Oxidative Phosphorylation

BIOCHEMICAL EFFECTS AND PROPERTIES OF TRIALKYLTINS

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Many workers have produced evidence that in rat-liver mitochondria oxidative phosphorylation, adenosine triphosphatase and swelling are related activities (for reviews see Lehninger, 1962*a*; Racker, 1961). Oxidative phosphorylation of rat-liver mitochondria is inhibited by low concentrations of trialkyltins (Aldridge & Cremer, 1955; Aldridge, 1958; Aldridge & Threlfall, 1961; Moore & Brody, 1961*a*). Adenosine-triphosphatase activity stimulated by 2,4-dinitrophenol and the [^{32}P]phosphate–ATP exchange reaction, both of which are considered to involve some of the reactions of oxidative phosphorylation, are inhibited by the same concentrations of trialkyltins that inhibit oxidative phosphorylation. Adenosine-triphosphatase activity is also stimulated by trialkyltins (Aldridge, 1958), but the degree to which it is stimulated varies with the particular trialkyltin and is never more than 40 % of the activity obtainable with 2,4-dinitrophenol.

In the present paper it is shown that trialkyltins cause swelling of mitochondria and that this occurs at the same concentrations that cause stimulation of adenosine-triphosphatase activity and inhibition of oxidative phosphorylation. The effects of trialkyltins on other biochemical systems and also their chemical properties have been studied to try to gain some insight in chemical terms of their activity against oxidative phosphorylation.

MATERIALS AND METHODS

Rat-liver mitochondria. Rat-liver mitochondria were prepared as described by Aldridge (1957, 1958) by using an homogenizer with a total clearance of 0·02 in. and a speed of rotation of 1100 rev./min. The construction of the homogenizer has been described by Aldridge, Emery & Street (1960). Oxidative phosphorylation and respiration in the absence of hexokinase and glucose were measured according to the method of Aldridge (1958) by using pyruvate (0·01 M) with fumarate (1 mM) as substrate. Uptake of inorganic phosphate was measured by using initial concentrations of potassium phosphate of 15 and 7 mM and over the times 10–22 min. and 0–10 min. respectively. The degree of inhibition of phosphate uptake by any of the trialkyltins studied was unaffected by these changes of procedure. Adenosine-triphosphatase activity was measured with the medium and methods described by Aldridge & Stoner (1960).

Microsomal fraction from rat brain. Rat brain was homogenized and the microsomal fraction isolated as described by Aldridge (1962). The hydrolysis of ATP in the presence and absence of Na^+ and K^+ ions was determined as described by Aldridge (1962). Phosphatidate phosphatase was also measured in the medium used for the assay of adenosine-triphosphatase activity in the presence of NaCl (150 mM) and KCl (15 mM). After incubation for 1 hr. at 37°, 10 mg. of bovine serum albumin was added and the reaction stopped by adding perchloric acid (final concn. 0·5 N). After centrifuging, the inorganic phosphate was determined in the clear supernatant by the method of Bartlett (1959).

Swelling of mitochondria. The swelling of mitochondria was followed by the decrease in extinction at 520 mμ (measured with a Unicam D.G. spectrophotometer) in the medium described by Lehninger (1959*b*) or in the medium used for assaying adenosine-triphosphatase activity (Aldridge & Stoner, 1960).

Inhibitors. The trialkyltin acetates were supplied by Dr G. J. M. Van der Kerk. These compounds were synthesized by published methods (Van der Kerk & Luijten, 1956). Solutions were prepared in dimethylformamide and a sample (0·03 ml.) was added to the medium such that a final concentration of 1 % (v/v) was never exceeded. This concentration of dimethylformamide has a negligible effect on the activity of the controls. Occasionally triethyltin sulphate was used, and this was prepared as described by Aldridge & Cremer (1955) from triethyltin hydroxide supplied by the Tin Research Institute, Greenford, Middx. This was added to media as a solution in water neutralized to pH 7 with KOH.

Special chemicals. The following chemicals were obtained from the sources stated: diolein (from Distillation Products Industries, Rochester, N.Y., U.S.A.); sheep-brain kephalin, $\beta\gamma$-dipalmitoyl-DL-α-lecithin, ovalbumin, sphingomyelin (containing 3·3 % of phosphorus, by analysis) and L-thyroxine (from L. Light and Co. Ltd., Colnbrook, Bucks.); salmine sulphate, clupeine sulphate and heparin (from British Drug Houses Ltd., Poole, Dorset); yeast RNA and egg phosvitin (from Nutritional Biochemicals Corp., Cleveland 28, Ohio, U.S.A.); dextran and dextran sulphate (from Glaxo Laboratories Ltd., Greenford, Middx.); α-lipoic acid (from Sigma Chemical Co., St Louis 18, Mo., U.S.A.); calf-thymus DNA (from Mann Research Laboratories Inc., New York, N.Y., U.S.A.); and lysolecithin (kindly supplied by Professor R. H. S. Thompson). Ox-brain sulpholipid was prepared by the method of Lees, Folch, Sloane-Stanley & Carr (1959); the sulphur content

after decomposition (Davison & Gregson, 1962) and determination of the sulphate by a turbimetric method (Dodgson, 1961) was 3·4% (calc. for potassium cerebron sulphate: 3·38%). Dioleoylphosphatidic acid was prepared by standard methods from diolein and $POCl_3$ with pyridine as catalyst. After the product had been dissolved in ether, the solution washed repeatedly with N-HCl and the ether removed, the product was neutralized to pH 7 with NaOH. The emulsion of sodium phosphatidate was dialysed free from inorganic phosphate and was stored at 5°.

