Biochem. J. (1972) **129**, 39–54
Printed in Great Britain

Biochemical Effects of the Hypoglycaemic Compound Diphenyleneiodonium

CATALYSIS OF ANION–HYDROXYL ION EXCHANGE ACROSS THE INNER MEMBRANE OF RAT LIVER MITOCHONDRIA AND EFFECTS ON OXYGEN UPTAKE

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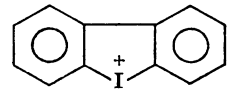
(*Received* 6 *March* 1972)

1. The hypoglycaemic compound diphenyleneiodonium causes rapid and extensive swelling of rat liver mitochondria suspended in 150mM-NH_4Cl, and in 150mM-KCl in the presence of 2,4-dinitrophenol and valinomycin. This indicates that diphenyleneiodonium catalyses a compulsory exchange of OH^- for Cl^- across the mitochondrial inner membrane. Br^- and SCN^- were the only other anions found whose exchange for OH^- is catalysed by diphenyleneiodonium. 2. Diphenyleneiodonium inhibited state 3 respiration of mitochondria and slightly stimulated state 4 respiration with succinate or glutamate as substrate in a standard Cl^--containing medium. 3. Diphenyleneiodonium did not inhibit state 3 respiration significantly in two Cl^--free media (based on glycerol 2-phosphate or sucrose) but caused some stimulation of state 4. 4. In Cl^--containing medium diphenyleneiodonium only slightly inhibited the 2,4-dinitrophenol-stimulated adenosine triphosphatase and it had little effect in the absence of Cl^-. 5. The inhibition of respiration in the presence of Cl^- is dependent on the Cl^-–OH^- exchange. 2,4-Dichlorodiphenyleneiodonium is ten times as active as diphenyleneiodonium both in causing swelling of mitochondria suspended in 150mM-NH_4Cl and in inhibiting state 3 respiration in Cl^--containing medium. Indirect evidence suggests that the Cl^-–OH^- exchange impairs the rate of uptake of substrate anions. 6. It is proposed that stimulation of state 4 respiration in the absence of Cl^- depends, at least in part, on an electrogenic uptake of diphenyleneiodonium cations. 7. Tripropyl-lead acetate, methylmercuric iodide and nine substituted diphenyleneiodonium derivatives also catalyse Cl^-–OH^- exchange across the mitochondrial membrane. 8. Diphenyleneiodonium is compared with the trialkyltin compounds, which are also known to mediate Cl^-–OH^- exchange and which have in addition strong oligomycin-like effects on respiration. It is concluded that diphenyleneiodonium is specific for catalysing anion–OH^- exchange and will be a useful reagent for investigating membrane-dependent systems.

Diphenyleneiodonium (I) has been reported briefly to be a very active hypoglycaemic compound causing extreme irreversible hypoglycaemia in several animal species with a dose of only 4mg/kg body wt. [T. Hanley, R. W. J. Neville, G. A. Stewart & F. W. Webb, unpublished work, quoted by Stewart & Hanley (1969)]. We investigated the effects of diphenyleneiodonium on mitochondrial reactions to see whether its mechanism of action was consistent with our earlier generalization that several unrelated hypoglycaemic compounds having effects independent of insulin impair gluconeogenesis secondarily to inhibition of ATP or NADH formation or both by mitochondria (Senior & Sherratt, 1968*b*; Sherratt, 1969; Sherratt *et al.*, 1971). During this investigation we found that diphenyleneiodonium has the unusual property of catalysing a compulsory exchange of chloride ions and hydroxyl ions across the inner mitochondrial membrane.

About this time Selwyn *et al.* (1970) independently reported that several trialkyltin compounds and phenylmercuric acetate (Watling & Selwyn, 1970) mediated linked chloride–hydroxyl ion exchanges across cellular membranes. Trialkyltin compounds have a strong 'oligomycin-like' effect on mitochond-



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rial respiration (Aldridge, 1958; Aldridge & Rose, 1969; Stockdale *et al.*, 1970) and phenylmercuric acetate is a well-known reagent for thiol groups. Diphenyleneiodonium, however, is a potentially very useful reagent for investigating membrane-dependent systems since no significant biochemical effects, other than those dependent on the promotion of an anion–hydroxyl ion exchange, have yet been found. Further, the mechanism of its hypoglycaemic effect and the relevance of the chloride–hydroxyl ion exchange to this remain to be elucidated. This paper describes the effects of diphenyleneiodonium on some reactions in rat liver mitochondria suspended in different media. Preliminary accounts of some of this work have already appeared (Holland & Sherratt, 1971*a*,*b*,*c*).

