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Oxidative Phosphorylation

THE SPECIFIC BINDING OF TRIMETHYLTIN AND TRIETHYLTIN TO RAT LIVER MITOCHONDRIA

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1. The binding of trimethyltin and triethyltin to rat liver mitochondria was determined and the results were analysed by the method of Scatchard (1949). 2. One binding site (site 1) has the correct characteristics for the site to which trimethyltin and triethyltin are attached when they inhibit oxidative phosphorylation. For each compound the concentration of site 1 is 0.8nmol/mg of protein and the ratios of their affinity constants are the same as the ratio of the concentrations inhibiting oxidative phosphorylation. 3. Binding site 1 is present in a fraction derived from mitochondria containing only 15% of the original protein. In this preparation ultrasonication rapidly destroyed site 1. 4. Dimethyltin and diethyltin do not prevent binding of triethyltin to rat liver mitochondria, whereas triethyl-lead does. 5. Trimethyltin and triethyltin bind to mitochondria from brown adipose tissue and the results indicate a binding site 1 similar to that in rat liver mitochondria. 6. The advantages and limitations of this approach to the study of inhibitors are discussed.

Oxidative phosphorylation is a process carried out by an enzymic system dependent on membranous structure, and because of this it has not been possible to understand its catalytic basis by the usual methods of division and purification of the separate components. The chemical basis of the mechanism whereby the energy derived from the oxidation of substrates is utilized for the synthesis of ATP from ADP and P_i is not known. Our present mental pictures of the integrated system are derived from the detailed examination of the effects produced by substances that perturb it. It is a reasonable hypothesis that those substances that inhibit the system do so by combination with a macromolecular component of the mitochondria. Therefore, if we understood the chemical basis for the interaction of an inhibitor of energy-conservation processes with a macromolecular component in the mitochondria, it may help towards an understanding of the chemical basis of oxidative phosphorylation.

Trialkyltins are potent inhibitors of oxidative phosphorylation and act on energy-conservation processes (Aldridge & Cremer, 1955; Aldridge, 1958; Aldridge & Threlfall, 1961; Moore & Brody, 1961; Aldridge & Street, 1964). These substances are suitable for this approach because they combine a high biological activity with a very limited chemical reactivity (Aldridge & Cremer, 1955; Rose & Aldridge, 1968). In addition they appear to be biologically very specific since only oxidative phosphorylation and photosynthesis (Kahn, 1968; Lynn, 1968) are affected by low concentrations. They may also be labelled with a convenient radioisotope, ^{113}Sn, and since they are stable measurement of the isotope is a measure of the trialkyltin. Thus it seems possible that a binding site for these compounds exists in mitochondria and this site is relevant to their inhibitory activity. Experiments with ^{113}Sn-labelled trimethyltin and triethyltin are described in this paper.

Preliminary accounts of some of this work have been published (Aldridge, 1968; Aldridge & Rose, 1969).

METHODS AND MATERIALS

Special chemicals. Triethyltin chloride and trimethyltin chloride labelled with ^{113}Sn were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. The specific radioactivities were 6mCi/mmol and 4mCi/mmol respectively. Stock solutions in ethanol were diluted with water for use.

Preparation of mitochondrial fractions. Mitochondria were prepared from rat liver as previously described (Aldridge & Street, 1964) by using a homogenizer with a total clearance of 0.5mm and a speed of rotation of

1100 rev./min (Aldridge, Emery & Street, 1960; Webster & Smith, 1964) and 0.3M-sucrose. Mitochondria from rat brown adipose tissue were isolated as described by Aldridge & Street (1968).

Fractionation of rat liver mitochondria. Rat liver mitochondria isolated as described above were suspended in 0.15M-KCl to a volume of 1.5ml/g of original liver. Equal volumes of this suspension and 67mM-Na_2HPO_4–KH_2PO_4 buffer, pH7.4, containing 2% (w/v) of Triton X-100 were mixed and incubated for 30min at 37°C. After centrifugation at 40000*g* for 30min, the supernatant and pellet were separated. The pellet was resuspended in 0.15M-KCl or 14mM-Na_2HPO_4–KH_2PO_4 buffer, pH7.0, to approx. 0.5ml/g of original liver. This fraction is called the 'TX-100 pellet' throughout this paper.

For the preparation of 'Lubrol membranes' by the method of Winkler & Lehninger (1968) Lubrol W Flakes (Imperial Chemical Industries Ltd., London S.W.1, U.K.) was used instead of Lubrol WX.

