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THE INFLUENCE OF pH ON THE INHIBITION OF OXIDATIVE PHOSPHORYLATION AND ELECTRON TRANSPORT BY TRIETHYLTIN

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SUMMARY

The ability of triethyltin to inhibit oxidative phosphorylation and electron transport in tightly coupled rat liver mitochondria is very dependent on the pH and the ionic constitution of the assay medium.

1. In an assay medium containing Cl^- at an alkaline pH, above 7.1, triethyltin inhibited both the ADP stimulated rate of oxygen uptake and the dinitrophenol-induced ATPase (EC 3.6.1.3) but had no effect on the dinitrophenol-stimulated rate of oxygen uptake. If the pH was reduced to below 6.9 the pattern of inhibition changed and both the ADP and dinitrophenol-stimulated rates of oxygen uptake were inhibited by triethyltin.

2. In the absence of Cl^- in the medium triethyltin inhibited both the ADP-stimulated rate of oxygen uptake and dinitrophenol-induced ATPase and had no effect on the dinitrophenol-stimulated rate of oxygen uptake at either pH 7.4 or 6.6.

3. In either the presence or absence of Cl^- the ability of triethyltin to inhibit ATP synthesis appears to markedly decrease as the pH is lowered from 7.4 to 6.6.

4. The significance of these observations is discussed in relation to the operation of a Cl^-/OH^- antiport in the coupling membrane.

INTRODUCTION

Trialkyltin compounds have been shown to inhibit oxidative phosphorylation by acting directly on the energy conserving processes[1-4]. ALDRIDGE AND ROSE[5,6] have shown that the oxidation of pyruvate stimulated by ADP or dinitrophenol and the ATPase (EC 3.6.1.3) induced by dinitrophenol are all equally inhibited by triethyltin. However, they observed that the ATP synthesis coupled to the oxidation of ascorbate + *N,N,N',N'*-tetramethylphenylenediamine (TMPD) was very much less sensitive to triethyltin. These observations together with extensive studies concerning the site to which triethyltin binds in the mitochondrion have lead ALDRIDGE AND ROSE[6] to propose a new model to explain the mechanism of oxidative phosphorylation. It is implicit from this model that triethyltin acts on an intermediate involved in energy

Abbreviations: TMPD, *N,N,N',N'*-tetramethylphenylenediamine; FCCP, *p*-trifluoromethoxyphenylhydrazone.

conservation which is situated between the electron transport chain and the site of action of dinitrophenol.

Work in other laboratories, in particular that of SONE AND HAGIHARA[4] and STOCKDALE *et al.*[7] has shown that the pattern of inhibition produced by trialkyltin compounds is similar to that produced by oligomycin, in which the ADP-stimulated rate of electron transport is readily inhibited whilst the dinitrophenol-stimulated rate is much less sensitive. At present there is no adequate explanation why these two groups of investigators obtain such divergent results.

In addition to inhibiting oxidative phosphorylation trialkyltin compounds have other effects on the metabolism of mitochondria not exhibited by oligomycin. MANGER[8] has shown that trialkyltin can inhibit the intra-mitochondrial accumulation of substrate anions and SELWYN *et al.*[9] have demonstrated that trialkyltin compounds mediate a Cl^-/OH^- antiport across the inner mitochondrial membrane.

Work on triethyltin was initiated in our laboratory in the hope that we would be able to impose respiratory control in uncoupled mitochondria, early experiments in which the redox state of the cytochromes was measured showed that this was not possible. Subsequent experiments showed that the inhibitory pattern caused by triethyltin varied with the composition of the assay medium; the results are reported in this paper and could explain why different laboratories have, in the past, obtained different results.

MATERIALS AND METHODS

Rat liver mitochondria were obtained from adult Sprague–Dawley rats using the method of WEINBACH[10]. Triethyltin sulphate was prepared by the method of ALDRIDGE AND CREMER[11] from triethyltin hydroxide, a gift from the Tin Research Institute.

Oxygen uptake was measured in a Gilson Oxygraph oxygen electrode. The reaction medium normally used (volume 1.0 ml) contained 100 μmoles KCl, 10 μmoles $MgCl_2$, 25 μmoles sucrose, 1 μmole EDTA, 5 μmoles *N*-Tris-(hydroxymethyl)-methyl-2-aminoethane sulphonic acid, 10 μmoles KH_2PO_4 and mitochondria containing 2 mg protein, at 25°. The pH was adjusted to between 7.6 and 6.6 using KOH. Other additions were made as indicated in the text. When measuring respiration in the absence of Cl^-, a medium containing 250 μmoles sucrose, 1 μmole EDTA, 5 μmoles *N*-Tris-(hydroxymethyl)-methyl-2-amino ethanesulfonic acid, 10 μmoles KH_2PO_4 was used.

