The Action of Trialkyltin Compounds on Mitochondrial Respiration THE EFFECT OF pH

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1. Inhibition of 2,4-dinitrophenol-stimulated respiration by trialkyltins is dependent on the presence of Cl^- in the assay medium and is only apparent at acid pH values. It appears to be a result of the Cl^- - OH^- exchange mediated by trialkyltins. 2. In a KCl medium at alkaline pH values, the maximum rate of respiration produced by uncouplers is further increased by the presence of trialkyltins. 3. The inhibition of uncoupled succinate oxidation at acid pH values is not reversed by increasing the external substrate concentration, suggesting that depletion of intramitochondrial succinate is not an important factor in the inhibition. 4. It is suggested that the probable explanation for these observations is that in the presence of Cl^- trialkyltins alter the internal pH to a more acid value and this directly affects the activity of one or more steps in succinate oxidation. 5. The oligomycin-like action of trialkyltins in a Cl^- -free medium shows considerable pH-dependence over the pH range 6.6-7.6 in the presence of 10 mM-phosphate, but very much less pH-dependence in the presence of 1 mM-phosphate. 6. The binding of triethyltin to mitochondria shows a pK at pH 6.3 and does not change greatly over the pH range 6.6-7.6. 7. It is suggested that the pH-dependence of the oligomycin-like action described by Coleman & Palmer (1971) is the result of the pH-dependence of the formation of a hydrophilic complex between trialkyltins and P_i .

Aldridge (1958) found that trialkyltin compounds are potent inhibitors of ADP-stimulated respiration and also of 2,4-dinitrophenol-stimulated respiration. Sone & Hagihara (1964) and Stockdale *et al.* (1970) found inhibition of ADP-stimulated respiration but only very slight time-dependent inhibition of dinitrophenol-stimulated respiration. These contradictory results have been reconciled by the findings of Coleman & Palmer (1971), who showed that the inhibition of dinitrophenol-stimulated respiration by triethyltin is very pH-dependent and also depends on the ionic composition of the assay medium. At pH values below 7.0 in a medium containing Cl^- , the conditions used by Aldridge (1958), there is considerable inhibition of dinitrophenol-stimulated oxidation of succinate or NAD^+ -linked substrates, but not of ascorbate linked to tetramethyl-*p*-phenylenediamine. At the more alkaline pH values (7.4-7.6) used by Sone & Hagihara (1964) and by Stockdale *et al.* (1970) the inhibition is very much less. In a sucrose or potassium isethionate medium there is no inhibition even at acid pH values (Coleman & Palmer, 1971; Rose & Aldridge, 1972). The requirement of the presence of Cl^- for the inhibition of uncoupled respiration strongly suggests the involvement of the Cl^- - OH^- antiport catalysed by trialkyltins across the inner mitochondrial membrane (Selwyn *et al.*, 1970a). Coleman & Palmer (1971) found that

triethyltin was more effective at catalysing the Cl^- - OH^- exchange at more acid pH values and suggested this as a basis for the pH-dependence of the inhibition of uncoupled respiration, since the entry of Cl^- in exchange for OH^- would be expected to compete with the accumulation of substrate anions (Manger, 1969; Stockdale *et al.*, 1970).

A further effect of pH on the action of triethyltin on mitochondria is that in a sucrose medium, where complications due to the Cl^- - OH^- exchange are minimized, triethyltin is much less effective as an inhibitor of ADP-stimulated succinate oxidation at pH 6.6 than at 7.4 (Coleman & Palmer, 1971). This pH-dependence is the same for phosphorylation coupled to the oxidation of ascorbate-tetramethyl-*p*-phenylenediamine but in this case can also be observed in a Cl^- -containing medium.

The results in the present paper confirm and extend the observations of Coleman & Palmer (1971) on both aspects of the pH-dependence of the action of trialkyltin compounds on mitochondrial respiration. The observations reported here suggest that the inhibition of uncoupled respiration by trialkyltins may be due to changes in intramitochondrial pH values having a direct effect on the activity of the electron-transport chain or on the rate of utilization of intramitochondrial substrate rather than an effect on substrate accumulation. The effects of pH on the

inhibition of phosphorylation in a sucrose medium seem to be largely due to the formation of a lipid-insoluble complex between P_i and trialkyltins (Rose, 1969).

