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Effects of Triphenylsulphonium Ions on Mitochondria

INHIBITION OF ADENOSINE TRIPHOSPHATASE ACTIVITY

By ROBERT H. BARRETT and MICHAEL J. SELWYN
School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, U.K.

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Triphenylsulphonium ions inhibit mitochondrial oxidative phosphorylation and adenosine triphosphatase activity. The site of action is on the soluble F_1 adenosine triphosphatase component. Triphenylsulphonium ions also inhibit electron transfer in the NAD–cytochrome *b* region of the respiratory chain. In both types of inhibition, triphenylsulphonium ions are effective at low concentrations, half-maximal inhibition being produced by a concentration of about 20–30μM. These effects resemble the effects of alkylguanidines on mitochondria and are discussed in relation to the effects of alkylguanidines and other lipophilic cations such as ethidium and dibenzyldimethylammonium ions. A modification of the purification procedure for the soluble mitochondrial adenosine triphosphatase [Beechey, Hubbard, Linnett, Mitchell & Munn (1975) *Biochem. J.* **148**, 533–537] is described, which yields a preparation with a higher specific activity and showing fewer bands in gel electrophoresis.

A variety of inhibitors of the ADP-phosphorylating system of mitochondria have been used, but some of these, such as oligomycin, are of relatively unknown constitution and others are complex and their chemistry of action is difficult to determine.

Trialkyltins have been shown to be potent inhibitors of the mitochondrial oxidative phosphorylation system (Aldridge, 1958; Aldridge & Street, 1964). In our laboratory, we have made considerable investigation of the trialkyltins (Selwyn *et al.*, 1970; Stockdale *et al.*, 1970; Dawson & Selwyn, 1974*a*, 1975), and although trialkyltins are of simple constitution, little is known of the ligand requirements for high-affinity binding, other than the possibility of complexing between a pair of histidine residues, which is postulated to occur when trialkyltins bind to rat haemoglobin (Rose & Aldridge, 1968).

We considered the possibility that electrostatic interaction might be of importance, and tested triphenylsulphonium chloride as a possible analogue of the trialkyltins. Triphenylsulphonium chloride proved to be an inhibitor of the mitochondrial ATPase* system, but as described here, its action is on the soluble ATPase component and on electron transfer between NAD and cytochrome *b* and thus resembles the action of the alkylguanidines (Chance & Hollunger, 1963; Chappell, 1963; Pressman, 1963; Papa *et al.*, 1975), and ethidium bromide (Miko & Chance, 1975) rather than the action of the trialkyltins.

* Abbreviation: ATPase, adenosine triphosphatase.

An additional comparison can be made with the diphenylene iodonium compounds that mediate a chloride–hydroxide exchange across membranes and inhibit electron transport, but have little direct effect on the phosphorylating system (Holland & Sherratt, 1972; Holland *et al.*, 1973).

Materials

NADH was from Sigma (London) Chemical Co., Kingston-upom-Thames, Surrey, U.K. Triphenylsulphonium chloride was from Aldrich Chemical Co., Wembley, Middx., U.K. ATP was from Boehringer Corp. (London) Ltd., Lewes, East Sussex, U.K. Hepes [2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid] was from Hopkin and Williams, Chadwell Heath, Essex, U.K. All other chemicals and reagents were of A.R. grade or of the highest purity available.

Methods

Preparation of ox heart mitochondria

Preparations were by the method of Selwyn (1967); the mitochondria were stored in 0.25M-sucrose/10mM-Tris/H_2SO_4/1mM-EDTA, pH7.6, in a deep-freeze at −20°C, until required.

Preparation of ox heart submitochondrial particles

Ox heart submitochondrial particles were prepared essentially as described by Hansen & Smith (1964), the only differences being a smaller scale of

preparation and the type of sonicator, which was an MSE 50W ultrasonic disintegrator.

Ox heart EDTA particles were prepared as described by Lee *et al.* (1964), as modified by Dawson & Selwyn (1975), the major difference between the methods of preparation of particles being the pH at which sonication of mitochondria is carried out.

