appears to be a high-affinity component which is consistent with the loss of ATPase activity[3,11,12] and a low-affinity component, the function of which is open to question.[12]

The chemical nature of the triorganotin binding site has been studied on the basis of organotin interactions with various proteins and suggestions that histidine and thiol/dithiol groups are involved have been made.[5,13–16] Functional indications as to the nature of the triorganotin binding site have come from experiments where mono- and di-thiols have been used to specifically reverse the inhibitory activities of the triorganotins towards the ATP-synthetase of submitochondrial particles.[11,17,18] It has been suggested from such experiments that dithiols may play an important role in the conduction of protons through the F_0 moiety of the mitochondrial ATPase complex.

In this paper we report the activities of various triorganotins on the ATP synthetic and hydrolytic functions of rat liver mitochondria, together with estimates of the parameters of protonmotive force (Δp) during inhibition. Evidence will be presented that triorganotins may act as potent 'oligomycin-like' inhibitors or potent uncouplers independent of chloride/hydroxide exchange. The relative concentrations at which these events take place are in

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid. F_1 and F_0 are components of the mitochondrial ATPase complex (E.C. 3.6.1.3).

the order ATPase < ATP-synthase < Δp reduction. The relevance of these observations will be discussed in the light of current theory and knowledge of the H^+–ATPase complex.

EXPERIMENTAL

Materials

Trialkyl- and triaryl-tin chlorides, triethyltin sulphate and dibutylchloromethyltin chloride [DBCT, $Bu_2(CH_2Cl)SnCl$] were obtained as described previously.[19] Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), oligomycin, rotenone and valinomycin were purchased from Sigma. 2-(dimethylaminostyryl)-1-methylpyridinium iodide (DSMP-I) and methyltriphenylphosphonium iodide (TPMP-I) were purchased from Aldrich Chemicals. Rubidium sulphate (Rb_2SO_4) was produced from the carbonate (Rb_2CO_3) purchased from Fisons. ATP and ADP were purchased from Boehringer–Mannheim, FRG. Tritiated water, [^{3}H]TPMP-Br, [^{14}C]sucrose, [^{14}C]mannitol, [^{14}C]lactate and ^{86}RbCl were all purchased from Amersham International, UK. [^{3}H]DSMP-I was a gift from Professor J Rafael, Heidelberg, FRG. All other reagents were of AR grade.

Methods

Rat liver mitochondria were prepared from adult male Wistar rats starved for 24 h before sacrifice. Liver mitochondria were isolated by differential contrifugation essentially as described by Selwyn *et al.*[8] The mitochondria were finally suspended in 250 mmol dm^{-3} sucrose, 10 mmol dm^{-3} Hepes (pH 7.5) at a 70 mg cm^{-3} protein concentration and kept at 0°C before use. ATP synthesis was assayed in a glucose hexokinase trap system, containing suspension buffer plus 20 mmol dm^{-3} glucose, 5 mmol dm^{-3} potassium phosphate, 2 mmol dm^{-3} magnesium sulphate ($MgSO_4$), 2 mmol dm^{-3} ADP, 5 mmol dm^{-3} succinate and 5 units of yeast hexokinase (Sigma type F300). ATP synthesis was measured by following the disappearance of inorganic phosphate. ATP hydrolysis was measured in phosphorylation buffer minus hexokinase, glucose and ADP by the release of inorganic phosphate liberated from 5 mmol dm^{-3} ATP. Reactions were terminated with 5% trichloroacetic acid, and inorganic phosphate was determined in the acid extracts by the method of Fiske and Subbarow.[20]

The ion distribution methods of Chappell and Crompton[21] were used to measure the mitochondrial volumes and the components of protonmotive force (Δp). For mitochondrial volumes, tritiated water was used to determine the water space of the pellet; the external space was monitored using [^{14}C]sucrose and [^{14}C]mannitol in separate determinations. The pH gradient was determined using the distribution of [^{14}C]lactate in the same incubation as the permeable ions ([^{3}H]triphenylmethylphosphonium and $^{86}Rb^+$) in the presence of 0.2 μg valinomycin. These incubations were performed in 250 mmol dm^{-3} sucrose, 5 mmol dm^{-3} Hepes (pH 7.4), 5 mmol dm^{-3} succinate and 8 μmol dm^{-3} rotenone at 25°C. Mitochondrial protein was determined by the biuret method of Gornall *et al.*[22] with bovine serum ablumin as standard.

