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The Action of Tributyltin on Energy Coupling in Coupling-Factor-Deficient Submitochondrial Particles

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1. Tributyltin at concentrations of approx. 1 nmol/mg of protein induces respiratory control and lessens the proton permeability of coupling-factor-deficient submitochondrial particles. 2. At these concentrations or lower, it increases the P/O ratio of the particles to a small extent and inhibits the adenosine triphosphatase activity without greatly increasing its sensitivity to uncoupling agents. 3. It fails to stimulate ATP-driven reversed electron transport or transhydrogenase, but stimulates the transhydrogenase driven by aerobic succinate oxidation. 4. The results indicate that, unlike oligomycin, tributyltin does not discriminate between damaged and intact ATP-synthesizing complexes. 5. The relationship between the oligomycin- and tributyltin-binding sites is discussed.

The action of trialkyltin compounds on mitochondria has been shown to be twofold (Stockdale *et al.*, 1970; Rose & Aldridge, 1972). The first mode of action, which results in some uncoupling (Stockdale *et al.*, 1970) and expulsion (Manger, 1969) of mitochondrial substrates, is the catalysis of an anion–hydroxide exchange across the inner mitochondrial membrane (Selwyn *et al.*, 1970). The second mode of action is similar to that of oligomycin, resulting in inhibition of phosphorylation and of the mitochondrial ATPase* (Stockdale *et al.*, 1970; Rose & Aldridge, 1972; Selwyn *et al.*, 1972). In media which are free of Cl^- and other anions active in the anion–hydroxide exchange, the second mode of action can be studied in isolation. This oligomycin-like activity has been shown to be correlated with the saturation of a high-affinity binding site for trialkyltins on the mitochondrial membrane (Rose & Aldridge, 1972). For rat liver mitochondria, the concentration of this binding site appears to be of the order of 0.8 nmol/mg of mitochondrial protein.

In view of their simple chemical nature and the relative ease with which binding parameters can be determined, trialkyltins are potentially useful probes for studying the mechanism of action of the mitochondrial proton-translocating ATPase. However, although it has been established that these compounds inhibit the membrane-bound ATPase but not the isolated F_1 ATPase (Kagawa & Racker, 1966; Selwyn *et al.*, 1972), their site of action has not been further localized and is difficult to relate to the action of, for example, oligomycin or dicyclohexylcarbodi-imide.

In order to clarify this situation, we have studied the effect of trialkyltin compounds on the energy-linked functions of coupling-factor-deficient ox heart submitochondrial particles. The action of oligomycin (Lee & Ernster, 1965, 1966), and dicyclohexylcarbodi-imide (Roberton *et al.*, 1968) is well established. Both compounds, at concentrations of less than 1 nmol/mg of protein, induce respiratory control and stimulate reversed electron transport, driven by either respiration or ATP hydrolysis. In addition, oligomycin has been shown to decrease the proton permeability of submitochondrial particles (Papa *et al.*, 1970; Hinkle & Horstman, 1971) and to stimulate oxidative phosphorylation (Lee & Ernster, 1966). The compound which we have used for the study is tributyltin, which is the most potent of the trialkyltins in terms of its oligomycin-like action (Stockdale *et al.*, 1970). Under the conditions described in the present paper, it is effective in the same concentration range as oligomycin and dicyclohexylcarbodi-imide, i.e. up to about 1 nmol/mg of protein.

* Abbreviation: ATPase, adenosine triphosphatase.

Materials and Methods

Materials

Oligomycin, NAD^+, NADH, $NADP^+$ and catalase (EC 1.11.1.6) were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. ADP, ATP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, yeast alcohol dehydrogenase (EC 1.1.1.1), hexokinase (EC 2.7.1.1) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) were purchased from Boehringer Corp. (London) Ltd., Lewes, East Sussex, U.K. Tributyltin chloride was from Aldrich Chemical Co., Wembley, Middx. HA0 1PY, U.K., and valinomycin was purchased from Calbiochem Ltd., London W1H 1AS, U.K. Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid] was from Hopkin and Williams Ltd., Chadwell

Heath, Essex, U.K. All other reagents were of A.R. or highest available grade.

Methods

Preparation of mitochondria. Heavy ox heart mitochondria were prepared by the Waring-Blendor method of Smith (1967), except that succinate was omitted from the isolation medium.