When measuring ATP synthesis accompanying ascorbate oxidation the phosphate was labelled with 200 μC ^{32}P per mmole and the ATP formed was measured using the method of AVRON[12].

Dinitrophenol-stimulated ATPase was measured by incubating mitochondria containing 2.0 mg of protein for 5 min in 1.0 ml of media similar to that used in the oxygen electrode, except that the phosphate was replaced by 2 μmoles of ATP and 20 nmoles dinitrophenol. The inorganic phosphate released was measured colorimetrically using the method of ERNSTER *et al.*[13].

Volume changes were measured in a medium (1.0 ml) containing 100 μmoles NH_4Cl, 10 μmoles *N*-Tris-(hydroxymethyl)-methyl-2-aminoethane sulfonic acid, 10 μg antimycin A, 1 μmole EDTA and mitochondria containing 1 mg protein. The

absorbance was measured at 500 nm in a Unicam SP 500 spectrophotometer attached to a Gilson absorbance converter.

Mitochondrial protein was determined using the biuret reagent after solubilization in deoxycholate[14].

RESULTS

Inhibition of electron transport

A preliminary series of experiments revealed that the pattern of inhibition of electron transport caused by triethyltin was dependent on the pH of the assay medium and the nature of the electron donor. Data in Fig. 1 show that if the pH was more acid than 7.0 triethyltin inhibits both the ADP and dinitrophenol-stimulated rates of oxygen uptake (*cf.* ALDRIDGE AND ROSE[6]). If, however, the pH of the medium was increased above 7.2 triethyltin appears to inhibit electron transport indirectly by imposing respiratory control, occurring as the result of inhibition of oxidative phosphorylation. It is apparent that triethyltin inhibits oxidative phosphorylation in a manner similar to oligomycin, causing inhibition of the ADP-stimulated rate of oxygen uptake but has no effect on the uncoupled rate of oxygen uptake (*cf.* SONE AND HAGIHARA[4]). The rate of oxidation of ascorbate *plus* TMPD in the presence of dinitrophenol was not inhibited at any pH used which is in contrast to data obtained using succinate as the electron donor. The ADP-stimulated rate of oxidation of ascorbate + TMPD was slightly inhibited over the whole pH range; in this respect it is similar to succinate. However, the rapid rate of oxidation and low level of respiratory control obtainable with this substrate made it difficult to assess accurately the effect of pH on the level of inhibition. It was possible to measure accurately the level of ATP synthesis coupled to the oxidation of ascorbate + TMPD and it can be seen from the data presented in Fig. 1 that the inhibitory activity of triethyltin increased as the pH of the medium was increased. The observation that below pH 7.0 triethyltin inhibited

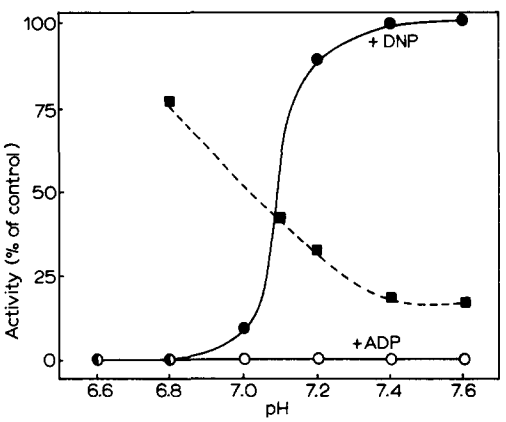

Fig. 1. The influence of pH on the inhibitory activity of triethyltin (10 μM). Parameters presented are: ○—○, oxygen uptake stimulated by adding ADP, 10 mM succinate used as the substrate; ●—●, oxygen uptake stimulated by adding 0.2 mM dinitrophenol (DNP), 10 mM succinate was used as the substrate; ■---■, ATP synthesis accompanying the oxidation of 16 mM ascorbate + 0.33 mM TMPD.

the uncoupled rate of succinate oxidation but was without effect on the uncoupled rate of ascorbate–TMPD oxidation is consistent with the suggestion that triethyltin may directly inhibit electron transport prior to cytochrome *c*. These data do not appear to be consistent with the model of oxidative phosphorylation proposed by ALDRIDGE AND ROSE[6] which would predict that the uncoupled rate of ascorbate–TMPD oxidation should be more sensitive to triethyltin than the coupled rate.