Preparation of soluble ATPase

The first stage of preparation of the soluble ATPase from frozen stored ox heart mitochondria was as described by Beechey *et al.* (1975). However, an extension of this procedure was carried out. Approx. 10ml of freshly prepared submitochondrial particles (Hansen & Smith, 1964), at a protein concentration of 5mg/ml in 0.25M-sucrose/10mM-Tris/H_2SO_4, pH7.6, were shaken vigorously with 0.5vol. of A.R. chloroform in a glass centrifuge tube for 15s at room temperature (20°C). The tubes were centrifuged for 2min, on a bench centrifuge, at 3000g to break the suspension, then the upper aqueous layer was spun on an MSE 50 centrifuge at 32000rev./min (70000$g_{av.}$) for 50min at 20°C. The supernatant was assayed for ATPase activity and protein content. Specific activities of between 10 and 20μmol of phosphate/min per mg of protein were obtained. Further purification was carried out by passing the enzyme through a column (1cm diam. ×2.5cm high) of DEAE-Sephadex [A-50 grade Sephadex, from Pharmacia (G.B.) Ltd., London W.5, U.K.], equilibrated with 20mM-Tris/H_2SO_4/2mM-EDTA buffer, pH8.0. Usually a layer of Sephadex G-25 was layered on top of the column, to prevent disturbance of the DEAE-Sephadex surface. ATPase solution, in 0.25M-sucrose/10mM-Tris/H_2SO_4 buffer, pH8.0, was applied slowly to the column, and elution proceeded, first with 20mM-Tris/H_2SO_4/2mM-EDTA/0.1M-Na_2SO_4, pH8.0 (approx. 50ml), then with 20mM-Tris/H_2SO_4/2mM-EDTA/0.2M-Na_2SO_4, pH8.0. Fractions (5ml) were collected throughout the elution, and these were assayed for both ATPase activity and protein content. A typical elution profile is shown in Fig. 1.

Fractions containing ATPase at a specific activity of at least 50μmol of phosphate/min per mg of protein (usually at least 75% of the recovered activity) were pooled, and the enzyme was precipitated by $(NH_4)_2SO_4$ at 60% saturation. The precipitate was collected by centrifugation at 12000g for 15min in an MSE 18 centrifuge. The pellet was resuspended in a small volume of 50% (v/v) glycerol/20mM-Tris/H_2SO_4/2mM-EDTA buffer, pH8.0. This procedure usually gave a preparation of ATPase solution with a specific activity of between 50 and 80μmol of phosphate/min per mg of protein. The preparation could be stored for several weeks in 50% glycerol/20mM-Tris/H_2SO_4/2mM-EDTA, pH8.0, at −20°C. Half the total activity is lost during storage in 3 weeks.

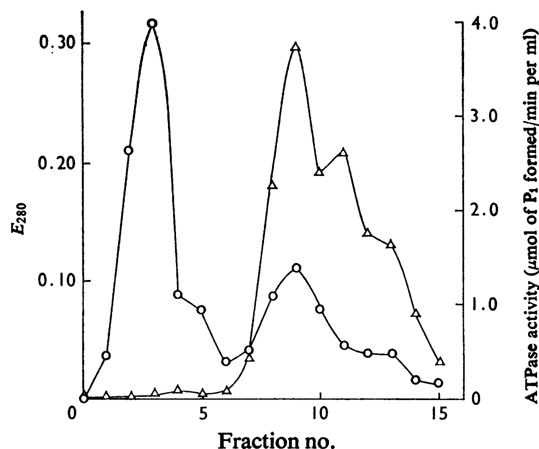

Fig. 1. *Elution profile of protein and ATPase activity from an A-50 DEAE-Sephadex column*

The column was set up as described in the Methods section. ATPase activity was eluted with 20mM-Tris/H_2SO_4/2mM-EDTA/0.2M-Na_2SO_4, pH8.0. ○, Protein was assayed by following the E_{280} and by using an extinction coefficient of 0.53 for a protein concentration of 1mg/ml. △, ATPase activity was followed by measuring liberated P_i as described in the Methods section; 5μl samples were assayed from each 5ml fraction collected.