Submitochondrial particles. 'Alkaline-EDTA' particles (defined by the method of preparation) were prepared by the method of Lee *et al.* (1964). Ox heart heavy mitochondria (20mg of protein/ml) were suspended in 250mM-sucrose–1mM-EDTA adjusted to pH8.5 with KOH. Sonication was for 1min at maximum output on an M.S.E. 50W ultrasonic disintegrator, the sample being cooled in an ice–ethanol bath. 'Alkaline-EDTA' particles were isolated by differential centrifugation and finally suspended at a concentration of approx. 20mg of protein/ml in 250mM-sucrose–1mM-EDTA, adjusted to pH7.6 with KOH.

'Ammonia' particles (defined by the method of preparation) were prepared by the method of Fessenden & Racker (1967), except that the sonication conditions were as described above for 'alkaline-EDTA' particles.

ATPase assays. The medium for the assay of ATPase activity of submitochondrial particles contained, in a volume of 1ml: 120mM-sucrose, 33mM-Tris–acetate buffer, pH7.5, 4mM-$MgSO_4$ and about 0.1mg of particle protein. The reaction was started by the addition of 5mM-ATP and incubation was for 5min at 30°C. The reaction was stopped by the addition of 0.5ml of 10% (w/v) trichloroacetic acid and, after centrifugation, P_i was measured by the method of Fiske & SubbaRow (1925). Where present, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (1μM final concentration), oligomycin and tributyltin were added as small volumes of ethanolic solutions before the submitochondrial particles.

Measurement of pH changes, oxygen uptake and P/O ratios. pH changes produced on addition of O_2 to submitochondrial particles (Mitchell & Moyle, 1965) were measured by using the apparatus described by Stockdale *et al.* (1970). The recorder had a full-scale deflexion of 0.01 pH unit. The reaction medium contained, in a volume of 5ml: 250mM-sucrose, 10mM-potassium hydroxyethyl sulphonate (isethionate), 1mM-Hepes–KOH, pH7.5, 10mM-potassium succinate, 0.4mg of catalase, 2μg of valinomycin and 4 mg of submitochondrial-particle protein. The oxygen pulse was provided by the addition of 1μl of 0.2% H_2O_2. Proton uptake was calibrated by the addition of HCl. The temperature was 30°C.

Oxygen uptake was measured with an oxygen electrode. For the measurement of P/O ratios the reaction mixture contained, in a volume of 4ml: 145mM-sucrose, 40mM-Tris–acetate, pH7.5, 15mM-glucose, 2mM-$MgSO_4$, 5mM-ADP, 3mM-potassium phosphate, 1mM-NADH and 50μg (7 units) of yeast hexokinase. The reaction was started by the addition of submitochondrial particles (about 0.5mg of protein). After 3min, a 1ml sample was removed from the oxygen-electrode vessel and added to a tube containing 0.1ml of 35% (w/v) $HClO_4$. The supernatant was neutralized with $KHCO_3$ and, after centrifugation, glucose 6-phosphate was determined in a 1ml portion by the method of Lang & Michal (1974). $NADP^+$ reduction in the presence of glucose 6-phosphate dehydrogenase was followed at 340nm. Correction was made for ATP formation by myokinase activity by running an assay for the same length of time without NADH but otherwise as for the experimental determinations. Oligomycin or tributyltin chloride were added as ethanolic solutions at the start of the reaction.

Reversed electron transport and energy-linked transhydrogenase. The reduction of NAD^+ or $NADP^+$ was followed at 340nm in a recording spectrophotometer. The temperature was 30°C in both cases.

The reduction of NAD^+ by succinate in the presence of ATP was assayed as described by Ernster & Lee (1967). The reaction mixture contained, in a volume of 3ml: 140mM-sucrose, 40mM-Tris–acetate, pH7.5, 0.2mM-NAD^+, 5mM-succinate, 1mM-KCN, 10mM-$MgSO_4$ and approx. 0.5mg of submitochondrial-particle protein. The reaction was started by the addition of 3mM-ATP.

The energy-linked transhydrogenase was measured by the method of Danielson & Ernster (1963). The assay mixture contained 140mM-sucrose, 40mM-Tris–acetate, pH7.5, 50mM-ethanol, 10mM-$MgSO_4$, 0.02mM-NAD^+, 0.2mM-$NADP^+$, 0.25mg of alcohol dehydrogenase, 1μg of rotenone and about 0.5mg of submitochondrial-particle protein in a total volume of 3ml. The reaction was started by the addition of either 5mM-succinate or 3mM-ATP.