Mitochondrial volume changes in the presence of triethyltin

SELWYN *et al.*[9] have shown that many trialkyltin compounds cause rapid swelling of mitochondria suspended in a medium containing Cl^-. They suggest that this occurs as a result of the trialkyltin compounds activating a Cl^-/OH^- antiport across the inner membrane. This group of workers have, however, not produced any data for triethyltin. Data presented in Fig. 2 show the influence of pH on the ability of triethyltin to activate swelling in mitochondria suspended in 0.1 M NH_4Cl. Changing the pH from 7.4 to 6.8 results in an steady increase in both the initial rate and the final amplitude of the volume change. It thus seems apparent that acidification of the assay medium results in an increased activation of the Cl^-/OH^- antiport which could lead to a collapse of the pH gradient across the coupling membrane and inhibition of succinate accumulation.

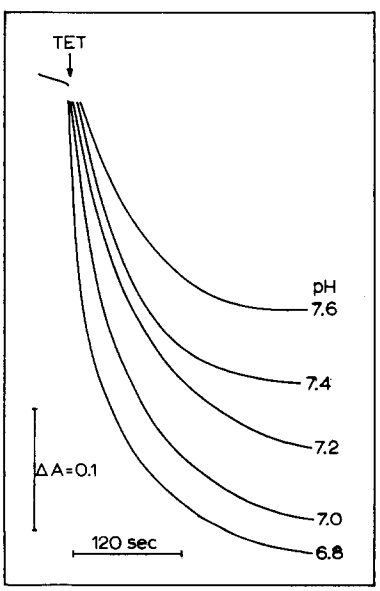

Fig. 2. The influence of pH on the swelling, measured by absorbance decrease at 500 nm, induced by addition of 0.5 nmoles triethyltin (TET) when the mitochondria were suspended in 0.1 M $(NH_4)Cl$ containing antimycin (10 μg/mg protein). Reaction volume, 1.0 ml.

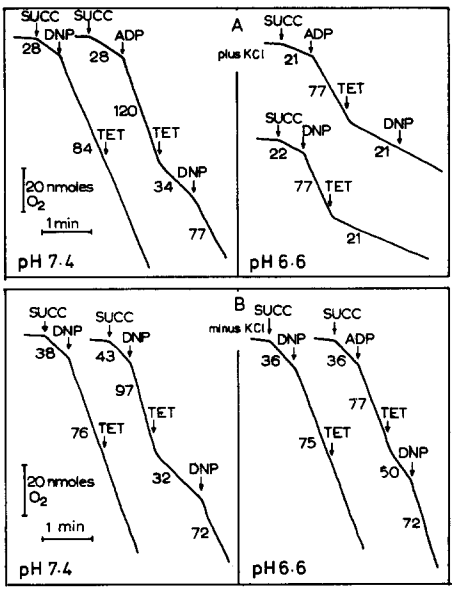

Fig. 3. An oxygen electrode trace showing the inhibitory effect of triethyltin (TET) on coupled and uncoupled oxygen uptake in (A) sucrose + KCl medium, and (B) a sucrose *minus* KCl medium. The figures under the traces are the rate of oxygen uptake in nmoles of oxygen/mg protein per min. DNP, dinitrophenol; SUCC, succinate (10 mM).

The inhibition of electron transport in the presence or absence of Cl^-

Since it has been shown[9] that trialkyltin compounds act specifically to activate a Cl^-/OH^- antiport, experiments were carried out to investigate the influence of the presence of Cl^- on the inhibitory activity of triethyltin shown in Fig. 1. The experiments were carried out at either pH 7.4 or 6.6 in the presence or absence of Cl^-. Data presented in Fig. 3A show the results obtained in the presence of 0.1 M KCl. At pH 7.4, triethyltin appeared to act like oligomycin, inhibiting the ADP stimulated rate of oxygen uptake which could be reversed on the addition of dinitrophenol. At pH 6.6, triethyltin inhibited both the ADP and dinitrophenol-stimulated rates of oxygen uptake. Data in Fig. 3B show that in the absence of KCl triethyltin appeared to act like oligomycin at either pH 7.4 or 6.6. It is also apparent from Fig. 3B that as the pH was decreased from 7.4 to 6.6 the inhibition of the ADP stimulated rate of electron transport by triethyltin was considerably decreased. This observation is in agreement with the similar reduction of inhibition of ATP synthesis accompanying the oxidation of ascorbate–TMPD shown in Fig. 1.