Polyacrylamide-gel electrophoresis

For this, 5% polyacrylamide gels were prepared and run as described by Knowles & Penefsky (1972). Between 20 and 30μg of protein was applied to the gels. Since dissociation of the F_1 component of the ATPase in dilute solutions is prevented by glycerol (Selwyn, 1967), some samples were run on gels containing 25% (v/v) glycerol with 25% glycerol also in the buffers in the reservoirs. ATPase activity on the gels was detected by the method of Wachstein & Meisel (1957). Protein was stained with Coomassie Brilliant Blue (Weber & Osborn, 1969).

Gels were run of enzyme before and after passage through a DEAE-Sephadex column. The untreated enzyme has two main protein bands and many other paler protein bands, whereas the untreated enzyme has two protein bands only. High ATPase activity is associated with the first band. The second band, which is thought to contain dissociation products of the first band, exhibits weak activity, but only in gels of enzyme that had been purified on a DEAE-Sephadex column. It is thought that the major purification achieved by the column treatment is the removal of dissociated ATPase fragments, corresponding to the paler bands seen in the untreated enzyme gels. It was also noted that gels run in

25% glycerol have sharper, more clearly defined bands than those gels without glycerol.

Preparation of rat liver mitochondria

The preparation was carried out in 0.25M-sucrose/5mM-Hepes (potassium salt), pH7.6, from albino Wistar rats killed by decapitation, the procedure being that of Selwyn *et al.* (1970).

Preparation of rat liver EDTA particles

Rat liver EDTA submitochondrial particles were prepared as for ox heart EDTA particles, as described by Dawson & Selwyn (1975), to give a final protein concentration of 5mg/ml.

Purification of triphenylsulphonium chloride

Triphenylsulphonium chloride, obtained as an aq. 50% (w/v) solution, was purified by recrystallization from chloroform/acetone, as described by Bonner (1952). The solid obtained was stored desiccated at room temperature, in the dark. Stock aqueous solutions were made up at 10mM and 1mM concentrations and stored at 4°C.

ATPase assays

The medium used for the assay of ATPase activity, of the soluble ATPase and ox heart frozen–thawed mitochondria, was 50mM-Tris/H_2SO_4 (pH7.4)/2.5mM-ATP/2.5mM-$MgSO_4$. A 5min preincubation of triphenylsulphonium chloride with either the soluble ATPase (0.5μg) or mitochondria (70μg) was carried out as a routine, the reaction being started by the addition of the ATP. The reaction was carried out in a 1ml volume at 30°C. To stop the reaction, 0.5ml of 10% (w/v) trichloroacetic acid was added, and the amount of phosphate liberated was determined by the method of Fiske & SubbaRow (1925). The pH changes (used to measure ATPase activity), oxygen uptake and light-scattering changes were recorded simultaneously when desired, by using the multichannel apparatus described by Stockdale *et al.* (1970). For pH measurements the recorder had a full-scale deflexion of 0.15pH unit. The reaction medium contained, in a 5ml volume, 50mM-KCl, 0.25mM-Hepes (potassium salt), pH7.6, 2.5mM-ATP and 2.5mM-$MgSO_4$, all at 30°C. Oxygen uptake was measured by using an oxygen electrode (Rank Brothers, Bottisham, Cambridge CB5 9DA, U.K.) in a 5ml volume, at 30°C, in a medium containing 150mM-KCl, 5mM-Hepes (potassium salt), pH7.6, and, as substrates, 10mM-potassium succinate, or 5mM-potassium glutamate+5mM-potassium malate, or 10mM-NADH. When succinate was the substrate, 3μg of rotenone was also added. Either 10mg of mitochondrial protein or 90μg of submitochondrial-particle protein was used in these experiments. In uncoupler-stimulated respiration, 2,4-dinitrophenol (final concn. 0.1mM) was added to start the reaction. In respiration supported by ADP and phosphate, the ADP (0.5mM) was present initially in the reaction medium and the reaction was started by addition of potassium phosphate to give a concentration of 10mM. Additions were made in this order, because in the presence of phosphate and triphenylsulphonium ions mitochondria swell to a significant extent unless ADP is also present.