Binding of trimethyltin and triethyltin to rat liver mitochondria and the TX-100 pellet. Rat liver mitochondria or the TX-100 pellet were incubated for 15min in polypropylene tubes in a medium (10ml) containing the tin compounds labelled with ^{113}Sn. The composition of the medium is defined in the text for the particular experiments. After incubation, the tubes were centrifuged at 40000*g* for 30min. The supernatant and pellet were separated and the tube was drained and wiped free from supernatant. The radioactivities of the whole of the resuspended pellet and duplicate 2.0ml portions of the supernatant were counted. In other experiments equilibrium dialysis was used. The content of triethyltin or trimethyltin was calculated from the counts by reference to a sealed standard solution prepared from the original material and whose radioactivity was always counted with the unknowns. The results were plotted by the procedure of Scatchard (1949) and analysed by trial and error or latterly by using a computer programme devised by Mrs P. Corney based on solving the simultaneous equation derived by Hart (1965).

Measurement of radioactivity. Radioactivity counting was carried out with a Packard Autogamma scintillation spectrometer with an efficiency of 16%. For more details of the decay process consult Rose & Aldridge (1968).

Analytical methods. The following methods were used: protein by the method of Robinson & Hogden (1940) as modified by Aldridge (1962); monoamine oxidase and cytochrome oxidase as described by Schnaitman, Erwin & Greenawalt (1967); NADH–cytochrome *c* reductase (rotenone-insensitive) by the method described by Sottocasa, Kuylenstierna, Ernster & Bergstrand (1967); succinate–ferricyanide reductase by the method described by Aldridge & Johnson (1959) with the pretreatment advocated by Johnson (1962).

RESULTS

Binding of trimethyltin and triethyltin to rat liver mitochondria. In any study of binding in a heterogeneous system, a major difficulty is to distinguish between that binding which is relevant to the problem in hand and that which is not. Methods of evaluation are discussed by Edsall & Wyman (1958). We have plotted the results by using the method of Scatchard (1949) derived from the following equation:

$$\frac{B}{F} = K_1(n_1 - b_1) + K_2(n_2 - b_2) \ldots + K_i(n_i - b_i) \qquad (1)$$

where b_1, b_2 . . . b_i is the concentration of ligand bound to an infinite series of sites (1, 2 . . . i) of concentration n_1, n_2 . . . n_i and corresponding affinity constants K_1, K_2 . . . K_i. B and F are the total concentrations of bound and free ligand respectively.

When there is only one class of sites, i.e. they all possess the same affinity constant, then a graph relating B/F against B will yield a straight line; the intercept on the B/F axis (i.e. when $B = 0$) is nK, the intercept on the B axis (i.e. when $B/F = 0$) is n and the slope of the line is $-K$. With more than one class of sites a straight line is not obtained, but by curve-fitting the constants for each class of

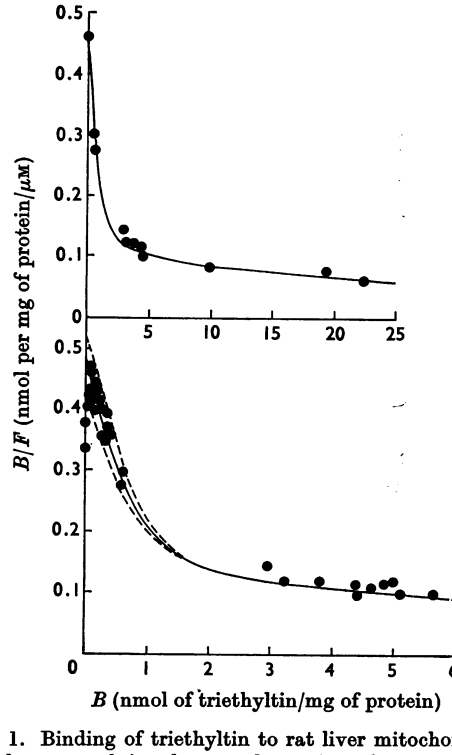

Fig. 1. Binding of triethyltin to rat liver mitochondria. The lower graph is a large-scale version of a part of the top graph. The solid line is that calculated for a two-component system of binding sites 1 and 2 with the following constants: n_1, 0.8nmol/mg of protein; K_1, $4.7 \times 10^5 M^{-1}$; n_2, 66nmol/mg of protein; K_2, $1.4 \times 10^3 M^{-1}$. For an explanation of the broken lines see the text.

sites may be calculated. In practice there are limitations to this method of analysis and the relative sizes of n_1, n_2 . . . and K_1, K_2 . . . greatly influence the accuracy of the procedure and the number of different sites that may be detected (see the Discussion section).

