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HALIDE-DEPENDENT AND HALIDE-INDEPENDENT EFFECTS OF TRIORGANOTIN AND TRIORGANOLEAD COMPOUNDS ON MITOCHONDRIAL FUNCTIONS

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1. Each of five triorganotin and five triorganolead compounds was shown to perturb mitochondrial functions in three different ways. One is dependent and two are independent of Cl^- in the medium. 2. Structure–activity relationships for the three interactions are described, and compounds suitable as tools for the separate study of each process are defined. 3. In a Cl^--containing medium trimethyltin, triethyltin, trimethyl-lead, triethyl-lead and tri-*n*-propyl-lead all produce the same maximum rate of ATP hydrolysis and O_2 uptake; this rate is much less than that produced by uncoupling agents such as 2,4-dinitrophenol. 4. Increases in ATP hydrolysis and O_2 uptake are measures of energy utilization when triorganotin and triorganolead compounds bring about an exchange of external Cl^- for intramitochondrial OH^- ions. Possible rate-limiting steps in this process are discussed. 5. In a Cl^--containing medium ATP synthesis linked to the oxidation of β-hydroxybutyrate or reduced cytochrome *c* is less inhibited by triethyltin or triethyl-lead than is ATP synthesis linked to the oxidation of succinate, pyruvate or L-glutamate. 6. The inhibition of ATP synthesis linked to the oxidation of both β-hydroxybutyrate and reduced cytochrome *c* consists of two processes: one is a limited uncoupling and is Cl^--dependent and the other is a Cl^--independent inhibition of the energy-conservation system. 7. The different sensitivities to inhibition by triethyltin of mitochondrial functions involving the oxidation of β-hydroxybutyrate and succinate are compared and discussed.

Triorganotin compounds mediate an exchange of halide for OH^- ions across membranes, including those of mitochondria (Selwyn *et al.*, 1970; Harris *et al.*, 1973; Skilleter, 1976). This property causes functional changes in mitochondria, and some of these have been described for triethyltin (Stockdale *et al.*, 1970; Rose & Aldridge, 1972). Triethyltin also interacts with mitochondria to produce other changes (Aldridge & Street, 1970; Rose & Aldridge, 1972) that have been described as oligomycin-like (Stockdale *et al.*, 1970; Manger, 1969) and do not require halide in the medium. In addition to the above two effects, the higher-molecular-weight triorganotins also cause gross swelling of mitochondria (Aldridge & Street, 1964); the dependence of this response on the composition of the medium has not been previously examined. The fact that these three effects may be distinguished as described above implies that they are brought about by different mechanisms, even though they may lead to the same end point, namely an inability of the mitochondria to synthesize ATP. In the present paper it is established that many triorganotin and triorganolead compounds interact with mitochondria in these three different ways.

From these experiments it has been possible to define the concentration of those compounds that are suitable for the study of each of these three effects without interference from the others.

Trialkyltin and trialkyl-lead compounds stimulate ATP hydrolysis by rat liver mitochondria (Aldridge, 1958; Aldridge *et al.*, 1962; Aldridge & Street, 1964, 1971), and this stimulation has been shown to depend on the presence of Cl^- in the medium (Coleman & Palmer, 1971; Rose & Aldridge, 1972). Trialkyltin and trialkyl-lead compounds increase State-4 O_2 uptake (Aldridge, 1958; Aldridge & Street, 1971; Manger, 1969), and this increase is also Cl^--dependent (Stockdale *et al.*, 1970; Rose & Aldridge, 1972; Skilleter, 1975). O_2 uptake of brain-cortex slices is increased by triethyltin (Cremer, 1957), and this increase is also Cl^--dependent (Lock, 1976). Other workers (Moore & Brody, 1961; Sone & Hagihara, 1964), who have failed to stimulate O_2 uptake of mitochondria, have used media containing very low concentrations of Cl^-.

Rapid and gross swelling of mitochondria when suspended in iso-osmotic solutions of NH_4Cl or NaCl containing trialkyltin compounds indicates that these organometals induce the penetration of Cl^-

into the mitochondrion (Selwyn *et al.*, 1970). Under conditions when utilization of energy derived from oxidation of substrates was not possible, Harris *et al.* (1973) showed by direct measurement that trialkyltin compounds cause Cl^- to move into the mitochondrion. Metabolically active mitochondria take up a much larger amount of Cl^-, and this uptake does not occur when either oxidation of substrates or hydrolysis of ATP is prevented by inhibitors (Skilleter, 1976). In the present study with suitable trialkyltin and trialkyl-lead compounds, the increase in ATP hydrolysis and O_2 uptake are shown to be a measure of energy utilized to bring about the uptake of Cl^- into the mitochondrion.

In a Cl^--containing medium ATP synthesis linked to oxidation of reduced cytochrome *c* is less sensitive to inhibition by trimethyltin and triethyltin than with other substrates such as pyruvate or succinate (Aldridge & Street, 1971). In a previous study in this laboratory ATP synthesis with β-hydroxybutyrate as substrate was less inhibited by 1 μM-triethyltin than when tricarboxylic acid-cycle substrates were used (Skilleter, 1975). In the present paper a more extensive study of this phenomenon is reported.

Materials and Methods

Special chemicals

Triethyltin hydroxide was supplied by the Tin Research Institute, Greenford, Middx., U.K., and triethyltin sulphate was prepared from it as described by Aldridge & Cremer (1955). Trimethyltin chloride was purchased from BDH Chemicals, Poole, Dorset, U.K. Trimethyltin hydroxide, tri-*n*-propyltin acetate, tri-*n*-butyltin acetate, triphenyltin acetate, trimethyl-lead acetate, triethyl-lead acetate, tri-*n*-propyl-lead acetate, tri-*n*-butyl-lead acetate and triphenyl-lead acetate were all supplied by Dr. G. J. M. van der Kerk, Institute for Organic Chemistry, Taegepast Natuurwetenschappalijk Onderzoek, Utrecht, The Netherlands. Rotenone was purchased from BDH Chemicals; ATP, sodium pyruvate, hexokinase (yeast), Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], Pipes (1,4-piperazinediethanesulphonic acid) and oligomycin were from Sigma Chemical Co., St. Louis, MO, U.S.A.; *NNN'N'*-tetramethyl-*p*-phenylenediamine hydrochloride was from Kodak, Liverpool L33 7UF, U.K. The rotenone and *NNN'N'*-tetramethyl-*p*-phenylenediamine were purified as previously described (Aldridge & Street, 1971). Sodium hydroxyethanesulphonate (isethionate) was purchased from Koch–Light Laboratories, Colnbrook, Bucks., U.K., and converted into the potassium salt as previously described (Rose & Aldridge, 1972). $Na^{36}Cl$ and triethyl[^{113}Sn]tin chloride were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Stock solutions of most of the triorganometal compounds were 10 mM in dimethylformamide. Triethyltin sulphate and trimethyltin hydroxide were dissolved in water. All stock solutions were stored cold and away from light.