The influence of triethyltin on the activity of the ATPase

Triethyltin has been reported to inhibit dinitrophenol-induced ATPase. Results presented in Fig. 4 show how this activity was influenced by the pH of the assay medium. Data in Fig. 4A were obtained in the presence of 100 mM KCl. It can be seen that as the pH was decreased the dinitrophenol-induced ATPase activity in the control treatment was reduced, due to the unfavourable H^+ concentration. At pH 7.4 triethyltin completely inhibited the dinitrophenol-induced ATPase but as the pH was reduced some ATPase activity was obtained in the presence of the inhibitor. However, it is apparent that an equal level of ATPase activity was obtained in the presence of triethyltin alone. If the experiment was repeated in the absence of KCl (Fig. 4B) it can be seen that the ATPase activity could only be obtained in the presence of tri-

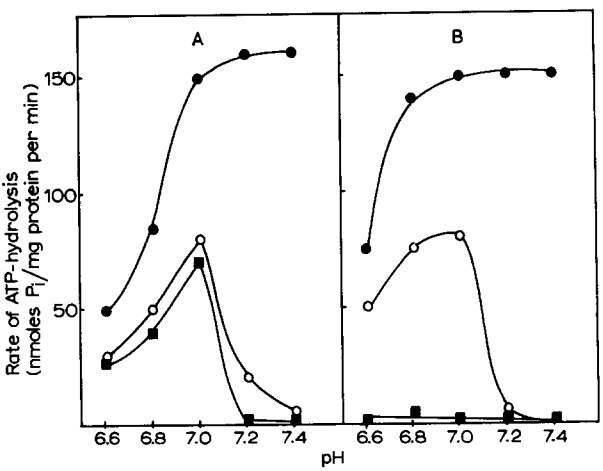

Fig. 4. The influence of pH on inhibition of dinitrophenol-stimulated ATPase by triethyltin. Data obtained in a sucrose *plus* Cl^- medium is given in section A, and data obtained in a sucrose *minus* Cl^- medium is given in section B. ●—●, rate of ATP hydrolysis in presence of 0.2 mM dinitrophenol; ○—○, rate of ATP hydrolysis in presence of 0.2 mM dinitrophenol + 10 μM triethyltin; ■—■, rate of ATP hydrolysis in presence of 10 μM triethyltin alone.

ethyltin below pH 7.0 and in the presence of dinitrophenol. Therefore triethyltin alone can only induce ATPase activity in the presence of Cl^- below pH 7.0 and presumably depends on the activation of Cl^-/OH^- antiport system. It is apparent that, in the absence of Cl^-, lowering the pH of the medium reduces the inhibitory activity of triethyltin on the phosphorylating system.

The effect of triethyltin on the redox state of cytochrome b

The inhibition of the dinitrophenol-stimulated rate of oxygen uptake by triethyltin required the presence of Cl^- (*cf.* STOCKDALE *et al.*[7]) and a slighlty acid pH, conditions which favour the operation of a Cl^-/OH^- antiport. The accumulation of Cl^- in exchange for OH^- would be expected to be competitive with the accumulation of substrate anions[8,9]. Thus the inhibition of electron transport under these conditions could result from the lack of electron donor. The alternative explanation for the inhibition of the uncoupled rate of electron transport could be that triethyltin reimposes respiratory control on the uncoupled system, as predicted by the model proposed by ALDRIDGE AND ROSE[6]. To investigate this possibility the redox state of cytochrome *b* was measured in a dual wavelength spectrophotometer. The trace presented in Fig. 5 shows that cytochrome *b* became reduced when succinate was added, the addition of a small amount of ADP caused a transient oxidation while it was being phosphorylated, indicating the existence of a control point between cytochrome *b* and *c*. The

