The Biochemistry of Organotin Compounds

TRIALKYLTINS AND OXIDATIVE PHOSPHORYLATION

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In a previous paper (Aldridge & Cremer, 1955) it was concluded that triethyltin was an inhibitor of oxidative phosphorylation. The inhibitory properties of triethyltin differed from diethyltin which, like phenylarsenious acid, was primarily an inhibitor of α-oxo acid oxidases.

The classical inhibitor of oxidative phosphorylation is 2:4-dinitrophenol (Loomis & Lipmann, 1948). The formation of adenosine triphosphate may be prevented with little or no diminution of oxygen consumption. This behaviour is typical of a group of phenols such as 2:4:6-trichloro- (Parker, 1958), pentachloro- (Weinbach, 1954), *p*-nitro- (Terner, 1954) and 2:4-dinitro-phenols (Loomis & Lipmann, 1948). Since oxidation is not inhibited there is no difficulty in deciding that these substances do not act upon the respiratory chain but rather at some point between electron transport and the phosphorylation of adenosine diphosphate (Slater, 1953; Lardy, 1955). Recently, however, claims have been made that a heterogeneous group of substances are inhibitors of oxidative phosphorylation. Examples of these are chlorpromazine (Andrejew & Rosenberg, 1956; Abood, 1955), barbiturates (Brody & Bain, 1954), bilirubin (Zetterström & Ernster, 1956), thyroxine (Hoch & Lipmann, 1954) and its derivatives (Dickens & Salmony, 1956). All of these compounds, like triethyltin, inhibit oxidation as well as producing a depression of the phosphorylation quotient (P/O ratio).

Mitochondria prepared in different ways and studied in different media show various properties. For instance some preparations oxidize substrate at a rate which cannot be increased by the addition of enzymes utilizing adenosine triphosphate (Aldridge & Cremer, 1955). Mitochondria may now be prepared (Siekevitz & Potter, 1953; Maley & Lardy, 1955; Aldridge, 1957) with a low oxidative rate, which can be increased by the addition of systems utilizing adenosine triphosphate. The mitochondria used in this work have a low oxidative rate in an electrolyte medium at 37°, which may be increased three- to four-fold by the addition of apyrase, hexokinase and glucose or 2:4-dinitrophenol (Aldridge, 1957). Consideration of the two extremes of behaviour of different preparations of mitochondria indicate that it can be difficult to reach a firm conclusion that a substance, which decreases oxidation, is primarily an inhibitor of oxidative phosphorylation. On the one hand, when oxidation is rapid and not stimulated by apyrase or 2:4-dinitrophenol the mitochondria usually have high adenosine triphosphatase activity (Potter & Recknagel, 1951) thus giving low control P/O ratios. Under these conditions it is probable that an inhibitor of the respiratory chain will produce a lowering of the P/O ratio. On the other hand, if oxidation cannot proceed without the extramitochondrial utilization of adenosine triphosphate then it is difficult to understand why an inhibition of an energy-transferring step leading to the phosphorylation of adenosine diphosphate should not also produce the same inhibition of oxygen consumption. In view of these possibilities it was thought necessary to re-examine the effects of triethyltin and a series of homologues on oxidative phosphorylation. The results in this paper show that trialkyltins are inhibitors of a reaction which occurs between electron transport and before the formation of adenosine triphosphate. Many are highly active inhibitors, the most active being triethyltin, which can produce demonstrable effects at 10^{-7} M-concentration.

METHODS

Preparation of mitochondria. Mitochondria were isolated from rat liver, as described previously (Aldridge, 1957), except that a Potter–Elvehjem-type homogenizer, with a smooth glass tube and Perspex pestle with a total clearance of 0·02 in. instead of 0·03 in., was used.

Manometric technique. The reasons for the composition and the pH of the medium have been previously discussed (Aldridge, 1957). Each flask contained 3 ml. of a solution containing adenosine 5-phosphate (0·00115 M), adenosine triphosphate (ATP) (0·00106 M), KCl (0·1 M), $MgCl_2$ (0·014 M) ethylenediaminetetra-acetic acid (EDTA) (0·001 M), potassium phosphate (0·015 M), sucrose (0·025 M) and substrates [(0·01 M; except fumarate (0·001 M)]. For studies of oxidative phosphorylation each flask contained in addition glucose (180 μmoles), glycylglycine (50 μmoles) and hexokinase (200–400 units). This medium was adjusted to pH 6·7–6·8 with KOH, a glass electrode being

used, and all experiments were carried out at 37°. Carbon dioxide was absorbed by 0·15 ml. of 5 M-KOH, the gas phase was air and 0·25–0·30 ml. of a mitochondrial suspension in 0·3 M-sucrose diluted to a volume (ml.) equal to twice the weight of the liver (g.) was used for all experiments.

Determinaton of adenosine triphosphatase activity. Each beaker contained 3 ml. of a solution containing ATP (0·003 M), KCl (0·1 M), $MgCl_2$ (0·014 M), EDTA (0·001 M) and sucrose (0·03 M). The beakers were shaken at 37° in a Dubnoff metabolic shaking incubator. After temperature equilibration mitochondria were added and the mixture was incubated for 10 min. The reaction was stopped by the addition of perchloric acid and inorganic phosphate was determined.

Analytical methods. Inorganic phosphate was determined by the method of Fiske & Subbarow (1925). Protein was measured by the biuret method of Robinson & Hogden (1940) modified as described by Aldridge (1957). Mitochondrial protein has been expressed as mg. of albumin.