A preliminary account of these findings has appeared (Dawson *et al.*, 1972).

Experimental

Materials

Triethyltin sulphate was prepared from triethyltin hydroxide donated by the Tin Research Institute, Greenford, Middx., U.K., as described by Aldridge & Cremer (1955). Triethyltin chloride labelled with ^{113}Sn was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. at a specific radioactivity of $5\mu\text{Ci}/\mu\text{mol}$. Trimethyltin chloride, tripropyltin chloride and triphenyltin chloride were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. as was Mes buffer [2-(*N*-morpholino)ethanesulphonic acid]. Tributyltin chloride was from Ralph N. Emmanuel Ltd., Wembley, Middx., U.K. Hepes [2-(*N*-2-hydroxyethylpiperazine-*N'*-yl)ethanesulphuric acid], rotenone and succinic acid were obtained from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K. ADP was purchased from Boehringer Corp. (London) Ltd., London W.5, U.K. All other reagents were of A. R. grade.

Methods

Preparation of mitochondria. Rat liver mitochondria were prepared as described by Selwyn *et al.* (1970a). They were finally suspended in 0.25M-sucrose, 5mM-Hepes-KOH buffer, pH7.6, at a protein

concentration of 80mg/ml. Protein concentration was measured by the biuret method (Gornall *et al.*, 1949) after clarification with Triton X-100.

Simultaneous measurements of light-scattering, O_2 consumption and pH changes. These were carried out in the apparatus described by Stockdale *et al.* (1970). The apparatus was thermostatically controlled at 30°C.

Measurement of triethyltin binding. This was carried out by the method of Aldridge & Street (1970). Mitochondria (approximately 20 mg of protein) were incubated for 5 min at 20°C in 10 ml of experimental medium containing ^{113}Sn -labelled triethyltin. The tubes were then centrifuged at 40000g for 20 min at 4°C, the supernatants were decanted and the interior of the tubes was thoroughly wiped. The mitochondrial pellets were taken up in 2 ml of 0.5% Triton X-100. Samples (1.5 ml) of the dissolved pellets and of the supernatants were taken for liquid-scintillation counting. The scintillant used was 13.5 ml of Amersham-Searle PCS solubilizer (Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.). Counts were recorded on a Packard Tri-Carb scintillation counter. The counting efficiency obtained was 70% and at least 4000 counts above background were recorded for each sample. A ^{113}Sn -labelled triethyltin standard was included with each series of samples.

Results

Fig. 1 shows that, in agreement with the results of Coleman & Palmer (1971), instantaneous inhibition of dinitrophenol-stimulated respiration by trimethyltin is apparent at pH6.8 but not at pH7.6.

Fig. 1. Inhibition of dinitrophenol-stimulated succinate oxidation by trimethyltin

The medium contained, in a total volume of 6.5 ml: 0.1M-KCl; 10mM-potassium succinate; 5mM-Hepes-KOH buffer; 2μg of rotenone; 19mg of mitochondrial protein. (a) pH7.6; (b) pH6.8. Additions: (1) 0.125μmol of dinitrophenol; (2) 0.45μmol of trimethyltin chloride.

Fig. 2. Absence of inhibition of dinitrophenol-stimulated succinate oxidation by trimethyltin in a Cl^- -free medium

All assays contained, in 6.5 ml: 10 mM-succinate; 5 mM-Hepes-KOH buffer; 2 μg of rotenone; 19 mg of mitochondrial protein. (a) 0.25 M-sucrose, pH 7.6; (b) 0.25 M-sucrose, pH 6.8; (c) 0.1 M-potassium isethionate, pH 7.6; (d) 0.1 M-potassium isethionate, pH 6.8. Additions: (1) 0.125 μmol of dinitrophenol; (2) 0.45 μmol of trimethyltin chloride.