Fig. 1 shows the accumulated results from several experiments to determine the distribution of triethyl[^{113}Sn]tin between mitochondria and medium. The medium was that previously described for the oxidation of pyruvate (Aldridge & Stoner, 1960) and with necessary minor modifications is that used for all previous studies of the effects of trialkyltins on oxidative phosphorylation. A satisfactory fit to the results may be obtained by a minimum of two classes of sites (1 and 2) with constants $n_1 = 0.8$nmol/mg of protein, $K_1 = 4.7 \times 10^5$M^{-1}, and $n_2 = 66$nmol/mg of protein, $K_2 = 1.4 \times 10^3$M^{-1}. The line drawn is that calculated from these constants. We have been unable to obtain a statistical evaluation of the possible error in these values. However, marked in Fig. 1 are two interrupted lines covering the range of the results over which binding to site 1 is predominant. Acceptable fit within this area is obtained for values of n_1 of 0.74–0.86nmol/mg of protein and of K_1 of 4.4–5.1×10^5M^{-1}.

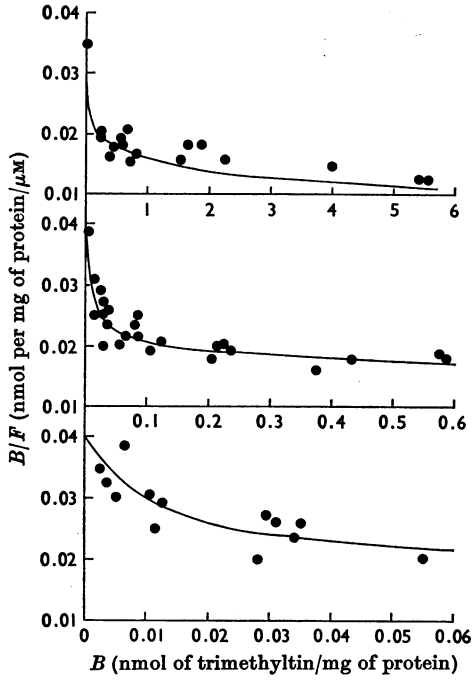

Fig. 2. Binding of trimethyltin to rat liver mitochondria. The lower two graphs are large-scale versions of parts of the top graph. The solid line is that calculated for a three-component system of binding sites 1, 2 and 3 with the following constants: n_1, 0.8nmol/mg of protein; K_1, 1.2×10^4M^{-1}; n_2, 120nmol/mg of protein; K_2, 1×10^2M^{-1}; n_3, 0.0042nmol/mg of protein; K_3, 4×10^6M^{-1}.

Triethyltin inhibits oxidative phosphorylation and activities considered to be part of or modifications of the energy-conservation mechanism (Aldridge & Cremer, 1955; Aldridge, 1958; Aldridge & Threlfall, 1961; Aldridge & Street, 1964). Effective concentrations have been from 0.1 to 10μM, thus indicating an affinity constant within the range 10^5–10^7M^{-1}. The affinity constant ($K_1 = 4.7 \times 10^5$M^{-1}) for a binding site for triethyltin in rat liver mitochondria indicates that this site is a possible candidate for the site involved when triethyltin inhibits oxidative phosphorylation.

Trimethyltin behaves, with respect to oxidative phosphorylation, qualitatively in the same way as triethyltin (Aldridge, 1958; Aldridge & Street, 1964). However, effective concentrations are 30–40-fold higher. Therefore the binding of trimethyltin to rat liver mitochondria was examined. The results of these experiments are shown in Fig. 2. A satisfactory fit is obtained with a minimum of three classes of sites with the following constants: $n_1 = 0.8$nmol/mg of protein, $K_1 = 1.2 \times 10^4$M^{-1}; $n_2 = 120$nmol/mg of protein, $K_2 = 1.0 \times 10^2$M^{-1}; $n_3 = 0.0042$nmol/mg of protein, $K_3 = 4 \times 10^6$M^{-1}. These results were obtained by using a 7000-fold range of concentrations (0.07–500μM). The results are rather scattered, but this is expected in view of the low values for the B/F ratio, i.e. the problems of contamination of the pellet by supernatant become greater. The value for the concentration of binding site 1 when both triethyltin and trimethyltin were used is identical with that obtained with either agent and the ratio of their affinity constants is that expected from the ratio of the concentrations that inhibit oxidative phosphorylation (Table 1). Therefore these results are consistent with this binding site being that to which trimethyltin and triethyltin are attached when oxidative phosphorylation is inhibited.