Fluorometric determination of membrance potential using the cationic dye 2-(dimethylaminostyryl)-1-methylpyridinium ($DSMP^+$) was performed as described by Mewes and Rafael[23] using [^{3}H]DSMP-I as calibrant in a Perkin–Elmer MPF 44 spectrofluorimeter (excitation at 479 nm, emission at 589 nm at 25°C). Routinely, mitochondria at 1 mg cm^{-3} were incubated with $DSMP^+$ (2 nmol mg^{-1} protein) under the conditions employed to measure oxidative phosphorylation. Uptake of $DSMP^+$ was followed by an increase in fluorescence until a maximum was obtained, approximating to an equivalent membrane potential ($\Delta\psi$) of −180 to −190 mV. The organotin was then added and the decrease in fluorescence monitored.

RESULTS

Mitochondrial volume

It has been proposed by Aldridge *et al.*[10] that chloride ion-dependent stimulation of ATP hydrolysis is related to small-scale mitochondrial swelling which precedes gross swelling as the concentration of the triorganotin is increased. To understand these effects and any possible effects on membrane potential determination, the mitochondrial volumes of progressively inhibited samples were measured under similar conditions to those employed for the determination of ATP-hydrolase activity, in the presence and absence of 0.15 mol dm^{-3} sodium chloride solution.

A chloride-dependent ATPase activity was in fact

Table 1 Effect of triethyltin sulphate on mitochondrial ATPase activity and volume

Triethyltin sulphate (nmol mg^{-1} mitochondria)	Mitochondrial volume ($\mu L\ mg^{-1}$ mitochondrial protein)		ATPase (nmol $mg^{-1}\ min^{-1}$)	
	$-\ Cl^-$	$+\ Cl^-$	$-\ Cl^-$	$+\ Cl^-$
0	0.70	0.68	3.2	3.2
0.25	0.71	0.78	3.0	4.2
0.50	0.71	0.97	2.5	4.9
1.00	0.70	1.20	2.2	3.8
2.00	0.76	1.98	1.5	1.8
5.00	0.77	–	0.2	0.8
10.00	0.85	–	0.0	0.0
15.00	0.96	–	0.0	0.0
20.00	1.65	–	0.0	0.0

These determinations of mitochondrial volume were made in triplicate using [^{14}C]mannitol and ^3H_2O. Both volume and ATPase determinations were performed as described in the Methods section. Mitochondria were incubated with various titres of triethyltin on ice, before dilution into assay medium at 4 mg cm^{-3} and 0.2 mg cm^{-3} for volumetric and ATPase assays respectively. Similar determinations were made using [^{14}C]sucrose; these results essentially displayed the same trend as those related above but differences in the control and experimental values of approximately 0.1–0.12 $\mu L\ mg^{-1}$ were consistently observed; these figures were considered when calculating Δp. The determination made in the presence of chloride, contained 0.15 mol dm^{-3} of sodium chloride.

observed by us with triethyltin sulphate at concentrations ranging between 0.25 and 1.00 nmol mg^{-1} of mitochondrial protein (Table 1). Within this range the mitochondrial volume appears to increase steadily before determinations become variable, presumably as the integrity of the membrane is disrupted. These phenomena presumably result from chloride/hydroxide exchange, as we do not observe them in minimal halide media. These results are in agreement with the functions proposed by Aldridge *et al.*[10] for mitochondria suspended in chloride-containing media. The mitochondria when suspended in low-halide media did not effectively swell at such low concentrations of triethyltin but did increase rapidly in volume after 15 nmol mg^{-1} concentrations of triethyltin. Further additions appeared to perturbate the integrity of the membrane in a non-specific manner. Concentrations of triorganotin over 25 nmol mg^{-1} of mitochondrial protein have been previously reported to cause gross swelling and release of soluble protein, suggesting that triorganotins induce lysis and/or dissociation of structural elements of the mitochondrial membrane.[26] The prediction that high concentrations of triorganotins cause gross perturbations of the integrity of the mitochondrial membrane is consistent with the above findings. The concomitant increase in ATPase activity following large-scale swelling reported for trimethyltin[10] was not observed in these experiments, presumably due to the enhanced 'oligomycin-like' activity of triethyltin species over that of trimethyltins. It is clear from these results that concentrations of triorganotins less than 20 nmol mg^{-1} of mitochondrial protein in the absence of exogenous halide should not perturbate the integrity of the membrane unduly.