Mitochondrial ATP synthesis, ATP hydrolysis and O_2 uptake

The preparation of the mitochondrial fraction from rat liver and the determination of ATP synthesis, O_2 uptake, ATP hydrolysis, P_i and protein were essentially as described previously (Aldridge & Street, 1971). Except where stated otherwise the substrates and their concentrations used were DL-β-hydroxybutyrate (20 mM), pyruvate+fumarate (10 mM+1 mM), succinate (10 mM), L-glutamate (10 mM) and, for the oxidation of reduced cytochrome *c*, ascorbate(40 mM)+*NNN'N'*-tetramethyl-*p*-phenylenediamine (1 mM). ATP synthesis linked to the oxidation of a particular substrate is designated throughout as ATP synthesis (substrate). For ATP hydrolysis the medium contained ATP (9 mM), KCl or potassium isethionate (100 mM), $MgCl_2$ or magnesium isethionate (14 mM), Hepes (20 mM), EDTA (1 mM) and rotenone (1.1 μM), and the pH was adjusted to 6.8, or as required, with KOH, HCl or isethionic acid. All experiments were carried out at 37°C, except for ATP synthesis linked to reduced cytochrome *c*, which was usually at 25°C. The rate of ATP synthesis with this substrate is more linear at 25°C than at 37°C; experiments showed that the percentage inhibition by triorganotin compounds was the same at both temperatures.

Mitochondrial swelling

For the measurement of energy-dependent mitochondrial swelling the medium used for ATP hydrolysis was used and A_{530} was measured after shaking in air for 2 min at 37°C. The concentration of mitochondrial protein was the same as that used for the examination of other mitochondrial functions, and 0.5 cm light-path was used. Changes in absorbance were calculated relative to an absorbance of 1.0 for the control mitochondrial suspension. For the measurement of the rate of swelling in NaCl the medium contained NaCl (0.15 M), Tris (10 mM) and rotenone (1.1 μM), and the pH was adjusted to 6.8 with HCl. The rate of change of A_{530} with 0.5 cm light-path was measured on a recording spectrophotometer. The initial rate of swelling was calculated as a percentage of that produced in the presence of triethyltin (2 μM). This swelling is independent of an energy source, since Na^+ ions readily penetrate rat liver mitochondria (Mitchell & Moyle, 1969; Selwyn *et al.*, 1970).

Distribution of triethyltin

The distribution of Cl^- between mitochondria and medium was determined by using $^{36}Cl^-$ as previously

described (Skilleter, 1976). The counting of triethyl-[^{113}Sn]tin radioactivity was carried out with the precautions defined by Rose & Aldridge (1968). The solvent/water distribution of triethyltin [^{36}Cl]-chloride or triethyl[^{113}Sn]tin hydroxide was measured between equal volumes of either benzene or hexane and buffer containingNaCl (0.1 M), Hepes (10 mM) and Pipes (10 mM) and the pH adjusted as required with HCl or NaOH. The distribution of triethyl[^{113}Sn]tin was determined between chloroform and the following buffers: Tris (0.1 M)/H_2SO_4, Tris (0.1 M)/HNO_3, $Na_4P_2O_7$ (0.1 M), Na_2HPO_4 (0.1 M)/KH_2PO_4 (0.1 M) and Na_2ATP/KOH.

Expression of results

The amounts of the organometals that perturb mitochondrial functions may be expressed in two ways: the concentration of the compound in the medium to which the mitochondria have been added or the quantity of compound per unit of mitochondrial protein. In the present paper effective concentrations are always expressed as the concentration of compound in the medium to which the mitochondria have been added. In each case, in addition, the concentration of mitochondrial protein is stated so that, if desired, the amount of compound per unit of protein may be calculated.

The summary of a collection of results in each case is given as mean±S.E.M. (number of observations).

Results

Effects of triorganotin and triorganolead compounds on mitochondrial functions

Three processes by which triorganotins influence mitochondrial functions can be illustrated by the effects of a range of concentrations of trimethyltin on ATP hydrolysis in a medium, the bulk anions being Cl^- or isethionate (Fig. 1). As the concentration of trimethyltin is raised in a chloride medium, ATP hydrolysis increases (Fig. 1*a*). This increase is Cl^--dependent because it does not occur in an isethionate medium. As the concentration of trimethyltin is further increased, the rate of ATP hydrolysis decreases (Fig. 1*a*). Substantial Cl^- uptake in the presence of organotins and organoleads requires the consumption of energy (Table 5 in Skilleter, 1976), and the decrease in ATP hydrolysis is due to inhibition of energy conservation by trimethyltin; this property of organotin compounds has been described as being like that of oligomycin (Stockdale *et al.*, 1970; Manger, 1969). Cl^--dependent ATP hydrolysis is correlated with small-scale swelling of the mitochondria (Fig. 1*b*). At higher concentrations of trimethyltin gross swelling occurs in both a chloride and an isethionate medium (Fig. 1*b*). Such gross physical change in mitochondria leads to increased ATP hydrolysis (Fig. 1*a*).

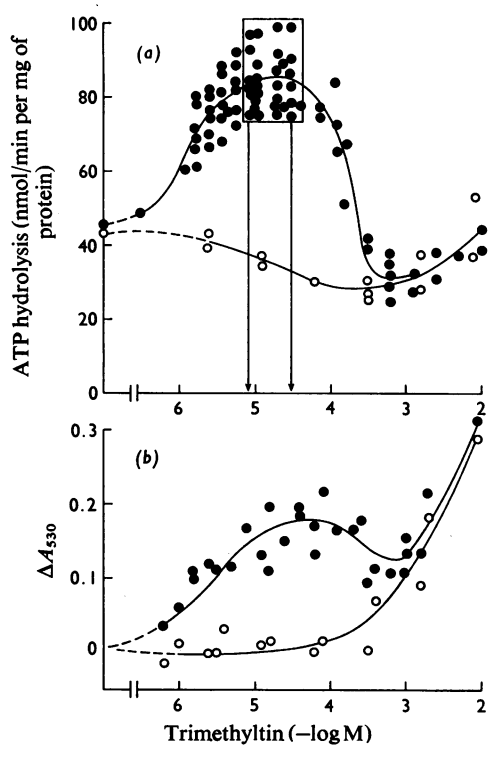


Fig. 1. *Influence of trimethyltin on various mitochondrial functions in a chloride* (●) *and a isethionate* (○) *medium* (*a*) ATP hydrolysis. The results for the chloride medium are from 12 experiments and those for the isethionate medium from two experiments. The vertical arrows indicate the range of concentrations of trimethyltin producing the maximum rate (cf. Table 1). (*b*) Swelling of mitochondria. The medium was that used for the measurement of ATP hydrolysis. The results for a chloride medium are from six experiments and those for an isethionate medium are from two experiments.