The affinity constants derived from the concentrations of trimethyltin and triethyltin necessary for 50% inhibition of oxidative phosphorylation are lower than those derived from measurements of binding (Table 1). It has been shown that phosphorylation linked to the oxidation of cytochrome *c* (ascorbate as substrate) requires higher concentrations for 50% inhibition, indicating affinity constants close to those obtained from binding measurements (Aldridge & Rose, 1969).

Binding of trimethyltin and triethyltin to the TX-100 pellet from rat liver mitochondria. Even though the above results are consistent with the view that binding site 1 could be involved in oxidative phosphorylation, the physical conditions in

Table 1. *Binding of trimethyltin and triethyltin to the TX-100 pellet from rat liver mitochondria*

For the preparation of the TX-100 pellet and for the determination of binding see the Methods and Materials section. The results are expressed as mean ± S.E.M. (no. of observations). n_1 and n_2 are the concentrations of binding sites 1 and 2 and K_1 and K_2 are their affinity constants. I_{50} is the concentration causing 50% inhibition of oxidative phosphorylation.

	Triethyltin	Trimethyltin
n_1 (nmol/mg of protein)	5.64±0.49 (5)	6.17 (2)
K_1 (M^{-1})	$3.94\pm0.32\times10^5$ (5)	1.03×10^4 (2)
n_2 (nmol/mg of protein)	146 ±55 (5)	57.5 (2)
K_2 (M^{-1})	$7.12\pm2.29\times10^3$ (5)	8×10^2 (2)
Protein recovered in the fraction (%)	15.6±0.98 (5)	16.8
I_{50} (μM)	0.20*	6.9*
Affinity constant derived from I_{50} (M^{-1})	5×10^6	1.4×10^5

* Taken from Aldridge (1958) and Aldridge & Street (1964).

suspensions of intact mitochondria are obviously very complex. Many of the problems of permeability and compartmentation were excluded by using a fraction prepared by disruption of mitochondria with Triton X-100 in phosphate buffer. By this treatment 80–85% of the protein is solubilized and only 15% is sedimented by centrifugation at 40000*g* for 30min to yield an insoluble residue containing all of binding site 1 (Table 1; compare Fig. 5 showing similar results obtained with an equilibrium-dialysis technique). A typical curve relating B/F against B is shown in Fig. 3. The binding of trimethyltin was compared with that of triethyltin; for both compounds the concentration of binding site 1 is the same and the affinity constants approximate to those obtained with intact mitochondria, so that the ratio between them is that expected from their inhibitory power against oxidative phosphorylation. Since 15–17% of the protein is recovered in the TX-100 pellet and the values of n_1 have increased from 0.8 to 5.6–6.2nmol/mg of protein (i.e. seven- to eight-fold) almost all of binding site 1 in the original mitochondria is accounted for in the TX-100 pellet. The values of n_2 and K_2 for site 2, as shown by the large standard errors, were rather variable. Therefore the results obtained for intact mitochondria and for the TX-100 pellet strongly support the view that site 1 is that site to which trimethyltin and triethyltin combine when they inhibit oxidative phosphorylation.

Winkler & Lehninger (1968) have used another detergent, Lubrol WX, to prepare a membrane fraction containing the atractyloside-binding site from rat liver mitochondria. In our hands this preparation, which contains no intact mitochondria, has approximately double the specific activity of adenosine triphosphatase and succinate–ferricyanide reductase when compared with the original mitochondria (Table 2). The binding of triethyltin to this preparation has been examined; its affinity

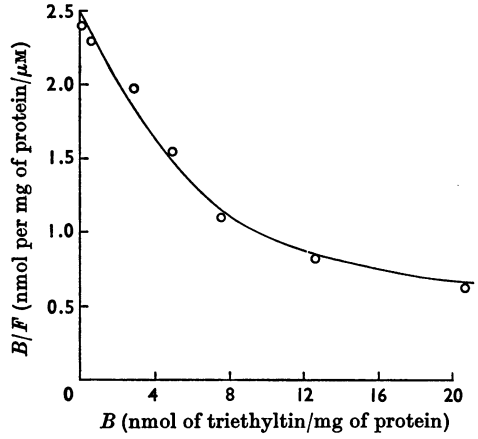

Fig. 3. Binding of triethyltin to the TX-100 pellet. The solid line is that calculated for a two-component system of binding sites 1 and 2 with the following constants: n_1, 6.5nmol/mg of protein; K_1, $3.1\times10^5\,M^{-1}$; n_2, 278nmol/mg of protein; K_2, $1.8\times10^3\,M^{-1}$.