Fig. 3. Effect of trimethyltin concentration on the inhibition of dinitrophenol-stimulated succinate oxidation

Measurements were carried out in a total volume of 8.5 ml, containing 0.1 M-KCl, 10 mM-succinate, 19 μM -dinitrophenol, 2 mM-Hepes-KOH buffer, pH 6.8, 2 μg of rotenone and 14 mg of mitochondrial protein.

stimulated succinate oxidation with the ability to catalyse the Cl^- - OH^- exchange and with the effectiveness as an oligomycin-like agent. It can be seen that there is very good correlation with the former but not the latter property. There thus seems little doubt that the inhibition of dinitrophenol-stimulated respiration arises from the operation of the Cl^- - OH^- exchange, or at least depends on a very closely related chemical property of the trialkyltin which also requires the presence of Cl^- .

Although Coleman & Palmer (1971) suggested the pH-dependence of the Cl^- - OH^- exchange activity as the basis for the pH-dependence of the inhibition, this does not seem an entirely satisfactory explanation. First, it is apparent that even under conditions where no inhibition of respiration occurs (e.g., Fig. 1a) the Cl^- - OH^- exchange is still operative as judged by light-scattering changes. Secondly, although the Cl^- - OH^- exchange activity is only increased about fivefold as the pH value is lowered from 7.6 to 6.8 (Dr. A. S. Watling, personal communication), a trimethyltin concentration 50 times as great as that required for half-maximal inhibition at pH 6.8 has no inhibitory action at pH 7.6 (Fig. 1). Thirdly, if the pH-dependence of the inhibition is examined over a wide range (Fig. 5), it is found that above pH 7.6 there is stimulation of respiration on addition of trialkyltin compounds. This is still manifested at alkaline pH values when the dinitrophenol concentration used is optimum for release of respiratory control at that pH (Fig. 6), so that this phenomenon cannot apparently be ascribed to an uncoupling effect of the trialkyltin (Stockdale *et al.*, 1970) summing

Further, it depends upon the presence of Cl^- ions in the assay medium, since there is no inhibition at either pH value in a medium containing 0.25 M-sucrose or 0.1 M-potassium isethionate (Fig. 2). Isethionate is an anion to which rat liver mitochondria are impermeable (Selwyn *et al.*, 1970b) and which is ineffective in the trialkyltin-catalysed anion-hydroxide exchange (Rose & Aldridge, 1972). The degree of inhibition obtained under the conditions of Fig. 1(b) depends upon the concentration of trimethyltin used (Fig. 3). Half-maximal inhibition is obtained at approximately 1.5 μM -trimethyltin. In the case of the two other previously reported activities of the trialkyltins, the anion-hydroxide exchange and the oligomycin-like action, the concentration required to produce an effect depends on the chain length of the alkyl group of the trialkyltin used. We have obtained results similar to those shown in Fig. 3 for a variety of trialkyltin compounds and Fig. 4 shows the correlation of activity in inhibition of dinitrophenol-

Fig. 4. Correlation of inhibition of dinitrophenol-stimulated respiration with (a) activity in the Cl^- - OH^- exchange and (b) the oligomycin-like activity for various trialkyltin compounds

I_{50} is the concentration giving 50% of maximum inhibition of dinitrophenol-stimulated succinate oxidation under the conditions of Fig. 3. c is the concentration required to give a passive rate of swelling of 0.03 absorbance unit/min in 0.1 M-NaCl. P_{50} is the concentration causing 50% of maximum inhibition of phosphorylation in a 0.25 M-sucrose medium. (1) Triethyltin; (2) tripropyltin; (3) tributyltin; (4) triphenyltin; (5) trimethyltin. Data for c and P_{50} are taken from Fig. 2 of Stockdale *et al.* (1970), except for the values for triethyltin, which were obtained under similar conditions.

Fig. 5. pH-dependence of the inhibition of dinitrophenol-stimulated succinate oxidation by trimethyltin chloride

All assays contained, in 8.5 ml: 0.1 M-KCl; 10 mM-succinate; 2 mM-Hepes buffer; 2 μg of rotenone; 15 mg of mitochondrial protein. ○, 19 μM -Dinitrophenol; ●, 19 μM -dinitrophenol + 62 μM -trimethyltin chloride. pH was adjusted to the required value with HCl or KOH and monitored continuously.

with suboptimal uncoupling by dinitrophenol. Very similar results to those shown in Fig. 5 are obtained if the experiment is repeated with 0.10 mM-dinitrophenol throughout the pH range.