Inhibition of ATP synthase and hydrolase activities

As reported previously for beef heart submitochondrial particles, triorganotins cause differential inhibition of the ATP synthetic and hydrolytic activities of the F_1F_0-ATPase.[17] This differential activity is also noted in rat liver mitochondria, where specific concentrations of triorganotins will completely prevent ATP hydrolysis, despite allowing almost control-level ATP synthesis, which is titratable by non-additive amounts of oligomycin. The sensitivities of these reactions under low-halide conditions to various triorganotins are presented as I_{50} values in Table 2. Under these conditions the data presented in Table 2 represent the abilities of various triorganotins to inhibit directly the mitochondrial ATPase complex.

The most active of these triorganotin compounds is DBCT, previously described by Cain *et al.*[19] The

Table 2 Sensitivities of mitochondrial ATPase, ATP-synthase and membrane potential functions to triorganotins

	I_{50} (nmol mg^{-1} protein) ±SD		
Triorganotin species	ATPase	ATP-synthase	$\Delta\psi = +100$ mV[a]
Trimethyltin chloride (2)	>100	>100	300±20
Triethyltin sulphate (5)	2.8±0.4	9.8±0.5	19±2
Tri-n-propyltin chloride (4)	1.8±0.2	7.6±0.5	30±5
Tri-n-butyltin chloride (6)	2.0±0.2	7.8±0.5	12±2
Tricyclohexyltin hydroxide (2)	3.0±0.5	9.0±0.4	11±3
Triphenyltin chloride (6)	1.8±0.4	7.8±0.4	25±5
DBCT (6)	1.0±0.2	2.5±0.4	4.5±0.5

As above, mitochondrial suspensions in 250 mmol dm^{-3} sucrose 10 mmol dm^{-3} Hepes were preincubated on ice with various titres of triorganotin for 15 min before dilution into assay media. ATPase- and succinate-driven ATP systhesis were measured as described in the Methods section. The numbers in parentheses correspond to the numbers of separate duplicate experiments. Control ATPase and ATP-synthase rates ranged between 30–40 nmol mg^{-1} min^{-1} and 80–100 nmol mg^{-1} min^{-1} respectively. [a]These assays were carried out as described by Mewes and Rafael[23] under conditions similar to these mentioned in the legend to Fig. 2. Fluorescent changes were calibrated against standard ion distributions using [^{3}H]DSMP$^+$.

compound will completely inhibit ATPase activity at 1.0 nmol mg^{-1} but allow continued ATP synthesis until a titre of 5.0 nmol mg^{-1} of mitochondrial protein. This property is not unique to DBCT and extends to other trialkyl- and triaryl-tins. The titration curves for DBCT are presented in Fig. 1.

Under reduced-halide conditions these compounds are observed to produce 'oligomycin-like' effects, but at these relatively low concentrations of triorganotin these should not be compromised by any non-specific swelling. Less potent triorganotin species do not appear to cause 100% ATPase inhibition but allow residual enzymic activity. However, at high inhibition titres no resistant residual ATP synthetic activity was registered.

Further energetic functions

Considering the inhibitory activities of the triorganotins, experiments were performed to rationalize these functions with those components of protonmotive force which drive ATP synthesis.[24] Initial experiments were performed to measure mitochondrial membrane potential with the cationic fluorescent probe DSMP$^+$. This cationic dye will distribute across the membrane according to the imposed potential and yield a fluorescent signal, the yield of which is proportional to the membrane potential.[23] Energization of the mitochondrial membrane was achieved via succinate-driven respiration in the presence of rotenone. This yielded a specific burst of fluorescence which equilibrated according to respiration-generated membrane potential in a time-dependent manner. Additions of uncouplers to the system (e.g. CCCP) caused a depolarization of the membrane potential and consequent loss of the fluorescent signal. Triorganotins also have the capacity to bring about this change in a concentration-dependent manner. After additions of limiting amounts of a triorganotin species the membrane potential equilibrated at a particular level, the residual potential being sensitive to established uncouplers or further additions of triorganotin. These effects were always additive in nature, the net drop in potential as a function of concentration being triorganotin-specific. The potency of particular triorganotin species and their ability to cause a drop of +100 mV in membrane potential ($\Delta\psi$), as estimated by [^{3}H]DMSP$^+$ calibrated fluorescence,[23] is presented in Table 2. The effect of chloride on this function was observed when exogenous 0.15 mol dm^{-3} NaCl solution was added to the incubation medium. Exogenous chloride ion did not appreciably alter the rate or steady-state estimates of triorganotin-mediated membrane potential depolarization. This result was not unexpected when one considers the mechanism of chloride-imposed uncoupling by chloride/hydroxide (Cl^-/OH^-) exchange, which is essentially an electroneutral effect directed at the pH component of protonmotive force, Δp.[28] Preincubation with triorganotins prior to