RESULTS

Effect of trialkyltins on rat-liver mitochondria

Adenosine-triphosphatase activity and swelling. During determinations of adenosine-triphosphatase activity it was noticed that at some concentrations of trimethyltin a slight decrease in the opalescence of the solution occurred. Since this could indicate some mitochondrial swelling (Cleland, 1952), the extinction of the mitochondrial suspensions was measured in the presence of various concentrations of trimethyltin in the medium used for the adenosine-triphosphatase assay. As increasing concentrations of trimethyltin were used up to 30 μM [$-\log$ (concentration) = 4·5], the extinction decreased (Fig. 1). With higher concentrations the extinction increased. Swelling therefore ran parallel to the adenosine-triphosphatase activity which also increased to a maximum at approx. 30 μM and then decreased with higher concentrations. Aldridge (1958) has established that the degree of stimulation of adenosine-triphosphatase activity is dependent on the particular trialkyltin used. Extinction measurements were therefore made with triethyltin, tri-*n*-propyltin and tri-*n*-butyltin, and the results are given in Figs. 2–4. With all the compounds there is a relationship between the extinction changes and the adenosine-triphosphatase activity,

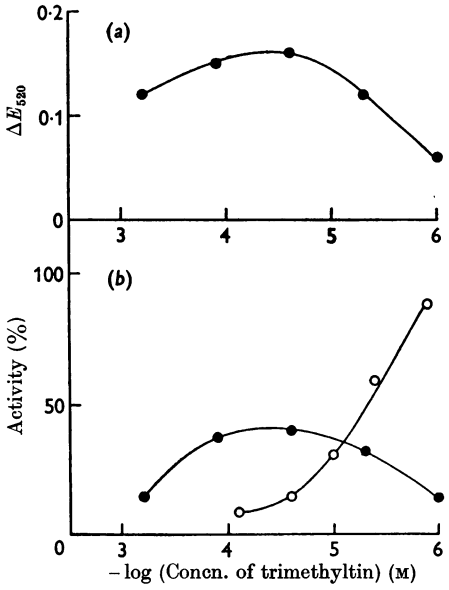

Fig. 1. Effect of trimethyltin on swelling, adenosine-triphosphatase activity and oxidative phosphorylation of rat-liver mitochondria. The determinations were carried out as described in the Materials and Methods section. Mitochondria were added to the medium containing trimethyltin. (*a*) Changes in extinction measured with mitochondrial suspensions containing 1·33 and 1·48 mg. of mitochondrial protein/ml. (*b*) Adenosine-triphosphatase activity (●) expressed as the percentage of the maximum activity obtainable with 2,4-dinitrophenol (2 experiments with control activities of 0·10 and 0·42, and in the presence of 30 μM-2,4-dinitrophenol of 12·0 and 13·6 μg.atoms of P/mg. of protein/hr. respectively); and oxidative phosphorylation (○) expressed as the percentage of the control rate (2 experiments with activities of 20·3 and 19·7 μg.atoms of P/mg. of protein/hr.).

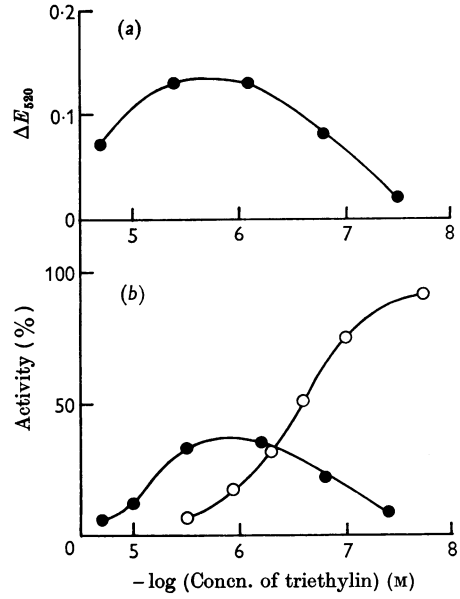

Fig. 2. Effect of triethyltin on swelling, adenosine-triphosphatase activity and oxidative phosphorylation of rat-liver mitochondria. The determinations were carried out as described in the Materials and Methods section. Mitochondria were added to the medium containing triethyltin. (*a*) Changes in extinction measured with mitochondrial suspensions containing 1·35, 1·43 and 1·55 mg. of mitochondrial protein/ml. (*b*) Adenosine-triphosphatase activity (●) expressed as the percentage of the maximum activity obtainable with 2,4-dinitrophenol (3 experiments with control activities of 0·40, 0·29 and 1·0, and in the presence of 30 μM-2,4-dinitrophenol of 12·5, 12·3 and 13·9 μg.-atoms of P/mg. of protein/hr. respectively); and oxidative phosphorylation (○) expressed as the percentage of the control rate (3 experiments with activities of 23·0, 25·9 and 21·5 μg.atoms of P/mg. of protein/hr.).

for with increasing concentrations of trialkyltin, and whenever adenosine-triphosphatase activity increased, the extinction decreased. The changes are more complex with tri-*n*-propyltin and tri-*n*-butyltin than with the lower homologues. Starting from the lowest effective concentrations the extinction and adenosine-triphosphatase activity exhibited qualitatively the same changes as for trimethyltin and triethyltin. However, with higher concentrations there was a second much larger decrease in extinction and a concomitant increase in adenosine-triphosphatase activity.

Changes in extinction of mitochondrial suspensions are used to indicate swelling of mitochondria (Cleland, 1952; Tedeschi & Harris, 1955). Although it has been demonstrated that, in a medium consisting of potassium chloride and tris buffer, extinction changes follow changes in water content (Price, Fonnesu & Davies, 1956; Lehninger, 1959*a*, 1961, 1962*b*), the changes of extinction in the present study were obtained with a medium (containing ATP, high Mg^{2+} ion concentration and EDTA) that usually prevents swelling and causes shrinkage of the mitochondria after swelling has progressed (Lehninger, 1959*b*). It was therefore important to confirm that changes in water content occurred. Table 1 shows the changes in water content after treatment with a range of concentrations of triethyltin or tri-*n*-propyltin. Maximum increase in water content of mitochondria was obtained with approx. 0·8 μM-triethyltin, and this agreed with that producing maximal change in extinction (Fig. 2). When mitochondria were treated with tri-*n*-propyltin within the concentra-

