Biochem. J. (1971) **124**, 221–234
Printed in Great Britain

Oxidative Phosphorylation

THE RELATION BETWEEN THE SPECIFIC BINDING OF TRIMETHLYTIN AND TRIETHYLTIN TO MITOCHONDRIA AND THEIR EFFECTS ON VARIOUS MITOCHONDRIAL FUNCTIONS

BY W. N. ALDRIDGE AND B. W. STREET
Biochemical Mechanisms Section, Medical Research Council Toxicology Unit, Woodmansterne Road, Carshalton, Surrey, U.K.

(*Received* 18 *January* 1971)

1. A binding site (site 1) is present in mitochondria with affinity for trimethyltin and triethyltin adequate for a site to which they could be attached when the processes of energy conservation are inhibited. 2. The quantitative relationships between the binding of trimethyltin and triethyltin to site 1 and their effects on various mitochondrial functions have been examined. 3. ATP synthesis linked to the oxidation of pyruvate, succinate and intramitochondrial substrate, ATP synthesis and oxygen uptake (succinate or pyruvate as substrate) stimulated by uncoupling agents are all inhibited by trimethyltin and triethyltin; when inhibition is less than 50% the ratio (percentage inhibition)/(percentage of binding site 1 complexed) is approx. 10:1. 4. ATP synthesis linked to the oxidation of reduced cytochrome *c* (ascorbate+*NNN'N'*-tetramethyl-*p*-phenylenediamine), ATP hydrolysis and oxygen uptake in the presence of low concentrations of trimethyltin and triethyltin approach zero activity as the proportion of binding site 1 complexed approaches 100%. 5. Possible interpretations of these findings are discussed with reference to published arrangements for coupling of electron transport to ATP synthesis and also to our present knowledge of the chemical and biological specificity of trialkyltin compounds.

Trialkyltins inhibit oxidative phosphorylation and act on energy conservation processes (Aldridge & Cremer, 1955; Aldridge, 1958; Aldridge & Threlfall, 1961; Moore & Brody, 1961; Aldridge & Street, 1964). Inhibition of an enzymic system may be due to an inhibitor combining with a macromolecular component of the system. Aldridge & Street (1970), have shown that triethyltin and trimethyltin combine with a site (binding site 1) in rat liver mitochondria with affinity constants of $4.7\times10^5\,M^{-1}$ and $1.2\times10^4\,M^{-1}$ respectively. This binding site 1 is also present in an insoluble fraction from mitochondria, this being almost certainly derived from the inner membrane. Thus it was concluded that binding site 1 could be the site to which trimethyltin and triethyltin are attached when oxidative phosphorylation is inhibited (Aldridge & Street, 1970).

If the above conclusion is accepted then a precise quantitative relation is required between the complexing of trimethyltin and triethyltin to binding site 1 and inhibition of oxidative phosphorylation. The experiments described in this paper explore the relationship between such binding and inhibition of mitochondrial functions involving processes of energy conservation. Our results are discussed with respect to existing postulated arrangements for the coupling of the respiratory chain (electron transport) to the formation of ATP.

MATERIALS AND METHODS

Special chemicals. Triethyltin sulphate was prepared from triethyltin hydroxide supplied by the Tin Research Institute, Greenford, Middx., U.K., as described by Aldridge & Cremer (1955). The following gifts are acknowledged: oligomycin from Dr G. D. Greville, trimethyltin acetate from Dr G. J. M. Van der Kerk, antimycin A from Boots Pure Drug Co., Nottingham, U.K., and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone from Dr P. G. Heytler of E.I. Du Pont de Nemour and Co., Wilmington, Del., U.S.A. The following were purchased: trimethyltin chloride and rotenone from British Drug Houses Ltd., Poole, Dorset, U.K.; ATP, sodium pyruvate and hexokinase (yeast) from Sigma Chemical Co., St Louis, Mo., U.S.A.; *NNN'N'*-tetramethyl-*p*-phenylenediamine hydrochloride from Kodak Ltd., London, U.K. Rotenone was recrystallized from ethanol–water. *NNN'N'*-Tetramethyl-*p*-phenylenediamine hydrochloride was purified by converting it into

the free base, followed by extraction with chloroform and washing with 0.1M-EDTA, pH 8. After evaporation of the chloroform solution the base was dissolved in ethanol and crystallized as the hydrochloride.

Preparation of the mitochondrial fraction. Rat liver (8g) was homogenized in 30ml of ice-cold 0.3M-sucrose in a homogenizer with a difference in diameter between pestle and tube of 0.5mm and the pestle rotating at 1100rev./min (Aldridge, Emery & Street, 1960; Webster & Smith, 1964). The homogenate was diluted to 80ml with ice-cold 0.3M-sucrose and centrifuged at 850*g* for 10min. The supernatant was then centrifuged at 5000*g* for 15min. The mitochondrial pellet was washed once by resuspending it in ice-cold 0.3M-sucrose and centrifuging again. The pellet, depending on the experiment, was suspended and diluted to 8ml or 16ml with ice-cold 0.3M-sucrose. For most experiments these suspensions of mitochondria were used immediately.

Determination of ATP synthesis. ATP synthesis was determined by measuring P_i uptake in the presence of hexokinase and glucose. The medium (3ml in each flask) contained (final concentrations) KCl (0.1M), $MgCl_2$ (14mM), EDTA (1mM), potassium phosphate (6.6mM), ATP (2.3mM), glucose (60mM), glycylglycine (16.7mM), sucrose (15–30mM, depending on the volume of mitochondrial suspension used) and hexokinase (capable of converting ATP into ADP at 40μmol/min at 25°C). Substrates used were the following: pyruvate (10mM)+fumarate (1mM) or pyruvate (10mM)+L-malate (1mM) or succinate (10mM)+rotenone (3.4nmol/flask) or ascorbate (40mM)+*NNN'N'*-tetramethyl-*p*-phenylenediamine hydrochloride (1mM). The pH was adjusted to 6.8. The process was started by the addition of mitochondrial suspension (3–4mg of protein) to the medium previously warmed to 37°C and the mixture was shaken in air. The reaction was stopped by the addition of 7ml of ice-cold perchloric acid (0.725M).

Determination of oxygen uptake. This was measured either manometrically with 3ml of medium in each flask or polarographically with 1ml of medium in the cell. The medium was as described for the determination of oxidative phosphorylation except for the omission of hexokinase or the omission of hexokinase+glucose+glycylglycine. Identical results were obtained in either case. The concentration of mitochondrial protein for both methods of measurement of oxygen uptake was 1–1.3mg/ml and the temperature was 37°C.

ATP hydrolysis. ATP hydrolysis was measured in a medium (3ml) containing (final concentrations) KCl (0.1M), $MgCl_2$ (14mM), EDTA (1mM), ATP (3mM), glycylglycine (16.7mM) and sucrose (15–30mM, depending on the volume of mitochondrial suspension used). When required, rotenone (3.4nmol/flask) was added. The process was started by the addition of mitochondria (3–4mg of protein) and the flasks were shaken in air at 37°C. The reaction was stopped by adding 7ml of ice-cold perchloric acid (0.725M).

Determination of inorganic phosphate. The method of Fiske & Subbarow (1925) was used. Depending on the particular experiment, various volumes of ice-cold protein-free solutions containing perchloric acid were added to the ice-cold molybdate reagent. After addition of reducing reagent and dilution to the required volume, the colour was developed at 17°C for 30min.

Determination of protein. Protein was determined by using the biuret method of Robinson & Hogden (1940) as modified by Aldridge (1962).

Inhibitors. Triethyltin sulphate and potassium cyanide were used as solutions in water. Triethyltin acetate or chloride, oligomycin, rotenone, Amytal or hexobarbital (as free acids), antimycin A and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone were used as solutions in dimethylformamide. For most experiments when inhibitors dissolved in dimethylformamide were used, the final concentration of dimethylformamide was kept constant and not more than 1% (v/v).

RESULTS

Development of methods

General condition of experiments. Oxidative phosphorylation is a co-ordinated process depending on the structure of mitochondria. Inhibitors have been discovered that produce defined effects on different mitochondrial functions. It is important to know if these effects are produced by the same primary action of the inhibitor. One way to examine this is to correlate the degree of effect on the different systems by various concentrations of inhibitor. There is a major difficulty in this approach, for it is well known that the environment of the mitochondria alters their capacity to carry out oxidative phosphorylation. Therefore ideally all the mitochondrial functions should be examined in the same medium, but this is obviously impossible.