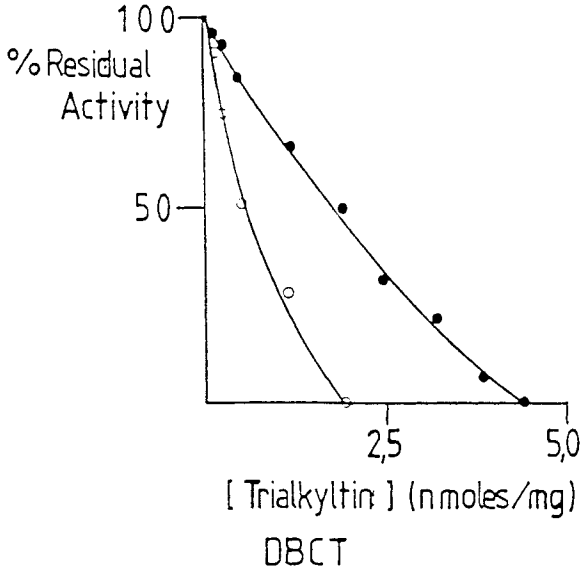

Figure 1 Inhibition of ATP synthesis and hydrolysis in mitochondria by DBCT [$Bu_2(CH_2Cl)SnCl$]. Estimates of ATP synthesis (●) and hydrolysis (○) were performed as described in the Methods section and expressed as percentiles of control rates without the addition of specific quantities of DBCT (38 nmol mg^{-1} min^{-1} and 79 nmol mg^{-1} min^{-1} respectively).

membrane potential ($\Delta\psi$) estimates caused a marked reduction in the energization capacity of the membrane; the depression in the maximal obtained fluorescent signal corresponded to the expected titre of triorganotin.

As may be seen in Table 2, certain triorganotin compounds studied here have the capacity to cause depolarization at concentrations which should not perturbate the integrity of the membrane. The exceptions to this are trimethyltin (which remains largely ineffective), tripropyltin and triphenyltin species. These compounds, although having potent effects on oxidative phosphorylation, does not cause depolarization as readily as other compounds tested here. This suggests these compounds probably have a truly 'oligomycin-like' effect in minimal halide media and merit further attention. However, triorganotins which undergo uncoupling activities may have a dual mode of action, even when assayed in the absence of chloride. They may simultaneously inhibit the ATPase complex in addition to causing profound uncoupling effects in mitochondria which may affect estimations of their ability to cause enzymic inhibition. The extremely active compound DBCT (5.0 nmol mg^{-1} protein to produce a +100 mV drop in Δp) will certainly affect oxidative phosphorylation as an uncoupler. In fact the concentration required to totally inhibit ATP synthesis is very similar to that required to depolarize the membrane potential. Uncoupling may even be its primary mode of action on oxidative phosphorylation. However, it remains a potent inhibitor of the ATP-hydrolase reaction.

Further experiments were performed to measure steady-state estimates of $\Delta\psi$ and pH utilizing the distributions of [^{3}H]TPMP$^+$, ^{86}Rb (in the presence of valinomycin) and [^{14}C]lactate. In general the $\Delta\psi$ estimates obtained from DSMP$^+$ fluorescence appeared higher than the latter two methods, particularly at lower membrane potentials. A typical titration with tributyltin chloride is presented in Fig. 2, where the estimates of $\Delta\psi$ differ by some 40 mV between the lowest estimates with ^{86}Rb$^+$ and the highest with DSMP$^+$. This phenomenon had been observed earlier by Mews and Rafael[23] (in a more exaggerated form) on decreasing the mitochondrial membrane potential by valinomycin-mediated potassium ion uptake. Certain differences in estimates may be inherent to each system rather than being solely due to artefacts of high potassium concentration as suggested by the previous workers.