The same range of concentration of trimethyltin that brings about the increase in ATP hydrolysis in a chloride medium also affects several other mitochondrial functions (Fig. 2). All of these effects are brought about by the primary Cl^-/OH^- exchange. This generalization is also true for triethyltin, trimethyl-lead, triethyl-lead and tri-*n*-propyl-lead.

The biphasic trimethyltin effect shown in Fig. 1(*a*) represents two antagonistic actions, i.e. trimethyltin-stimulated ATP hydrolysis in a chloride medium is prevented by higher concentrations of trimethyltin. For some organometal compounds the concentrations effective in these two ways overlap (cf. Aldridge & Street, 1964). In these cases the maximum rate of ATP hydrolysis can either be less, e.g. with tri-*n*-propyltin, tri-*n*-butyltin or tri-*n*-butyl-lead, or

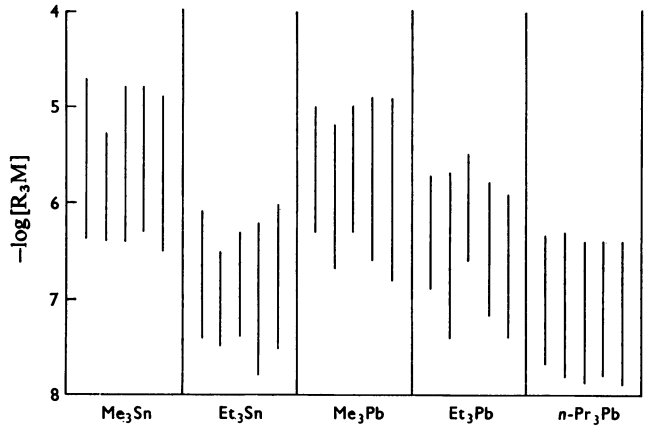


Fig. 2. *Concentrations of triorganotin and triorganolead compounds that affect various mitochondrial functions in a chloride medium*

For each system the range of concentration is that producing 10–90% of the maximum change. In each section the mitochondrial functions examined are from left to right: (1) increase in O_2 uptake, (2) increase in ATP hydrolysis, (3) small-scale swelling in the medium used for ATP hydrolysis, (4) inhibition of ATP synthesis linked to oxidation of succinate, and (5) rate of swelling in 0.15M-NaCl.

Table 1. *Rates of ATP hydrolysis and O_2 uptake in a chloride medium in the presence of triorganotin and triorganolead compounds*

[R_3M] is the concentration of triorganometal causing maximum ATP hydrolysis. The numbers in parentheses after ATP hydrolysis (No R_3M) indicate the numbers of mitochondrial preparations. The numbers in parentheses after the name of the organometal and after ATP hydrolysis (+R_3M) indicate the numbers of observations over the range of concentrations stated (cf. Fig. 1*a*).

Organometal	Mean concn. of protein (mg/ml)	[R_3M] or [dinitrophenol] (μM)	ATP hydrolysis (nmol/min per mg of protein)	
			No R_3M or dinitrophenol	+R_3M or dinitrophenol
ATP hydrolysis				
Trimethyltin (37)	1.19	8.3–32		
Trimethyltin (21)	1.14	0.4–1.3		
Trimethyl-lead (13)	1.28	5–40	41.5±1.3 (40)	83.0±1.2 (102)
Triethyl-lead (15)	1.28	1.6–63		
Tri-*n*-propyl-lead (16)	1.22	0.5–8.1		
2,4-Dinitrophenol*		30	42.5±1.3 (29)	336±18 (6)
			O_2 uptake (ng-atoms/min per mg of protein)	
			No R_3M or dinitrophenol	+R_3M or dinitrophenol
Pyruvate+fumarate oxidation				
Trimethyltin (5)	1.18	40–80		
Triethyltin (5)	1.16	0.8–6.3		
Trimethyl-lead (7)	1.22	20–63	39.0±1.5 (27)	64.0±1.7 (34)
Triethyl-lead (7)	1.22	2.2–6.3		
Tri-*n*-propyl-lead (3)	1.30	0.4–1.6		
2,4-Dinitrophenol	1.21	20	42.0±2.0 (14)	131±6 (14)
Succinate oxidation				
Triethyltin (5)	1.26	1–5	78.6±3.5 (5)	121±5 (5)
2,4-Dinitrophenol	1.25	20		356±18 (4)
β-Hydroxybutyrate				
Triethyltin (3)	1.19	1.3–4	44.8±3.6 (3)	73.2±7.4 (3)
2,4-Dinitrophenol	1.19	20		118±22 (3)

* Data taken from Aldridge & Street (1971).

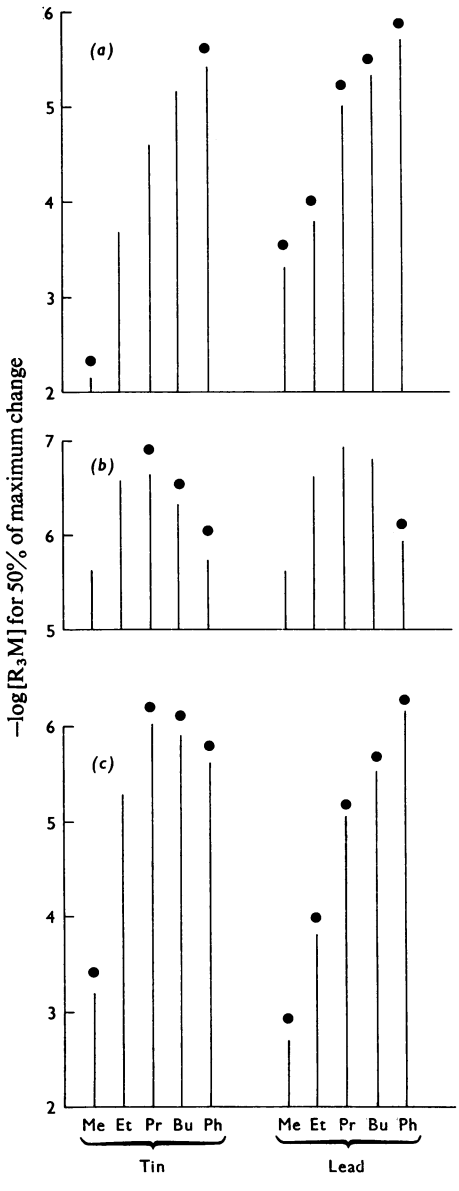


Fig. 3. *Ability of triorganotin and triorganolead compounds to change mitochondrial functions, by three mechanisms*
The measurements made are (*a*) large-scale swelling in chloride or isethionate medium, (*b*) the Cl^-/OH^- exchange measured by the rate of swelling in 0.15M-NaCl and (*c*) ATP synthesis linked to oxidation of succinate in an isethionate medium. Those columns marked ● indicate the systems for which inhibitory concentrations overlap. Those columns not marked indicate that the compounds may be used as tools for the study of the particular system.