For the comparisons made in this work the following conditions have been used. (1) The medium was that described by Aldridge (1957). (2) The concentration of substrates was high (usually 10mM). (3) Comparison of the effects of inhibitor on various mitochondrial functions were always made when activity was linear with respect to time, both in the presence and in the absence of inhibitor. (4) Phosphorylation of ADP was measured by the loss of P_i from the medium when hexokinase and glucose were used to regenerate ADP. The concentration of hexokinase added was at least 30–40 times that required on the basis of the rate of production of ATP. With these general experimental conditions (described in detail in the Materials and Methods section) the mean rate for the processes studies are given in Table 1.

ATP synthesis linked to oxidation of pyruvate. Both in the presence and in the absence of triethyltin there was a delay in P_i uptake of about 3–4min before a linear uptake was achieved. Under these conditions P_i uptake with pyruvate (10mM) alone as substrate was very slow compared with pyruvate (10mM)+fumarate (1mM). The delay also occurred if fumarate was replaced by L-malate. For most experiments inhibition by trimethyltin or triethyltin was measured over the linear portion of the curve,

Table 1. *Rates of various mitochondrial functions*

Results are expressed as means±S.E.M. with numbers of observations in parentheses. The Materials and Methods section and the relevant text and figures should be consulted for experimental details. Abbreviation: TMPD, *NNN'N'*-tetramethyl-*p*-phenylenediamine.

System measured	Temp. (°C)	Activity (nmol of P_i/min per mg of protein)
Oxidative phosphorylation (pyruvate+fumarate)	37	279±12 (16)
Oxidative phosphorylation (succinate in the presence of rotenone)	37	294±7 (14)
Oxidative phosphorylation (ascorbate in the presence of TMPD)	37	317±14 (8)
Oxidative phosphorylation (ascorbate in the presence of TMPD)	25	203±9 (14)
ATP hydrolysis (in the presence of rotenone)	37	42.5±1.3 (29)
ATP hydrolysis (stimulated by 20 μM-dinitrophenol)	37	336±18 (6)
ATP hydrolysis (stimulated by 0.063 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone)	37	287±17 (5)

although the same percentage inhibition was obtained when P_i uptake from zero time was used. The mean rate of P_i uptake for the controls by using this procedure was 279±12 (16) nmol of P_i/min per mg of protein (Table 1).

ATP synthesis linked to oxidation of succinate. Previous experiments with succinate as substrate (Aldridge, 1958) were made over 30min and oxidation of the products of oxidation of succinate must have occurred. In the present study the rate of P_i uptake was determined in the presence of sufficient rotenone (1nmol/mg of protein) to prevent oxygen uptake with substrates oxidized by NAD-requiring dehydrogenases. The rate of P_i uptake was linear for 8min and was for the controls 294±7 (14) nmol of P_i/min per mg of protein (Table 1). This rate is similar to that obtained with pyruvate+fumarate but in contrast there was no initial delay.

ATP synthesis linked to the oxidation of ascorbate. The oxidation of ascorbate in the presence of *NNN'N'*-tetramethyl-*p*-phenylenediamine leads to the phosphorylation of ADP and is considered to be phosphorylation linked to the oxidation of reduced cytochrome *c* (Jacobs, 1960; Howland, 1963; Rossi & Lehninger, 1963; Packer & Mustafa, 1966; Chamalaun & Tager, 1969).

With the concentrations of ascorbate and *NNN'N'*-tetramethyl-*p*-phenylenediamine used the rate of P_i uptake was comparable with that obtained for the oxidation of succinate or pyruvate (Table 1). However, whatever concentrations were used at 37°C the rate of P_i uptake always decreased rapidly and was linear for only 4min. When experiments were done at 25°C there was a longer linear phase (12min) at a lower rate (Table 1). Most of our experiments were done at the lower temperature and only sufficient at 37°C to confirm that similar results were obtained.

ATP hydrolysis stimulated by uncoupling agents. In previous studies (Aldridge, 1958) of the influence of triethyltin on 2,4-dinitrophenol-stimulated ATP hydrolysis, measurements were made over 10min and a correction was made for the stimulation of ATP hydrolysis by triethyltin itself; the correction was large and could be as much as 40% of the measured ATP hydrolysis stimulated by 2,4-dinitrophenol.

Two aspects of the original technique required alteration. First, in the presence of 30 μM-2,4-dinitrophenol the rate of hydrolysis was not linear and rapidly decreased after 3 μmol of the 9 μmol of ATP had been hydrolysed. The initial rate was 530nmol of P_i/min per mg of protein compared with 220nmol of P_i/min per mg of protein when the average rate over 10min was determined (Aldridge & Stoner, 1960). A second source of error concerns stimulation of ATP hydrolysis by triethyltin itself. Approximately one-half of the ATP hydrolysis found in the presence of triethyltin is due to inhibition of resynthesis of ATP linked to the oxidation of intramitochondrial substrate (see below); this ATP hydrolysis may be unmasked by rotenone and other substances that inhibit oxidative phosphorylation (Table 2), and triethyltin or trimethyltin only increase this rate by approx. 40nmol/min per mg of protein. In the experiments described in the present paper measurements of ATP hydrolysis with and without 2,4-dinitrophenol

were always made in the presence of rotenone (1nmol/mg of protein) and over a 2min period. Under these conditions the ATP hydrolysis stimulated by triethyltin is only approx. 10% of that stimulated by 30μM-2,4-dinitrophenol instead of the 40% previously reported.

The concentration of 2,4-dinitrophenol used for most of the experiments was 20μM; this concentration produced a rate of ATP hydrolysis the same as that for the rate of ATP synthesis with pyruvate, succinate or ascorbate as substrate (Table 1). A similar rate of ATP hydrolysis is obtained by 63nM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (Table 1).

The percentage activity in the presence of triethyltin or trimethyltin was calculated as follows:

$$100\ (A-B)/(C-D)$$

where the symbols indicate ATP hydrolysis in the presence of: *A*, 20μM-2,4-dinitrophenol and trialkyltin; *B*, trialkyltin; *C*, 20μM-2,4-dinitrophenol; *D*, no additions.

ATP synthesis linked to the oxidation of endogenous substrates. If mitochondrial ATP hydrolysis was measured in air for 10min at 37°C a very low rate was apparent (Table 2; Aldridge, 1958; Aldridge & Parker, 1960; Aldridge & Stoner, 1960). When rotenone (1nmol/mg of protein) was added a higher rate was obtained (Table 2). Since rotenone is a rather specific inhibitor of the oxidation of substrates by NAD-requiring dehydrogenases in mitochondria, it seemed likely that the increase of ATP hydrolysis was associated with this effect. Rotenone may unmask ATP hydrolysis by preventing its resynthesis linked to the oxidation of endogenous substrate. The same explanation has been given for the stimulation of ATP hydrolysis by arsenate and cyanide (Ter Welle & Slater, 1964). In agreement with this view other inhibitors of the processes of oxidative phosphorylation produce a similar increase in ATP hydrolysis (Table 2). A similar rate was found with rotenone, Amytal and cyanide and when oxygen was excluded. The same rate of ATP hydrolysis was found with a range of 0.1–1.0nmol of rotenone/mg of protein.

The time-course of ATP hydrolysis in the presence and in the absence of rotenone is shown in Fig. 1. In the presence of rotenone (1nmol/mg of protein) the liberation of P_i was linear for 60min and the rate was 42nmol/min per mg of protein. In the absence of added rotenone there was at first a small decrease in the P_i present in the medium followed by a gradual increase in rate until from 30min to 60min the rate was the same as that in the presence of rotenone. This delay before the rate in the absence of rotenone becomes equal to that in its presence is probably due to the oxidation of intramitochondrial substrates; these should be exhausted after incubation for 30min. After incubation for 40min with and without rotenone the addition of succinate causes a rapid uptake of P_i, showing that in both cases the capacity for oxidative phosphorylation is still substantial (Fig. 1). The difference between the two parallel lines show that approx. 0.9μmol of ATP/mg of mitochondrial protein was synthesized before all intramitochondrial substrate was utilized.

The reactions involved are summarized in Scheme 1. It is not known if reaction B, the hydrolysis of

Table 2. *ATP hydrolysis in the presence of various inhibitors of oxidative phosphorylation*

The reaction was started by the addition of mitochondria. For experiments with cyanide or under N_2 stoppered flasks were used. The results are expressed as means ±S.E.M. with numbers of observations in parentheses.