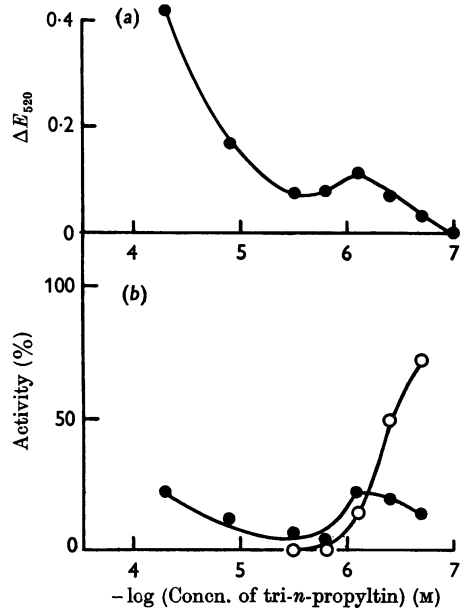

Fig. 3. Effect of tri-*n*-propyltin on swelling, adenosine-triphosphatase activity and oxidative phosphorylation of rat-liver mitochondria. The determinations were carried out as described in the Materials and Methods section. Mitochondria were added to the medium containing tri-*n*-propyltin. (*a*) Changes in extinction measured with mitochondrial suspensions containing 1·15 and 1·33 mg. of mitochondrial protein/ml. (*b*) Adenosine-triphosphatase activity (●) expressed as the percentage of the maximum activity obtainable with 2,4-dinitrophenol (2 experiments with control activities of 0·12 and 0·51, and in the presence of 30 μM-2,4-dinitrophenol of 13·0 and 12·7 μg.atoms of P/mg. of protein/hr. respectively); and oxidative phosphorylation (○) expressed as the percentage of the control rate (2 experiments with activities of 22·8 and 19·5 μg.-atoms of P/mg. of protein/hr.).

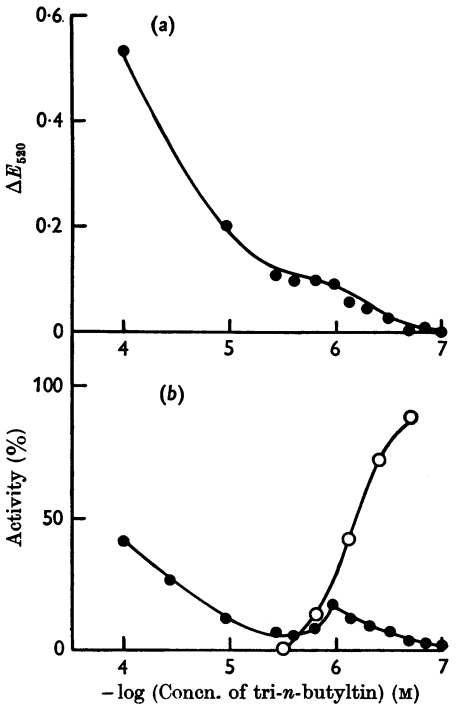

Fig. 4. Effect of tri-*n*-butyltin on swelling, adenosine-triphosphatase activity and oxidative phosphorylation of rat-liver mitochondria. The determinations were carried out as described in the Materials and Methods section. Mitochondria were added to the medium containing tri-*n*-butyltin. (*a*) Changes in extinction were measured with a mitochondrial suspension containing 1·29 mg. of mitochondrial protein/ml. (*b*) Adenosine-triphosphatase activity (●) expressed as the percentage of the maximum activity obtainable with 2,4-dinitrophenol (1 experiment with control activity of 0·60, and in the presence of 30 μM-2,4-dinitrophenol of 12·1 μg.atoms of P/mg. of protein/hr.); and oxidative phosphorylation (○) expressed as the percentage of the control rate (2 experiments with activities of 24·0 and 21·5 μg.atoms of P/mg. of protein/hr.).

tion range 0·13–2·0 μM, maximum increase in water content occurred at 0·3–0·8 μM, agreeing with the maximum fall in extinction at the same concentration (Fig. 3). With concentrations of tri-*n*-propyltin of 4·0 μM and above, the decrease of extinction was much larger (Fig. 3), until at 50 μM the mitochondrial suspension was almost clear. There were corresponding increases in water content (Table 1). Therefore the measurements of changes in extinction under these experimental conditions show changes in water content and indicate an uptake of water and a swelling of the mitochondria.

Table 1. *Changes in extinction and water content of liver mitochondria in the presence of triethyltin and tri-*n*-propyltin*

Extinctions were measured in 1 cm. circular tubes after incubation for 10 min. at 37° in the medium (3 ml.) used for the determination of adenosine-triphosphatase activity (Aldridge & Stoner, 1960). The fall in extinction, ΔE_{520}, is the difference between the mitochondrial suspension with and without trialkyltin. For the determination of water content the mitochondrial suspension (15 ml.) was centrifuged in stainless-steel tubes at 35 000*g* for 15 min. at 15–20° after incubation for 5 min. at 37°. After thorough draining the wet wt. of the pellet and its dry wt. after drying overnight at 105° were determined. The water content of the control mitochondria was 2·69 and 3·02 mg. of water/mg. dry wt. The mitochondrial suspensions contained 0·9–1·55 mg. of mitochondrial protein/ml.

Concn. of trialkyltin (μM)	ΔE_{520}	Change in water content (mg./mg. dry wt.)
Triethyltin		
20	0·09	0·25
4	0·13	0·39
0·8	0·14	0·51
0·16	0·07	0·44
0·032	0·01	0·16
Tri-*n*-propyltin		
20	0·25	0·78
5	—	0·22
3·15	0·065	—
2·0	0·08	0·14
0·79	0·10	0·47
0·35	0·07	0·54
0·18	0·03	—
0·13	—	0·10

Table 2. *Effect of 2,4-dinitrophenol on mitochondrial swelling produced by trimethyltin and tri-*n*-propyltin*

The methods were as given in Table 1. The mitochondrial suspensions contained 1·5 and 1·41 mg. of mitochondrial protein/ml.