completely overlapped by the inhibition, so that there is no stimulation of adenosine triphosphatase, e.g. with triphenyltin or triphenyl-lead.* This is illustrated by the fact that, in the presence of tri *n*-butyltin, ATP hydrolysis cannot be increased by trimethyltin,* since tri-*n*-butyltin is preventing the consumption of energy (ATP) necessary for the Cl^-/OH^- exchange (see below). Hence, for an unambiguous method to measure the activity of organometals bringing about the Cl^-/OH^- exchange, a system independent of a source of energy must be used.

It is suggested, therefore, that determination of the ability of the organometals to bring about the activities described above can be made by the following three methods: (1) the rate of swelling of mitochondrial suspensions in 0.15M-NaCl to measure the Cl^-/OH^- exchange; (2) inhibition of ATP synthesis linked to the oxidation of succinate in an isethionate medium to measure inhibition of energy conservation by the 'oligomycin-like' action; (3) optical changes in the medium used for ATP hydrolysis containing either Cl^- or isethionate as the bulk anion to measure gross swelling. The results of such measurements are summarized in Fig. 3.

Stimulation of ATP hydrolysis and O_2 consumption of liver mitochondria by triorganotin and triorganolead compounds in halide-containing media

ATP hydrolysis is stimulated to the same maximum rate by trimethyltin, triethyltin, trimethyl-lead, triethyl-lead and tri-*n*-propyl-lead (Table 1). This maximum rate of ATP hydrolysis of 83nmol/min per mg of protein is much less than that produced by 30μM-2,4-dinitrophenol (336nmol/min per mg of protein). The increase in the rate of ATP hydrolysis produced by triethyltin was unaffected by the concentration of ATP (a range of 1–9mM was examined)* and was also unaffected by the nature of the bulk cation (KCl, NaCl, choline chloride and tetramethylammonium chloride were tested).*

If rates of ATP hydrolysis are compared when Cl^- is changed to Br^- (Table 2), a slightly higher rate is obtained. The rate of ATP hydrolysis for all the organometals listed is the same in the presence of Br^-, as was previously shown for Cl^- (Table 1). In other experiments the concentration of Cl^- or Br^- was varied and the osmolarity of the medium kept constant by replacing KCl or KBr by potassium isethionate.* Analysis of the data indicated that the maximum rate of ATP hydrolysis would be 90 and 104nmol/min per mg of protein for Cl^- and Br^- respectively and with both triethyltin and triethyl-lead. The concentrations of Cl^- necessary to produce

* Those passages in the text marked with an asterisk signify experimental results that have been seen by the Editorial Board but that are not published in detail in this paper. These experimental results may be obtained from the authors on request.

Table 2. *Increase in ATP hydrolysis by triorganotin and triorganolead compounds in the presence of Cl^- and Br^-*

Compounds	Mean concn. of protein (mg/ml)	$[R_3M]$ (μM)	ATP hydrolysis (nmol/min per mg of protein)	
			No R_3M	$+R_3M$
Chloride medium				
Trimethyltin	1.11	6.3–40		
Triethyltin	1.11	0.4–1.3		
Trimethyl-lead	1.22	16–100	40.2±1.6 (5)	78.6±0.4 (5)
Triethyl-lead	1.22	1–100		
Tri-*n*-propyl-lead	1.40	0.4–3.2		
Bromide medium				
Trimethyltin	1.11	5–40		
Triethyltin	1.11	0.3–1.0		
Trimethyl-lead	1.22	4–50	41.4±0.5 (5)	90.4±0.8 (5)
Triethyl-lead	1.22	0.8–13		
Tri-*n*-propyl-lead	1.40	0.1–0.6		

Table 3. *Effects of trimethyltin, triethyltin and triethyl-lead on the Cl^- content of mitochondria*

The medium used was that used for the measurement of ATP hydrolysis. Cl^- content was measured by using $^{36}Cl^-$. For details of methods see Skilleter (1976).

$[R_3M]$ (μM)	Increase in Cl^- content (ng-atoms/mg of protein)		
	Me_3Sn	Et_3Sn*	Et_3Pb
0.01	—	9	10
0.1	17	34	30
1	45	135	60
10	103	67	100
100	116	105	97
1000	89	—	—

* Data taken from Skilleter (1976).

half the maximum increase in rate of ATP hydrolysis by triethyltin and triethyl-lead are 80mM and 38mM respectively. The corresponding values for bromide are 30mM and 18mM.* The fact that with triethyl-lead an increase of over 5-fold in bromide concentration (18 to 100mM) produces only a rate of ATP hydrolysis of 104nmol/min per mg of protein illustrates that 100mM-Br^- cannot be rate-limiting.

Trimethyltin, triethyltin, trimethyl-lead, triethyl-lead and tri-*n*-propyl-lead also increase to the same extent the rate of O_2 uptake with pyruvate+fumarate as substrate (Table 1). Triethyltin increases to a greater extent the oxidation of succinate (Table 1). In either case the rate is much less than that obtained with 20μM-2,4-dinitrophenol. Other experiments have shown that the increase in rate of O_2 uptake is not affected by the concentration of succinate (1–30mM)* nor by the concentration of pyruvate (0.6–30mM) either with a [pyruvate]/[fumarate] ratio kept constant at 10 or with a constant concentration of fumarate of 1mM.*

Increase in Cl^- content of mitochondria

In previous work (Skilleter, 1976) triethyltin was shown to increase the Cl^- content of mitochondria either in a medium containing ATP or when the mitochondria were actively oxidizing substrates. Other triorgano-tin and -lead compounds also increase the Cl^- content, and the effective concentrations are those leading to maximum rates of ATP hydrolysis (Table 3; cf. Table 1).