Addition	ATP hydrolysis (nmol of P_i/min per mg of protein)
None	6.9±1.0 (18)
Rotenone (1nmol/mg of protein)	42.5±1.3 (29)
Amytal (0.8mM)	46.6 (1)
Cyanide (0.5–1.0mM)	46.2±2.8 (6)
Nitrogen	44.5 (2)
Trimethyltin (20μM)	86.5±2.1 (3)
Trimethyltin (20μM)+rotenone (1nmol/mg of protein)	86.4±3.2 (7)
Triethyltin (1.0μM)	73.3±2.0 (6)
Triethyltin (1.0μM)+rotenone (1nmol/mg of protein)	86.9±3.9 (7)

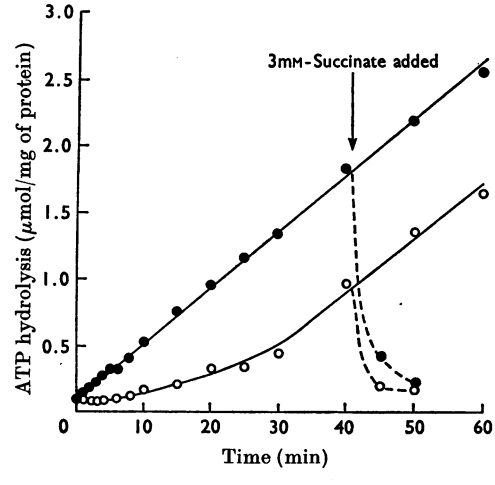
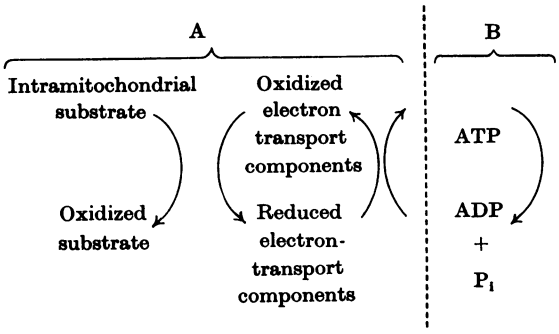


Fig. 1. Rate of ATP hydrolysis by mitochondria in the presence and in the absence of rotenone. The reaction was started by the addition of mitochondria. ○, Rotenone absent; ●, rotenone present (1.07nmol/mg of protein).



Scheme 1. Reactions occurring when ATP hydrolysis by mitochondria is measured in air.

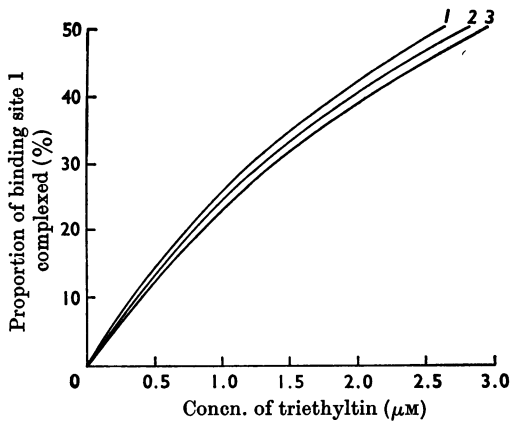


Fig. 2. Relation between the total concentration of triethyltin added to mitochondrial suspensions and the degree of complexing of binding site 1. This relationship was calculated from the constants derived by Aldridge & Street (1970): n_1, 0.8nmol/mg of protein; K_1, $4.75\times10^5\,M^{-1}$; n_2, 66nmol/mg of protein; K_2, $1.4\times10^3\,M^{-1}$. Curve 1, 1.0mg of mitochondrial protein/ml; curve 2, 1.25mg of mitochondrial protein/ml; curve 3, 1.5mg of mitochondrial protein/ml.

ATP, is brought about by a reversal of part of the energy-conservation process or even if it is one process only; and for the purpose described it is not necessary to know. The measurement of the apparent stimulation of ATP hydrolysis (reactions A and B) can therefore be used as an indication of the inhibition of oxidative phosphorylation linked to the oxidation of endogenous (intramitochondrial) substrate (reaction A).

Calculation of binding site 1 complexed with trimethyltin and triethyltin. The binding of trimethyltin and triethyltin to intact rat liver mitochondria was measured in the medium used for the examination of the processes of oxidative phosphorylation (Aldridge & Street, 1970). The results were analysed by using the relationship devised by Scatchard (1949) and the following constants were derived: for trimethyltin, n_1, 0.8nmol/mg of protein; K_1, $1.2\times10^4\,M^{-1}$; n_2, 120nmol/mg of protein; K_2, $1\times10^2\,M^{-1}$; n_3, 0.0042nmol/mg of protein; K_3, $4\times10^6\,M^{-1}$; for triethyltin, n_1, 0.8nmol/mg of protein; K_1, $4.7\times10^5\,M^{-1}$; n_2, 66nmol/mg of protein; K_2, $1.4\times10^3\,M^{-1}$. With this information the relationship between binding to binding site 1 (as defined by the constants for n_1 and K_1 for trimethyltin and triethyltin) and the overall concentration of tin compound added may be calculated for various concentrations of mitochondrial protein.

Examples of such calculations are plotted in Fig. 2. Given the concentration of mitochondrial protein used in a particular experiment, the percentage of binding site 1 complexed may be derived from the concentration of tin compound added.

The correlation made in this work between inhibition of various mitochondrial functions and binding to site 1 shows considerable scatter. The values for inhibition are necessarily derived from different preparations of mitochondria. The values for the derived constants n_1 and K_1 are the means of many experiments. Thus some scatter is expected when the values of inhibition for individual preparations are correlated with binding to site 1 derived from these mean constants.

Influence of trimethyltin and triethyltin on various mitochondrial functions

ATP synthesis linked to the oxidation of pyruvate. When the experimental results from which previously published information had been derived (Aldridge, 1958; Aldridge & Street, 1964) were plotted so as to correlate the percentage inhibition against percentage of binding site 1 complexed, an unexpected result was obtained (Fig. 3). If binding site 1 was a single site per molecule involved in the processes of oxidative phosphorylation (as in a molecule of enzyme containing one catalytic centre), then the 1:1 relationship shown by the broken line would be obtained. In contrast, about 10 times more inhibition was obtained than expected up to about 50% inhibition, which was reached at about 5% coverage of binding site 1 (Fig. 3). Such a result could indicate that binding site 1 is heterogeneous, that binding site 1 is not involved in oxidative phosphorylation or that triethyltin has more than one mode of action. Trimethyltin is 30–40 times less active as an inhibitor of oxidative phosphorylation, and yet when binding to site 1 by both compounds was correlated with the inhibition of oxidative phosphorylation the same result was obtained (Fig. 3). We therefore considered that we

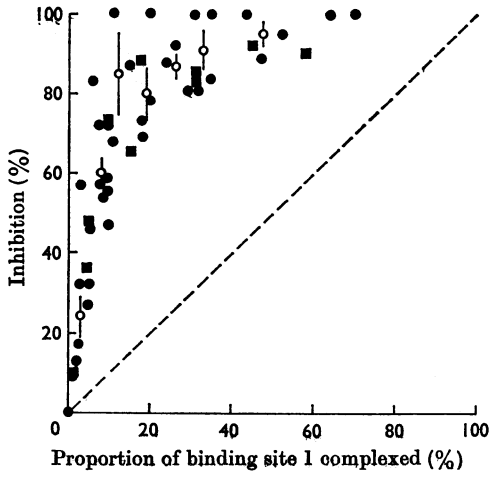


Fig. 3. Inhibition of oxidative phosphorylation and the complexing by triethyltin and trimethyltin of binding site 1. The results are taken from manometric experiments previously published (Aldridge, 1958; Aldridge & Street, 1964). Individual results for triethyltin and trimethyltin are shown by the symbols ● and ■ respectively, and the grouped results by the means (○) with the vertical lines indicating the S.E.M. The broken line indicates a 1:1 relationship.

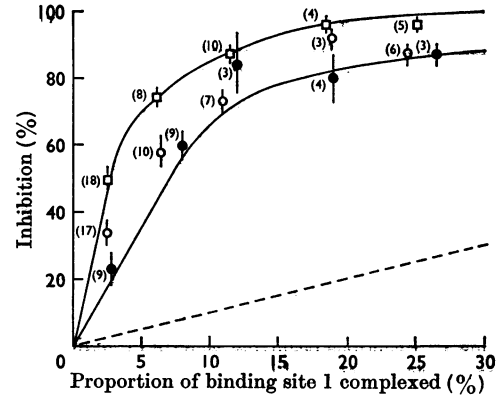


Fig. 4. Inhibition of phosphorylation linked to the oxidation of pyruvate and succinate and complexing by triethyltin of binding site 1. The results are plotted as means±S.E.M. (shown as the vertical lines) by the symbol □ for the oxidation of succinate in the presence of rotenone, and ○ and ● for the present and previous experiments respectively with pyruvate+fumarate as substrate. The means are calculated over the ranges 0–5%, 5–10%, 10–15%, 15–20% and 20–30% of binding site 1 complexed. The broken line indicates a 1:1 relationship and the continuous lines indicate the range of the three different means. The values in parentheses are the numbers of experiments used to obtain each point.

should not immediately conclude that binding site 1 was not involved in oxidative phosphorylation.