Estimation of $\Delta\psi$, ΔpH and the resulting Δp are presented in Fig. 3 for titrations with various triorganotins. Two appropriate modes of action can be observed in Fig. 3, represented by DBCT and

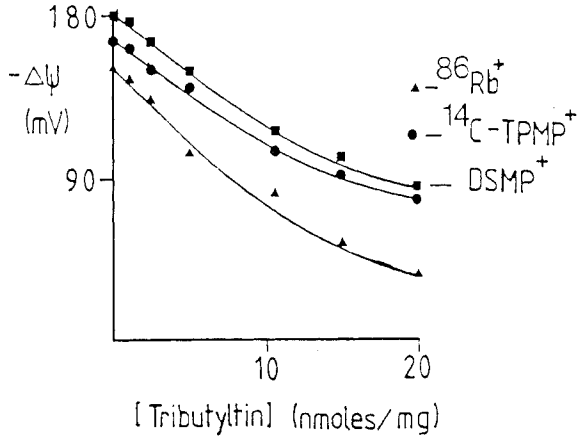

Figure 2 Variation in $\Delta\psi$ determination with ^{86}Rb$^+$, [^{3}H]TPMP$^+$ and DSMP$^+$ based on titration with tri-n-butyltin chloride. Mitochondria were preincubated with tributyltin chloride and diluted into the assay. The respective assays were performed as described in the Methods section. TPMP$^+$ (● , 2 μmol dm^{-3}, 1 μCi) and ^{86}Rb$^+$ (▲ , 50 μmol dm^{-3}, 0.2 μCi) were utilized in steady-state estimates and DSMP$^+$ (■ , 1 μmol dm^{-3}) was used in time-dependent fluorescence experiments.

triphenyltin chloride. DBCT is observed to titrate Δp from the onset of inhibition, whereas triphenyltin chloride has a more passive function until 10 nmol mg^{-1} protein, by which time ATP synthesis is only about 20% active. These observed differences are diagnostic of the two contrasting modes of action: triphenyltin acting as an 'oligomycin-like' inhibitor of oxidative phosphorylation, and DBCT operating principally by its uncoupling activity. The uncoupling function of triphenyltin does not reach an optimum until 25 nmol mg^{-1} protein are imposed upon the system, at which point the mitochondrial membrane may be disrupted and the enzymic functions of the ATPase complex has long since decreased.

DISCUSSION

It is clear from these results that inhibitory mechanisms of triorganotin species are complex. Triorganotins have been shown to inhibit proton conductivity in sub-mitochondrial particles and chloroplasts,[5,7,25] presumably by interaction with the F_0 moieties of mitochondrial and chloroplast $^+$H-ATPases. These effects should occur at low titres of triorganotin (i.e. those which will affect ATPase activity) since the findings reported here clearly show that higher titres of triorganotins depolarize membrane potential. The antagonistic effects may be rationalized when one considers the compounds which are relatively poor uncouplers and which in fact may show an increase in Δp at low titres of 2–3 nmol mg^{-1} protein. This finding suggests that the inhibitors may be preventing proton conduction, which in turn directly affects the hydrolysis or synthesis of ATP. Other triorganotins may produce similar effects but will be less apparent as other inhibitory functions also occur. Nevertheless, it appears triorganotin compounds arrest proton conduction at the level of the H^+-ATPase at low titres; whereas at higher titres they cause specific uncoupling and ultimately destroy the integrity of the membrane, resulting in the collapse of most energetic functions. The latter severe action is synonymous with the release of soluble proteins and possible lysis.[26]

The tin–anion bond (where anions such as chloride, acetate, phosphate and malate bound to tri-n-butyltin have all yielded similar results) appears as a possible candidate for the on-and-off movement of hydroxyl groups which may transverse the membrane and cause uncoupling. The probability that these reactions are