Experimental

Materials

Chemicals. Most biochemicals were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, except oligomycin and antimycin, which were obtained from Mann Laboratories Inc., New York, N.Y., U.S.A., valinomycin (from Calbiochem, Los Angeles, Calif., U.S.A.) and gramicidin D, rotenone, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonate and glycerol 2-phosphate (sodium salt) (from the Sigma Chemical Co., London S.W.6, U.K.). Glycerol 2-phosphoric acid was prepared from its sodium salt as described by Aldridge (1957). Methylmercuric iodide was obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K., acetazolamide from Cyanamide of Great Britain Ltd., Gosport, Hants., U.K., tetra-*n*-butylphosphonium chloride and diphenyliodonium nitrate from the Aldrich Chemical Co., Milwaukee, Wis., U.S.A., and tri-*n*-propyl-lead acetate from R. N. Emanuel Ltd., Wembley, Middx., U.K. Other chemicals were purchased from British Drug Houses Ltd., Poole, Dorset, U.K., and A.R.-grade chemicals were used where possible. Diphenyleneiodonium nitrate and 2,2-dimethylisoarsindoline iodide were gifts from Imperial Chemical Industries Ltd., Pharmaceuticals Division, Alderley Park, Cheshire, U.K., through the courtesy of Dr. D. C. N. Earl, and were used as 5 or 10mM solutions in aq. 50% (v/v) ethanol. Substituted diphenyleneiodonium derivatives were gifts from Eli Lilly and Co. Ltd., Indianapolis, Ind., U.S.A.

Apparatus. Polarographic measurements of oxygen activity were made with an oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio, U.S.A.). Spectrophotometric determinations and optical measurements of mitochondrial swelling were made with a Zeiss PMQ II spectrophotometer with a temperature-controlled cell-holder fitted with a T.E. converter and Servoscribe chart recorder. Fluorescence measurements were made with a Zeiss PMQ II spectrophotometer fitted with the ZFM fluorimeter attachment containing a mercury lamp, by using a temperature-controlled cell-holder and recorder.

Methods

Chemical determinations. Protein was determined by the method of Layne (1957) and orthophosphate by that of King (1926).

Animals. Male Wistar rats (180–300g) from a local inbred strain maintained on a standard diet were used throughout.

Preparation of mitochondria. Mitochondria were prepared as described by Senior & Sherratt (1968*a*) in 0.3M-sucrose–2mM-EDTA, adjusted to pH7.0 with either tris for measurements of swelling or KOH for measurements of oxygen uptake.

Measurement of oxygen uptake by mitochondria. Oxygen concentration was recorded at pH7.0, 30°C, in various media in a final volume of 3.0 or 6.0ml with about 1.0mg of mitochondrial protein/ml. Standard medium contained: 120mM-KCl, 5mM-$MgCl_2$, 20mM-tris, 2mM-EDTA and 2.5mM-P_i adjusted to pH7.0 with HCl. Glycerol 2-phosphate medium contained: 80mM-glycerol 2-phosphoric acid, 5mM-$MgSO_4$, 20mM-tris, 2mM-EDTA and 2.5mM-P_i adjusted to pH7.0 with KOH. Sucrose medium contained: 240mM-sucrose, 5mM-$MgSO_4$, 2mM-*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonate, 2mM-EDTA and 2.5mM-P_i adjusted to pH7.0 with tris. Thiocyanate medium contained: 150mM-KSCN, 5mM-$MgSO_4$, 2mM-*N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonate, 2mM-EDTA and 2.5mM-P_i adjusted to pH7.0 with KOH. The oxygen concentration in standard medium at 30°C was determined as described by Chappell (1964). Rotenone (1μg/ml) was included in all experiments where succinate was used as substrate. Respiration is designated as state 3 or state 4 (Chance & Williams, 1956) except that state 3 is also used for mitochondria in the presence of 20μM-2,4-dinitrophenol.

Adenosine triphosphatase (EC 3.6.1.4) activity of mitochondria. Both the latent and 2,4-dinitrophenol-stimulated mitochondrial adenosine triphosphatase were determined in the Cl^--, sucrose- and glycerol 2-phosphate-containing media used for the measurement of oxygen uptake, except that P_i was omitted. Mitochondria (about 2mg of protein) were added to 3.0ml of medium containing 3mM-ATP, with or without 20μM-2,4-dinitrophenol, and incubated at 30°C for 10min. Rotenone (2μg/ml) was included when 2,4-dinitrophenol was omitted (Aldridge & Street, 1971). The reaction was stopped by the addition of 1.0ml of 30% (v/v) $HClO_4$ and the total P_i liberated was determined after correction for enzyme and substrate blanks. The results are expressed as μmol of P_i liberated/10min per mg of protein, since

the reaction is not necessarily linear with time (Aldridge, 1958; Aldridge & Street, 1971), although deviation from linearity at 30°C was not great when P_i release was followed in a few experiments in Cl^--containing or sucrose-containing medium with an automatic pH-recording apparatus (Radiometer Ltd., Copenhagen, Denmark).