Since all the results given in Fig. 3 were obtained from long-term manometric experiments, i.e. phosphorylation was measured up to 30 min after the reaction had started, it is possible that the inhibition might reflect secondary processes rather than the primary events. A re-examination of the effect of trimethyltin and triethyltin at early times was therefore made.

The delay found before the maximum rate of P_i uptake was the same in the presence and in the absence of triethyltin. This indicates that triethyltin exerts its effects quickly, since the degree of inhibition was the same whether the average rate of P_i uptake was calculated over the linear or the curved portions of the graph. The mean results of similar experiments with a wide range of concentrations of triethyltin are shown in Fig. 4. The percentage inhibition of P_i uptake is much more than the percentage of site 1 complexed. A similar result was found for trimethyltin (Fig. 5). Several conclusions may be drawn from these results: (1) triethyltin and trimethyltin inhibit the phosphorylation of ADP after a short time of contact; (2) for a given percentage of binding site 1 complexed the inhibition obtained is similar whether it is measured over the first 10 min (Fig. 4) or over a 10–30 min period (Fig. 3): the system must be in a steady

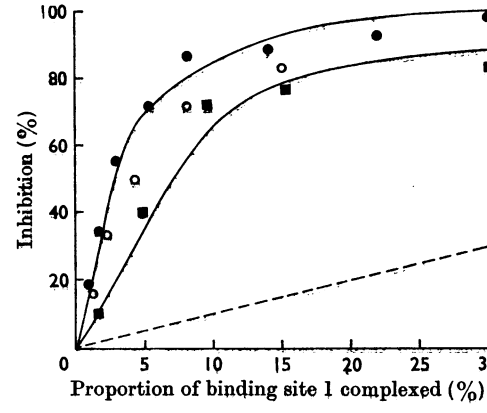


Fig. 5. Inhibition of phosphorylation linked to oxidation of pyruvate or succinate and complexing by trimethyltin of binding site 1. The results with succinate as substrate (●) in the presence of rotenone are the means of the results of six experiments, and with pyruvate+fumarate the means of the results of two experiments in the present study (○) and two experiments from previous work (■). The lines are taken from Fig. 4.

state with respect to inhibition; (3) the concentrations of trimethyltin and triethyltin that we calculate will lead to the same percentage of

binding site 1 complexed, also lead to the same degree of inhibition.

ATP synthesis linked to the oxidation of succinate. Phosphorylation linked to the single-step oxidation of succinate to fumarate is linear for 8min, and the inhibition by triethyltin is immediate and does not increase over this period. The results from these experiments are shown in Figs. 4 and 5. Again percentage inhibition is much greater for a given percentage of binding site 1 complexed and the same results are obtained with both triethyltin and trimethyltin. There is a general tendency with both compounds that for a given percentage of binding site 1 complexed inhibition of P_i uptake with succinate as substrate is more than that found with pyruvate as substrate.

Pyruvate oxidation involves more than one step of the tricarboxylic acid cycle, in contrast with the one-step oxidation of succinate in the presence of rotenone. The yield of ATP from oxidation of 1mol of pyruvate is higher than that from 1mol of succinate. Both these facts indicate that for a given rate of P_i uptake (Table 1) the rate of penetration of succinate into the mitochondria will require to be much greater than that of pyruvate. If substrate penetration is an energy-requiring process then the required higher rate of penetration of succinate may be more vulnerable to the inhibition of the supply of energy. Some evidence has been published that the penetration of succinate is influenced by triethyltin (Manger, 1970); however, the concentration of succinate used by him (0.5mM) was much lower than ours (10mM) and his concentration of triethyltin was at the top end of our range (0.5μM). The effects of changes in succinate concentration were therefore examined. Increase in succinate concentration from 3mM to 30mM increased the rate of P_i uptake and decreased the inhibition by triethyltin and trimethyltin (Fig. 6). For the highest concentrations of succinate the results are very similar to those obtained with pyruvate as substrate (Figs. 4 and 5). Therefore although the increased sensitivity of phosphorylation linked to the oxidation of succinate may be due to a limiting rate of penetration by succinate, there is no reason to consider the difference between the percentage inhibition and percentage of binding site 1 complexed to be due to this.

In all graphs the lines that enclose most of the results (Fig. 4) will be used to assess whether results obtained with other mitochondrial parameters fall into the same pattern.

ATP synthesis linked to oxidation of ascorbate. Triethyltin was much less inhibitory for phosphorylation linked to the oxidation of ascorbate (in the presence of *NNN'N'*-tetramethyl-*p*-phenylenediamine) than to that linked to oxidation of pyruvate or succinate (Fig. 7). Although the

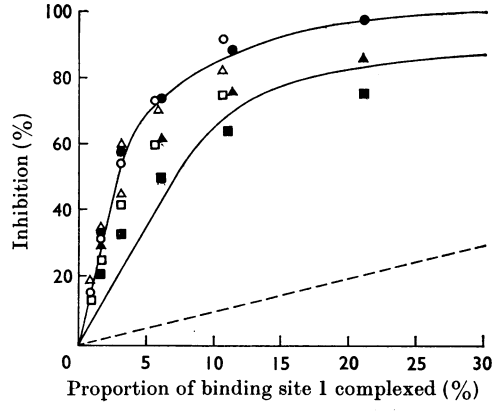


Fig. 6. Inhibition of phosphorylation linked to oxidation of various concentrations of succinate and complexing by trimethyltin and triethyltin of binding site 1. The open symbols indicate trimethyltin and the filled symbols triethyltin. The concentrations of succinate were 3mM (● and ○), 10mM (▲ and △) and 30mM (■ and □). The mean rates of P_i uptake were: for 3mM-succinate, 296nmol/min per mg of protein; for 10mM-succinate, 356nmol/min per mg of protein; for 30mM-succinate, 340nmol/min per mg of protein. The lines are taken from Fig. 4.

points fall on a slightly sigmoid curve there was an approximately 1:1 relationship between percentage inhibition and percentage of binding site 1 complexed. A few results obtained at 37°C instead of 25°C show the same relationship (Fig. 7). A more pronounced sigmoid curve is obtained when trimethyltin is used, but again it is sigmoid about a line for the 1:1 relationship (Fig. 8).

The concentration of triethyltin required to produce 90% inhibition of ATP synthesis linked to oxidation of succinate or pyruvate is about 0.8μM, whereas for that linked to oxidation of ascorbate it is 23μM. The corresponding concentrations for trimethyltin are 20μM and 1.5mM respectively. The higher concentrations required for inhibition of ATP synthesis linked to ascorbate agree with the concentrations calculated to be necessary to complex 90% of binding site 1 with either compound. Therefore, assuming that phosphorylation of ADP linked to oxidation of ascorbate involves the pathway through which phosphorylation of ADP linked to oxidation of succinate or pyruvate must pass, these results provide evidence that the whole of binding site 1 is involved in oxidative phosphorylation.

ATP synthesis linked to endogenous substrate. A variety of substances that inhibit oxidative phosphorylation have been shown to stimulate to a limited extent ATP hydrolysis by mitochondria

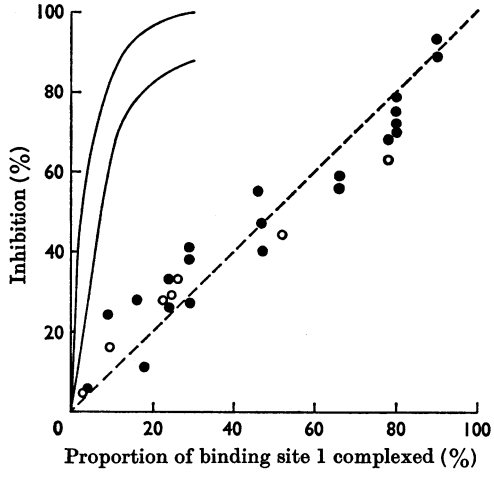


Fig. 7. Inhibition of phosphorylation linked to oxidation of ascorbate and complexing by triethyltin of binding site 1. The results are from nine experiments, each with a different preparation of mitochondria in a medium containing ascorbate (40mM) and *NNN'N'*-tetramethyl-*p*-phenylenediamine (1mM) at 25°C (●) and at 37°C (○). The broken line indicates a 1:1 relationship and the continuous lines are taken from Fig. 4.