Assay of protein. Protein was assayed by the biuret method (Gornall *et al.*, 1949) after solubilization with Triton X-100.

Results

Fig. 1 shows that the addition of tributyltin chloride to 'ammonia' particles in a medium free of anions that are active in the anion–hydroxide exchange results in an inhibition of NADH oxidation. The resulting slower rate of oxygen uptake can be restored to its previous value by the addition of an uncoupling agent. The dependence of the inhibition on the quantity of tributyltin added is shown in Fig. 2. A maximal effect is obtained at approx. 1nmol/mg of protein.

Oligomycin has been shown to increase the extent of inward proton translocation by anaerobic submitochondrial particles in response to an oxygen pulse,

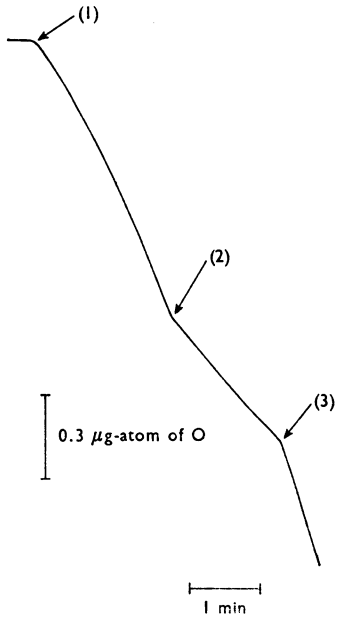

Fig. 1. *Effect of tributyltin on the rate of oxygen uptake of 'ammonia' particles*

The assay mixture contained in 4ml: 180mM-sucrose, 50mM-Tris–acetate, pH7.6, and 1mg of protein of 'ammonia' particles. Additions: (1) 2.5mM-NADH, (2) 2.4nmol of tributyltin, (3) 1μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

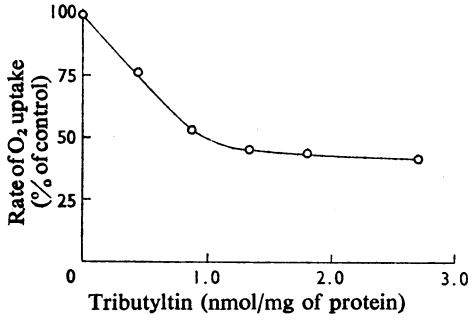

Fig. 2. *Dependence of restoration of respiratory control on tributyltin concentration*

Conditions were as described for Fig. 1. Each assay contained 1mg of protein of 'ammonia' particles.

particularly in the presence of a permeant counter-ion such as K^+ (plus valinomycin) (Papa *et al.*, 1970; Hinkle & Horstman, 1971). Oligomycin also causes a decrease in the rate at which protons re-equilibrate

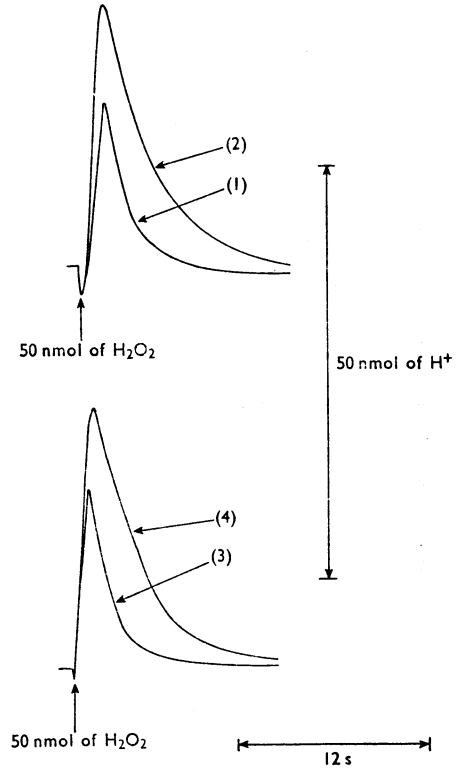

Fig. 3. *Effect of tributyltin and oligomycin on proton translocation in response to oxygen pulses*

The composition of the medium was as described under 'Methods'. Each assay contained 3.6mg of 'ammonia'-particle protein. Traces (1) and (3), no further additions; trace (2), after addition of 1nmol of tributyltin/mg of protein; trace (4), after addition of 1nmol of oligomycin/mg of protein. Half-times for the decay of the pH change after exhaustion of O_2 were: trace (1), 0.7s; trace (2), 2.2s; trace (3), 0.7s; trace (4), 2.2s.