Osmotic-swelling experiments

These were carried out in the apparatus described by Selwyn *et al.* (1970), by using 2.5ml of iso-osmotic solutions of ammonium salts (chloride, isethionate, nitrate and thiocyanate) containing 2μg of rotenone, 1μg of antimycin A and 5mg of mitochondrial protein.

Protein determinations

These were performed on mitochondrial and submitochondrial-particle preparations by the biuret method of Gornall *et al.* (1949), after solubilization with Triton X-100. Protein concentrations of preparations of the soluble ATPase were calculated from E_{280}, assuming an extinction coefficient of 0.53 for a protein concentration of 1mg/ml (Penefsky & Warner, 1965).

Results

Inhibition of the ATPase activity of 'alkaline-EDTA' particles from ox heart mitochondria by triphenylsulphonium chloride is shown in Fig. 2. Half-maximal inhibition is observed at a triphenylsulphonium ion concentration of 15μM. This inhibition appeared to be instantaneous, on the time-scale of these experiments, since identical curves were obtained with and without a period of incubation of the submitochondrial particles and inhibitor before the assay. The ATPase activity of submitochondrial particles prepared from rat liver mitochondria by the 'alkaline-EDTA' treatment was also inhibited by triphenylsulphonium ions (Fig. 2), though the inhibition may not be so complete and requires slightly higher concentrations of triphenylsulphonium ions.

The ATPase activity of ox heart mitochondria is also inhibited by triphenylsulphonium ions, but, as shown in Fig. 2, higher concentrations of the inhibitor are required than with the submitochondrial particles prepared from these mitochondria. Although these mitochondria were stored frozen, this lower sensitivity to the inhibitor may indicate that the site of action of the inhibitor is on the inner face of the mitochondrial membrane and is less accessible in whole mitochondria than in the inverted submitochondrial particles.

These observations established that the triphenylsulphonium ion is a potent inhibitor of the mito-

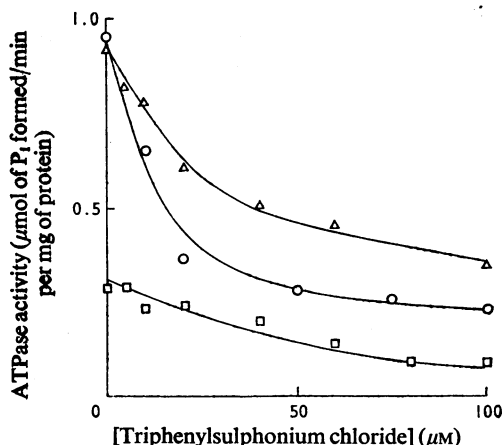

Fig. 2. *Inhibition of ATPase activity of particulate mitochondrial systems by triphenylsulphonium chloride*

Ox heart EDTA submitochondrial particles (○) (36μg) and rat liver EDTA submitochondrial particles (△) (60μg) were assayed for ATPase activity as described in the Methods section. Frozen–thawed ox heart mitochondria (□) (70μg) were assayed for ATPase activity by measuring liberated P_i as described in the Methods section. A 5min preincubation with triphenylsulphonium chloride was carried out.

chondrial energy-coupling system when it is assayed by ATPase activity, but, before dealing with other aspects of its inhibitory action, another potential activity of triphenylsulphonium ions needs to be considered. Triphenyltin and trialkyltin compounds mediate an anion exchange across the mitochondrial membrane (Selwyn *et al.*, 1970), which complicates their inhibitory action on mitochondrial processes (Stockdale *et al.*, 1970; Dawson & Selwyn, 1974*a*). Although sulphonium compounds are always fully ionic, it was considered possible that certain anions might act as counterions for the hydrophobic lipid-soluble triphenylsulphonium ion and their movement across biological membranes might thereby be facilitated.