for binding site 1 is unchanged but its concentration has increased to approximately the same extent as the increase in the specific activities of adenosine triphosphatase and succinate–ferricyanide reductase. Winkler & Lehninger (1968) consider that this preparation is enriched in inner membrane; our results support this view (see below).

Binding of trimethyltin and triethyltin to mitochondria from brown adipose tissue. Trimethyltin and triethyltin bind to mitochondria from brown adipose tissue of the rat. The values for n_1 and K_1 approximate to those obtained for binding site 1 in rat liver mitochondria (Table 3). These measurements were made because it was suggested that such mitochondria, although they contained densely packed cristae, were unable to synthesize ATP by an electron-transport-coupled system

Table 2. *Succinate–ferricyanide reductase, adenosine triphosphatase and the binding of triethyltin to 'Lubrol membranes'*

The membranes were prepared and adenosine triphosphatase activity was determined as described by Winkler & Lehninger (1968).

	Original mitochondrial fraction	Mitochondria +Lubrol W	'Lubrol membranes'	Supernatant
Succinate–ferricyanide reductase (μl of CO_2/min per mg of protein)	2.6	2.1	5.4	0
Adenosine triphosphatase (μg atom of P_i/min per mg of protein)	—	0.30	0.66	0.016
Binding of triethyltin				
n_1 (nmol/mg of protein)	0.8	—	2.0	—
K_1 (M^{-1})	4.75×10^5	—	4.3×10^5	—
n_2 (nmol/mg of protein)	66	—	50	—
K_2 (M^{-1})	1.4×10^3	—	5×10^3	—

Table 3. *Binding of trimethyltin and triethyltin to mitochondria from brown adipose tissue*

Mitochondria were prepared as described by Aldridge & Street (1968).

		Trimethyltin	
	Triethyltin	(1)	(2)
n_1 (nmol/mg of protein)	0.62	0.53	0.71
K_1 (M^{-1})	1.94×10^6	3×10^4	3.5×10^4
n_2 (nmol/mg of protein)	20	70	72
K_2 (M^{-1})	1×10^4	4×10^2	4×10^2

(Smith, Roberts & Hittelman, 1966; Lindberg, De Pierre, Rylander & Afzelius, 1967). However, subsequent work has shown that mitochondria from brown adipose tissues are able to synthesize ATP coupled to electron transport (Joel, Neaves & Rabb, 1967; Aldridge & Street, 1968; Hohorst & Rafael, 1968; Guillory & Racker, 1968; Christiansen, Pedersen & Grav, 1969) and are inhibited by triethyltin (Aldridge & Street, 1968).

Influence of other substances on the binding of triethyltin to rat liver mitochondria. Triethyltin and diethyltin have different biological actions. Diethyltin inhibits the oxidation of α-oxo acids in a similar way to arsenicals, by possessing a high affinity for dithiols (Aldridge & Cremer, 1955). Other studies of a series of dialkyltin homologues have confirmed this conclusion and no biochemical properties common to both dialkyltins and trialkyltins have been found (W. N. Aldridge, unpublished work). This biological specificity is reflected in a lack of influence of dialkyltins on the binding of trialkyltins to rat liver mitochondria (Fig. 4). For these experiments 10 μM-diethyltin or 10 μM-dimethyltin was added to a range of concentrations of triethyltin (0.08–44 μM, i.e. [dialkyltin]/[triethyltin] ratio 0.23–125). In contrast triethyl-lead, which, although less effective, behaves similarly to triethyltin in its effects on oxidative phosphorylation (Aldridge, Cremer & Threlfall, 1962), does decrease the binding of triethyltin (Fig. 4). At all concentrations of triethyltin when the [triethyl-lead]/[triethyltin] ratio is 10, the binding of triethyltin is less than the controls.