Concn. of trialkyltin (μM)	ΔE_{520} Without 2,4-dinitrophenol	ΔE_{520} With 30 μM-2,4-dinitrophenol
Trimethyltin		
794	0·10	0·01
158	0·14	0
31·6	0·145	0·01
6·3	0·12	0
1·2	0·07	+0·01
Tri-*n*-propyltin		
50	0·475	0·44
12·5	0·17	0·06
3·12	0·07	0·02
1·56	0·08	0·02
0·78	0·11	0
0·39	0·07	0·01
0·195	0·03	0·01
0·049	0	0

In initial experiments to determine the water content of mitochondria after treatment with trialkyltins it was difficult to obtain evidence that there was a significant increase in water content. The procedure at this time was to incubate mitochondria for 10 min. at 37°, cool to 0° and then centrifuge at 0° for 1 hr. It was only when the mitochondrial suspensions were centrifuged at 15–20° that the results given in Table 1 were obtained. By using extinction measurements, the swelling process was shown to be temperature-dependent, the rate of fall in extinction being at least ten times smaller at 0° than at 37°. If after swelling had begun (a fall of extinction from 0·8 to 0·64 in 10 min.) the suspension was cooled there was a slight increase in extinction. Although not proved, it seems probable that the long cooling down and centrifuging at 0° allowed water to be extruded. The conditions of incubation finally used, namely 5 min. at 37° followed by 15 min. centrifuging at 15–20°, were taken to be approximately equivalent to the 10 min. at 37° used for the extinction measurements. Except for the high concentrations of tri-*n*-propyltin and tri-*n*-butyltin the changes in extinction were less and slower than those produced by other agents (Lehninger, 1959*b*).

2,4-Dinitrophenol will prevent swelling occurring in the presence of a variety of different compounds (Tapley, 1956; Witter & Cottone, 1956; Hunter *et al.* 1959*a*). Table 2 shows the effect of adding 2,4-dinitrophenol (30 μM) to the medium containing trimethyltin or tri-*n*-butyltin. In each case 2,4-dinitrophenol prevented the fall in extinction except for the gross change produced by high concentrations of tri-*n*-propyltin (cf. Table 1).

Adenosine-triphosphatase activity and inhibition of oxidative phosphorylation. Trialkyltins, as well as stimulating adenosine-triphosphatase activity, inhibit oxidative phosphorylation (Aldridge, 1958). 2,4-Dinitrophenol uncouples respiration from phosphorylation and also increases the adenosine-triphosphatase activity of the mitochondria, and there appears to be a correlation between these two phenomena (Parker, 1958; cf. Aldridge & Parker, 1960, for literature). The quantitative relationship between adenosine-triphosphatase activity, phosphate uptake and concentration of 2,4-dinitrophenol is shown in Fig. 5. With increasing concentrations of 2,4-dinitrophenol, adenosine-triphos-

phatase activity increased to a maximum at 30 μM and then decreased. The mechanism of the decrease with concentrations higher than 30 μM is not understood. When oxidative phosphorylation is measured with increasing concentrations of 2,4-dinitrophenol, phosphate uptake decreases until it is zero at 30 μM. The curves cross over at 50 % inhibition of phosphate uptake and 50 % of maximum stimulation of adenosine-triphosphatase activity.

Thus the possibility of a relationship between the adenosine-triphosphatase activity and the inhibition of phosphate uptake by trialkyltins must be examined. Only the adenosine-triphosphatase activity stimulated by the lower concentrations of tri-*n*-propyltin and tri-*n*-butyltin is considered, since with higher concentrations the mitochondria do not respire. Comparison of Figs. 1–4 shows that: (*a*) the maximum to which adenosine-triphosphatase activity is stimulated varies with each homologue; (*b*) each homologue elicits maximum stimulation at a different concentration; (*c*) the adenosine-triphosphatase activity is never more than 40 % of that obtainable with 2,4-dinitrophenol; (*d*) in every case inhibition of phosphate uptake occurs over the range of concentrations stimulating adenosine-triphosphatase activity; (*e*) in every case concentrations of trialkyltins that produce the highest adenosine-triphosphatase activity also inhibit phosphate uptake by 80–90 %. Thus, although there is no quantitative relationship between the adenosine-triphosphatase activity and the inhibition of phosphate uptake, nevertheless it is difficult to evade the conclusion that these two phenomena are related to one another. Such a conclusion implies no opinion as to whether either of such phenomena is primary event or 'biochemical symptom' of some other action by the trialkyltins.

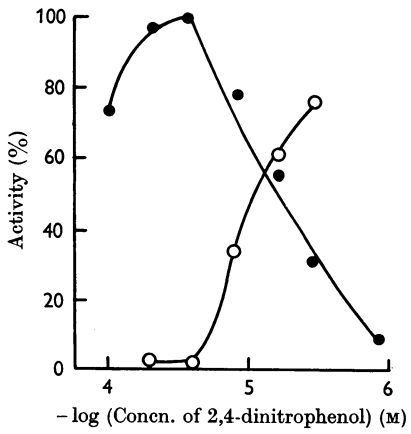

Fig. 5. Effect of 2,4-dinitrophenol on adenosine-triphosphatase activity and oxidative phosphorylation of rat-liver mitochondria. The determinations were carried out as described in the Materials and Methods section. (●), Adenosine-triphosphatase activity expressed as the percentage of the maximum activity obtained (2 experiments with maximum activities of 13·7 and 13·9 μg.atoms of P/mg. of protein/hr.); ○, oxidative phosphorylation expressed as the percentage of the control rate (2 experiments with activities of 26·2 and 26·4 μg.atoms of P/mg. of protein/hr.).

Chemical properties of trialkyltins

The high biological activity of the trialkyltins should indicate some chemical affinity or reactivity. The outstanding feature of this group of compounds is their lack of chemical reactivity (Aldridge, Cremer & Threlfall, 1962). Trialkyltins have little affinity for thiol groups (Aldridge & Cremer, 1955) and do not form complexes with EDTA (Aldridge & Cremer, 1957). They do form complexes with diphenylthiocarbazone (dithizone) (Aldridge & Cremer, 1957), though not with a high affinity (Aldridge *et al.* 1962). This complex has been used as a method for the determination of trialkyltins (Aldridge & Cremer, 1957). The procedure is to shake an aqueous solution of the trialkyltin with a chloroform solution of dithizone, and the yellow triethyltin–dithizone complex is formed in the chloroform. This is a convenient system for testing whether trialkyltins react with other substances. In Table 3 is a list of substances that did not prevent the formation of the triethyltin–dithizone complex. The hydrophilic substances were all ineffective. Some lipophilic substances were also ineffective, e.g. diolein, triolein, liquid paraffin, cholesterol, aniline and dimethylaniline. Those preventing the formation of the triethyltin–dithizone complex were phosphatidic acid, kephalin, lecithin, α-lipoic acid and oleic acid. The common factor among these active compounds is that they are lipophilic and are negatively charged. It was possible that this type of compound was exerting its effect after itself passing into the chloroform, and it seemed important to find out if they reacted with the trialkyltins in aqueous medium. A new technique was therefore used for the lipophilic substances. Triethyltin was added to a suspension of the substance, the suspension centrifuged down and the clear supernatant tested for the presence of triethyltin by the dithizone technique. Table 4 shows a rough measure of the quantity of triethyltin taken up by the substance. Lecithin, phosphatidate, α-lipoic acid and kephalin reacted with triethyltin, whereas brain sulphatide and sphingomyelin did not. Tri-*n*-heptylamine, a basic lipophilic substance, had to be tested by this method because it altered the dithizone itself; it did not react with triethyltin. Diethyltin, which does not