Effect of pH on the distribution of Cl^- and triethyltin between water and either benzene or hexane

At neutral pH in a medium containing 0.1M-$Na^{36}Cl$, triethyltin caused Cl^- to distribute from aqueous medium into benzene or hexane. The concentration of Cl^- in the solvents decreased with increasing pH above 6. At pH10 no Cl^- is found in the solvent.* By comparison with other experiments, with triethyl[^{113}Sn]tin, it was shown that at pH6 all the triethyltin in the solvents is triethyltin chloride, whereas at pH10 it must all be as triethyltin hydroxide.* The dissociation constant for triethyltin hydroxide is 6×10^{-8}M (Tobias, 1966). These results show that, for the conditions used to examine various mitochondrial functions (0.1M-chloride, pH6.8), Cl^- can compete effectively with OH^- for the triethyltin. The dissociation constant of triethyltin chloride is, however, very large compared with that for triethyltin hydroxide and must be of the order of 0.1M.

Effect of triethyltin on ATP synthesis linked to the oxidation of various substrates

Triethyltin is a compound suitable for the demonstration of three different processes brought about by

Table 4. *Inhibition by triethyltin of ATP synthesis linked to the oxidation of various substrates*
For all experiments the mitochondrial protein was between 0.97 and 1.35mg/ml. The numbers in parentheses after the substrate indicate the numbers of experiments. For details of methods see the Materials and Methods section.

	Control rate of ATP synthesis (nmol/min per mg of protein)	[Triethyltin] for 50% inhibition (μM)	Range of [triethyltin] for 10–90% inhibition (μM)
Chloride medium			
Pyruvate+fumarate (10)	226	0.25	0.04–1.2
Succinate (9)	312	0.09	0.02–0.6
L-Glutamate (1)	311	0.14	0.03–3.1
β-Hydroxybutyrate (8)	262	2.5	0.10–20
Reduced cytochrome *c* (9)	293 (37°C) 210 (25°C)	4.0	0.16–30
Isethionate medium			
Pyruvate+fumarate (10)	180	9	2.8–40
Succinate (9)	293	6	1.2–30
β-Hydroxybutyrate (3)	284	11	2.8–40
Reduced cytochrome *c* (2)*	—	9	1.6–30

* Results taken from Rose & Aldridge (1972).

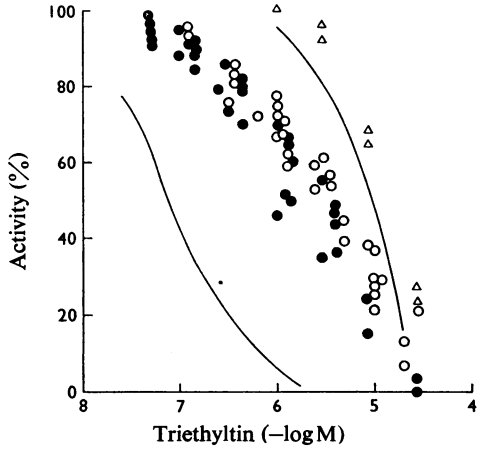


Fig. 4. *Inhibition of ATP synthesis by triethyltin*
The left-hand and right-hand lines are ATP synthesis (succinate) in a chloride and an isethionate medium respectively. The symbols refer to the ATP synthesis linked to the oxidation of the following substrates: ○, reduced cytochrome *c* in a chloride medium; ●, β-hydroxybutyrate in a chloride medium; △, β-hydroxybutyrate in an isethionate medium.

triorganotin compounds (Fig. 3). For example, in a 0.13M-chloride medium (see the Materials and Methods section) 0.02–0.6μM-triethyltin inhibits ATP synthesis (succinate) and this is due to the exchange of intramitochondrial OH^- for Cl^- (Table 4 and Fig. 2). In contrast, much higher concentrations (1.2–30μM) are required to inhibit ATP synthesis (succinate) in an isethionate medium (Table 4 and Fig. 3). In a chloride medium much higher concentrations of triethyltin are required to inhibit by 50% ATP synthesis (reduced cytochrome *c* or β-hydroxybutyrate) than that linked to the oxidation of pyruvate, succinate or L-glutamate (Table 4). In contrast, in an isethionate medium the same but even higher concentrations are necessary to inhibit ATP synthesis of all substrates examined (pyruvate, succinate, reduced cytochrome *c* and β-hydroxybutyrate; Table 4). For such comparisons the composition of the medium must be as far as possible the same, because triethyltin combines with phosphate and ATP* (cf. Rose, 1969).

ATP synthesis linked to the oxidation of β-hydroxybutyrate and succinate differ in other respects. The concentrations of triethyltin that inhibit ATP synthesis (succinate) in chloride medium do not overlap those needed in an isethionate medium (Fig. 4 and Table 4). In contrast, the range of concentrations of triethyltin required to inhibit ATP synthesis (β-hydroxybutyrate) in a chloride medium is much larger (Fig. 4 and Table 4), and covers the whole range of concentrations causing inhibition with succinate in chloride and isethionate media (Fig. 4). This anomalous behaviour for ATP (β-hydroxybutyrate) is also shown for the inhibition of ATP synthesis (reduced cytochrome *c*) by triethyltin (Fig. 4).

Since inhibition by triethyltin of ATP synthesis (succinate) in a chloride or isethionate medium is brought about by two different mechanisms, complete inhibition of ATP synthesis (β-hydroxybutyrate) appears to involve both mechanisms. Further experiments have been designed to try to explain why 2μM-triethyltin inhibits ATP synthesis (β-hydroxybutyrate) by only 30%, whereas that with succinate ATP synthesis is completely prevented. The range of concentration (0.01–2μM) that increases ATP hydrolysis to a maximum rate in a chloride medium also causes up to 100% inhibition of ATP synthesis

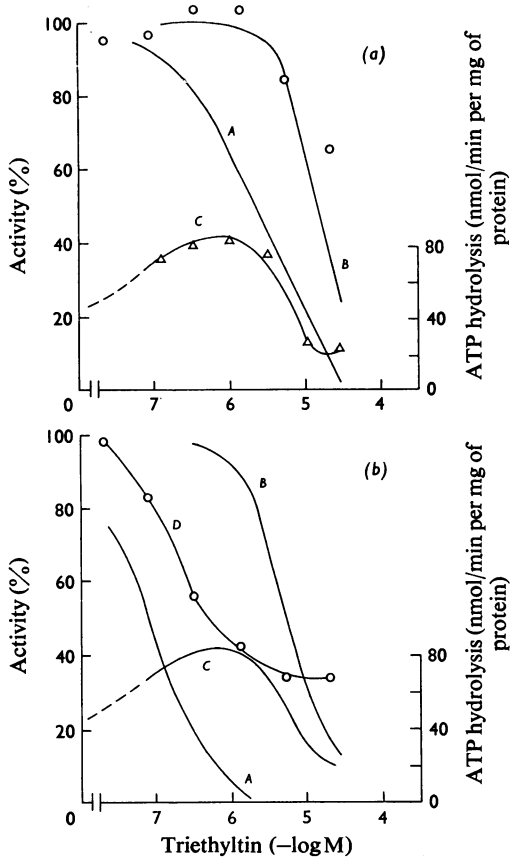


Fig. 5. *Effect of triethyltin on ATP synthesis and O_2 uptake with β-hydroxybutyrate or succinate as substrate and on ATP hydrolysis*