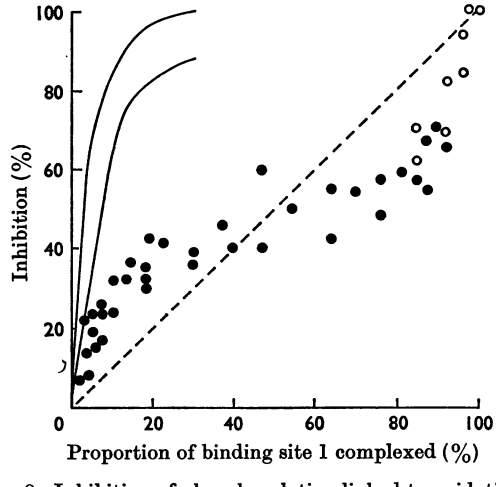


Fig. 8. Inhibition of phosphorylation linked to oxidation of ascorbate and complexing by trimethyltin of binding site 1. The results are from eight experiments, each with a different preparation of mitochondria in a medium containing ascorbate (40mM) and *NNN'N'*-tetramethyl-*p*-phenylenediamine (1mM) at 25°C. Trimethyltin acetate dissolved in dimethylformamide was added so that the final medium contained 1% (v/v) (●) or 4% (v/v) (○) of solvent. The latter was necessary because of the limiting solubility of trimethyltin acetate in the solvent. The broken line indicates a 1:1 relationship and the continuous lines are taken from Fig. 4.

(Table 2). Evidence has been produced to show that the rate of this ATP hydrolysis (expressed as a percentage of the maximum rate) may be correlated with the ability of the substances to inhibit oxidative phosphorylation. Trimethyltin and triethyltin stimulate ATP hydrolysis (Table 2 and Fig. 13). The measured rate of ATP hydrolysis in the presence of trimethyltin or triethyltin should therefore be a measure of their ability to inhibit ATP synthesis linked to oxidation of intramitochondrial substrates (Scheme 1). The range of concentrations of trimethyltin and triethyltin that stimulate ATP hydrolysis are those that inhibit ATP synthesis linked to oxidation of pyruvate or succinate. When the rate of ATP hydrolysis, expressed as a percentage of the maximum rate, is plotted against percentage of binding site 1 complexed then the relationship approximates to that found for other mitochondrial functions (Fig. 9; cf. Figs. 4, 5, 10 and 11). Therefore ATP synthesis linked to the oxidation of endogenous (intramitochondrial) substrates is inhibited by concentrations of triethyltin and trimethyltin similar to those that inhibit ATP synthesis linked to exogenous substrates.

ATP hydrolysis stimulated by uncoupling agents. Triethyltin stimulates a small amount of ATP hydrolysis and inhibits ATP hydrolysis stimulated by 2,4-dinitrophenol (Fig. 12). The slight delay in the action of triethyltin on ATP hydrolysis in the presence of 2,4-dinitrophenol was not more than 10s. Other experiments indicate that the rate of liberation of P_i was linear for 2min in the presence of

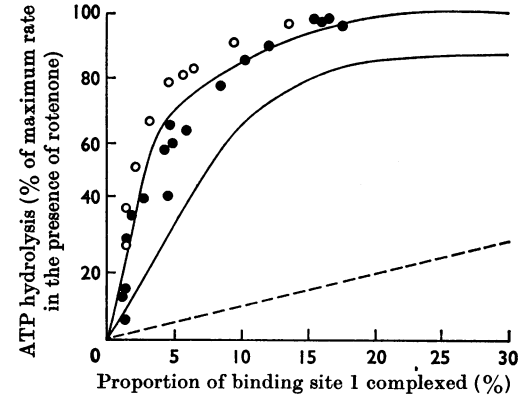


Fig. 9. ATP hydrolysis unmasked by trimethyltin and triethyltin and their complexing of binding site 1. ATP hydrolysis in the presence of tin compounds was calculated as a percentage of the maximum rate. For the experiments with trimethyltin (○) and triethyltin (●) this rate was 85.0 and 71.7nmol/min per mg of protein respectively. The broken line indicates a 1:1 relationship and the continuous lines are taken from Fig. 4.

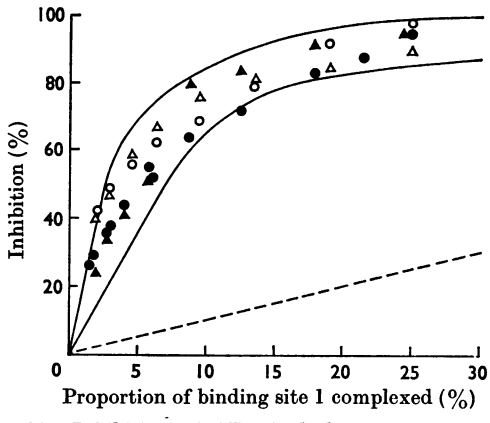


Fig. 10. Inhibition of ATP hydrolysis stimulated by uncoupling agents and complexing by trimethyltin and triethyltin to binding site 1. Rotenone (approx. 1 nmol/mg of protein) was present in all flasks, and the concentration of 2,4-dinitrophenol (○ and ●) was 20 μM and that of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (△ and ▲) was 0.063 μM. The open symbols indicate trimethyltin and the filled symbols triethyltin. The lines are taken from Fig. 4. The method of calculation of results is given in the text.

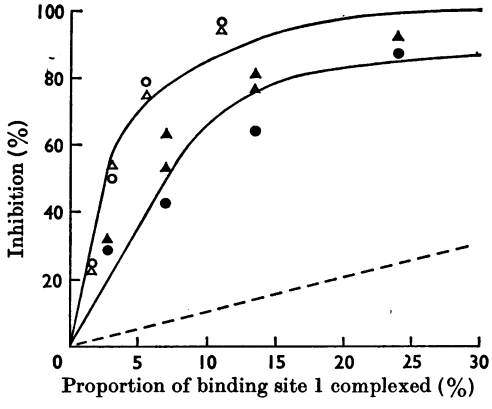


Fig. 11. Inhibition of oxygen uptake stimulated by 2,4-dinitrophenol and complexing by trimethyltin and triethyltin of binding site 1. The concentration of 2,4-dinitrophenol was 20 μM and the oxygen uptake was measured polarographically. The order of addition of reactants was mitochondria, 2,4-dinitrophenol and tin compound or mitochondria and tin compound (see Fig. 14). The results for trimethyltin (○ and △) and triethyltin (● and ▲) were obtained with a medium containing pyruvate (10 mM) + fumarate (1 mM) (△ and ▲) or succinate (10 mM) in the presence of rotenone (○ and ●). The lines are taken from Fig. 4.

2,4-dinitrophenol with or without triethyltin and for at least 10 min in the presence of triethyltin alone.

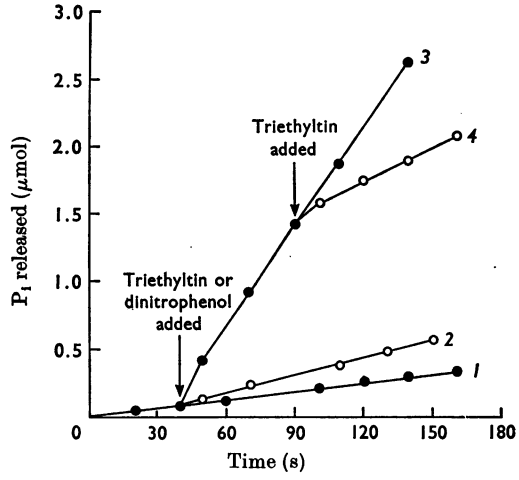


Fig. 12. Effect of 2,4-dinitrophenol and triethyltin on ATP hydrolysis by mitochondria. The reaction was started by the addition of mitochondria (2.86 mg of protein). Rotenone (1.2 nmol/mg of protein) was present in all flasks, and the concentration of 2,4-dinitrophenol was 30 μM and that of triethyltin 0.25 μM. Curve 1, control; curve 2, triethyltin added; curve 3, 2,4-dinitrophenol added; curve 4, 2,4-dinitrophenol + triethyltin added.

The relationship between percentage inhibition by triethyltin or trimethyltin of ATP hydrolysis stimulated by 2,4-dinitrophenol is shown in Fig. 10. All results for both triethyltin and trimethyltin fall within the range obtained for phosphorylation linked to the oxidation of pyruvate and succinate.

Inhibition of ATP hydrolysis stimulated by 2,4-dinitrophenol is not a special case, for it has been shown that the ATP hydrolysis stimulated by carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone is also inhibited in the same way. 2,4-Dinitrophenol at 20 μM was used, and this is much higher than the concentrations of triethyltin that inhibit. Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone was chosen because the concentration necessary to produce the same ATP hydrolysis as 20 μM-2,4-dinitrophenol is less than the inhibitory concentration of triethyltin. The relationship shown by Fig. 10 may be expected with all uncouplers of this type (see Parker, 1965).