Table 3. *Reaction of various substances with triethyltin*

Buffer (5 ml. of borate–EDTA; Aldridge & Cremer, 1957) containing triethyltin (0·03 mM) was shaken with 6 ml. of chloroform containing dithizone (0·00067 %). The yellow triethyltin–dithizone complex forms in the chloroform. The substance to be tested was then added to give a concentration of approx. 0·2 mM in the aqueous phase, except where stated otherwise as total amount added, and the phases were reshaken. No change in the colour of the chloroform indicates no reaction of the substance with triethyltin. A change of the colour from yellow to green may indicate reaction.

Substances that cause no colour change:	
α-Tocopherol phosphate	Sodium tauroglycocholate
NAD	Albumin (20 mg.)
Sodium pyrophosphate	Casein (10 mg.)
Glucose 1-phosphate	Salmine (5 mg.)
Glucose 6-phosphate	Clupeine (5 mg.)
Sodium β-glycerophosphate	Ovalbumin (25 mg.)
ATP	Phosvitin (5 mg.)
Monoisopropyl phosphate	Glutathione
Monoisobutyl phosphate	Arginine
RNA (10 mg.)	Lysine
DNA (1·25 mg.)	Liquid paraffin (75 mg.)
Heparin (10 mg.)	Cholesterol
Dextran sulphate (10 mg.)	Olive oil
Glycerol	Diolein
L-Ascorbic acid	Tri-*o*-cresyl phosphate
Glucose	Sphingomyelin
Glycogen (5 mg.)	Lysolecithin
Sodium citrate	Aniline
Dextran	Dimethylaniline
Substances that cause a colour change:	
Sodium dioleoyl-phosphatidate	Lecithin
Kephalin	α-Lipoic acid
	Sodium oleate

Table 4. *Reaction of triethyltin with various lipophilic substances*

Suspensions of various substances (8 ml.; 0·25 mM) in borate–EDTA buffer were shaken in the presence of a range of concentrations of triethyltin. After centrifuging 5 ml. of the clear supernatant was shaken with 6 ml. of chloroform containing dithizone. By comparison with controls an assessment was made of the amount of triethyltin that had been removed with the insoluble material centrifuged down.

Substance tested	Approximate concn. of triethyltin removed (μM)
Reaction with triethyltin	
Lecithin	25
Kephalin	20
Phosphatidate	20
α-Lipoic acid	30
Sulphatide (from ox brain)	< 3
Sphingomyelin	< 3
Tri-*n*-heptylamine	< 3
Reaction with diethyltin	
Lecithin	3·5
Kephalin	5·0

behave biochemically like triethyltin (Aldridge & Cremer, 1955), also combines with lecithin and kephalin, though not so strongly. Although these tests were carried out for convenience in a borate–EDTA buffer, pH 8·3 (Aldridge & Cremer, 1957), the same result was obtained with lecithin and triethyltin in phosphate buffer, pH 7, without EDTA.

Effect of trialkyltins on systems involving membranes

Trialkyltins inhibit oxidative phosphorylation at low concentrations. Mitochondria have a highly organized structure of membranes which are largely lipoprotein (Lehninger, 1962*a*) and on which oxidative phosphorylation is dependent. Fragments of mitochondria that have retained some ability to carry out oxidative phosphorylation also retain structure and contain phospholipid (Cooper & Lehninger, 1956; Green, 1956–57); trialkyltins inhibit oxidative phosphorylation in such particles (A. L. Lehninger & C. L. Wadkins, unpublished work). It was therefore decided to examine other enzyme systems operating in membranes.

Adenosine-triphosphatase activity of a microsomal fraction from rat brain. A microsomal fraction from brain hydrolyses ATP, the activity being increased by the presence of Na^+ and K^+ ions together but not separately (Aldridge, 1962). This enzymic activity is present in many fractions thought to have been derived from membranes and is considered to be involved in the active transport of Na^+ ions (Post, Merritt, Kinsolving & Albright, 1960; Skou, 1960*a*; Whittam, 1962; Dunham & Glynn, 1961). The hydrolysis of ATP by a rat-brain microsomal fraction was inhibited by trialkyltins (Table 5), and both the hydrolysis in the absence of added Na^+ and K^+ ions and that stimulated by the addition of Na^+ and K^+ ions were affected. No increase in inhibition by triethyltin was obtained by preincubation with the microsomal fraction before the addition of ATP. The inhibitory concentrations of trialkyltins required were much higher than those necessary to inhibit oxidative phosphorylation. At a given concentration the inhibition obtained increased from trimethyltin to tri-*n*-butyltin, i.e. as lipophilic properties increase (Aldridge *et al.* 1962). However, not all fat-soluble substances inhibit this enzyme, for less than 15 % inhibition was obtained in the presence of thiopental, Amytal, dieldrin or tri-*o*-tolyl phosphate (each at 1 mM); with the exception of Amytal all of these substances are far more lipophilic than the trialkyltins.

Fragility of erythrocytes. Triethyltin only increases the fragility of rabbit erythrocytes at higher concentrations than those effective against oxidative phosphorylation. Triethyltin (1 mM) increased the haemolysis 2–3-fold. This finding is in contrast with the effects found with chlorpromazine (Freeman & Spirtes, 1962, 1963), namely that concentrations

that are active against oxidative phosphorylation (Dawkins, Judah & Rees, 1960) protect erythrocytes against haemolysis by hypo-osmotic solutions. No evidence of such a change was found with triethyltin even though chlorpromazine has been shown to be adsorbed on and into a lecithin–cholesterol monolayer (Bangham, Rees & Shotlander, 1962).