(*a*) β-Hydroxybutyrate. Curve *A* is ATP synthesis in a chloride medium, curve *B* is ATP synthesis in an isethionate medium and shows the mean of the results of experiments summarized in Table 4, and curve *C* is ATP hydrolysis in a chloride medium. The symbols indicate the following: ○, O_2 uptake stimulated by ADP (mean control rate±ADP was 130 and 49ng-atoms/min per mg of protein); △, ATP hydrolysis measured in the presence of β-hydroxybutyrate (+rotenone) in a chloride medium. Additional experiments have shown that the rates of ATP hydrolysis±2μM-triethyltin are the same± 1.7mM-P_i. (*b*) Succinate. Curve *A* is ATP synthesis in a chloride medium, curve *B* is ATP synthesis in an isethionate medium, curve *C* is ATP hydrolysis in a chloride medium and curve *D* is O_2 uptake stimulated by ADP (mean control rates±ADP were 320 and 78ng-atoms of O/min per mg of protein).

(succinate) (Fig. 5*b*). O_2 uptake in the presence of excess of ADP (conditions as for ATP synthesis) is also inhibited (Fig. 5*b*). Analogous experiments with β-hydroxybutyrate are shown in Fig. 5(*a*). Triethyltin (0.01–2μM) causes only partial inhibition (30%) of ATP synthesis and no inhibition of ADP-stimulated O_2 uptake. Thus when β-hydroxybutyrate is used as substrate an uncoupling action of triethyltin is seen: a decrease in ATP synthesis, increase in ATP hydrolysis and no inhibition of O_2 uptake. Such an uncoupling action only results in partial inhibition of ATP synthesis (compare rates of ATP hydrolysis with 2μM-triethyltin and 30μM-2,4-dinitrophenol; Table 1). The utilization of energy for the Cl^-/OH^- exchange should result in a decrease in the yield of ATP whether β-hydroxybutyrate or succinate is oxidized. However, with ATP synthesis (succinate) the Cl^-/OH^- exchange leads to secondary effects leading to complete inhibition, in contrast with the effects of triethyltin on ATP synthesis (β-hydroxybutyrate).

ATP synthesis with β-hydroxybutyrate and succinate present separately or as mixture of substrate

ATP synthesis with a mixture of β-hydroxybutyrate and succinate has a sensitivity to triethyltin indicated

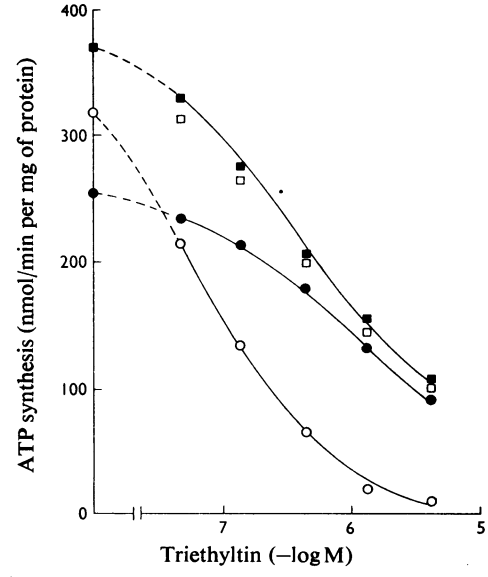


Fig. 6. *Inhibition of ATP synthesis linked to oxidation of succinate and/or β-hydroxybutyrate by triethyltin in a chloride medium*

The symbols represent ATP synthesis linked to the oxidation of: ●, β-hydroxybutyrate (20mM); ○, succinate (10mM); ■, β-hydroxybutyrate (20mM)+ succinate (10mM); □, results calculated from the mixture of the two substrates (see the text). The control activity with β-hydroxybutyrate was 255, with succinate 320 and with both β-hydroxybutyrate and succinate 369nmol/min per mg of protein. It was assumed that the additional ATP synthesis of the mixture (369−255 = 114) was due to succinate.

by their separate sensitivities, if it is assumed that oxidation of β-hydroxybutyrate takes precedence. For example, the rate of ATP synthesis (succinate) is 320 and ATP synthesis (β-hydroxybutyrate) is 255 nmol/min per mg of protein. With the two substrates together the rate is 369 nmol/min per mg of protein (in contrast with the total of 575 nmol/min per mg of protein for the two substrates separately). The relationship between ATP synthesis and concentration of triethyltin, calculated on the assumption that with the two substrates together ATP synthesis of 255 nmol/min per mg of protein is due to oxidation of β-hydroxybutyrate and 369–255 = 114 nmol/min per mg of protein is due to succinate, agrees with that found experimentally (Fig. 6).

Discussion

From the studies of five triorganotin and five triorganolead compounds on mitochondria it may be concluded that they bring about three mechanistically different processes. They are (1) physical disruption of the mitochondria brought about by large-scale swelling in either KCl or potassium isethionate media, (2) Cl^--dependent effects brought about by an exchange of intramitochondrial OH^- for extramitochondrial Cl^-, best demonstrated by the rate of swelling of mitochondria in an NaCl medium, and (3) Cl^--independent effects similar to those brought about by oligomycin, demonstrated by inhibition of mitochondrial ATP synthesis in an isethionate medium.

If triorganotin or triorganolead compounds are to be used as tools to study each of these mechanisms, then they are not all suitable. From Fig. 3 the most suitable compound for the study of each process can be chosen. All three mechanisms may be separately examined with different concentrations of triethyltin. Trimethyltin, triethyltin, trimethyl-lead, triethyl-lead, tri-*n*-propyl-lead and tri-*n*-butyl-lead are suitable for the study of mitochondrial functions influenced by the Cl^-/OH^- exchange. Triethyltin, tri-*n*-propyltin and tri-*n*-butyltin are suitable for the study of large-scale swelling. Only triethyltin and possibly trimethyltin are suitable for the study of the halide-independent effects not associated with large-scale swelling.