Properties of the TX-100 pellet. Since it seems probable that binding site 1 in intact mitochondria or the TX-100 pellet is involved in the process of oxidative phosphorylation it is important to know from what part of the mitochondria it arises. The treatment to isolate the TX-100 pellet is a very effective way of solubilizing mitochondrial protein for 80–85% is not sedimented when centrifuged at 40000*g* for 30min (Table 1). The distribution of certain enzymes, generally accepted as markers for inner membrane and outer membrane, in the TX-100 pellet and the supernatant fraction was determined. Interpretation of the results is difficult because the treatment itself lowers enzyme activity, the reason for which is not known (Table 4). Of the activity recovered, almost all the succinate–ferricyanide reductase, the rotenone-insensitive

NADH–cytochrome *c* reductase and monoamine oxidase is in the supernatant fraction. Cytochrome oxidase, however, is present in both the insoluble and supernatant fractions, and although the proportions in this fraction are variable the pellet always has the higher specific activity. These facts suggest that the TX-100 pellet is a component of the inner membrane (Sottocasa *et al.* 1967; Schnaitman *et al.* 1967) with little or no contamination with outer membrane.

Adenosine triphosphatase activity in the TX-100 pellet is always low. After removal of phosphate from the TX-100 supernatant fraction, adenosine triphosphatase activity may be demonstrated. Good recovery of adenosine triphosphatase activity is difficult to achieve after most treatments of mitochondria. The results of an experiment are shown in Table 5 and indicate that, in contrast with treatment with water, after treatment with Triton X-100 in phosphate buffer adenosine triphosphatase activity is found in the supernatant fraction. This has been a constant finding in many experiments even though the actual adenosine triphosphatase activity found varies; this suggests that the binding site 1 for trimethyltin and triethyltin in the TX-100 pellet is not a protein or proteins possessing adenosine triphosphatase activity.

Ultrasonication of the TX-100 pellet showed that binding of triethyltin to site 1 was rapidly destroyed whereas that to site 2 was not (Fig. 5). No capacity to bind triethyltin appeared in the suspension medium after such treatment. The implication of these findings is that in the TX-100 pellet tertiary structure is necessary for the binding of triethyltin to site 1. This tertiary structure that survives the treatment with Triton X-100 is apparently easily destroyed by ultrasonication.

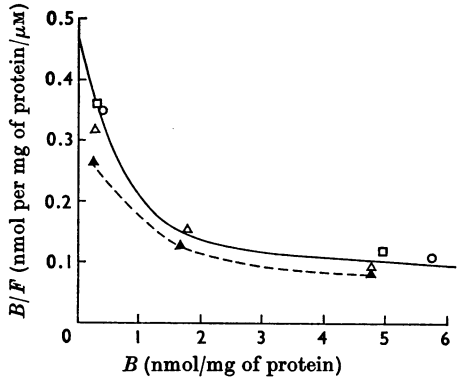

Fig. 4. Effect of dimethyltin, diethyltin and triethyl-lead on the binding of triethyltin to rat liver mitochondria. The solid line is taken from Fig. 1. Diethyltin (○) and dimethyltin (□) were present in 10μM concentration throughout. Triethyl-lead was present so that the [triethyl-lead]/[triethyltin] ratio was 1 (△) and 10 (▲).

DISCUSSION

The binding of triethyltin to rat liver mitochondria has been determined. The results, when analysed by the method of Scatchard (1949), may be described by a minimum of two classes of sites. One class of binding site (site 1) has an affinity of approx. $5 \times 10^5 M^{-1}$ and a concentration of 0.8nmol/mg of protein. The other class of site is of much lower affinity and higher concentration. Binding site 1 survives treatment of rat liver mitochondria with Triton X-100 in phosphate buffer. By this treatment 85% of the mitochondrial protein is solubilized and the concentration of binding site 1 in the pellet becomes 5.6nmol/mg of protein, thus accounting for all of site 1 found in the original

Table 4. *Activity of some enzymes in fractions obtained by treatment of rat liver mitochondria with Triton X-100 in phosphate buffer*

The sums of the activities (TX-100 pellet + supernatant) compared with those in the 'mitochondria + Triton X-100' were 92% for succinate–ferricyanide reductase, 95% for cytochrome oxidase, 95% for NADH–cytochrome *c* reductase and 91% for monoamine oxidase.