Inhibitors. Phenylarsine oxide was synthesized as follows: phenylarsonic acid was prepared (Vogel, 1948) followed by reduction by sodium bisulphite (A. H. Ford-Moore, personal communication). Assay by titration with iodine in the presence of bicarbonate indicated 99·7% of $C_6H_5 \cdot AsO$. The compound had an m.p. of 121–122° (uncorrected) in agreement with Nesmejanow & Freidlina (1934) (122°), but not with Blicke & Smith (1929) (144–146°). Diethyltin dichloride and all 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). Dimethylformamide is an excellent solvent for the trialkyltins, allowing stock solutions (usually 0·02 M) to be prepared. A sample (0·03 ml.) of these solutions was added to each flask, final concentrations of 1% (v/v) dimethylformamide never being exceeded. This concentration has been found to produce a negligible effect upon oxidation and only a very slight stimulation of adenosine triphosphatase activity (Table 7).

2:4-Dinitrophenol (British Drug Houses Ltd.) was used from a stock solution (0·01 M) neutralized to approx. pH 7.

Special chemicals and reagents. The following chemicals were obtained from the sources indicated: adenosine 5-phosphate, glycylglycine, sodium pyruvate, cocarboxylase (Roche Products Ltd.), disodium salt of ATP, coenzyme A, flavinadenine dinucleotide (Sigma Chemical Co., St Louis, Mo., U.S.A.), glucose, sodium fumarate (British Drug Houses Ltd.) and lipoic acid (L. Light and Co. Ltd.). All free acids were neutralized to approx. pH 6·8 with KOH before use.

Coenzyme I, cytochrome *c* and coenzyme II were prepared and assayed as previously described (Aldridge & Cremer, 1955; Aldridge, 1957). Potato apyrase was prepared by the method of Lee & Eiler (1951) and assayed by the method of Krishnan (1949); the preparation had an activity of 11 000 units/ml.

Hexokinase was prepared from baker's yeast by a modification by V. H. Parker (unpublished work) of the method of Berger, Slein Colowick & Cori (1946). The preparation was taken to the equivalent of their step 3*a* and when assayed by their procedure (but at 37° instead of 30°) had an activity of 3500 units/ml.

Units used. The oxidative activity of the mitochondria (q_{O_2}) is expressed as the μl. of O_2/mg. of protein/hr. Adenosine triphosphatase activity is given as μmoles of P liberated/mg. of protein/hr. The inhibitory power of substances is given as pI_{50} values, which is the negative logarithm of the molar concentration which will produce 50% inhibition.

RESULTS

Unstimulated and stimulated oxidation. In the usual mitochondrial preparation the rate of oxidation of a variety of substances must be regulated by the extra-mitochondrial utilization of the ATP formed (Potter & Recknagel, 1951; Siekevitz & Potter, 1953; Chance & Williams, 1955; Aldridge, 1957). The rate of oxidation of pyruvate by the present preparation of mitochondria is greatly increased by the addition of hexokinase and glucose, potato apyrase or 2:4-dinitrophenol (Aldridge, 1957). In the work to be described unstimulated oxidation (A) is the rate of oxygen uptake with no additions to the standard medium other than substrate, and stimulated oxidation (B) is the rate of oxygen uptake in the presence of apyrase, 2:4-dinitrophenol or hexokinase and glucose. The increase in oxygen uptake induced by these additions is the difference between the stimulated and unstimulated oxidation ($B-A$). The addition of apyrase brings about a three- to four-fold increase in the rate of oxygen uptake with pyruvate as substrate. Apyrase removes the terminal phosphorus of ATP 30 times faster than the other phosphorus atoms (Lee & Eiler, 1951) and the activity of apyrase in the medium used for the manometric experiments was 660 μg. atom of P liberated/ml./hr. Therefore the rate of dephosphorylation of ATP by the apyrase used (0·1 ml.) was 1·1 μg.atom/min. The mean rate of oxygen uptake in these experiments was 0·46 μg.atom/min. and the mean stimulation due to apyrase was 3·3-fold, indicating an oxygen uptake of 0·32 μg.atom/min. due to the addition of apyrase. A P/O ratio of 3 indicates a rate of phosphorylation of ADP of 0·96 μg.atom/min., a value in agreement with the rate of dephosphorylation by the apyrase. It is concluded therefore that the additional oxygen uptake in the presence of apyrase (i.e. oxidation induced by apyrase) is oxidation which is coupled to the phosphorylation of ADP. The unstimulated preparation which shows no adenosine triphosphatase activity (Table 7) nevertheless does oxidize pyruvate slowly, but whether the oxidative and energy-transferring processes involved are essentially the same as those in the stimulated preparation is not known. Since a large proportion (70–80%) of the total stimulated oxidation is oxidation induced by apyrase it has been possible to obtain accurate values for the inhibitory power (pI_{50}) of trialkyltins

Table 1. *Influence of trialkyltins on unstimulated and stimulated oxidation of pyruvate by mitochondria*

Mitochondria were added to the Warburg vessels containing the medium with or without apyrase (1100 units) and trialkyltins. After temperature equilibration readings were taken at 10 and 50 min. pI_{50} = − log M concentration of inhibitor to produce 50% inhibition, pC = − log M-concentration. These values were obtained graphically with results obtained with a range of concentrations forming a geometric progression with a common ratio of 4. The pI_{50} values for the oxidation induced by apyrase were calculated as described in the text and Figs. 1 and 2. The mean q_{O_2} for the unstimulated and stimulated oxidations were 26·8±1·5 (10) and 85·1±2·9 (10) respectively, indicating a mean 3·3-fold stimulation.

	Inhibition of O_2 uptake (pI_{50})			Stimulation of O_2 uptake by trialkyltins	
Compound	Unstimulated oxidation (A)	Stimulated oxidation (B)	Oxidation induced by apyrase ($B-A$)	Increase in rate of oxidation (q_{O_2})	Range of concentration of trialkyltin producing maximum stimulation (pC)
Trimethyltin	2·6	4·2	5·0	26–49	4·0–4·4
Triethyltin	4·2	5·5	6·6	30–55	5·5–5·8
Tri-*n*-propyltin	5·1	5·8	6·2	19–32	5·6–6·0
Tri-*iso*propyltin	5·1	5·7	6·3	36–45	5·7–6·0
Tri-*n*-butyltin	5·3	5·8	6·0	Nil	No stimulation
Tri-*n*-hexyltin	*	4·9	5·0	Nil	No stimulation
Tri-*n*-octyltin		No inhibition		Nil	No stimulation
Phenylarsenious acid	6·4	6·8	7·1	Nil	No stimulation
Diethyltin	4·6	4·9	5·1	Nil	No stimulation

* Less than 50% inhibition with a saturated solution.