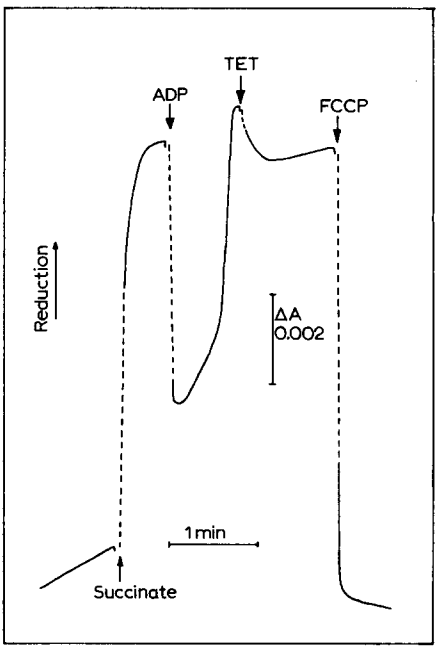

Fig. 5. The level of cytochrome *b* reduction in the presence of triethyltin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP). 3 mg of mitochondrial protein were suspended in 2.5 ml of medium containing KCl at pH 6.8, and the redox state of cytochrome *b* was measured using the wavelength pair 434 and 410 nm. The following additions were made in this order, 30 μmoles of succinate, 0.3 μmoles of ADP, 0.05 μmoles of triethyltin (TET) and 10 nmoles of FCCP.

addition of triethyltin has very little effect on the redox state, causing a slight oxidation; subsequent addition of *p*-trifluoro-methoxyphenylhydrazone (FCCP) resulted in the complete oxidation of cytochrome *b*. This observation is not in agreement with the predictions of ALDRIDGE AND ROSE[6] that triethyltin inhibits the uncoupled electron transport by reimposing respiratory control. The data are not inconsistent with the suggestion that electron transport is inhibited by triethyltin at a point in the electron transport chain prior to cytochrome *b* since the respiratory activity was not increased by adding FCCP under these conditions.

DISCUSSION

The results presented in this paper show that in the presence of Cl^- triethyltin can exhibit two patterns of inhibition of mitochondrial metabolism depending on the pH of the assay medium. Above pH 7.0 triethyltin acted like oligomycin and inhibited the ADP-stimulated rate of electron transport and the dinitrophenol-induced ATPase. However, below pH 7.0 triethyltin inhibited both the ADP and dinitrophenol-stimulated rates of oxygen uptake and actually induced an ATPase activity. In the absence of Cl^- in the medium triethyltin acted like oligomycin at all the pH values tested; the inhibition exerted on the phosphorylating system appeared to become less as the pH was reduced, which was particularly noticeable when measuring the inhibition of the ATPase activity (Fig. 4B). In order to obtain an equal inhibition of the ADP and dinitriphenol-stimulated rates of electron transport and the dinitrophenol-induced ATPase necessary to support the model proposed by ALDRIDGE AND ROSE[6] the experiment has to becarried out in medium containing Cl^- at a pH below 7.0. These conditions favour the efficient operation of the Cl^-/OH^- antiport activated by triethyltin, which would allow Cl^- to compete with substrate anions for uptake into the mitochondrion[9]. This process could lead to an inhibition of electron transport from electron donors such as pyruvate or succinate which have to enter the mitochondria before being oxidised[15] while it would have no effect on the oxidation of ascorbate–TMPD which donates electrons to cytochrome *c* on the outside of the inner membrane[16]. Such a situation offers an alternative explanation to the one advanced by ALDRIDGE AND ROSE[6] to account for the apparent difference in sensitivity to triethyltin of phosphorylation coupled to the oxidation of ascorbate–TMPD and pyruvate.

It is apparent that the pattern of inhibition caused by triethyltin changes around pH 7.0; it is significant to note that ROSE[11] reported that the pK of the group responsible for binding triethyltin to rat haemoglobin is 7.1. Thus it is possible that the changing pattern of inhibition exhibited as the pH reduced from 7.4 to 6.6 may reflect the changing affinity of the binding group for triethyltin as the degree of dissociation alters, the binding being less strong at the acid pH resulting in a reduction in the level of inhibition of phosphorylation and an increase in the concentration of triethyltin which is available to participate in other types of inhibitory activity. The dependence of the inhibitory activity of the uncoupled rate of electron transport on the pH of the medium appears to be too abrupt to be solely accounted for by the changing degree of dissocation of a single ionisable group and may mean that more than one type of binding group is involved in bringing about the inhibitory patterns described in this paper.

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