	Original mitochondrial fraction	Mitochondria +Triton X-100	TX-100 pellet	Supernatant
Succinate–ferricyanide reductase (μl of CO_2/min per mg of protein)	9.5–13.4	2.1–3.9	0–0.9	1.7–4.4
Cytochrome oxidase (μg-atom of O/min per mg of protein)	0.85	0.23	0.76	0.12
NADH–cytochrome *c* reductase (rotenone-insensitive) (E^{1cm}_{549}/min per mg of protein)	1.74	0.61	0.015	0.69
Monoamine oxidase (E^{1cm}_{250}/min per mg of protein)	0.043	0.022	0.0056	0.023

Table 5. *Distribution of adenosine triphosphatase between fractions from rat liver mitochondria*

For the treatment with water, 1 vol. of mitochondrial suspension (15.2mg of protein/ml) was added to 3 vol. of water. The treatment of mitochondria with Triton X-100 in phosphate buffer is described in the Methods and Materials section. After incubation of both suspensions for 30min at 37°C, they were separated into supernatant and pellet fractions by centrifugation at 40000*g* for 30min. Phosphate was removed from the TX-100 supernatant by passage through a column of Amberlite IRC-400 (acetate form). Adenosine triphosphatase was determined as described by Aldridge & Stoner (1960) except that, because of the presence of Triton X-100, P_i was measured by the turbidimetric method of Eibl & Lands (1969).

Treatment	Adenosine triphosphatase activity (μg-atom of P/min per mg of protein)	Adenosine triphosphatase activity (μg-atom of P/min per ml of original mitochondrial suspension)	Protein (mg/ml of original mitochondrial suspension)
Water			
Supernatant	0	0	6.5
Pellet	0.024	0.24	10.0
Triton X-100–phosphate			
Supernatant	0.0063	0.083	13.2
Pellet	0.0057	0.0143	2.5

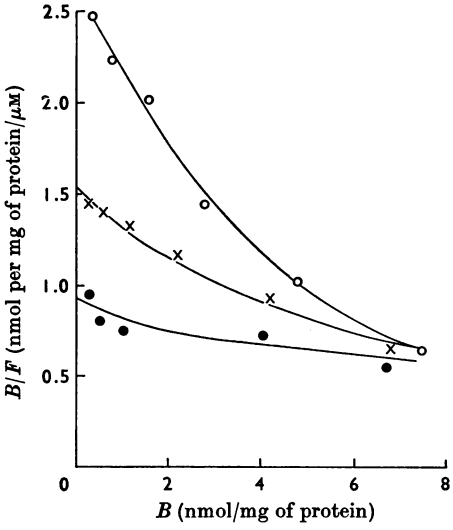

Fig. 5. Effect of ultrasonication on the binding of triethyltin to the TX-100 pellet. The suspensions were treated for 1min (×) or 3min (●) in ice. The binding of triethyltin to the control (○) and to the treated TX-100 pellets was determined by equilibrium dialysis.

mitochondria. The binding of trimethyltin to intact mitochondria and to the TX-100 pellet also yields concentrations of binding site 1 of respectively 0.8 and 6.2nmol/mg of protein. The affinity constant of the binding of trimethyltin is about $1.1 \times 10^4 M^{-1}$. This is about 40-fold lower than that derived from the binding of triethyltin. This difference is that expected from the inhibitory power of the two compounds against oxidative phosphorylation. Thus the affinity constants for trimethyltin and triethyltin have the relationship to one another predicted from their biological activity; in contrast the affinity constants for the binding of these two organotins to rat haemoglobin do not differ greatly, their affinities being 3.5×10^5 and $2.8 \times 10^5 M^{-1}$ for triethyltin and trimethyltin respectively (Rose, 1968, 1969). Mitochondria from brown adipose tissue also bind triethyltin; this is consistent with the now accepted view that they can carry out oxidative phosphorylation. The binding of triethyltin to rat liver mitochondria is unaffected by the presence of diethyltin or dimethyltin; this is in accord with their differing biological properties. In contrast triethyl-lead, which affects oxidative phosphorylation in a similar way to triethyltin, does lower the apparent affinity of triethyltin for mitochondria. Thus all the evidence so far available is in favour of the view that binding site 1 is that site to which triethyltin and trimethyltin are attached when they inhibit phosphorylation. Studies of the relationship between inhibition and binding to site 1 have also provided evidence to support this view (Aldridge & Rose, 1969).