Swelling of liver mitochondria by phosphate and L-*thyroxine.* Swelling of rat-liver mitochondria induced by a variety of agents is reversed by the addition of ATP, Mg^{2+} ions and albumin (Lehninger, 1959*b*). The effect of triethyltin on the shrinking of mitochondria by ATP after swelling by phosphate is shown in Table 6. In medium A, used by Lehninger (1959*b*), triethyltin inhibits the shrinking of the swollen mitochondria at concentrations of 0·2 μM and higher. In medium B, which resembles more the medium used for the determination of adenosine-triphosphatase activity (see the Materials and Methods section), triethyltin also inhibits shrinking and, although both swelling and shrinking are much faster at higher temperatures (19° increased to 37°), the inhibition occurs at approximately the same concentrations. Medium B contains higher concentrations of Mg^{2+} ions (14 mM instead of 3 mM). With these conditions EDTA or albumin (or both) is not necessary for maximal contraction; these additions are beneficial with medium A (Lehninger, 1959*b*). In other experiments neither EDTA nor albumin influenced the inhibition of shrinking by triethyltin. Triethyltin also prevents the shrinking by ATP after swelling in the presence of L-thyroxine (medium C, Table 6; Moore & Brody, 1961*b*) and the concentrations are approximately those effective after swelling by phosphate. It has been shown by Lehninger (1959*b*, 1962*b*), with medium A and medium C, that changes in extinction indicate changes in the water content of rat-liver mitochondria.

Table 5. *Action of trialkyltins on brain-microsomal adenosine-triphosphatase activity*

Microsomal suspensions were added to the medium without ATP but containing the trialkyltin. After incubation for 10 min. at 0°, ATP was added and the enzymic reaction started by placing in a bath at 37°. After 10 min. the reaction was stopped by the addition of ice-cold perchloric acid. For details of procedure see Aldridge (1962). The enzymic activity in the absence of Na^+ and K^+ ions was 16·9–22·5 and the additional activity in the presence of NaCl (150 mM) and KCl (15 mM) was 18·0–21·4 μg.atoms of P/mg. of protein/hr.

Concn. of trialkyltin (mM)	Inhibition (%): Activity in absence of Na^+ and K^+ ions	Inhibition (%): Activity stimulated by Na^+ and K^+ ions
Trimethyltin		
0·1	13	12
1·0	25	49
Triethyltin		
0·01	23, 10, 19	6, 3, 8
0·1	35, 11, 27	40, 47, 28
1·0	47, 35, 44	87, 90, 80
Tri-*n*-propyltin		
0·01	22	14
0·1	36	78
1·0	43	89
Tri-*n*-butyltin		
0·001	11	21
0·01	35	50
0·1	55	92

DISCUSSION

The present studies extend our information about the effects of trialkyltins on liver mitochondria. Aldridge (1958) suggested that the stimulation of adenosine-triphosphatase activity was not associated with the inhibition of oxidative phosphorylation, but the present work shows that this is only true for the activity stimulated by high concentrations of tri-*n*-propyltin and tri-*n*-butyltin. Such adenosine-triphosphatase activity is accompanied by gross swelling of the mitochondria and a complete absence of respiration. At much lower concentrations all the trialkyltins induced adenosine-triphosphatase activity; the relationship between the concentration of trialkyltin and level of adenosine-triphosphatase activity was unusual in that higher concentrations lead to a decreased hydrolysis of ATP (cf. Figs. 1–4). This activation–inhibition phenomenon must consist of two processes. However, it always occurs over the range of concentrations that inhibit oxidative phosphorylation, and it is therefore difficult to avoid the conclusion that they involve common processes. At these same concentrations swelling and stimulation of respiration occur (Aldridge, 1958). Since the conditions used for those experiments have been identical as far as the particular experiment allows, the conclusion that swelling, effect on adenosine-triphosphatase activity (either activation or inhibition) and the inhibition of oxidative phosphorylation by trialkyltins have a common origin can be more certainly reached. Such a conclusion does not, however, indicate which are primary or secondary events.

In the following discussion the term 'swelling' refers only to that produced by trimethyltin and triethyltin and by the lower concentrations of tri-*n*-propyltin and tri-*n*-butyltin. The qualitative correlation between adenosine-triphosphatase activity and swelling is interesting, for swelling induced by phosphate has been known for many years (Raaflaub, 1953*a*; Hunter & Ford, 1955). The swelling shown is temperature-dependent as well as the adenosine-

Table 6. *Effect of triethyltin on the reversal of phosphate- and* L-*thyroxine-induced swelling of rat-liver mitochondria*

Medium A contained KCl (0·125 M), tris–tris hydrochloride buffer, pH 7·4 (0·02 M), and potassium phosphate buffer, pH 7·4 (0·01 M). Shrinking was caused by the addition of ATP (5 mM), $MgCl_2$ (3 mM) and crystalline bovine serum albumin (2·0 mg./ml.). Medium B contained KCl (0·1 M) and potassium phosphate buffer, pH 7·4 (0·015 M). Shrinking was caused by the addition of ATP (3 mM), $MgCl_2$ (14 mM) and EDTA (1 mM). Medium C contained KCl (0·125 M), tris–tris hydrochloride buffer, pH 7·4 (0·02 M) and L-thyroxine (1·25 μM); mitochondria were added to the KCl–tris buffer mixture followed immediately by the L-thyroxine. Shrinking was caused by the addition of ATP (5 mM), $MgCl_2$ (3 mM), EDTA (1 mM) and albumin (2 mg./ml.). An aqueous solution of triethyltin sulphate was added to the mixture containing ATP before addition to the mitochondrial suspension. The values for extinction at 520 mμ are corrected for the dilution caused. Expts. 1, 2 and 4 were carried out at 18–20°, and Expt. 3 at 37°. Mitochondria were always used within 30 min. of preparation.