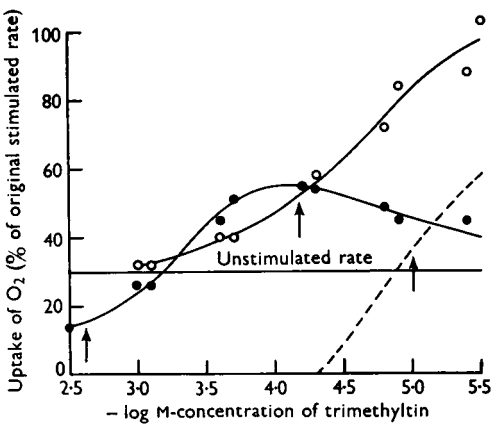

Fig. 1. Effect of trimethyltin on the stimulated and unstimulated oxidation of pyruvate by mitochondria. Each curve is derived from two experiments, each experiment contributing alternate points. ●, Unstimulated oxidation; ○ oxidation in the presence of apyrase (1100 units). Mitochondria were added to the Warburg flasks containing the medium and trimethyltin; readings were taken at 10 and 50 min. after placing the flasks in the bath at 37°. The broken line is the difference between the two curves. The pI_{50} (5·0) for the inhibition of the additional uptake of oxygen induced by apyrase has been derived from this line. (Arrows indicate the concentrations where oxidation is 50% inhibited.) q_{O_2} values for stimulated oxidation for the two experiments were 74 and 100.

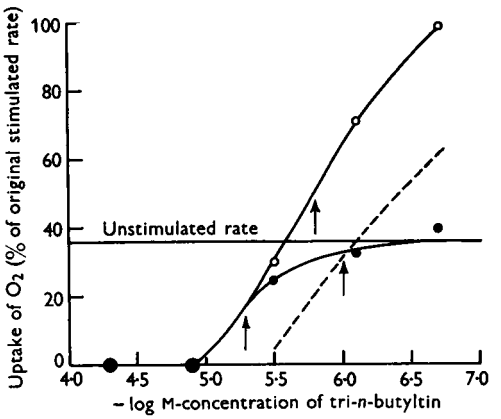

Fig. 2. Effect of tri-*n*-butyltin on the stimulated and unstimulated oxidation of pyruvate by mitochondria. ●, Unstimulated oxidation; ○, oxidation in the presence of apyrase (1100 units). Mitochondria were added to Warburg flasks containing the medium and tri-*n*-butyltin; readings were taken at 10 and 50 min. after placing the flasks in the bath. The broken line is the difference between the two solid curves. The pI_{50} (6·0) for the inhibition of the additional uptake of oxygen induced by apyrase has been derived from this line. (Arrows indicate the concentrations where oxidation is 50% inhibited.) q_{O_2} for the stimulated oxidation was 73.

against this induced oxidation, which is certainly coupled to phosphorylation. The method whereby these values have been derived from the experimental results is shown in Figs. 1 and 2. Triethyltin inhibits the oxidation of a variety of substrates by liver mitochondria (Aldridge & Cremer, 1955). The mitochondrial preparation used for that work must have been damaged since little or no increase in oxidation was obtained upon the addition of

hexokinase and glucose. With the present preparation it was found that the stimulated oxidation of pyruvate was prevented by lower concentrations of trialkyltin (pI_{50} 5·5, Table 1) than was originally found (pI_{50} approx. 4·7; Aldridge & Cremer, 1955). Examination of a series of trialkyltin compounds has shown that with the exception of tri-*n*-octyltin they inhibit both the unstimulated and the stimulated oxidation of pyruvate.

However, the unstimulated oxidation required a higher concentration of trialkyltin to produce a given inhibition. This is not a peculiarity of trialkyltins, for respiratory inhibitors such as phenylarsenious acid (Peters, 1955) and diethyltin (Aldridge & Cremer, 1955) behave similarly. The difference in the concentrations required to inhibit 50 % of the unstimulated and stimulated oxidation is largest with trimethyl and decreases in the order trimethyl, triethyl, tripropyl until at tri-*n*-butyltin it is approximately the same as the difference found for phenylarsenious acid and diethyltin.

During the examination of the sensitivity to trialkyltins of the unstimulated oxidation it was observed that the lower homologues produced considerable stimulation of oxidation. In Fig. 1 the behaviour of trimethyltin is illustrated. Over a considerable range of concentration an increase in oxygen uptake was observed. Trimethyltin and triethyltin produced comparable stimulation but the extent of the stimulation decreased with tri-*n*-propyl and tri-*iso*propyl until with tri-*n*-butyl and higher homologues it was undetectable (Table 1). Fig. 2 shows the behaviour of tri-*n*-butyltin, which is typical of the higher homologues.

Triethyl-, tri-*n*-propyl-, tri-*iso*propyl- and tri-*n*-butyl-tin all inhibit stimulated oxidation at approx. 10^{-6} M. There is a decrease in activity on either side of this series, trimethyl and tri-*n*-hexyl being less active and tri-*n*-octyl being completely inactive, owing perhaps to its insolubility in water.