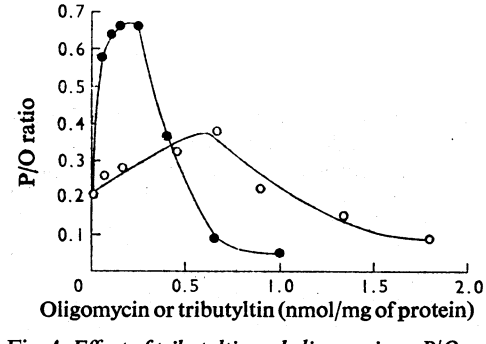

Fig. 4. *Effect of tributyltin and oligomycin on P/O ratio*

P/O ratios were determined as described under 'Methods'. Each assay contained 0.45mg of protein of 'alkaline-EDTA' particles in the original 4ml volume. ○, Tributyltin; ●, oligomycin.

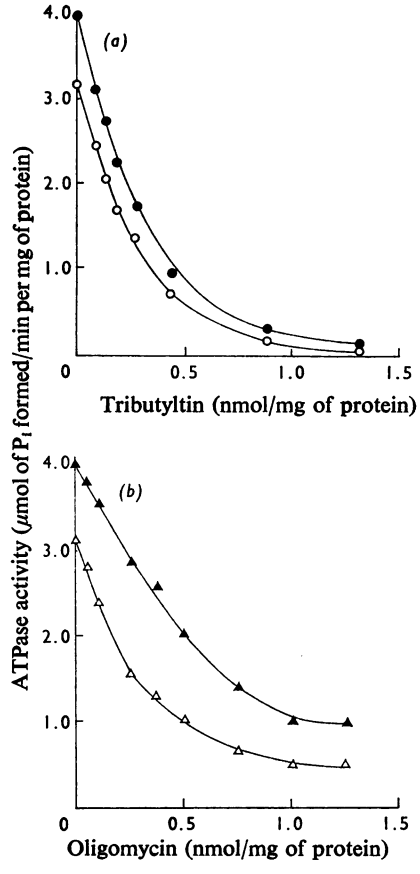

Fig. 5. *Inhibition of ATPase activity of 'alkaline-EDTA' particles by tributyltin and oligomycin*

ATPase activity was assayed as described under 'Methods'. Each assay contained 0.09mg of particle protein. (*a*) ○, Tributyltin; ●, tributyltin+1 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; (*b*) △, oligomycin; ▲, oligomycin+1 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

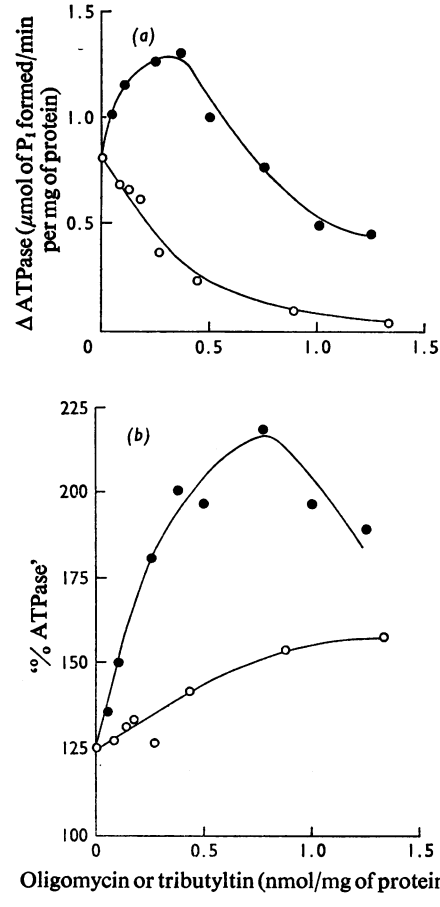

Fig. 6. *Effects of tributyltin and oligomycin on the uncoupler-sensitivity of the ATPase of 'alkaline-EDTA' particles*