Expt. no.	Concn. of triethyltin (μM) 0	0·04	0·2	1·0	5·0
1 (medium A)					
E_{520} at 0 min.	0·77	0·77	0·77	0·77	0·78
ΔE_{520} (0–20 min.)	−0·32	−0·32	−0·34	−0·34	−0·36
ATP, Mg^{2+} ions, albumin and triethyltin added					
ΔE_{520} (20–25 min.)	+0·13	+0·14	+0·11	+0·06	+0
ΔE_{520} (20–40 min.)	+0·29	+0·29	+0·26	+0·15	+0·02
2 (medium B)					
E_{520} at 0 min.	0·875	0·86	0·87	0·86	—
ΔE_{520} (0–15 min.)	−0·37	−0·36	−0·365	−0·355	—
ATP, Mg^{2+} ions, EDTA and triethyltin added					
ΔE_{520} (15–30 min.)	+0·31	+0·29	+0·245	+0·14	—
3 (medium B)					
E_{520} at 0 min.	0·805	—	0·80	0·78	—
ΔE_{520} (0–3 min.)	−0·255	—	−0·25	−0·26	—
ATP, Mg^{2+} ions, EDTA and triethyltin added					
ΔE_{520} (3–7 min.)	+0·195	—	+0·17	+0·13	—
4 (medium C)					
E_{520} at 0 min.	0·68	0·67	0·68	0·67	0·67
ΔE_{520} (0–25 min.)	−0·28	−0·28	−0·29	−0·31	−0·31
ATP, Mg^{2+} ions, EDTA, albumin and triethyltin added					
ΔE_{520} (25–30 min.)	+0·28	+0·27	+0·25	+0·09	−0·01

triphosphatase activity. Swelling induced by phosphate is also temperature-dependent (Hunter & Ford, 1955; Di Sabato, 1959; Hunter *et al.* 1959*a*; Neubert, Foster & Lehninger, 1962). The swelling induced by trialkyltins is prevented by 2,4-dinitrophenol (Table 2; Moore & Brody, 1961*b*) and so is the swelling by phosphate (Lehninger, 1956; Hunter *et al.* 1959*a*). Swelling is not induced by phosphate in the presence of ATP, Mg^{2+} ions and EDTA (Hunter & Ford, 1955; Lehninger, 1959*b*; Price *et al.* 1956). However, Figs. 1–4 indicate an approximately quantitative relationship between the degree of swelling and the adenosine-triphosphatase activity (and therefore the inorganic phosphate concentration) in the presence of the various trialkyltins. Triethyltin prevents the shrinking by ATP, Mg^{2+} ions and albumin of mitochondria swollen by phosphate (0·2 μM and higher concentrations; cf. Table 6, medium B). Therefore, this swelling obtained in the presence of triethyltin, ATP and Mg^{2+} ions could be due to the phosphate liberated from ATP by the adenosine triphosphatase. However, other experiments indicated that there are other possibilities. Although the amount of phosphate liberated from ATP (Fig. 2) can cause swelling, triethyltin causes swelling of mitochondria in the medium used for adenosine-triphosphatase assay (but without ATP). This swelling is not reversed by ATP and is temperature-dependent. Therefore an explanation of the relationship between swelling and adenosine-triphosphatase activity cannot be given.

During phosphate-induced swelling adenine nucleotides are liberated (Siekevitz & Potter, 1955; Raaflaub, 1953*b*; Hunter & Ford, 1955; Hunter, Malison, Bridgers, Schutz & Achison, 1959*b*; Hunter, Davis & Carlat, 1956; Kaufmann & Kaplan, 1959, 1960; Kaplan, Swartz, Frech & Ciotti, 1956), but the phosphorylating mechanism is not rapidly inactivated by this procedure (Hunter *et al.* 1956). The effects of trialkyltins on respiration and oxidative phosphorylation are not due to a loss of known essential coenzymes because: (*a*) the addition of NAD, NADP, FAD, cytochrome *c*, cocarboxylase, lipoic acid and CoA has no influence on the inhibition of oxidative phosphorylation (Aldridge, 1958), whereas the addition of NAD to mitochondria grossly swollen after phosphate

reinstates oxidative phosphorylation (Hunter *et al.* 1959*b*); (*b*) the degree of swelling is small (after trimethyltin only 20–30 % of the maximum obtainable fall in extinction); (*c*) although all trialkyltins inhibit oxidative phosphorylation the degree of swelling obtained varies with the different homologues.

To gain information of possible chemical groups that might be involved in their action the chemical affinities of the trialkyltins have been studied. The only substances that possessed a demonstrable affinity for the trialkyltins were those that were lipophilic and were negatively charged. Not all such substances gave a positive reaction in the rather crude test employed, e.g. sphingomyelin and sulpholipid. Phospholipids are the most obvious biologically important compounds with such properties, but many substances that combine with phospholipids do not have the specific actions of the trialkyltins, e.g. Ca^{2+} ions (Kimizuka & Koketsu, 1962) or diethyltin (cf. Table 4). It is rather the absence of reactivity with other substances (either water-soluble or neutral or basic lipids) that suggests the view that combination with phospholipid might be involved in their biological action. Other observations that could agree with this view are:

(1) Mitochondria that contain much phospholipid also extract trialkyltins from aqueous solution (Aldridge & Threlfall, 1961).

(2) The inhibition of the contraction of mitochondria swollen by treatment with L-thyroxine. Phospholipids are involved for, during contraction, fatty acid is incorporated into the phosphatidic acid fraction in mitochondria (Wojtczak & Lehninger, 1961). A major component of this fraction in mitochondria and phosphorylating particles from mitochondria is cardiolipin, a polyglycerol phosphatide (Macfarlane, Gray & Wheeldon, 1960; Getz & Bartley, 1959, 1961; Biran & Bartley, 1961; Getz, Bartley, Stirpe, Notton & Renshaw, 1962; Bartley, Getz, Notton & Renshaw, 1962). This argument loses some of its force since shrinking by ATP after swelling of mitochondria by phosphate is also prevented by concentrations of trialkyltin that are effective after swelling by L-thyroxine. This might indicate that shrinking by ATP after these two swelling agents have something in common, even though the processes can be clearly distinguished; for instance, fatty acid or 'U factor' is involved in swelling by L-thyroxine but not by phosphate (Lehninger, 1962*a*). Of particular interest is the demonstration by Vignais, Vignais & Lehninger (1963) that phosphatidylinositol can restore the ability of ATP to contract 'aged' mitochondria swollen either by L-thyroxine or by inorganic phosphate.