Large-scale swelling in both KCl and potassium isethionate media

The ability to bring about such swelling increases for both tin and lead compounds with the number of carbons attached to the metal (Fig. 3). Maximum activity (<3 μM) is reached with butyl and phenyl groups. The lower and less lipophilic members of the series require much higher concentrations, e.g. for trimethyltin 1–10 mM. The mechanism whereby this large-scale swelling is brought about is not known. It may be by combination with the low-affinity binding sites previously detected in mitochondria (Aldridge & Street, 1970). The dissociation constants of 1.2–10 mM and 140–710 μM for trimethyltin and triethyltin approximate to the concentrations required to bring about such swelling (Fig. 3). For the lipophilic members such as tri-*n*-butyltin or triphenyltin, which are probably almost all bound to the mitochondria (Aldridge & Threlfall, 1961), the concentrations necessary to saturate the low-affinity sites (Aldridge & Street, 1970) may be calculated to be 40–90 μM; this range of concentration approximates to the experimental results (Fig. 3). It is possible therefore that the production of large-scale swelling by both the triorganotins and triorganoleads involves the low-affinity binding sites (cf. Wulf & Byington, 1975).

Dependence of the Cl^-/OH^- exchange on consumption of energy

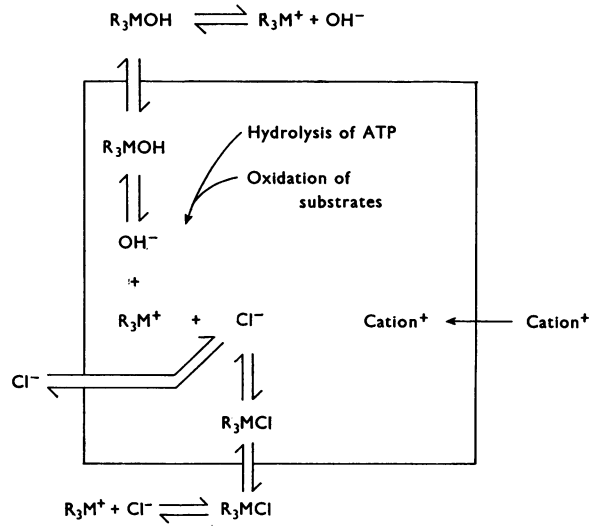
Many of the compounds studied in the present work are more effective in inhibiting mitochondrial functions in a chloride than in an isethionate medium. There is no prior reason why this should always be so, since the effects in the two media are brought about by different processes. Many secondary effects result from the exchange of external Cl^- for intramitochondrial OH^- (Fig. 2). Limited swelling, stimulation of ATP hydrolysis and O_2 uptake are associated phenomena (Aldridge & Street, 1964; Fig. 2). Unlike the large-scale swelling that is not Cl^--dependent, limited swelling in a chloride medium is prevented by uncoupling agents. Parker (1965) showed that a variety of uncoupling agents prevented limited swelling by trimethyltin, and we have shown by other experiments that such swelling by the lower-molecular-weight triorganotins and triorganoleads is prevented by 2,4-dinitrophenol and 5,6-dichlorobenzimidazole.* The simplest explanation of these results is that the movement of significant quantities of osmotically active Cl^- into the mitochondrion requires the utilization of energy (Skilleter, 1976). Swelling is a consequence of the former, and the increase in ATP hydrolysis and O_2 uptake is a measure of the latter (Table 1). Calculations shown in Table 5 indicate that for a given steady-state Cl^- content of the mitochondria the expected relationship between ATP hydrolysis and O_2 uptake is obtained.

In the presence of triorganotin or triorganolead compounds the Cl^- content of mitochondria is in steady state (Skilleter, 1976), whereas the hydrolysis of ATP or oxidation of substrates is continuous (Table 1). If one ATP can yield 1, 2 (Mitchell & Moyle, 1965; Mitchell, 1969) or 4 OH^- ions (Lehninger *et al.*, 1975) to be exchanged for 1, 2 or 4 Cl^- ions then the flux of Cl^- across the mitochondrial membrane could be 41, 83 or 124 ng-atoms/min per mg of protein. A question requiring an answer is why the rate of con-

Table 5. *Relationship between the utilization of energy and the Cl^- content of mitochondria in the presence of triorganotin and triorganolead compounds*

The values in the Table are taken from Table 1 and Skilleter (1976) (Figs. 1 and 2). For details of methods see the Materials and Methods section and Skilleter (1976).

Energy source	Steady-state Cl^- content (nmol/mg of protein) (*A*)	ΔO_2 uptake of ATP hydrolysis (nmol of ATP or ng-atoms of O/min per mg of protein) (*B*)	O or ATP/ unit of Cl^- (*B*/*A*)	ATP/O ratio
Pyruvate+fumarate	220	25.0	0.114	2.5
β-Hydroxybutyrate	245	28.4	0.116	2.5
Succinate	255	42.4	0.166	1.7
ATP	145	41.5	0.287	



Scheme 1. *Diagrammatic representation of reactions occurring in mitochondria in the presence of triorganotin or triorganolead compounds (R_3M), Cl^- and an energy source*

sumption of energy as shown by ATP hydrolysis and O_2 uptake is limited, and does not approach the rates obtained with 2,4-dinitrophenol and other uncouplers. It is not dependent on the particular organometal (Table 1), and the concentrations of halide, ATP or substrates are not likely to be rate-limiting. The rate was also unaffected by the nature of the bulk cation.

Exchange of Cl^- for OH^- leads to an acidification of the inside of the mitochondrion (Selwyn *et al.*, 1970; Dawson & Selwyn, 1974). Distribution studies between solvents and aqueous media indicate that an equilibrium between triethyltin hydroxide and triethyltin chloride exists in solution and is of course influenced by the relative concentrations of Cl^- and OH^- ions. Therefore, as the intramitochondrial conditions change owing to the action of triorganotin and triorganolead compounds, the steady-state concentration of Cl^- will be an equilibrium between the exchange of the organometal chloride for OH^- (see Scheme 1) and the electrogenic outward diffusion of Cl^- from the slightly swollen mitochondrion (Stockdale *et al.*, 1970).

Preliminary experiments based on addition of trace amounts of $^{36}Cl^-$ to the ATP-containing medium after the steady state has been reached indicate that a rapid equilibration of Cl^- occurs across the mitochondrial membrane and that this process is not markedly inhibited by oligomycin or 2,4-dinitrophenol (D. N. Skilleter, unpublished work). On the basis that in the presence of triorganotin and triorganolead compounds ATP can be used to achieve maximum uptake of Cl^- (Skilleter, 1976; Table 3) and maintenance of the steady state, then these measurements indicate that a non-energy-dependent exchange of Cl^- inside for Cl^- outside must be a rapid process compared with the ATP-dependent Cl^-/OH^- exchange (see Scheme 1).