(*a*) ΔATPase is the absolute difference between the ATPase activities measured in the presence and absence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. Data are taken from Fig. 5; ○, tributyltin (from Fig. 5*a*); ●, oligomycin (from Fig. 5*b*). (*b*) '% ATPase' is the ATPase activity in the presence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone as a percentage of the activity in its absence. Data are taken from Fig. 5; ○, tributyltin (from Fig. 5*a*); ●, oligomycin (from Fig. 5*b*).

across the membrane when the oxygen pulse is exhausted, and Hinkle & Horstman (1971) have shown a correlation between the half-time for this process and the rate of respiration. Since in terms of the chemiosmotic hypothesis it is the passive permeability of the membrane to protons which determines the resting rate of respiration, it would be expected that tributyltin, which has a similar effect to that of oligomycin in restoring respiratory control, would also have a similar effect on proton translocation. Fig. 3 shows that this is indeed the case. Optimum quantities of oligomycin and tributyltin added to 'ammonia' particles cause similar increases in the extent of proton uptake in response to an oxygen pulse compared with the control. Both compounds increase the half-time for the decay process from 0.7s to 2.2s. The addition of tributyltin and oligomycin together in these quantities does not enhance the effect observed with either inhibitor alone.

Compared with oligomycin (Lee & Ernster, 1965, 1966) tributyltin is relatively ineffective at increasing the P/O ratio of coupling-actor-deficient submitochondrial particles (Fig. 4). Maximum enhancement of the P/O ratio by tributyltin is produced by about

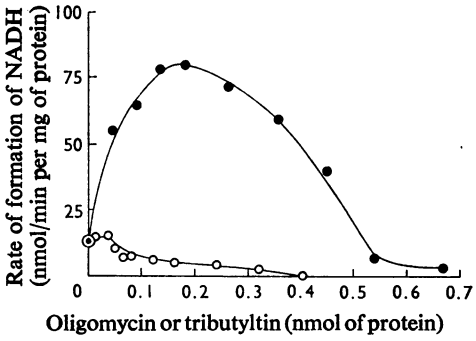

Fig. 7. *Effects of tributyltin and oligomycin on the rate of reversed electron transport from succinate to NAD$^+$ driven by ATP hydrolysis*

The reaction mixture contained 0.45mg of protein of 'ammonia' particles. Other conditions were as described in the text. ○, Tributyltin; ●, oligomycin.

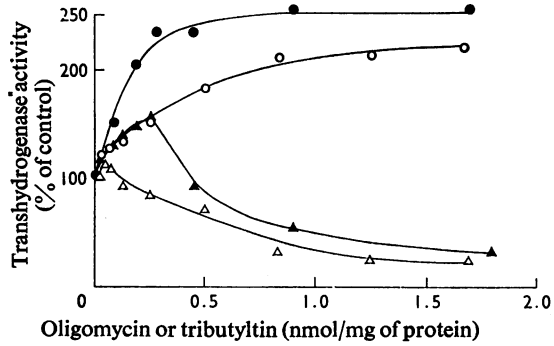

Fig. 8. *Effects of tributyltin and oligomycin on the transhydrogenase activity of 'alkaline-EDTA' particles*

Each assay contained 0.48mg of particle protein. Other conditions were as described in the text. ○, Tributyltin, 5mM-succinate as energy source; ●, oligomycin, 5mM-succinate as energy source; ▲, tributyltin, 3mM-ATP as energy source; △, oligomycin, 3mM-ATP as energy source. The rates of NADPH production were not corrected for the non-energy-dependent rate.

0.7nmol/mg of protein, which is considerably greater than the amount of oligomycin required (0.3nmol/mg of protein, for the same batch of particles). At this maximum, the P/O ratio obtained with tributyltin is only about 0.4, compared with a value of approx. 0.7 obtained with oligomycin. The increase in P/O ratio found for tributyltin is largely due to a decrease in oxygen uptake, rather than an increase in the net rate of ATP synthesis (results not shown), whereas with oligomycin, changes in both parameters contribute to the increase in the P/O ratio.