ATP hydrolysis. Triethyltin and trimethyltin bring about an increased rate of ATP hydrolysis (Aldridge, 1958; Aldridge & Street, 1964). In a previous section it has been shown that in the presence of rotenone alone an increased rate of ATP hydrolysis is found and that this is due to the prevention of ATP synthesis linked to the oxidation of intramitochondrial substrates. The effects of triethyltin and trimethyltin on ATP hydrolysis in the presence and in the absence of rotenone were

therefore re-examined (Fig. 13). Triethyltin and trimethyltin increase the rate of ATP hydrolysis above that obtained in the presence of rotenone alone (Table 2 and Fig. 13). The extent of this stimulation above that produced by rotenone is about 40nmol/min per mg of protein, i.e. approx. 10% of the maximum rate of ATP hydrolysis obtained in the presence of 2,4-dinitrophenol (50μM) or carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (0.1μM). The rate of ATP hydrolysis increased to a maximum over the range of concentrations of triethyltin necessary to complex 20–30% of binding site 1 (Fig. 13). When 20–30% of binding site 1 is complexed other mitochondrial functions would be approx. 90% inhibited (Figs. 4, 5, 10 and 11). If the concentrations of triethyltin are increased over this then ATP hydrolysis decreases and approaches zero as complete complexing of binding site 1 was approached (Fig. 13). Other experiments indicate that essentially similar results are obtained with trimethyltin.

Oxygen uptake stimulated by 2,4-dinitrophenol. There is little doubt that the stimulation of oxygen uptake on addition of 2,4-dinitrophenol to mitochondria is brought about by the same primary action as that stimulating ATP hydrolysis (Parker, 1958, 1965). This additional respiration has been reported to be inhibited by triethyltin (Aldridge, 1958). However, these results were from manometric measurements over long periods (10–50min), and others have published results claiming that respiration stimulated by 2,4-dinitrophenol is less inhibited by triethyltin and that inhibition is delayed (Sone & Hagihara, 1964). A re-examination was therefore made by using the polarographic method for oxygen-uptake measurements, so that initial rates were measured. The results obtained when pyruvate was substrate are illustrated in Fig. 14; similar results were obtained with succinate. This reconstructed tracing shows that triethyltin inhibited oxygen uptake stimulated by 2,4-dinitrophenol and also stimulated some additional oxygen uptake itself. There was a delay of about 10s before inhibition had reached its maximum.

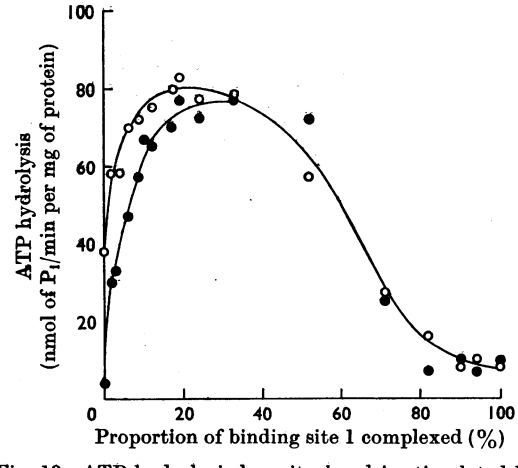


Fig. 13. ATP hydrolysis by mitochondria stimulated by triethyltin in the presence and in the absence of rotenone. P_i released was measured 10min after the addition of mitochondria. Results in the presence and in the absence of rotenone (1nmol/mg of protein) are indicated by ○ and ● respectively.

Calculations of the percentage activity in the presence of trimethyltin and triethyltin were made as follows:

$$100\ (A-B)/(C-D)$$

where the symbols indicate oxygen uptake in the presence of: *A*, 20μM-2,4-dinitrophenol and trialkyltin; *B*, trialkyltin; *C*, 20μM-2,4-dinitrophenol; *D*, no additions.

The collected results are plotted in Fig. 11 and show that oxygen uptake stimulated by 2,4-dinitrophenol is also inhibited by trimethyltin or triethyltin to an extent similar to that of the stimulated ATP hydrolysis (Fig. 10).

Oxygen uptake. Under the experimental conditions described in this paper oxygen uptake by mitochondria with pyruvate as substrate (State 4) is stimulated 3–4-fold by the addition of systems utilizing ATP (Aldridge, 1957). Both trimethyltin and triethyltin stimulate ATP hydrolysis (Fig. 13 and Table 2) and therefore an increased oxygen uptake in State 4 should be found.

This is found whether the manometric or the polarographic method is used. The pattern of effects is very similar to that found for ATP hydrolysis. The rate of oxygen uptake increased over the same range of complexing of binding site 1 (Figs. 13 and 15), and when the concentration of both trimethyltin and triethyltin was further increased oxygen uptake decreased and approached zero as complete complexing of binding site 1 was obtained. A similar relationship was obtained for the stimulation by trimethyltin and triethyltin on oxygen uptake in State 4 when succinate was used as substrate. Again there was close agreement between polarographic and manometric measurements.

Influence of oligomycin on various mitochondrial functions

Oligomycin is a powerful inhibitor of oxidative phosphorylation (Lardy, Johnson & McMurray, 1958) and is a key inhibitor in our understanding of this enzymic system. Because it has been suggested that triethyltin behaves like oligomycin (Sone & Hagihara, 1964; Manger, 1970; Tyler, 1969; Stockdale, Dawson & Selwyn, 1970), the effects of

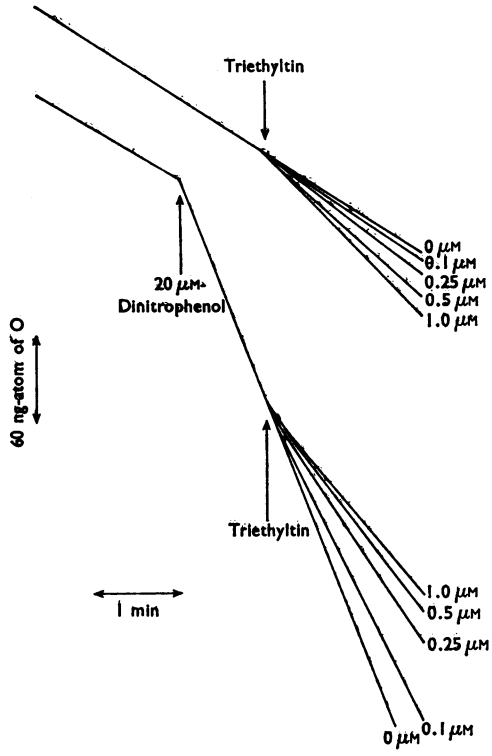


Fig. 14. Inhibition of oxygen uptake stimulated by 2,4-dinitrophenol by triethyltin. This figure contains a reconstruction of and an amalgamation of many tracings. The reaction was started by the addition of mitochondria (3.9 mg of protein). The substrate was pyruvate (10 mM) + fumarate (1 mM).

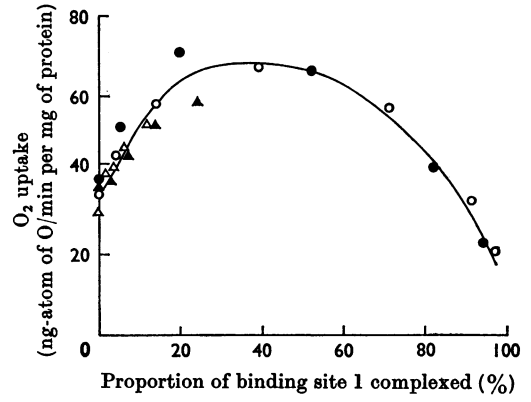


Fig. 15. Oxygen uptake and complexing by trimethyltin and triethyltin of binding site 1. The results for trimethyltin (○ and △) and for triethyltin (● and ▲) were determined by the manometric method (○ and ●) and by the polarographic method (△ and ▲) with pyruvate (10 mM) + fumarate (1 mM) as substrate.

oligomycin on the mitochondrial functions described in the present paper were determined. Because inhibition is progressive a meaningful comparison is not easy to make (Bertina, 1970). For example, measurements of P_i uptake with the different substrates must be made over a longer time-period than ATP hydrolysis stimulated by 2,4-dinitrophenol and this will influence the degree of inhibition obtained; for example, the inhibition of this ATP hydrolysis by 0.16 μg of oligomycin/ml was 20% when measured from 0 min to 2 min and 90% measured from 6 min to 8 min after the addition of oligomycin. However, under the conditions described in Fig. 16, no major difference in the sensitivity of the four systems was found; 6 μg of oligomycin/ml had no effect on oxygen uptake stimulated by 2,4-dinitrophenol.