The hypothesis that inhibition of respiration arises from operation of the Cl^- - OH^- exchange leading to expulsion of intramitochondrial substrate owing to acidification of the matrix space (Manger, 1969) does not therefore explain the pH-dependence of the inhibition. Further, it predicts that the inhibition should be reversible by increasing the external substrate concentration. The observations of Aldridge

& Street (1971) are at variance with this prediction, as are the data in Fig. 7. There is only very slight release of the inhibition on increasing the external succinate concentration to approx. 50 mM.

An alternative interpretation of these phenomena rests on the predictable effects of trialkyltin compounds on the internal pH of the mitochondria. First, trialkyltins allow rapid titration of the matrix space of mitochondria by externally added acid in the presence of Cl^- ions (Selwyn *et al.*, 1970a). Secondly, the Cl^- - OH^- exchange, coupled with the Cl^- concentration of 100 mM outside the mitochondria, should cause the internal pH of the mitochondria to be skewed to a more acid value than the external pH. If the internal pH of the mitochondria influences the activity of one or more components of the electron-transport chain or the rate of utilization of intramitochondrial substrate, then these properties of the trialkyltin compounds would result in an apparent shift of the pH optimum for respiration to a more alkaline region. Such a shift would explain both the inhibition of respiration by trialkyltins at acid pH values and the stimulation at alkaline pH values. It would also explain why the inhibition at acid pH values is not complete (Fig. 3), since once sufficient trialkyltin is present to equilibrate the Cl^- and OH^- gradients, the internal pH value will be independent of the trialkyltin concentration. The amount of inhibition would then be determined by the activity of respiration at the new internal pH value.

According to the chemiosmotic hypothesis (Mitchell, 1966), normally respiring mitochondria would be expected to maintain the pH of the matrix space at a slightly alkaline value with respect to the external medium, the magnitude of this difference in pH being determined by the permeability properties

Fig. 6. Stimulation of dinitrophenol-stimulated respiration by trimethyltin at pH 8.2

The reaction medium contained, in 6.5 ml: 0.1 M-KCl; 10 mM-succinate; 5 mM-Hepes-KOH buffer, pH 8.2; 2 μ g of rotenone; 9.5 mg of mitochondrial protein. Additions: (1) 7.5 μ mol of dinitrophenol; (2) 0.45 μ mol of trimethyltin chloride; (3) 2.5 μ mol of dinitrophenol. Numbers in parentheses on the traces refer to O_2 uptake rate in ng-atoms of O_2 /min per mg of protein on the adjacent portion of the trace.

Fig. 7. Effect of succinate concentration on the degree of inhibition of dinitrophenol-stimulated respiration by trimethyltin

The total volume was 8.5 ml, containing 0.1 M-KCl, 5 mM-Hepes-KOH buffer, pH 6.8, 6.5 mg of mitochondrial protein, 19 μ M-dinitrophenol and 2 μ g of rotenone. ○, No addition; ●, 6.5 μ M-trimethyltin chloride.

Fig. 8. pH versus activity curve for frozen-thawed mitochondria

Experimental conditions were as described for Fig. 5. ○, 19 μ M-Dinitrophenol; ●, 19 μ M-dinitrophenol + 62 μ M-trimethyltin chloride.

of the inner mitochondrial membrane for the various ionic species present in the assay medium and by the metabolic state of the mitochondria. Thus the pH optimum as determined in the medium for the

respiration of coupled or only partially uncoupled mitochondria would not necessarily be a true measure of the optimum internal pH value. Evidence for this view comes from the observation (Fig. 8) that the pH optimum for succinate oxidation by mitochondria fragmented by freezing and thawing is at a considerably more alkaline value than that for whole mitochondria oxidizing succinate in the presence of a low