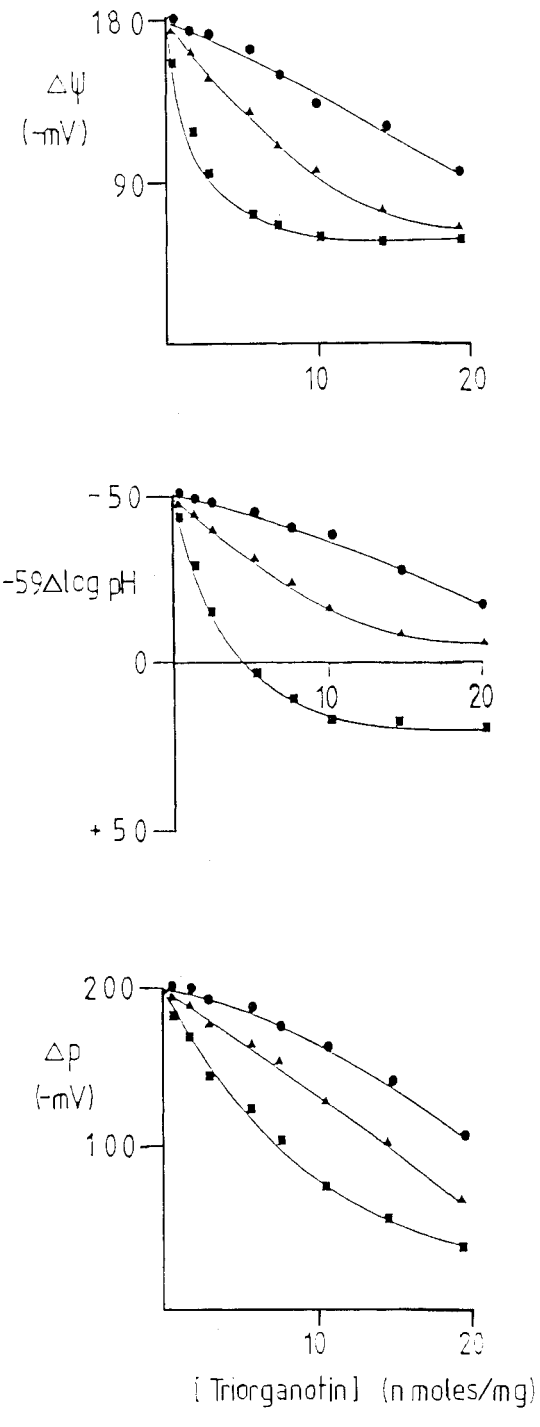

Figure 3 The effect of triorganotins on $\Delta\psi$, ΔpH and Δp in minimal halide media. Estimates of $\Delta\psi$ and Δp were based on those obtained with [^{3}H]TPMP$^+$. The distributions of 2 μmol dm^{-3} (1 μCi) [^{3}H]TPMP$^+$ and 25 μmol dm^{-3} (0.5 μCi) [^{14}C]lactate were measured as described in the Methods section. ■, DBCT; ●, triphenyltin chloride; ▲, tri-n-butyltin chloride.

pH- and/or lipid phase-dependent also exists. Therefore counter-equilibria may exist which will ultimately re-equilibriate protonic imbalances across the membrane, although any such scheme should result in a net change in charge to facilitate the fall in membrane potential. The chemical nature of these interactions remains unknown since the solvolysis of triorganotins may yield several molecular or ionic species. Clearly further chemical analyses are required to secure the exact nature of organotin related effects on membranes.

The phenomenon of continued ATP synthesis in the absence of the hydrolysis reaction had been predicted by Ferguson and Parsonage[27] for an inhibitor of the ATPase complex. They have postulated that an increase in Δp potentiated by the inhibition of proton conductance through F_0 may cause uninhibited ATP-synthase enzymes to increase their turnover rate and result in a compensated rate of oxidative phosphorylation over ATP hydrolytic activities. This is indeed possible when one considers the net stimulation of Δp at low concentrations of tri-n-propyl- and triphenyltins. However, the rate of ATP synthesis by the uninhibited enzymes would also have to be considerable.

In summary the inhibitory actions of triorganotin compounds on mitochondrial energy conservation are multi-faceted, ranging from a precise interaction with the F_0 portion of the ATPase complex through specific uncoupling to gross swelling and possible disaggregation. The uncoupling effect of triorganotin compounds is not solely attributable to halide/hydroxyl exchange. These actions must all occur simultaneously; the contribution of any given mechanism will depend upon the triorganotin species in use, the relative concentration of the compound and the conditions under which the mitochondria are subjected to the inhibitor.

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