DISCUSSION

General approach. It is accepted that oxidative phosphorylation is dependent on the membranous structure of mitochondria. The activity of this system is also affected by the environment in which the experiments are done. The present work is part of an extensive study of the chemical basis for the inhibition of oxidative phosphorylation by trialkyltins. A binding site for trimethyltin and triethyltin has been detected in rat liver mitochondria (Aldridge & Street, 1970). In this attempt to correlate binding with inhibitory effects by both compounds, changes in the composition of the media used for the study of each mitochondrial function have been kept to a minimum. This rigidity in the experimental conditions is intended to minimize variation in the quantitative responses of the different functions not due to the trialkyltins.

Biological and chemical specificity of trimethyltin and triethyltin. The only enzymic systems that have to date been shown to be sensitive to trialkyltins are oxidative phosphorylation in mitochondria and photophosphorylation by chloroplasts (Lynn, 1968; Kahn, 1968, 1970). Anaerobic glycolysis is not inhibited (Aldridge & Cremer, 1955; Cremer, 1962), nor are lactate dehydrogenase, L-glutamate dehydrogenase, L-malate dehydrogenase, succinate oxidase, yeast hexokinase or apyrase from potatoes (Aldridge & Cremer, 1955; Aldridge, 1958). The ATP hydrolysis by microsomal fraction from brain stimulated by Na^+ and K^+ ions is slightly inhibited at very much higher concentrations of triethyltin than those inhibiting oxidative phosphorylation (Aldridge & Street, 1964).

Triethyltin and trimethyltin combine with very few proteins (Rose & Aldridge, 1968; Rose, 1969, 1970). They have little or no affinity for thiols or dithiols (Aldridge & Cremer, 1955), and are not

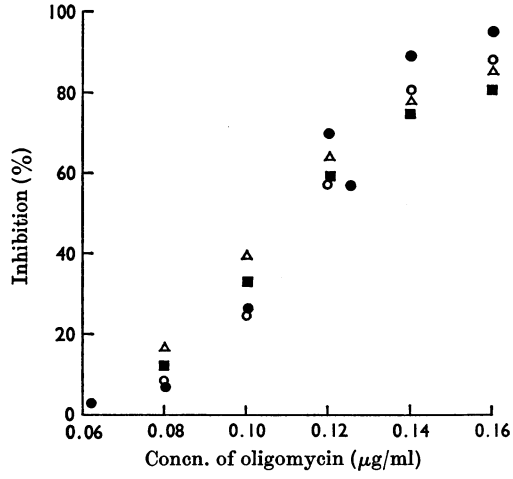


Fig. 16. Inhibition by oligomycin of various mitochondrial functions. Oxidative phosphorylation with pyruvate (10mM)+fumarate (1mM) (●) was measured over 0–13min, with succinate (10mM) in the presence of rotenone (○) over 0–8min, and with ascorbate (40mM) in the presence of *NNN'N'*-tetramethyl-*p*-phenylenediamine (1mM) (■) over 0–6min. ATP hydrolysis in the presence of 20μM-2,4-dinitrophenol (△) was measured over 6–8min. The concentration of mitochondrial protein was 1.3–1.4mg/ml. Under the same conditions oxygen uptake stimulated by 20μM-2,4-dinitrophenol with either pyruvate+fumarate or succinate in the presence of rotenone as substrate was not inhibited by 6μg of oligomycin/ml.

complexed by EDTA (Aldridge & Cremer, 1955) or by many other chemicals (Aldridge & Street, 1964). So far only two proteins have been found that complex triethyltin with an affinity of 10^5–$10^6 M^{-1}$, namely rat haemoglobin and a protein in liver supernatant fractions. Analysis of the binding data for rat haemoglobin indicate that binding is to one class of sites only (Rose, 1969). Triethyltin does have affinity for acidic lipids (Aldridge & Street, 1964), but the affinity is probably low (M. S. Rose, unpublished work). It is now concluded that triethyltin and trimethyltin, when they complex with macromolecules, react with two histidine residues/binding site in such a conformation that 5-co-ordination can take place (Rose, 1969, 1970). Such a conclusion is in agreement with the known chemistry of these compounds (Pollar, 1965; Luijten, Janssen & Van der Kerk, 1962).

In addition, it has been reported that trialkyltins mediate a Cl^-–OH^- exchange by virtue of the movement of the un-ionized trialkyltin chlorides into the mitochondrion (Selwyn, Dawson, Stockdale & Gains, 1970; Stockdale *et al.* 1970).

Do triethyltin and trimethyltin behave like oligomycin? It has been suggested that triethyltin behaves like oligomycin (Sone & Hagihara, 1964; Tyler, 1969; Manger, 1970; Stockdale *et al.* 1970). There are several ways in which the action of oligomycin and triethyltin or trimethyltin differ. With the experimental conditions used in the present paper: (1) high concentrations of oligomycin do not inhibit oxygen uptake stimulated by 2,4-dinitrophenol or other uncouplers, whereas triethyltin and trimethyltin do; oligomycin and the trialkyltins under the same conditions inhibit the complete oxidative phosphorylation system and also ATP hydrolysis stimulated by 2,4-dinitrophenol (Figs. 4, 10 and 16); (2) inhibition by oligomycin is slow and progressive (Bertina, 1970); inhibition by trimethyltin and triethyltin rapidly reaches a steady state (Figs. 12 and 14); (3) oligomycin inhibits to the same degree, at the same concentration, phosphorylation linked to oxidation of pyruvate, succinate and ascorbate; trimethyltin and triethyltin inhibit phosphorylation linked to oxidation of pyruvate and succinate at concentrations lower than those required to inhibit that linked to ascorbate. Thus the pattern of effects produced by oligomycin may be differentiated from that of trimethyltin and triethyltin.

Is binding site 1 involved in energy-conservation processes? Aldridge & Street (1970) concluded that a binding site in rat liver mitochondria had the correct properties for a site to which trimethyltin and triethyltin could be attached when oxidative phosphorylation is inhibited. By using the affinity constants for trimethyltin and triethyltin and the concentration of binding site 1 derived from the binding data, the proportion of binding site 1 complexed can be calculated from the added concentration of the respective tin compound. Identical results are obtained for both trimethyltin and triethyltin when the relationship between percentage inhibition and percentage of binding site 1 complexed is examined for all the parameters we have studied. This is true for those systems that are very sensitive (Figs. 3, 4, 5, 6, 9, 10 and 11) and for those less sensitive (Figs. 7, 8, 13 and 15). Thus although a concentration of trimethyltin 30–40 times higher than that of triethyltin is required, by the use of the independently determined binding constants a common relationship between inhibition and binding to site 1 is obtained for both compounds. Thus we conclude that binding site 1 is involved in oxidative phosphorylation and that it is involved in the energy-conservation process.

Is the whole of binding site 1 involved in energy conservation processes? The following mitochondrial activities were examined and show that, when inhibition of less than 50% is considered the ratio

(percentage inhibition)/(percentage of binding site 1 complexed) is approx. 10:1, and almost complete inhibition is obtained when 20–30% of binding site 1 is complexed by either trimethyltin or triethyltin: (1) phosphorylation linked to oxidation of pyruvate; (2) phosphorylation linked to oxidation of succinate; (3) ATP hydrolysis stimulated by uncoupling agents; (4) oxygen uptake stimulated by 2,4-dinitrophenol with pyruvate or succinate as substrate; (5) phosphorylation linked to oxidation of endogenous (intramitochondrial) substrates. This could suggest that only a part of what has been defined as a homogeneous binding site 1 is involved in these processes. However, the following observations suggest that the whole of binding site 1 is involved: (*a*) the ratio (percentage inhibition)/(percentage of binding site 1 complexed) for phosphorylation linked to oxidation of ascorbate is about 1:1; (*b*) the ATP hydrolysis stimulated by trimethyltin and triethyltin decreases with higher concentrations and approaches zero as the proportion of binding site 1 complexed approaches 100%; (*c*) the oxygen uptake (State 4) stimulated by both tin compounds decreases with increasing concentration and approaches zero as the proportion of binding site 1 complexed approaches 100%; this is true for oxidation of both pyruvate and succinate.

We therefore conclude that the whole of binding site 1 is involved in oxidative phosphorylation.