Fig. 9. pH changes caused by the addition of triethyltin

All assays contained, in 6.5 ml: 0.1 M-KCl; 10 mM-succinate; 1.6 mM-Hepes-KOH buffer; 19 μ M-dinitrophenol; 2 μ g of rotenone; 24 mg of mitochondrial protein. Additions: (1) 2 μ g of antimycin A; (2) 5 nmol of triethyltin sulphate; (3) 100 μ l of 5% (w/v) Triton X-100. The addition of triethyltin after Triton X-100 caused no pH change. Traces (a)–(c), pH 7.6. Traces (d)–(f), pH 6.8.

dinitrophenol concentration (Fig. 5). The curve shown in Fig. 8 is very similar to that obtained for whole mitochondria when both dinitrophenol and trimethyltin are present together (Fig. 5). Dinitrophenol and trimethyltin together or individually were without effect on frozen-thawed mitochondrial preparations.

By the use of a lightly buffered external medium it is possible to measure the pH changes caused by the addition of trialkyltin compounds under a variety of conditions. An experiment of this sort is shown in Fig. 9. At both pH 6.8 and 7.6, in a KCl medium containing 19 μ M-dinitrophenol, respiration maintains a disequilibrium of H^+ across the membrane, since either anaerobiosis or, in the case shown in the figure, addition of antimycin A, leads to the uptake of H^+ . This uptake amounts to 16.5 μ mol of H^+ /g of protein at pH 6.8 and 8.2 μ mol of H^+ /g of protein at pH 7.6. Lysis of the mitochondria by addition of Triton X-100 after this has essentially no effect on the pH value of the medium. However, addition of triethyltin sulphate after the cessation of respiration causes the apparent uptake of 11.0 μ mol of H^+ /g of protein at pH 6.8 and 7.7 μ mol/g at pH 7.6. At both pH values, this alkalinization of the external medium due to the addition of triethyltin is reversed by lysing the mitochondria by Triton X-100 and can therefore be ascribed to the pH imbalance resulting from the operation of Cl^- - OH^- exchange in the

presence of a Cl^- concentration gradient. Triethyltin has different effects on respiring mitochondria at the two pH values. At pH 6.8 it causes the uptake of 24.0 μ mol of H^+ /g of protein and there is only a very small further H^+ uptake on the addition of antimycin A. Of the H^+ taken up, 9.3 μ mol/g is released on lysis with Triton X-100. At pH 7.6, addition of triethyltin results in the uptake of 9.1 μ mol of H^+ /g, and subsequent addition of antimycin A causes the uptake of a further 7.7 μ mol/g. The difference between the results at the two pH values is explicable in terms of the inhibition of respiration by triethyltin at pH 6.8. It appears that respiration maintains a pH imbalance (alkaline inside) equivalent to 16.5 μ mol of H^+ /g of protein at pH 6.8 and 8.2 μ mol/g at pH 7.6. This gradient can apparently be maintained even in the presence of triethyltin, provided that respiration is not inhibited (e.g. at pH 7.6 in the absence of antimycin A). However, at both pH 6.8 and 7.6 addition of triethyltin skews the internal pH value towards the acid region owing to the operation of the Cl^- - OH^- exchange.

Effect of trialkyltins on phosphorylation in a sucrose medium

Fig. 10 compares the effect of trimethyltin on ADP-stimulated respiration at pH 6.8 and 7.6 for mitochondria oxidizing succinate in a sucrose medium

Fig. 10. Inhibition of ADP-stimulated O_2 uptake by trimethyltin chloride

The reaction medium contained, in 8.5 ml: 0.25M-sucrose; 2mM-Hepes-KOH buffer; 1 mM-EDTA; 10mM-succinate; 0.15mM-ADP; 14mg of mitochondrial protein. $\circ$, 10mM-Phosphate, pH 7.6; $\bullet$, 10mM-phosphate, pH 6.8; Δ , 1mM-phosphate, pH 7.6; $\blacktriangle$, 1mM-phosphate, pH 6.8. Phosphate and succinate were present as the potassium salts.

containing 10mM-potassium phosphate. Trimethyltin is very much less effective as an inhibitor at the lower pH value, in agreement with the results of Coleman & Palmer (1971). However, if the phosphate concentration is decreased to 1.0mM, the inhibitor is equally effective at the two pH values. Very similar results have been obtained for triethyltin.