(3) The inhibition of the adenosine-triphosphatase activity stimulated by Na^+ and K^+ ions. This enzyme is considered to be involved in the transport of Na^+ ions (Skou, 1960*a*) and, although it is not established that a lipid is involved (McIlwain, 1962; Heald, 1962; Järnefelt, 1961; Ahmed & Judah, 1962), there is evidence that a phospholipid may be important in this process (Hokin & Hokin, 1960; Schatzmann, 1962; Skou, 1960*b*).

(4) Trialkyltins have many effects that are probably caused through actions on membranes. In addition to those mentioned above, they cause oedema of the white matter of the central nervous system (Magee, Stoner & Barnes, 1957; Barnes & Stoner, 1958; Smith, McLaurin, Nichols & Asbury, 1960; Torack, Terry & Zimmerman, 1960), cause various substances to leak out of brain slices [creatine (Cremer, 1961); amino acids (Cremer, 1964)] and inhibit the movement of Na^+ ions between plasma, brain and cerebrospinal fluid (B. J. Parsons, unpublished work).

(5) After injection of triethyltin into rats followed by injection of inorganic [^{32}P]phosphate, the earliest and only change was a depression in the relative specific radioactivity of the lipid phosphorus (relative to the acid-soluble phosphorus) from brain and spinal cord and liver (Stoner & Threlfall, 1958).

With increasing concentrations of trimethyltin, adenosine-triphosphatase activity rises to a maximum at 30 μM. Higher concentrations produce less activity until at 600 μM it is only 40 % of the maximum. A similar relationship applies to triethyltin, tri-*n*-propyltin and tri-*n*-butyltin, but the concentrations at which the increase and decrease in activity occurs become closer together the higher the molecular weight and therefore the more lipophilic they are (cf. Table 7). If combination of trialkyltins with lipophilic components of the mitochondria is involved in their biological action then a straightforward distribution between each of

Table 7. *Concentration of trialkyltins inducing adenosine-triphosphatase activity in rat-liver mitochondria*

The concentrations are taken from Figs. 1–4. For tri-*n*-propyltin and tri-*n*-butyltin only the increase and the decrease of activity at the lowest concentrations are considered.

Trialkyltin	Concn. of trialkyltin (μM): Lower concn. producing 50 % of maximum activity (*a*)	Higher concn. producing 50 % of maximum activity (*b*)	*b*/*a* ratio
Trimethyltin	1·41	500	350
Triethyltin	0·125	7·07	56
Tri-*n*-propyltin	0·177	1·12	6·2
Tri-*n*-butyltin	0·45	1·41	3·2

the components (*a* and *b* of Table 7) and the aqueous phase is not possible. If this were so, the *b*/*a* ratio would remain constant. A more plausible model is a saturable component and the other obeying the simple distribution law; however, with such a complex lipoprotein structure as in mitochondria it is premature to speculate further. Affinity for lipids has also been shown to influence the potency of local anaesthetics in blocking nerve conduction (Skou, 1954, 1961) and the anaesthetic potency of barbiturates (Butler, 1950). The major difficulty is to envisage the necessary specificity in lipoprotein structures to account in chemical terms for the diverse pharmacological and biochemical properties of these drugs.

SUMMARY

1. Trimethyl-, triethyl-, tri-*n*-propyl- and tri-*n*-butyl-tin stimulate adenosine-triphosphatase activity and cause limited swelling of rat-liver mitochondria. These two effects occur at the same concentration of a particular trialkyltin.

2. The level of the adenosine-triphosphatase activity induced varies with each trialkyltin and is only 17–40% of that produced by 30 μM-2,4-dinitrophenol. Nevertheless, both stimulation of adenosine-triphosphatase activity and inhibition of oxidative phosphorylation occur at the same concentration of a particular trialkyltin.

3. Triethyltin inhibits the shrinking by ATP of mitochondria swollen by treatment with inorganic phosphate or L-thyroxine.

4. Triethyltin has affinity for substances that are lipophilic and are negatively charged.

5. The hypothesis that combination of trialkyltins with negatively charged lipids is involved in their biological action is discussed.

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The Arylesterases of Human Serum

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It has been apparent for some time that the hydrolysis of phenyl esters by human serum, first observed by McGuire & Falk (1934), is due to more than one enzyme. Huggins & Lapides (1947) found that most of the activity was insensitive to concentrations of eserine sufficient to inhibit serum cholinesterase. Subsequently Mounter & Whittaker (1953) found that the hydrolysis of phenyl acetate by plasma was only slightly sensitive to inhibition by DFP.* In addition, the ratio of the rate of DFP- and eserine-sensitive hydrolysis of phenyl acetate by purified human serum cholinesterase to the rate of hydrolysis of acetylcholine was much lower than that of the total rate of hydrolysis of phenyl acetate to that of acetylcholine catalysed by whole serum, suggesting that a DFP- and eserine-insensitive phenyl acetate esterase had been removed by purification. Aldridge (1954) showed that the enzyme catalysing the DFP-insensitive hydrolysis of phenyl acetate by rabbit serum also catalysed the hydrolysis of diethyl *p*-nitrophenyl phosphate (E600) and showed that the enzyme was distinct from cholinesterase. He also found that E600 was hydrolysed by human plasma.

A clear resolution of the different arylesterase activities in plasma was first obtained by Augustinsson (1959), who assayed the components of human serum, separated by electrophoresis on a cellulose column, for esterase activity against several esters. He obtained a separation of the eserine- and organophosphorus-sensitive phenyl acetate-hydrolysing activity, which migrated with the cholinesterase between the α_2- and β-globulins, and the eserine- and organophosphorus-insensitive activity, which travelled with the albumin. The latter fraction contained by far the major part of the phenyl acetate-hydrolysing activity. Augustinsson (1959) thought that some human plasmata could contain an esterase 'running close to, but more rapidly than, the main peak fraction since this esterase peak was not uniform'.

When the arylesterase activity of human serum was separated by starch-gel electrophoresis and detected with β-naphthyl acetate as substrate, Dubbs, Vivonia & Hilburn (1960) found that most of this activity was separated from the albumin and

* The abbreviation used is: DFP, di-isopropyl phosphorofluoridate.