This was tested, by the technique of Chappell & Haarhoff (1967), by suspending mitochondria in iso-osmotic (0.1M) solutions of ammonium chloride, isethionate, nitrate and thiocyanate, and observing changes in light-scattering as described in the Methods section. In no case did triphenylsulphonium ions, at concentrations up to 0.6mM, produce a significant decrease in the light-scattering by the mitochondrial suspension. This shows that no swelling of the mitochondria occurred and hence that triphenylsulphonium ions do not mediate an anion–hydroxide exchange with these anions. With chloride and isethionate ions, mediation of anion uniport could also be eliminated, since no significant swelling was observed when 2,4-dinitrophenol was present as well as triphenylsulphonium ions.

Further investigation of the inhibitory action of triphenylsulphonium ions was made by studying its effect on the rate of respiration of mitochondrial preparations under a variety of conditions. Fig. 3 shows the inhibition of succinate oxidation by rat liver mitochondria in both state 3 (ADP- and phosphate-stimulated) (Chance & Williams, 1955) and uncoupler-stimulated conditions. The pattern of inhibition is markedly different in the two cases, in the presence of uncoupler the maximal inhibition is small, about 20%, but half-maximal inhibition is produced by about 12μM-triphenylsulphonium ions, whereas under phosphorylating conditions a considerably greater degree of inhibition is attained,

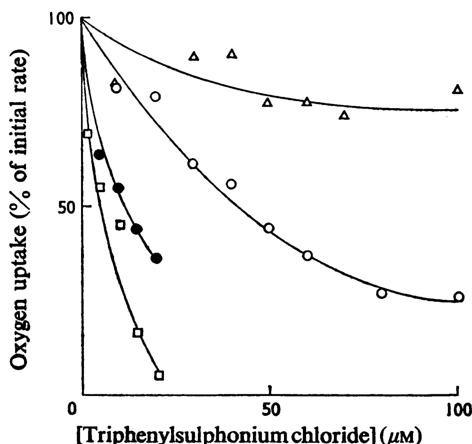

Fig. 3. *Effect of triphenylsulphonium chloride on succinate and glutamate+malate respiration by fresh rat liver mitochondria*

○, Mitochondria (10mg) were suspended in a medium containing 150mM-KCl, 5mM-Hepes (potassium salt; pH7.6), 10mM-succinate, 0.5mM-ADP and 4μg of rotenone (final vol. 5ml). The reaction was started by the addition of K_3PO_4 to a final concentration of 10mM. △, Mitochondria (10mg) were suspended in a medium containing 150mM-KCl, 5mM-Hepes (potassium salt; pH7.6), 10mM-succinate and 4μg of rotenone. The reaction was started by the addition of 50mM-2,4-dinitrophenol to a final concentration of 0.1mM. □, As for ○, but with 5mM-glutamate+5mM-malate instead of 10mM-succinate and with no rotenone present. ●, As for △, but with 5mM-glutamate+5mM-malate instead of 10mM-succinate and with no rotenone present. Triphenylsulphonium chloride was present in the medium before the addition of phosphate. Oxygen uptake was measured with an oxygen electrode. Rates of oxygen uptake (ng-atom of O/min per mg of protein) in the absence of inhibitor were: ○, 87; △, 131; □, 50; ●, 45.

but this requires higher concentrations of the inhibitor. In contrast, when glutamate+malate was used as the substrate (Fig. 3), a considerable degree of inhibition was observed for both uncoupler-stimulated and phosphorylating respiration; in the latter case almost total inhibition was observed with 20μM-triphenylsulphonium ions. In other experiments on this system, virtually complete inhibition of uncoupler-stimulated respiration was attained with 35μM-triphenylsulphonium ions.