In studies of interactions of a dissociable nature of small molecules with macromolecules, binding to different sites may only be distinguished if they have different affinity constants. The first requirement for such studies is an analytical method sufficiently sensitive for use at concentrations lower than those that are biologically active (cf. Winkler & Lehninger, 1968). In retrospect, we can appreciate the futility of our earlier experiments on binding (Aldridge & Threlfall, 1961). A chemical

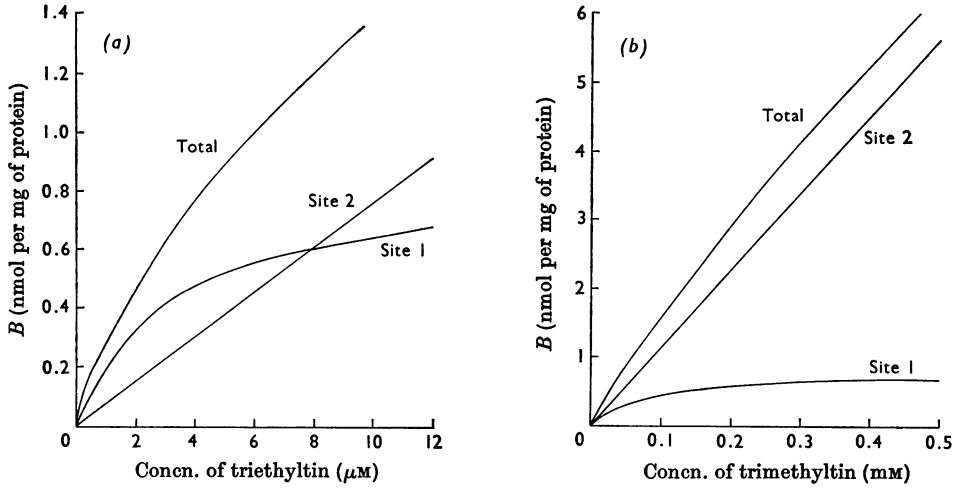

Fig. 6. Relationship of total amounts of bound organotin and that bound to sites 1 and 2 to the concentration used. (*a*) Curves calculated by using eqn. (1) and the constants for triethyltin of n_1, 0.8nmol/mg of protein, K_1 4.7×10^5M^{-1}, n_2 66nmol/mg of protein, K_2 1.4×10^3M^{-1} and a concentration of mitochondrial protein of 12.5mg/10ml. (*b*) Curves calculated by using eqn. (1) and the constants for trimethyltin of n_1 0.8nmol/mg of protein, K_1 1.2×10^4M^{-1}, n_2 120nmol/mg of protein and K_2 1×10^2M^{-1} and a concentration of mitochondrial protein of 12.5mg/10ml. The binding to site 3 has been neglected.

analytical method (Aldridge & Cremer, 1957) was used at the limit of its sensitivity. At the lowest concentration of triethyltin used (40μM) we now appreciate that approx. 80% of the organotin was bound to the binding site 2. For the present work triethyltin and trimethyltin that were sufficiently radioactive to measure concentrations of 10nM were used. A very wide range of concentrations must be examined. Even if such methods are available there are other criteria that must be satisfied if reasonably accurate results are to be obtained. So that different classes of sites may be differentiated, the magnitude of the product nK (cf. eqn. 1) ought to be greater for the relevant site than for others. When the binding of triethyltin to rat liver mitochondria was studied n_1K_1 was 0.380 and n_2K_2 was 0.092 (nmol/mg of protein$\times\mu$M^{-1}; cf. Fig. 1); this allowed a fairly accurate assessment of the characteristics of binding site 1. For similar studies with trimethyltin n_1K_1 was 0.0096 and n_2K_2 0.012 (cf. Fig. 2); assessment of the characteristics of binding site 1 is thus uncertain and is reflected in the scatter of the results. Irrespective of the accuracy of the derived values for the constants it is essential to attempt an analysis of the results in this way. As shown in Figs. 6(*a*) and 6(*b*), the total amount bound is not a measure of one particular site.

The position of binding site 1 in the structure of the mitochondria is not known, but our results indicate that it is probably part of the inner membrane. Whatever structure is necessary to bind triethyltin survives treatment of the mitochondria with Triton X-100 in phosphate buffer, a procedure that 'solubilizes' 85% of the protein. Treatment of this TX-100 pellet with ultrasonics removes the capacity to bind triethyltin and probably indicates further damage to an essential tertiary structure. The fact that binding of triethyltin (binding site 1) is in the insoluble fraction (TX-100 pellet) whereas much of the adenosine triphosphatase is in the supernatant probably indicates that triethyltin does not act on the adenosine triphosphatase directly, a conclusion that is consistent with previous views (Aldridge, 1958).

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