Consideration of the quantitative relationships between inhibition and complexing of binding site 1. It has been pointed out in the preceding section that there are two groups of mitochondrial functions one at least 10 times more sensitive to inhibition by trimethyltin and triethyltin than the other. The biological and chemical specificity of these trialkyltins suggests that some attempt ought to be made to explain the results on the basis of one site of action. However, other possibilities must be considered. Triethyltin may prevent the processes involved in movement of substrates into the inner membrane and matrix of the mitochondria (Manger, 1970). The concentration of substrate used when inhibition was found was much lower (0.5mM) than those found in this study. High concentrations of substrate (10mM) have been used, and even with 30mM-succinate very similar inhibition is obtained (Fig. 6). There must be major differences between the penetration of pyruvate or succinate and *NNN'N'* - tetramethyl - *p* - phenylenediamine for ascorbate oxidation. The interpretation of item 5 from the preceeding section is important with respect to transport of substrate in and out of the mitochondrion. One interpretation is that phosphorylation is linked to the oxidation of intramitochondrial substrate. It is also possible that in the presence of trimethyltin and triethyltin initially intramitochondrial substrate becomes distributed throughout the medium thus lowering its rate of oxidation and the consequent phosphorylation.

If the different sensitivities of the two groups of systems (1)–(5) and (*a*)–(*c*) above are taken to be due to two different binding sites then their required binding constants may be derived from the inhibitory data. If there is a 1:1 relationship between percentage inhibition and percentage complexing of both of these sites, then the affinity constants for the two sites may be calculated to be approx. $10^7 M^{-1}$ and $4 \times 10^5 M^{-1}$. If the Scatchard relationship for binding (Aldridge & Street, 1970) is calculated by using these constants, we find that the experimentally determined binding results and the calculated binding required by a two-site hypothesis are not compatible. Therefore an explanation of our experimental results on the basis of two binding sites with different affinities seems unlikely.

There is another possibility, namely that binding site 1 is involved in the inhibition of ATP synthesis linked to ascorbate and the other more sensitive mitochondrial functions are due to an effect of trimethyltin and triethyltin that does not involve a binding site. Such a possibility has been suggested by Stockdale *et al.* (1970) and Selwyn *et al.* (1970). They suggest that trialkyltins mediate an anion–OH^- exchange across lipid membranes that in media containing certain anions can produce uncoupling, swelling and lowering of intramitochondrial substrate and P_i concentrations, together with associated effects such as structural damage following gross swelling. This gross swelling is claimed to be the cause of the inhibition of oxygen uptake stimulated by uncoupling agents. None of the experimental conditions used in this paper lead to gross swelling. Such swelling that has been found is small and is well correlated with ATP hydrolysis and stimulation of oxygen uptake (Aldridge & Street, 1964; M. S. Rose, unpublished work). Therefore there are five reasons why we cannot accept that the inhibition of oxygen uptake stimulated by uncoupling agents is due to structural damage: (1) there is no gross swelling with our experimental conditions; (2) there is only a short delay of about 10s before maximum inhibition with both oxygen uptake and ATP hydrolysis stimulated by 2,4-dinitrophenol (Figs. 12 and 14); (3) after the short delay inhibition reaches a steady state; the agreement between the results obtained with the polarographic and the manometric methods indicates that this must be true; (4) oxygen uptake stimulated by 2,4-dinitrophenol is equally sensitive to triethyltin and to trimethyltin when either pyruvate or succinate is used as substrate; these two systems often show differences in sensitivity to structural damage; (5) Stockdale *et al.* (1970)

have used trialkyltins with higher molecular weight, and it is known that these homologues do produce gross swelling (Aldridge & Street, 1964).

Therefore there is evidence against the explanation put forward by Stockdale *et al.* (1970) being applicable to our conditions. Nevertheless, because of the ignorance about the co-ordination of the mitochondrial functions, it is not possible to reject the theory that trimethyltin and triethyltin exert an effect on ATP synthesis linked to oxidation of pyruvate, succinate or intramitochondrial substrate that is different from the effect they exert on ATP synthesis linked to oxidation of ascorbate.

Since both trimethyltin and triethyltin show a 1:1 or 1:10 relationship, as well as the chemical evidence of specificity and the absence of any positive evidence to indicate what any other effect might be, we must consider what arrangement is possible that would be compatible with our results and only one chemically distinct type of site. If binding site 1 is a homogeneous class of site and complexing of less than the whole of the site can lead to complete inhibition of phosphorylation linked to oxidation of pyruvate or succinate, then on a molecular basis there must be more than one binding site per energy-transfer unit. With values for inhibition less than 50%, the ratio of (percentage inhibition)/(percentage of binding site 1 complexed) is approx. 10:1. Our experimental results would therefore be explained if there were a chain of ten sites, only one of which had to be complexed with trimethyltin or triethyltin to prevent phosphorylation. Such an arrangement in which each binding site consists of two histidine residues has been previously suggested and fully discussed (Aldridge & Rose, 1969). Although this hypothesis appears to explain satisfactorily the results presented in the present paper, we cannot eliminate the possibility that both trimethyltin and triethyltin act in two different ways. The results of Selwyn *et al.* (1970) and Stockdale *et al.* (1970) have provided another parameter to be evaluated. The integration of their results and ours will require more experiments; if trimethyltin and triethyltin perturb mitochondrial functions in two ways, one requiring at least tenfold lower concentrations than the other, then one of them is associated with the binding of the trialkyltin to a site in mitochondria; on current evidence this seems likely to consist of two histidine residues/binding site.

REFERENCES

Aldridge, W. N. (1957). *Biochem. J.* **67**, 423.
Aldridge, W. N. (1958). *Biochem. J.* **69**, 367.
Aldridge, W. N. (1962). *Biochem. J.* **83**, 527.
Aldridge, W. N. & Cremer, J. E. (1955). *Biochem. J.* **61**, 406.
Aldridge, W. N., Emery, R. C. & Street, B. W. (1960). *Biochem. J.* **77**, 326.
Aldridge, W. N. & Parker, V. H. (1960). *Biochem. J.* **76**, 47.
Aldridge, W. N. & Rose, M. S. (1969). *FEBS Lett.* **4**, 61.
Aldridge, W. N. & Stoner, H. B. (1960). *Biochem. J.* **74**, 148.
Aldridge, W. N. & Street, B. W. (1964). *Biochem. J.* **91**, 287.
Aldridge, W. N. & Street, B. W. (1970). *Biochem. J.* **118**, 171.
Aldridge, W. N. & Threlfall, C. J. (1961). *Biochem. J.* **79**, 1961.
Bertina, R. M. (1970). *Biochem. J.* **116**, 9P.
Chamalaun, R. A. F. M. & Tager, J. M. (1969). *Biochim. biophys. Acta*, **207**, 204.
Cremer, J. E. (1962). *J. Neurochem.* **9**, 289.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Howland, J. L. (1963). *Biochim. biophys. Acta*, **77**, 419.
Jacobs, E. E. (1960). *Biochem. biophys. Res. Commun.* **3**, 536.
Kahn, J. S. (1968). *Biochim. biophys. Acta*, **153**, 203.
Kahn, J. S. (1970). *Biochem. J.* **116**, 55.
Lardy, H. A., Johnson, D. & McMurray, W. C. (1958). *Archs Biochem. Biophys.* **78**, 587.
Luijten, J. G. A., Janssen, M. J. & Van der Kerk, G. J. M. (1962). *Recl Trav. chim. Pays-Bas Belg.* **81**, 202.
Lynn, W. S. (1968). *Biochemistry, Easton*, **7**, 3811.
Manger, J. R. (1970). *FEBS Lett.* **5**, 331.
Moore, K. E. & Brody, T. M. (1961). *Biochem. Pharmac.* **6**, 125.
Packer, L. & Mustafa, M. G. (1966). *Biochim. biophys. Acta*, **113**, 1.
Parker, V. H. (1958). *Biochem. J.* **69**, 306.
Parker, V. H. (1965). *Biochem. J.* **97**, 658.
Pollar, R. S. (1965). *J. organomet. Chem.* **3**, 321.
Robinson, H. W. & Hogden, C. G. (1940). *J. biol. Chem.* **135**, 707.
Rose, M. S. (1969). *Biochem. J.* **111**, 129.
Rose, M. S. (1970). *Biochem. J.* **120**, 151.
Rose, M. S. & Aldridge, W. N. (1968). *Biochem. J.* **106**, 821.
Rossi, C. S. & Lehninger, A. L. (1963). *Biochem. Z.* **338**, 698.
Scatchard, G. (1949). *Ann. N.Y. Acad. Sci.* **51**, 660.
Selwyn, M. J., Dawson, A. P., Stockdale, M. & Gains, N. (1970). *Eur. J. Biochem.* **14**, 120.
Sone, N. & Hagihara, B. (1964). *J. Biochem., Tokyo*, **56**, 151.
Stockdale, M., Dawson, A. P. & Selwyn, M. J. (1970). *Eur. J. Biochem.* **15**, 342.
Ter Welle, H. F. & Slater, E. C. (1964). *Biochim. biophys. Acta*, **89**, 385.
Tyler, D. D. (1969). *Biochem. J.* **111**, 665.
Webster, G. R. & Smith, A. T. (1964). *Biochem. J.* **90**, 64.