Submitochondrial particles are able to utilize NADH as a respiratory substrate and exhibit a high rate of respiration in the absence of uncouplers and phosphorylation substrates. Fig. 4 shows the inhibition of NADH oxidation by 'alkaline-EDTA' particles from ox heart mitochondria. The pattern of inhibition resembles the inhibition of uncoupler-stimulated glutamate+malate oxidation shown in Fig. 3. Fig. 4 shows the effect of triphenylsulphonium ions on the oxidation of succinate catalysed by 'alkaline-EDTA' particles from ox heart mitochondria. The feebleness of the inhibition resembles the effect on uncoupler-stimulated succinate respiration with rat liver mitochondria. These results suggest that triphenylsulphonium ions have a direct inhibitory action on the respiratory chain in the region between NAD and cytochrome *b* in addition to their action on the phosphorylation process. The overall inhibition of respiration thus ranges from very little in uncoupler-stimulated succinate oxidation, where neither of the sensitive processes is involved, to very great inhibition in phosphorylation-supported oxidation of glutamate+malate, where both sensitive processes are involved.

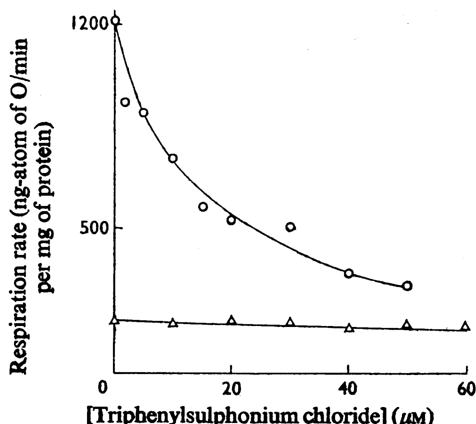

Fig. 4. *Effect of triphenylsulphonium chloride on respiration of ox heart submitochondrial particles with NADH and succinate as substrates*

The assay system was as described in Fig. 3. The reaction medium (5ml) contained 150mM-KCl, 5mM-Hepes (potassium salt; pH7.6) and 90μg of EDTA submitochondrial particles. The reaction was started by the addition of NADH to a final concentration of 10mM (○), or of succinate to a final concentration of 10mM (△). Rotenone (3μg) was present in the medium.

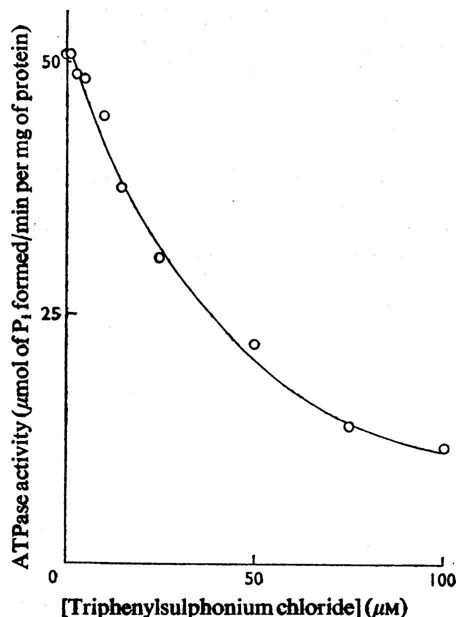

Fig. 5. *Inhibition of the soluble ATPase from ox heart mitochondria by triphenylsulphonium chloride*

ATPase activity was measured by assaying liberated P_i as described in the Methods section; 0.5μg of protein was added to the assay system, the reaction was started by the addition of ATP after a 5min preincubation.

That the inhibition of the phosphorylation process is on the phosphorylating reactions and not on the movement of ADP and ATP across the inner membrane of the mitochondria is suggested by the inhibition of the ATPase activity of submitochondrial particles described above. The type of particles used are inverted relative to whole mitochondria, that is the F_1 component of the ATPase is exposed to the external medium and is directly accessible to ATP. A further test of the site of action of triphenylsulphonium ions was made by studying the arsenate-stimulated respiration of intact rat liver mitochondria. Atractyloside, which inhibits the adenine nucleotide transporter (Chappell & Crofts, 1965), inhibits respiration stimulated by ADP and low concentrations of arsenate, but not respiration stimulated by high concentrations of arsenate in the absence of ADP. Triphenylsulphonium ions at 100μM produced 55% inhibition of respiration stimulation by 10mM-arsenate and 25% inhibition of respiration stimulated by 2mM-

arsenate+0.2mM-ADP. The similarity of degree of inhibition, and in fact the lesser inhibition of the arsenate+ADP stimulated respiration, is additional evidence for the view that triphenylsulphonium ions inhibit the phosphorylation process itself, i.e. the ATPase system.