Although production of a maximal P/O ratio requires a greater concentration of tribultyltin than of oligomycin, this does not reflect the relative potencies of the two compounds as inhibitors of the mitochondrial ATPase. Fig. 5 shows that tributyltin is slightly more effective as an inhibitor of the ATPase than is oligomycin, both in the absence and in the presence of an uncoupling agent. However, it has been found previously (Lee & Ernster, 1966) that oligomycin at low concentrations increases the effect of uncoupling agents on the ATPase of coupling factor-deficient particles. This can be seen both as an absolute increase in the amount of ATPase activity stimulated by uncouplers (ΔATPase) and also as an increase in the ATPase activity in the presence of the uncoupler expressed as a fraction of that in the absence of the uncoupler. In Fig. 6, data from Fig. 5 are expressed in these terms. At all of the concentrations used, tributyltin causes a decrease in ΔATPase, in contrast with oligomycin, which at low concentrations causes an increase in ΔATPase activity, the effect being maximal at 0.3–0.4nmol of oligomycin/mg of protein. Tributyltin does, however, cause an increase in uncoupler sensitivity in percentage terms, although its effect is small compared with that of oligomycin.

These results suggest that tributyltin should be less effective than oligomycin at stimulating ATP-driven energy-linked functions of submitochondrial particles, since it would be expected that the efficiency with which ATP could drive, for example, reversed electron transport or the transhydrogenase would be related to the uncoupler sensitivity of the ATPase. Fig. 7 shows the actions of oligomycin and tributyltin on ATP-driven reversed electron transport by 'ammonia' particles. Although optimum concentrations of oligomycin (0.2nmol/mg of protein) produce an approximately sevenfold stimulation of ATP-driven succinate-linked NAD$^+$ reduction, tributyltin has an inhibitory effect throughout the concentration range used. Similar results are obtained for the ATP-driven energy-linked transhydrogenase (Fig. 8), although tributyltin may cause a slight stimulation at very low concentration. However, Fig. 8 also shows that tributyltin is effective at stimulating the transhydrogenase driven by aerobic succinate oxidation. The maximal stimulation attained in this latter case is nearly as great as that brought about by oligomycin. However, although maximal stimulation is produced by the addition of about 0.4nmol of oligomycin/mg of protein, approx. 1nmol of tributyltin/mg of protein is required. This is a very similar concentration to that necessary for maximal inhibition of the ATP-driven energy-linked transhydrogenase, the restoration of respiratory control and the inhibition of the ATPase activity.

Discussion

The major difference between the effects of oligomycin and tributyltin on the energy-coupling reactions of submitochondrial particles appears to reside in the relative concentrations necessary to restore respiratory control and to inhibit the mitochondrial ATPase. For 'alkaline-EDTA' particles, Lee & Ernster (1966) showed that, for oligomycin, the concentration necessary to give maximum restoration of respiratory control or stimulation of the transhydrogenase driven by succinate oxidation was very much less than the concentration necessary to give complete inhibition of the ATPase activity. With tributyltin, maximum restoration of respiratory control, stimulation of the transhydrogenase driven by succinate oxidation and complete inhibition of the ATPase are all produced by the addition of about 1 nmol of inhibitor/mg of protein. The generally accepted interpretation of the effect of oligomycin on coupling of submitochondrial particles (Lee & Ernster, 1965) is that it interacts preferentially with damaged coupling sites and only at higher concentrations does it also block intact sites. In terms of the chemiosmotic hypothesis, it is proposed (Mitchell, 1966; Mitchell & Moyle, 1974) that oligomycin blocks proton conduction through the proton-conducting channel of damaged ATPase complexes in preference to blocking the passage of protons through the intact complexes. The evidence produced in the present paper suggests (Fig. 3) that tributyltin has a similar effect to that of oligomycin in lowering the proton permeability of coupling-factor-deficient submitochondrial particles. However, the findings described above suggest that tributyltin shows little or no selectivity between damaged and intact coupling sites. This suggestion is consistent with the failure to observe any stimulation of ATP-driven transhydrogenase or reversed electron transport. The restoration of energy coupling, as measured by respiratory control or the energy-linked transhydrogenase driven by oxidation of succinate, can be achieved only by adding sufficient inhibitor to give essentially complete inhibition of ATPase activity. The small effect on the P/O ratio may be explained on the basis that at the concentrations of tributyltin giving partial restoration of respiratory control, sufficient energy can be conserved to drive ATP synthesis via the small amount of residual ATPase. It should be noted in this context that the utilization of a respiration-generated high-energy state by residual ATPase does not imply that ATP hydrolysis via the residual ATPase should be able itself to generate a high-energy state. In the former case the ATPase is the rate-limiting step for energy utilization and in the second case that for energy production.