Since evidence has been produced that triethyltin is an inhibitor of oxidative phosphorylation (Aldridge & Cremer, 1955), it was important to obtain a value for the inhibitory power of the trialkyltins against oxidation which was coupled to phosphorylation. The pI_{50} values for the inhibitory action of trialkyltins upon this oxidation have been derived as shown in Figs. 1 and 2 and are listed in Table 1. It is clear that oxidation which is coupled to phosphorylation is very sensitive to trialkyltins, the concentration of triethyltin producing 50 % inhibition being $2·5 \times 10^{-7}$ M. With a preparation of mitochondria which would oxidize succinate at a similar rate with or without the addition of nucleotide, succinate oxidation has previously been shown to be unaffected by triethyltin (Aldridge & Cremer, 1955). This oxidation of succinate must have been unaccompanied by phosphorylation of nucleotide. Oxidation of succinate by the present preparation was at least partially coupled to phosphorylation for the unstimulated oxidation was stimulated two- or three-fold by the addition of apyrase (Aldridge, 1957). It was interesting to find that the oxidation of succinate had now become sensitive to triethyltin and inhibition of succinate oxidation induced by apyrase was obtained at the same concentrations which inhibit induced pyruvate oxidation (Tables 1, 2). Also the unstimulated oxidation of succinate was increased by triethyltin but only a slight stimulation was produced by tri-*n*-butyltin.

Oxidative phosphorylation. With the system for the measurement of oxidative phosphorylation, with an excess of hexokinase and glucose in order to trap any ATP formed, trialkyltins are potent inhibitors of oxidation and phosphorylation. With all the trialkyltins phosphorylation was inhibited more than oxidation with a given concentration of inhibitor. This was not so with the respiratory inhibitor phenylarsenious acid (Table 3 and Aldridge, 1957). It is clear, however, that the difference between the inhibition of oxygen and phosphate uptake is not great (16–26 %) and does not approach that obtained with 2:4-dinitrophenol. The P/O ratios were much reduced when oxygen uptake was 50 % inhibited by trialkyltins. It is, however, important to appreciate that P/O ratios give an exaggerated impression of the divergence of the inhibition of uptakes of oxygen and phosphorus determined experimentally. It is interesting to note that the pI_{50} values for trialkyltins against oxidation in the presence of a large excess of hexokinase agreed closely with those for the oxidation induced by apyrase (Table 6).

Table 2. *Influence of trialkyltin on the oxidation of succinate by mitochondria*

Mitochondria were added to the Warburg vessels containing the medium with or without apyrase (1100 units) and trialkyltin. Readings were taken at 10 and 40 min. after placing the vessels in the bath. $pI_{50} = -\log$ M-concentration of inhibitor to produce 50 % inhibition. These values were obtained graphically with results obtained with a range of five concentrations forming a geometric progression with a common ratio for triethyltin of 4 and for tri-*n*-butyltin of 3. The pI_{50} values for the oxidation induced by apyrase were calculated as described in the text and Figs. 1 and 2. The mean q_{O_2} for the unstimulated and stimulated oxidation were 47 and 122, indicating a mean 2·6-fold stimulation.

	Inhibition of oxidation (pI_{50})		
Trialkyltin	Unstimulated oxidation	Stimulated oxidation	Oxidation induced by apyrase
Ethyl	4·2	5·0	6·8
n-Butyl	5·1	5·5	5·9

Table 3. *Influence of trialkyltin compounds on oxidation of pyruvate and coupled phosphorylation*

Mitochondria were added to the Warburg vessels containing the medium and trialkyltin. After temperature equilibration readings were taken at 10 min., 13 min. 20 sec., 16 min. 40 sec. and 20 min. The rate of uptake of oxygen was calculated from the slope of the best straight line through these points. At 22 min. 0·5 ml. of 60% $HClO_4$ was added to each flask. To a control set of flasks 0·5 ml. of 60% $HClO_4$ was added at 10 min. pI_{50} values ($-\log$ M-concentration of inhibitor to produce 50% inhibition) were obtained graphically with the results obtained with a range of concentrations forming a geometric progression with a common ratio of 2. The inhibition of uptake of phosphorus (%) and the P/O ratios when uptake of oxygen was 50% inhibited were derived from these graphs.

Compound	Control (q_{O_2})	pI_{50} O_2 uptake	pI_{50} P uptake	Control P/O ratio	P/O ratio when O_2 uptake was 50% inhibited	% Inhibition of P uptake when O_2 uptake was 50% inhibited
Trimethyltin	95	5·0	5·4	2·4	1·2	76
Triethyltin	77	6·8	7·1	2·6	1·8	66
Tri-*n*-propyltin	88	6·2	6·5	2·9	1·6	72
Tri-*iso*propyltin	100	6·1	6·2	2·8	1·5	73
Tri-*n*-butyltin	98	6·0	6·2	2·7	1·4	74
Tri-*n*-hexyltin	97	5·5	5·6	2·8	1·8	68
Tri-*n*-octyltin	76	—	—	2·9	—	—
Phenylarsenious acid	104	6·9	6·9	2·8	2·6	54
2:4-Dinitrophenol	109	—	5·1	2·8	0·1*	92†

* P/O ratio when O_2 uptake was 17% inhibited.
† Inhibition of P uptake (%) when O_2 uptake was 17% inhibited.

Table 4. *Influence of coenzymes and trialkyltin on oxidation of pyruvate and coupled phosphorylation*

Cofactors [500 μg. of cytochrome *c*, 100 μg. of coenzyme I (Co I) and coenzyme II (Co II) and 10 μg. of coenzyme A (Co A), cocarboxylase, lipoic acid and flavinadenine dinucleotide (FAD)] were added to the flask when required. The triethyltin added is given below. Mitochondria were added last. After equilibration readings were taken at 10, 14, 18, 22 and 26 min. At 7, 11, 15, 19, 23 and 27 min. 0·5 ml. of 60% $HClO_4$ was added to a flask from each series and the concentration of inorganic P was determined. The rate of O_2 uptake and of P uptake was calculated from the slope of the best straight line through these points.