Further support for this view was obtained from experiments on the effect of triphenylsulphonium ions on the reaction catalysed by the soluble component of the mitochondrial ATPase system. This was prepared as described in the Methods section [by the method of Beechey *et al.* (1975)] and further purified by passage through a DEAE-Sephadex column. Fig. 5 shows that the ATPase activity of the purified soluble F_1 component is potently inhibited by triphenylsulphonium ions, half-maximal inhibition occurring at a concentration of about 30μM-triphenylsulphonium ions, which is similar to the value for the ATPase activity of 'alkaline-EDTA' particles from ox heart mitochondria. Thus although triphenylsulphonium ions do resemble the triorganotin compounds in inhibiting the proton-translocating ATPase of mitochondria, their site of action is different, the tin compounds blocking the proton-carrying membrane-bound component of the ATPase whereas the triphenylsulphonium ions inhibit the soluble F_1 component, and the triphenylsulphonium ion cannot be considered as an analogue of the tin compounds in its biochemical action.

Discussion

The effects of triphenylsulphonium ions reported in this paper, that is inhibition of the mitochondrial ATPase at a site in the soluble F_1 component and inhibition of electron transport at a site in the NAD–cytochrome *b* region of the respiratory chain, are qualitatively very similar to effects reported for alkyl- and aryl-substituted guanidines.

There are, however, some significant differences between the actions of the two types of compound. Inhibition of respiration by, for example, octylguanidine (Pressman, 1963) or 3-butenyl-4-methylguanidine (galegine) (Chappell, 1963) requires preincubation of the mitochondria with the inhibitor under conditions of controlled respiration, i.e. in the absence of ADP and uncouplers, and inhibition of oxidation of NAD-linked substrates is slowly reversed by addition of 2,4-dinitrophenol. These effects were originally interpreted to indicate that alkylguanidines are inhibitors of the phosphorylation process specifically for the phosphorylation site associated with the NAD–cytochrome *b* region of the respiratory chain. A different interpretation has been put forward by Mitchell (1966), who suggested that these alkylguanidines are inhibitors of electron transport in this region rather than inhibitors of phosphorylation; preincubation is required to accumulate the alkylguanidines to an inhibitory concentration within the mitochondria, the slow reversal of inhibition by addition of uncouplers being caused by the collapse of the membrane potential and consequent loss of the cationic alkylguanidines from the mitochondrial matrix. Davidoff (1971) has reported experimental evidence on the uptake of phenethylbiguanide that is in accord with Mitchell's (1966) proposals about uptake and release of guanidines.

There is also considerable variation in the effects produced by different guanidines. For example, galegine produces little or no inhibition of either uncoupler-stimulated or ADP-supported oxidation of succinate, whereas phenethylbiguanide under some conditions can produce greater inhibition of oxidation of succinate than of NAD-linked substrates. The effects of octylguanidine appear to resemble the effects of triphenylsulphonium ions most closely. Papa *et al.* (1975) have reported that octylguanidine inhibits the soluble F_1 component of the mitochondrial ATPase system and have also shown that part of the explanation for the apparent site specificity may be that observation of inhibition depends on whether electron transport or phosphorylation is the rate-limiting process before addition of inhibitor. However, octylguanidine is a much less effective inhibitor of the ATPase (half-maximal inhibition produced by about 300μM-octylguanidine) than of electron transport (half-maximal inhibition produced by 30μM-octylguanidine in whole mitochondria from rat liver; Papa *et al.*, 1975), and this must make a large contribution to the greater inhibition of oxidation of NAD-linked substrates. As reported in the present paper, triphenylsulphonium ions inhibit both the ATPase and electron transport at similar and low concentrations. It appears that triphenylsulphonium ions move rapidly through the mitochondrial membranes, since inhibition and uncoupler-induced reversal of inhibition are both virtually instantaneous. The pattern of effects produced by a compound, which inhibits both the mitochondrial ATPase and electron transport in the NAD–cytochrome *b* region of the respiratory chain, is thus more clearly discernable than with the alkylguanidines. It seems that the alkylguanidines move relatively slowly through the mitochondrial membranes, but if comparisons are made when steady rates have been established, i.e. after allowing time for reversal of inhibition by uncouplers as well as for onset of inhibition during preincubation, then the action of the alkylguanidines is very similar to that of triphenylsulphonium ions.