The effect of tributyltin on proton permeability of submitochondrial particles indicates that the mechanism by which it inhibits oxidative phosphorylation is very similar to that of oligomycin, i.e. by blocking proton flux through the coupling ATPase. However, the question arises as to whether or not it acts at the same site as oligomycin. In the present paper, it is shown that although sonication of ox heart mitochondria results in alteration of the inhibitory effect of oligomycin (such that it becomes more effective at the proton-conducting damaged ATPase sites compared with the intact ATPase complexes), there is no such alteration in the interaction of tributyltin with the membrane-bound ATPase. Together with the observations of Linnett *et al.* (1975), who showed that solubilization of the ATPase of ox heart submitochondrial particles with Triton X-100 resulted in maintenance of oligomycin sensitivity but variable loss of trialkyltin sensitivity, our results suggest that the two inhibitors bind to different sites. However, it is possible that alterations in a single site, brought about by sonication or Triton treatment, could affect the action of the inhibitors differently. For mammalian mitochondria, additional evidence that the two sites are different is that we have failed so far to detect any effect of oligomycin on the high-affinity binding of trialkyltins to rat liver mitochondria (A. P. Dawson & B. Farrow, unpublished work). For yeast mitochondria, Lancashire & Griffiths (1975) have shown that it is possible to isolate yeast mutants which are resistant to trialkyltins but not to oligomycin. Further, they suggest that the trialkyltin-binding site is on a different gene product from that of oligomycin. This lends strong support to the idea that the inhibitors bind to separate sites within the ATPase complex.

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References

Danielson, L. & Ernster, L. (1963) *Biochem. Biophys. Res. Commun.* **10**, 91–96

Ernster, L. & Lee, C. P. (1967) *Methods Enzymol.* **10**, 729–738

Fessenden, J. M. & Racker, E. (1967) *Methods Enzymol.* **10**, 194–197

Fiske, C. H. & SubbaRow, Y. (1925) *J. Biol. Chem.* **66**, 375–400

Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766

Hinkle, P. C. & Horstman, L. L. (1971) *J. Biol. Chem.* **246**, 6024–6028

Kagawa, Y. & Racker, E. (1966) *J. Biol. Chem.* **241**, 2461–2466

Lancashire, W. E. & Griffiths, D. E. (1975) *Eur. J. Biochem.* **51**, 377–392

Lang, G. & Michal, G. (1974) in *Methods of Enzymatic Analysis*, 2nd edn. (Bergmeyer, H. U., ed.), pp. 1238–1242, Academic Press, New York and London

Lee, C. P. & Ernster, L. (1965) *Biochem. Biophys. Res. Commun.* **18**, 523–529
Lee, C. P. & Ernster, L. (1966) in *Regulation of Metabolic Processes in Mitochondria* (Tager, J. M., Papa, S., Quagliariello, E. & Slater, E. C., eds.), pp. 218–234, Elsevier, Amsterdam, London and New York
Lee, C. P., Azzone, G. F. & Ernster, L. (1964) *Nature (London)* **201**, 152–155
Linnett, P. E., Mitchell, A. D. & Beechey, R. B. (1975) *FEBS Lett.* **53**, 180–183
Manger, J. R. (1969) *FEBS Lett.* **5**, 331–334
Mitchell, P. (1966) *Chemiosmotic Coupling in Oxidative and Photosynthetic Phosphorylation*, p. 130, Glynn Research, Bodmin
Mitchell, P. & Moyle, J. (1965) *Nature (London)* **208**, 1205–1206
Mitchell, P. & Moyle, J. (1974) *Biochem. Soc. Spec. Publ.* **4**, 91–111
Papa, S., Guerrieri, F., Rossi-Bernardi, L. & Tager, J. M. (1970) *Biochim. Biophys. Acta* **197**, 100–103
Roberton, A. M., Holloway, C. T., Knight, I. G. & Beechey, R. B. (1968) *Biochem. J.* **108**, 445–456
Rose, M. S. & Aldridge, W. N. (1972) *Biochem. J.* **127**, 51–59
Selwyn, M. J., Dawson, A. P., Stockdale, M. & Gains, N. (1970) *Eur. J. Biochem.* **14**, 120–126
Selwyn, M. J., Dunnett, S. J., Philo, R. D. & Dawson, A. P. (1972) *Biochem. J.* **127**, 66P–67P
Smith, A. L. (1967) *Methods Enzymol.* **10**, 81–86
Stockdale, M., Dawson, A. P. & Selwyn, M. J. (1970) *Eur. J. Biochem.* **15**, 342–351