Concentration of triethyltin (M)	q_{O_2}	O_2 uptake (μg.atom/min.)	P uptake (μg.atom/min.)	P/O
No coenzymes added				
Nil	99	0·475	1·355	2·85
$3·12 \times 10^{-7}$	49	0·234	0·37	1·58
Co I, Co II, FAD, cytochrome *c* added				
Nil	100	0·362	1·05	2·91
$3·12 \times 10^{-7}$	69	0·25	0·43	1·72
Co I, Co II, FAD, cytochrome *c*, cocarboxylase, lipoic acid, Co A added				
Nil	119	0·80	2·40	3·00
$3·12 \times 10^{-7}$	86	0·573	1·03	1·80

The possibility that the inhibition of oxidation was due to a loss of essential respiratory coenzymes was tested. The results in Table 4 show that although the addition of a variety of coenzymes slightly influenced the inhibition of oxidation, it has not influenced the lowering of phosphate uptake produced by the triethyltin. Parallel experiments with succinate as substrate gave similar results.

Oxidation stimulated by apyrase, hexokinase and 2:4-dinitrophenol

Oxidation stimulated by hexokinase and glucose (Table 3) is more sensitive to trimethyl and triethyltin than oxidation stimulated by apyrase (Table 1): for instance, the pI_{50} values for trimethyltin are 5·0 and 4·2 and for triethyltin 6·8 and 5·5 respectively. Consideration of the experiments showed that conditions were not identical. Hexokinase was added in large excess, in order to trap all ATP formed, whereas only sufficient apyrase was added to produce about the maximum rate of oxidation. In fact 14 units of hexokinase were sufficient to produce maximum rate of oxidation, whereas 350 units were added in the oxidative phosphorylation experiments. In Table 5 is shown a comparison of the inhibitory power of triethyltin upon oxidation stimulated by the minimal amounts

of apyrase, 2:4-dinitrophenol and hexokinase with oxidation stimulated by a large excess of hexokinase. The last system is the most sensitive to triethyltin and the others are all of the same sensitivity.

Oxidation stimulated by 2:4-dinitrophenol and by apyrase is equally inhibited by other trialkyltins (Table 6). Neither apyrase nor hexokinase was inhibited by the concentrations of trialkyltins used, and since the concentrations of 2:4-dinitrophenol were very much higher than the effective concentrations of many of the trialkyltins, any reaction of trialkyltins with 2:4-dinitrophenol cannot explain these results.

Mitochondrial adenosine triphosphatase. Since some trialkyltins, like 2:4-dinitrophenol, stimulate oxidation their influence upon the latent adenosine triphosphatase of liver mitochondria was examined. These mitochondria have a negligible adenosine

Table 5. *Inhibition by triethyltin of oxidation of pyruvate stimulated by various agents*

Mitochondria were added to the Warburg flasks containing the medium, triethyltin and apyrase (1100 units) or 2:4-dinitrophenol (2×10^{-5} M) or hexokinase (14 or 350 units). Readings were taken at 5 min. intervals from 10 to 40 min. after placing in the bath. Rate of uptake of oxygen was calculated from the best straight line through these points. q_{O_2} values: for unstimulated control, 20; for oxidation stimulated by apyrase, 72; for 2:4-dinitrophenol 71; for 14 units of hexokinase, 74, and for 350 units of hexokinase, 76.

	O_2 uptake (% of control)			
Concn. of triethyltin (M)	Hexokinase (350 units)	Hexokinase (14 units)	Apyrase	2:4-Dinitrophenol
$8{\cdot}65 \times 10^{-7}$	27	51	55	59
$2{\cdot}88 \times 10^{-7}$	49	68	74	—
$9{\cdot}6 \times 10^{-8}$	78	81	88	81

Table 6. *Inhibitory power of trialkyltins against oxidation stimulated by apyrase, 2:4-dinitrophenol and hexokinase and against 2:4-dinitrophenol-activated adenosine triphosphatase*

Oxidation was stimulated by 10^{-5} M-2:4-dinitrophenol. pI_{50} values were calculated for oxidation induced by 2:4-dinitrophenol as shown in Figs. 1 and 2. Other pI_{50} values are taken from Tables 1, 3 and 7.

	pI_{50}			
Trialkyltin	Oxidation induced by apyrase	Oxidation induced by 2:4-dinitrophenol	Oxidation in the presence of excess of hexokinase	2:4-Dinitrophenol-activated adenosine triphosphatase
Methyl	5·0	5·1	5·0	5·3
Ethyl	6·6	6·5	6·8	6·7
n-Propyl	6·2	6·1	6·2	6·4
*iso*Propyl	6·3	6·1	6·1	6·3
n-Butyl	6·0	5·9	6·0	6·1
n-Hexyl	5·0	4·8	5·5	4·5
n-Octyl	—	—	—	—

Table 7. *Action of trialkyltins on latent and 2:4-dinitrophenol-activated adenosine triphosphatase*

Liberation of inorganic phosphate was determined 10 min. after the addition of mitochondria to the medium containing trialkyltin. The adenosine triphosphatase (ATPase) activity of the mitochondria alone was negligible and in the presence of 1% (v/v) dimethylformamide and 10^{-5} M-2:4-dinitrophenol was $0{\cdot}35 \pm 0{\cdot}06$ (10) and $9{\cdot}0 \pm 0{\cdot}16$ (13) μmoles of P/mg. of protein/hr. respectively. Activity in the presence of dimethylformamide has been subtracted from the results. pI_{50} values were corrected for the activation of ATPase by trialkyltins as shown in Figs. 3 and 4.