Rose (1969) observed that trialkyltins form complexes with phosphate, as indicated by the ability of phosphate to decrease the partition of triethyltin from water into chloroform. This effect increased as the pH was decreased from 7.6 to 6.8. Since Coleman & Palmer (1971) used 10mM-phosphate in the medium for their phosphorylation experiments, it seemed possible that the pH-dependence they found may have been due to the pH-dependence of the stability of the trialkyltin-phosphate complex, rather than an effect of pH on the binding of trialkyltins to the inhibitory site on the mitochondria. The measurement of binding of trialkyltins to mitochondria is complicated by the presence of at least two classes of binding site (Aldridge & Street, 1970). The binding data can be fitted on the basis that there are a small number of high-affinity sites (0.8 nmol/mg of protein, association constant $4.7 \times 10^5 \text{ M}^{-1}$) and a large

Fig. 11. Effect of pH on the binding of triethyltin chloride to mitochondria

The incubation mixture was 10ml of 0.25M-sucrose, 10mM-Hepes-KOH buffer ($\circ$, Δ) or 10mM-Mes-KOH buffer ($\bullet$, $\blacktriangle$) and 21 mg of mitochondrial protein. ^{113}Sn -labelled triethyltin chloride was added to a final concentration of $0.5 \mu\text{M}$ ($\circ$, $\bullet$) or $16 \mu\text{M}$ (Δ , $\blacktriangle$). ^{113}Sn -labelled triethyltin gave approximately 1500c.p.m./nmol.

number of low-affinity sites (66 nmol/mg of protein, association constant $1.4 \times 10^3 \text{ M}^{-1}$). The large number of low-affinity sites means that even at low triethyltin concentrations, e.g., when less than 0.5 nmol of triethyltin is bound/mg of protein, the contributions to the binding by the low- and high-affinity sites are approximately equal. However, at higher concentrations of triethyltin, binding to the low-affinity sites greatly predominates. In looking at the effects of pH on binding it is therefore necessary to consider effects both on the high- and on the low-affinity sites. Rose & Aldridge (1972) have shown that the oligomycin-like activity of the trialkyltins is correlated with the degree of saturation of the high-affinity site, as long as anions active in the anion-hydroxide exchange are excluded from the medium. Fig. 11 shows a binding experiment carried out at two different triethyltin concentrations in a sucrose medium. At the low concentration, the maximum amount of triethyltin bound is approximately 0.08 nmol/mg of protein, whereas at the high concentration the maximum amount bound is about 2 nmol/mg . It can be seen that at the lower concentration the bound/free ratio changes considerably between pH 5.0 and 7.0, whereas at the higher concentration there is very much less change over this pH range. This indicates that the difference observed at the lower triethyltin concentration reflects a change in the number or affinity of the high-affinity sites and should therefore be reflected in a pH-dependence of the oligomycin-like action. However, the apparent pK

for the effect of pH on binding is at approximately pH 6.3 and the change in binding over the range pH 6.8–7.6 is relatively slight.

Discussion

As discussed above, the hypothesis put forward by Coleman & Palmer (1971), that the pH-dependence of the rate of the Cl^- – OH^- exchange is the basis for the pH-dependence of the inhibition of dinitrophenol-stimulated respiration, does not seem to be entirely satisfactory. However, the evidence is very strong that the inhibition of dinitrophenol-stimulated respiration by trialkyltins does arise through the operation of the Cl^- – OH^- exchange in a slightly acid medium. Since the inhibition cannot be relieved by increasing the external substrate concentration, it seems improbable that exclusion of the substrate by acidification of the matrix space is the fundamental cause of the inhibition. Harris *et al.* (1973) found that addition of trialkyltins to mitochondria treated with dinitrophenol in a Cl^- -containing medium results in the loss of some dinitrophenol from the mitochondria. However, recoupling due to exclusion of dinitrophenol is not a likely explanation of the observations, since the inhibition at acid pH values is not decreased by increasing the dinitrophenol concentration.