The effects of triphenylsulphonium ions can also be compared with those of ethidium bromide reported by Miko & Chance (1975). Ethidium bromide acts as an uncoupler, producing marked

Table 1. *Actions of organic cations on mitochondrial functions*

Where given, concentrations are for half-maximal inhibition. References: (1) Dawson & Selwyn (1974*b*), Skulachev *et al.* (1969); (2) Miko & Chance (1975); (3) Papa *et al.* (1975).

	Effect of organic cation			
Organic cation ...	Dibenzyldimethylammonium ion (1)	Ethidium ion (2)	Octylguanidinium ion (3)	Triphenylsulphonium ion
State 3 respiration				
(*a*) Succinate as substrate	Stimulation	Stimulation	Inhibition	Inhibition
(*b*) Glutamate+malate as substrates	—	Stimulation	Inhibition	Inhibition
Whole mitochondrial ATPase (in the absence of uncoupler)	Stimulation*	Stimulation	Inhibition	Inhibition
Inhibition of F_1 component of ATPase	—	200 μM	300 μM	30 μM
Inhibition of electron transport between NAD and cytochrome *b*	Non-inhibitory	Inhibitory ?†	90 μM	7 μM
Relative rate of movement through membrane	Fast*	Fast*	Slow*	Fast*

* Predicted property.

† Data of Miko & Chance (1975), showing much greater stimulation of succinate respiration than of glutamate+malate respiration, suggest inhibition of electron transport from NAD to cytochrome *b*.

stimulation of both succinate oxidation by, and the ATPase activity of, pigeon heart mitochondria, but rather less stimulation of glutamate oxidation. In uncoupling it appears to act as a permeant cation in a manner comparable with that of dibenzyl dimethyl ammonium ions (Skulachev *et al.*, 1969; Dawson & Selwyn, 1974*b*). Miko & Chance (1975) also reported that at high concentrations ethidium bromide inhibited the ATPase activity of pigeon heart mitochondria, and we have found that ethidium bromide inhibits the soluble ATPase component, producing half-maximal inhibition at 200 μM (preparations of ATPase as described in the Methods section).

It is our view that the effects of many lipophilic cations on mitochondrial processes can be rationalized on the basis of three fundamental effects: (i) rate of passage through the inner mitochondrial membrane; (ii) affinity for an inhibitory site on the soluble F_1 component of the mitochondrial ATPase; (iii) affinity for an inhibitory site in the NAD–cytochrome *b* region of the respiratory chain.

The ions, dibenzyldimethylammonium, ethidium, alkylguanidinium and triphenylsulphonium form a series of increasing inhibitory action, which increasingly masks the uncoupling effect shown clearly by the non-inhibitory dibenzyldimethylammonium ion. The properties and some predicted properties of these ions are summarized in Table 1. The properties of these ions can be contrasted with those of the trialkyltins and the diphenylene iodonium compounds, both of which mediate chloride–hydroxide exchange (Selwyn *et al.*, 1970; Holland & Sherratt, 1972), but are much more specific in their inhibitory actions; the former inhibiting the ATPase system (in the intrinsic membrane bound components), whereas the latter inhibits only at the electron-transport site (Holland *et al.*, 1973).

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