	Activation of ATPase		Inhibition of ATPase activated by 10^{-5} M-2:4-dinitrophenol	
Compound	Range of concn. producing maximum activation (pC)	Maximum activation produced (μmoles of P/mg. of protein/hr.)	pI_{50}	pI_{50} (corrected for stimulation of ATPase by trialkyltin)
Trimethyltin	4·1–4·6	5·1	3·8	5·3
Triethyltin	5·7–6·1	4·0	5·9	6·7
Tri-*n*-propyltin	6·1–6·3	2·9	6·3	6·4
Tri-*iso*propyltin	6·0–6·2	1·8	6·2	6·3
Tri-*n*-butyltin	5·8–5·9	1·6	6·1	6·2
Tri-*n*-hexyltin	*	*	4·3	4·5
Tri-*n*-octyltin	—	—	—	—

* ATPase activated at concentrations when tri-*n*-hexyltin was not all in solution.

triphosphatase activity in an electrolyte medium containing magnesium (cf. Lardy & Wellman, 1953; Potter & Recknagel, 1951). Dimethylformamide, the solvent used for the trialkyltins, induced a low, and $3{\cdot}3 \times 10^{-5}$ M-2:4-dinitrophenol a high activity (0·3 and 13·9 μmoles of P/μg. of protein/hr. respectively). With the exception of tri-*n*-octyltin all the trialkyltins tested produced some activation of adenosine triphosphatase. The largest activation was produced by trimethyl- and triethyl-tins but even with these compounds it was only one-third of the maximum activation of adenosine triphosphatase in the presence of $3{\cdot}3 \times 10^{-5}$ M-2:4-dinitrophenol or one-half of the activity with 10^{-5} M. Only those trialkyltins which stimulated oxidation also activated adenosine triphosphatase appreciably, and the order of the activity of the trialkyltins in the two systems is the same (Tables 1 and 7). The concentrations which give maximum activation of adenosine triphosphatase are slightly lower than those producing maximum stimulation of oxidation (Tables 1 and 7). It should be noted that tri-*n*-butyltin activated adenosine triphosphatase to a small extent, whereas no stimulation of oxidation was obtained. However, a small stimulation of oxidation would have been very difficult to detect in these experiments.

Activation of adenosine triphosphatase is also obtained with higher concentrations of tri-*n*-butyltin (Fig. 4) and this also occurs with tri-*n*-propyl-, tri-*iso*propyl- and tri-*n*-hexyl-tins at similar concentrations.

Trialkyltins inhibit oxidation stimulated by apyrase, hexokinase and glucose or 2:4-dinitrophenol (Table 6). The influence of trialkyltins upon adenosine triphosphatase stimulated by 2:4-dinitrophenol was therefore examined. It was found that all the trialkyltins with the exception of tri-*n*-octyltin prevent the activation of adenosine triphosphatase by 2:4-dinitrophenol (Table 7). The relation between concentrations of inhibitor and inhibition for triethyltin and tri-*n*-butyltin is shown in Figs. 3 and 4. The concentration where the activation by 10^{-5} M-2:4-dinitrophenol is 50% of its maximum has been calculated (cf. Figs. 3 and 4 for method of calculation) and the pI_{50} values are listed in Table 7. Since 10^{-5} M-2:4-dinitrophenol was used for the stimulation of oxidation for the purposes of comparison this concentration was used for these experiments upon adenosine triphosphatase. Inhibitors of respiration such as cyanide, phenylarsenious acid or diethyltin did not prevent the activation of adenosine triphosphatase by 2:4-dinitrophenol (Table 7).

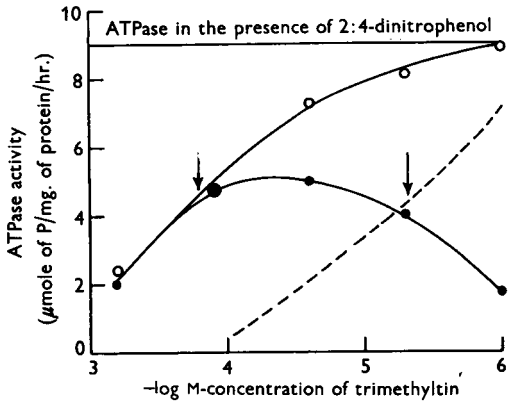

Fig. 3. Influence of trimethyltin on latent and 2:4-dinitrophenol-activated adenosine triphosphatase (ATPase). ●, Latent ATPase; ○, ATPase activated by 10^{-5} M-2:4-dinitrophenol. Mitochondria were added to the medium containing trimethyltin with or without 2:4-dinitrophenol. After 10 min. 0·5 ml. of 60% $HClO_4$ was added and the liberated phosphate determined. Control activity in the presence of 1% (v/v) dimethylformamide was 0·26 μmole of P/mg. of protein/hr. The broken line is the difference between the two solid curves. The pI_{50} for the inhibition by trimethyltin of the ATPase activated by 2:4-dinitrophenol (uncomplicated by ATPase activation by trimethyltin) has been derived from this line. Arrows indicate the concentration where ATPase is 50% inhibited.

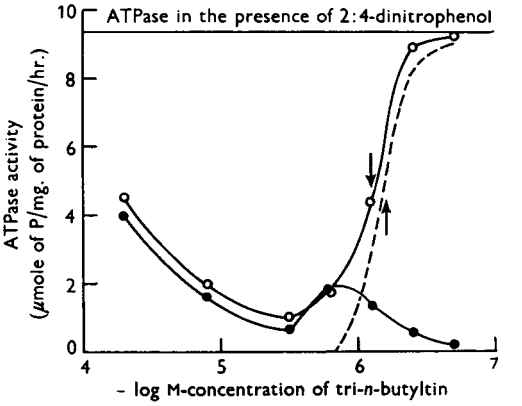

Fig. 4. Influence of tri-*n*-butyltin on latent and 2:4-dinitrophenol-activated adenosine triphosphatase (ATPase). ●, Latent ATPase; ○, ATPase activated by 10^{-5} M-2:4-dinitrophenol. Mitochondria were added to the medium containing tri-*n*-butyltin or tri-*n*-butyltin and 2:4-dinitrophenol. After 10 min. 0·5 ml. of 60% $HClO_4$ was added and the liberated phosphate determined. Control activity in the presence of 1% (v/v) dimethylformamide was 0·38 μmole of P/mg. of protein/hr. The broken line is the difference between the two solid curves. The pI_{50} for the inhibition by tri-*n*-butyltin of the ATPase activated by 2:4-dinitrophenol (uncomplicated by ATPase activation by tri-*n*-butyltin) has been calculated from this line. Arrows indicate the concentration where ATPase is 50% inhibited.