An alternative explanation is that there is a direct effect of the lowering of the pH value of the matrix space on the activity of either the transport systems or enzymes involved in substrate utilization or of the respiratory chain itself. The first question to be answered in this connexion is whether or not the internal pH change caused by the trialkyltin is sufficient to explain an apparent shift in the pH optimum for succinate oxidation from pH 6.9 to 7.5 (Fig. 5). Mitchell & Moyle (1969) calculated the internal buffering capacity of rat liver mitochondria to be of the order of $18 \mu\text{g}$ -ions of H^+ /pH unit per g of protein over this pH range. The H^+ uptake caused by the addition of triethyltin to respiring mitochondria at pH 7.6 is about $9 \mu\text{g}$ -ions of H^+ /g of protein, which should therefore be equivalent to an internal pH change of 0.5 unit, in good agreement with the observed shift in pH optimum of 0.6 pH unit.

A second point requiring explanation concerns the rates of O_2 uptake at the pH optima. In the absence of trialkyltin, the rate of O_2 uptake at the pH optimum, 6.9, is about $200 \mu\text{g}$ -atoms/min per g of protein under these conditions. In the presence of trialkyltin, at the new pH optimum of 7.5, the rate of O_2 uptake is only $130 \mu\text{g}$ -atoms/min per g of protein. It is clear that the pH versus activity curve is not merely shifted 0.6 pH unit towards the alkaline side, as is the case, for example, with the intramitochondrial glutaminase when a pH gradient is applied across the inner

membrane (Crompton *et al.*, 1973). If that were true, the rate of O_2 uptake at pH 7.6 in the presence of trialkyltin should be the same as that at pH 6.9 in the absence of trialkyltin. There is, however, a considerable body of evidence that supports the idea that the components of the respiratory chain are arranged in such a way that some are in contact with the intermembrane space and some with the matrix space (Mitchell, 1971). It would therefore be expected that both internal and external pH values would influence the rate of O_2 uptake. To that extent, the condition when the external pH is 6.9 is not directly comparable with that when the external pH is 7.6, even though the presence of trialkyltin at the higher pH value results in the internal pH values being similar under the two conditions. The pH versus activity curve for whole mitochondria in the presence of trimethyltin and dinitrophenol is remarkably similar to that for frozen-thawed mitochondria, and the absolute O_2 -uptake rates are also very comparable. This may be a reflexion of the inability of frozen-thawed mitochondria to maintain a pH difference across their inner membranes. However, the appearance or disappearance of titratable groups on disruption of the mitochondrial structure by freeze-thawing and the changes in permeability properties attendant on the process may also be involved in modifying the pH versus activity curve in this case.

Although the inhibition by trialkyltins of uncoupler-stimulated respiration at pH 6.8 appears to be the result of a decrease in the internal pH value of the mitochondria, it is still not clear which step is inhibited. Coleman & Palmer (1971) showed that the oxidation of both succinate and NAD^+ -linked substrates is affected, whereas that of tetramethyl-*p*-phenylenediamine and ascorbate is not. Further, they found that triethyltin caused a slight oxidation of cytochrome *b* under those conditions where it inhibited respiration. The site of inhibition appears therefore to be on the substrate side of cytochrome *b*.

The pH-dependence of the oligomycin-like action of the trialkyltins, which is best studied in a Cl^- -free medium, may be determined by two different factors. The first is that the binding of trialkyltin to phosphate, forming a hydrophilic complex, becomes greater at more acid pH values, being very significant at pH 7.0 and below. The second factor is the effect of pH on binding to the high-affinity binding site. Since pK for this second effect appears to be at about pH 6.3, it seems unlikely that this alone is the cause of the difference observed by Coleman & Palmer (1971) between pH 7.4 and 6.6 and that a large part of this difference can be ascribed to the binding of the trialkyltin to phosphate. This view is supported by the experiment described in Fig. 10, where at low phosphate concentrations the pH-dependence of the inhibition is decreased or abolished.

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