None of these considerations allow us to suggest why the intramitochondrial pH is so decreased when the rate of energy utilization from hydrolysis of ATP or from oxidation of substrates is not maximal. Maximal rates of ATP synthesis are about 300nmol/min per mg of protein (Table 4), and the maximum rate of ATP hydrolysis is also of the same magnitude (Table 1). The highest rate of ATP hydrolysis brought about in a chloride or bromide medium is little over 100nmol/min per mg of protein. There may be rate-limiting steps that our experiments have not uncovered, e.g. rate of cation movement, but one possibility is that the maximum rate of generation of OH^- within the mitochondrion is one-third of the maximum rate of energy utilization for other purposes.

ATP synthesis linked to the oxidation of β-hydroxybutyrate or reduced cytochrome c

Triethyltin can affect mitochondrial functions over the concentration range 0.05–40 μM by two different mechanisms, which can be demonstrated by their inhibition by different concentrations of triethyltin (Fig. 3). They are (1) an exchange of external Cl^- for intramitochondrial OH^- and (2) an interaction with the energy-conservation system to bring about an 'oligomycin-like' effect. With ATP synthesis (succinate) mechanism (1) leads to complete inhibition with 2 μM-triethyltin. With ATP synthesis (β-hydroxybutyrate) this mechanism only leads to partial uncoupling, and complete inhibition is only brought about by higher concentrations of triethyltin (40 μM) by mechanism (2). The relationship between ATP synthesis and the concentration of organometal necessary for inhibition is identical when either β-hydroxybutyrate or reduced cytochrome *c* is oxidized.

ATP synthesis linked to oxidation of β-hydroxybutyrate or succinate are interesting to compare, since the succinate system is one of the most sensitive to inhibition by triethyltin in a chloride medium, and β-hydroxybutyrate is the least. A comparison of the properties of other systems involving β-hydroxybutyrate or succinate is shown in Table 6.

Both of the hydrogenases are tightly membrane-bound, and in mitochondrial subfractionation studies follow the cristae material along with other components of the electron-transport chain (Werner & Neupert, 1972). Although these two dehydrogenases appear to be bound in membranous material sedimenting in the same way, they react differently to the action of triethyltin. A simple explanation of the results obtained in the present work would be that β-hydroxybutyrate dehydrogenase is located in the membrane, but is not in contact with the compartment whose composition is altered by triethyltin in a chloride medium. If the dehydrogenase oxidizes β-hydroxybutyrate in the external medium, the β-hydroxybutyrate that penetrates (Land & Clark, 1974; Skilleter, 1975) cannot be oxidized in the inner compartment in which succinate is oxidized.

An assumption implicit in such deductions is that there is one respiratory chain and one energy-conservation system into which reducing equivalents derived from all substrates are channelled. For example, in an isoethionate medium, triethyltin

Table 6. *Comparison of the properties of systems involving β-hydroxybutyrate and succinate oxidation*
References: (1) Lehninger *et al.* (1960); (2) Sottocasa *et al.* (1967); (3) Werner & Neupert (1972); (4) Harris & Manger (1969); (5) Harris *et al.* (1967); (6) Dawson & Selwyn (1974); (7) present paper; (8) Skilleter (1975); (9) Manger (1969); (10) Harris & Manger (1968); (11) Moret *et al.* (1967).

Effect	β-Hydroxybutyrate oxidation	Reference	Succinate oxidation	Reference
Dehydrogenase bound to membrane	Yes	(1)	Yes	(2)
pH optima of dehydrogenase	8–8.5	(1)	7.7	(6)
In cristae fraction	Yes	(3)	Yes	(3)
Increase in mitochondrial K^+ leads to increased substrate uptake	No	(4)	Yes	(5)
Inhibition of oxidation by other substrate	Yes	(4)	—	
Substrate uptake inhibited by other substrate	Yes	(4)	Yes	(4)
ATP synthesis: inhibition by triethyltin in a chloride medium	High concentrations required	(7)	Low concentrations required	(7)
Inhibition of substrate uptake by triethyltin in a chloride medium	Yes	(8)	Yes	(9)
Resynthesis of endogenous ADP	No Slight	(4) (11)	Yes	(4, 11)
Substrate uptake inhibited by malate, malonate and citrate	No	(4)	Yes	(4, 10)
Uptake kinetics	Linear	(4)	Saturation	(10)

(2–40μM) inhibits (0–100%) ATP synthesis linked to the oxidation of pyruvate, succinate, reduced cytochrome *c* and β-hydroxybutyrate by an oligomycin-like effect, i.e. by inhibition of the oligomycin-sensitive adenosine triphosphatase (see above and Fig. 4). In a chloride medium and 2μM-triethyltin ATP synthesis (succinate) is completely inhibited (Fig. 4), whereas ATP synthesis (β-hydroxybutyrate) is only partially uncoupled (see above and Fig. 4). If there is only one energy-conservation system, it cannot be inhibited in a chloride medium when ATP synthesis (succinate) is prevented, and the rate-limiting step must be sought elsewhere (cf. above). If more than one common energy-conservation system is considered a possibility, then the above argument cannot be sustained. There are other observations in the literature that may require such a possibility to be considered. Atractyloside prevents completely the phosphorylation of endogenous adenine nucleotide when β-hydroxybutyrate is the substrate being oxidized (Moret *et al.*, 1967), but when α-oxoglutarate was used phosphorylation was increased. Atractyloside prevents the diffusion of endogenous nucleotide out of the mitochondria (Moret *et al.*, 1964), and the implication must be that when β-hydroxybutyrate is oxidized endogenous nucleotide must leave the mitochondrion and be phosphorylated elsewhere. Estrado-O *et al.* (1967) have shown that the decrease in ATP hydrolysis by mitochondria in the presence of nigericin is greater for β-hydroxybutyrate and succinate together than separately. Similarly Wadkins & Lehninger (1959) showed that ATP hydrolysis and ATP/$^{32}P_i$ exchange is diminished when the electron chain is reduced by β-hydroxybutyrate or succinate. Together they are much more effective. The results shown in Fig. 6 could also be explained by separate energy-conservation systems attached to β-hydroxybutyrate dehydrogenase and succinate dehydrogenase.

An improvement in knowledge of the different sensitivities to triethyltin of ATP synthesis linked to the oxidation of β-hydroxybutyrate and succinate must await further understanding of the structural organization of the systems involved.

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