DISCUSSION

Six of the seven trialkyltin compounds tested are inhibitors of the oxidation of pyruvate by liver mitochondria. Some of the lower molecular-weight homologues also activate the adenosine triphosphatase of mitochondria. The question to be decided is whether the inhibition of oxidation is secondary to a primary inhibition of phosphorylation or whether trialkyltins have a dual action, one stimulating adenosine triphosphatase and the other inhibiting electron transport, their final actions being a combination of these two.

There are several reasons why the inhibition of electron transport is unlikely. Previous work (Aldridge & Cremer, 1955) indicated that in some preparations of mitochondria neither succinate oxidase nor diphosphopyridine nucleotide–cytochrome *c* reductase is inhibited by triethyltin. Assuming that in these mitochondria electron transport flows through the same respiratory chain as in other preparations in which oxidation is coupled to phosphorylation, these results would indicate that electron transport is not inhibited by triethyltin. In the presence of excess of hexokinase for a given inhibition of oxygen uptake, a similar inhibition of phosphate uptake and lowering of P/O ratio is obtained for all the trialkyltins (Table 3). Only trimethyl- and triethyl-tin produce an appreciable activation of adenosine triphosphatase. If the enzyme were an important factor in the apparent inhibition of phosphorylation by trialkyltins a larger difference would be found for the lower members. Oxidation is most sensitive to trialkyltins when nearly all the uptake of oxygen is being utilized for the esterification of adenosine diphosphate (i.e. in the presence of a large excess of hexokinase and a P/O ratio of 2·7 or more). When maximum oxygen uptake is obtained by the addition of minimal amounts of apyrase, hexokinase or 2:4-dinitrophenol oxidation is less inhibited. This is particularly so for trimethyl- and triethyl-tin and is probably associated with the liberation of adenosine triphosphatase activity by these compounds. Oxidation therefore can occur in the presence of concentrations of trialkyltins higher than those known to prevent phosphate esterification. Inhibitors of the respiratory chain like cyanide, phenylarsenious acid and diethyltin do not prevent the activation of mitochondrial adenosine triphosphatase by 2:4-dinitrophenol. Trialkyltins on the contrary are potent inhibitors of adenosine triphosphatase activated by 2:4-dinitrophenol or prevent the activation of it by 2:4-dinitrophenol. Therefore for a variety of reasons it seems impossible to formulate a hypothesis which will explain the biochemical properties of the trialkyltins in terms of a dual action involving electron transport and adenosine triphosphatase.

An alternative hypothesis, that the primary block is in the energy-trapping and -transferring reactions between electron transport and the formation of ATP, is one which fits the present experimental findings. Preparations of mitochondria in which oxidation and the phosphorylation of ATP are coupled are very sensitive to trialkyltins. Succinate oxidation, when it can be stimulated by apyrase and is therefore utilized in the phosphorylation of adenosine diphosphate, is inhibited by trialkyltins. There is good agreement between the concentrations of trialkyltin required to inhibit oxidation induced by apyrase, 2:4-dinitrophenol or hexokinase, oxidation in the presence of excess of hexokinase and adenosine triphosphatase activated by 2:4-dinitrophenol (Table 6). The agreement covers the seven trialkyltins studied. There seems no doubt that 2:4-dinitrophenol acts at some stage between electron transport and the formation of ATP. It has recently been shown in this Laboratory that there is a good correlation between the concentrations necessary for the stimulation of oxidation and the activation of adenosine triphosphatase by 2:4:6-trichloro-, pentachloro-, *p*-nitro- and 2:4-dinitro-phenols (Parker, 1958). Both phenomena therefore seem to be due to the same primary action. There is good agreement between the concentration of trialkyltins necessary to inhibit the forward (oxidation) and the back (adenosine triphosphatase) reactions induced by 2:4-dinitrophenol. It is therefore probable that trialkyltins are inhibiting the same step in the process studied in the different directions. A consequence of this conclusion is that the trialkyltins are blocking at the stage where 2:4-dinitrophenol acts. It must be emphasized that it is not suggested that 2:4-dinitrophenol and trialkyltin have the same mechanism of action. But if it may be accepted that trialkyltins inhibit a specific enzyme then it follows from these results that the mechanism of action of 2:4-dinitrophenol also involves this enzyme and is not a simple chemical reaction with a 'high-energy' intermediate (unless this is the enzyme). Rather 2:4-dinitrophenol may be acting as an acceptor for a group transferred by an enzyme and trialkyltins block this enzymic step. Hypothetical series of reactions have been postulated for the trapping of the energy of oxidation of reduced coenzymes (Slater, 1953; Lardy, 1955; Cooper & Lehninger, 1957; Chance & Hollunger, 1957). Since the nature of these reactions is at present unknown it is not possible to draw any conclusions about the stage at which a trialkyltin or 2:4-dinitrophenol acts.

In the above discussion the activation of adenosine triphosphatase by some trialkyltins is

not implicated in the primary mode of action of trialkyltins. Indeed the fact that activation of adenosine triphosphatase always occurs at concentrations higher than those necessary to prevent phosphate esterification in respiring mitochondria indicates that it is a different phenomenon. As shown in Fig. 4, as the concentration of tri-*n*-butyltin is increased from 10^{-7} M, adenosine triphosphatase is slightly activated at 10^{-6} M. As the concentration is further increased, a decrease in adenosine triphosphatase activity is followed by an appreciable activation. These results indicate that there are two types of adenosine triphosphatase activation. As pointed out, the stimulation of oxidation by trialkyltins parallels their ability to stimulate adenosine triphosphatase, but this correlation applies only to the stimulation at the lower concentrations of trialkyltins. Trimethyl- is the most active, followed in decreasing order by triethyl-, tri-*n*-propyl-, tri-*iso*propyl- and tri-*n*-butyl-tins. Fat-soluble substances modify mitochondrial membranes (Christie & Judah, 1954) and the trialkyltins are more soluble in organic solvents than in water. However, the most active member of the series is trimethyltin, the least soluble in organic solvents. It seems unlikely, therefore, that the adenosine triphosphatase accompanied by stimulation of oxidation is caused by structural damage to the mitochondria. The other type of activation of adenosine triphosphatase occurs at higher concentrations (10^{-4} M) of tri-*n*-propyl-, tri-*iso*propyl-, tri-*n*-butyl- and tri-*n*-hexyl-tins. At these concentrations oxidation is completely inhibited, and since these compounds are the more fat soluble it is possible that this is a structural damage to the mitochondria.

Of the trialkyltins examined only tri-*n*-octyltin is inactive. Experience with tri-*n*-hexyltin has shown that it is less consistent in its behaviour than the lower homologues. The behaviour of these higher molecular-weight homologues is probably due to their low solubility in water. The other trialkyltins are all very active and extremely consistent in their behaviour. Triethyltin is the most active (10^{-7} M) and is undoubtedly the most active inhibitor of oxidative phosphorylation known.

The results in Table 3 show that the difference between the inhibition of uptakes of oxygen and phosphate are not large. Theoretically if mitochondrial oxidation cannot occur without the utilization of ATP then inactivation of any enzymic step in this process must lead to the same lowering of uptake of oxygen and phosphate and must give a constant P/O ratio. If it is accepted that such a relationship between oxidation and phosphorylation exists in normal mitochondria then inhibitors such as the trialkyltins cannot be expected to produce profound lowering of P/O ratios. Indeed if the experiments were perfect no change should be found. With damaged mitochondria (possessing free adenosine triphosphatase activity) a lowering of P/O ratio is more likely, but then even a respiratory inhibitor may give such a result. For this reason it is important to use a standard respiratory inhibitor to check the constancy of the P/O ratio under conditions of lowered oxygen uptake with the particular preparation of mitochondria used. Phenylarsenious acid (Peters, 1955) is very suitable for this purpose. It is highly active and appears to be a specific inhibitor of the oxidation of α-oxo acids, for under all the conditions examined an inhibition of oxygen uptake is always accompanied by an accumulation of α-oxo acids. For up to 70% inhibition of oxygen uptake no change in the P/O ratio is obtained.

In the following paper (Stoner & Threlfall, 1958) attempts to apply these findings *in vitro* to the study of the toxicity of the trialkyltin compounds in the whole animal are described. The results show the difficulty of correlating findings *in vitro* and findings *in vivo*, for in the whole animal no unequivocal evidence was found with the techniques now available for a derangement of oxidative phosphorylation by triethyltin, and it would have been impossible to deduce that this was its action *in vitro*.

SUMMARY

1. Rat-liver mitochondria whose rate of oxidation of pyruvate may be increased three- to four-fold by the addition of potato apyrase, hexokinase and glucose and 2:4-dinitrophenol have been used to examine the biochemistry of trialkyltins.

2. Oxidation induced by apyrase, 2:4-dinitrophenol or hexokinase and glucose, oxidation in the presence of excess of hexokinase and glucose and adenosine triphosphatase stimulated by 2:4-dinitrophenol are all equally sensitive to inhibition by a trialkyltin. Seven trialkyltins have been tested against these processes.

3. The lower molecular-weight trialkyltins induce some adenosine triphosphatase activity in mitochondria and this has been correlated with a stimulation of the oxidation of pyruvate.

4. It is concluded that trialkyltins inhibit a step in the energy-transferring chain between electron transport and the formation of adenosine triphosphate.

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The Biochemistry of Organotin Compounds

EFFECT OF TRIETHYLTIN SULPHATE ON TISSUE PHOSPHATES IN THE RAT

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In the preceding paper Aldridge (1958) has shown that trialkyltin compounds interfere with the process of oxidative phosphorylation in liver mitochondria and he has also shown (personal communication) that brain mitochondria are equally sensitive. The toxicity of these compounds to the whole animal has been known for a long time (Jolyet & Cahours, 1869) and has recently been reinvestigated in this Laboratory (Stoner, Barnes & Duff, 1955; Barnes & Stoner, 1958). The parallel investigation of these compounds at different levels of biological organization has led to an attempt, so far unsuccessful, to explain the toxicity in biochemical terms. The main effect of these compounds *in vivo* is upon the central nervous system, which is also the site of the only significant pathological change, an interstitial oedema confined to the white matter of the brain and spinal cord (Magee, Stoner & Barnes, 1957). Difficulties in elucidating the biochemical mechanisms of their toxicity have already been encountered. Although liver and brain mitochondria are equally affected *in vitro*, Cremer (1957), working with tissue slices, found that it was only the uptake of oxygen by slices of nervous tissue which was inhibited whether the triethyltin was added *in vitro* or injected into the rat before it was killed. This was despite the fact that after the injection of triethyltin there was more in the liver than brain and that the temperature of both was depressed after the injection.

Triethyltin is the most active member of the series *in vitro* and *in vivo* and its effects on some phosphate components of rat tissues and on the distribution of injected ^{32}P has now been studied in the whole animal. Triethyltin affects the metabolism of both the nervous system and liver in the rat but the mechanism of the derangement cannot yet be deduced from these results nor can they be readily correlated with the previous findings *in vitro*.

METHODS

Male albino rats of the Porton strain (145 ± 10 g. body wt.) were used. They were fed either on M.R.C. Diet 41 (Bruce & Parkes, 1949) or more recently on Diet 41B (Bruce & Parkes, 1956) given *ad lib.* In the experiments on nephrectomized rats both kidneys were removed through a ventral