Results and Discussion

Effects of diphenyleneiodonium on mitochondrial reactions

In preliminary experiments it was found that oxygen uptake by rat liver mitochondria in state 3 with 3.3mM-succinate or 10mM-glutamate as substrate, stimulated by ADP or by 2,4-dinitrophenol, was strongly inhibited by 5μM-diphenyleneiodonium, and unstimulated respiration in state 4 was slightly stimulated. These effects of diphenyleneiodonium resemble those of the trialkyltin compounds (Aldridge, 1958), although in marked contrast diphenyleneiodonium causes little inhibition of the 2,4-dinitrophenol-stimulated mitochondrial adenosine triphosphatase at low concentrations. The possibility was therefore considered that diphenyleneiodonium inhibits state 3 respiration by impairing the transport of substrate anions into the mitochondrial inner space. This was investigated by using the P_i-induced swelling of mitochondria suspended in isoosmotic ammonium succinate or malate solutions buffered at pH7.4 with 5mM-tris – HCl (Chappell & Haarhoff, 1966; Chappell, 1968). No significant impairment was found with these conditions but it was noticed that addition of 10μM-diphenyleneiodonium before P_i causes a limited swelling of the mitochondria (Fig. 1). Systematic investigation of this swelling showed that this is related to the Cl^- present and that 10μM-diphenyleneiodonium causes a rapid and extensive swelling of mitochondria suspended in 150mM-NH_4Cl (Fig. 2), indicating that the compound transports Cl^- and OH^- across the mitochondrial membrane. The effects of diphenyleneiodonium on respiration, adenosine triphosphatase activity and swelling of mitochondria in various media were therefore investigated in some detail. Appropriate experiments were also done with tri-*n*-propyltin chloride to compare its similarities and differences with diphenyleneiodonium under our experimental conditions.

Anion–hydroxyl ion exchange. Diphenyleneiodonium causes a rapid and extensive swelling of

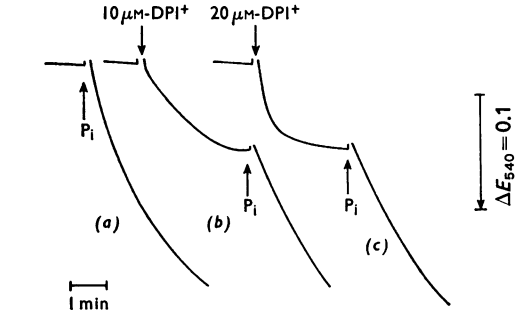


Fig. 1. *Effect of diphenyleneiodonium on the P_i-induced swelling of mitochondria in* 150mM-*ammonium succinate*

Mitochondria (1.7mg of protein) were suspended in 3.0ml of 150mM-ammonium succinate containing 0.5mM-EDTA and 5mM-tris – HCl buffer, adjusted to pH7.4, at 30°C. Rotenone (1μg/ml) and antimycin (1μg/ml) were also added to prevent oxidation of endogenous substrate or succinate and to prevent energy-linked volume changes. Swelling of mitochondria was followed by the rate of increase of E_{540} and was induced by the addition of 2mM-P_i: (*a*) control; (*b*) 10μM-diphenyleneiodonium (DPI^+) added; (*c*) 20μM-diphenyleneiodonium added.

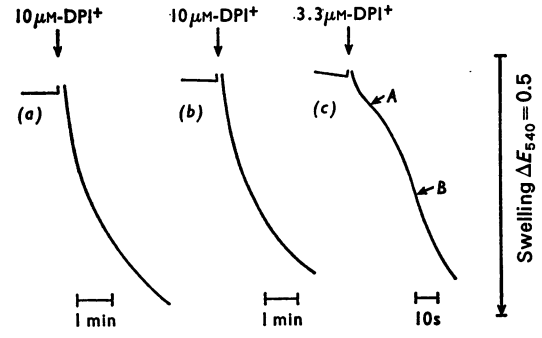


Fig. 2. *Swelling of mitochondria in ammonium chloride, ammonium bromide and ammonium thiocyanate solutions induced by diphenyleneiodonium*

Mitochondria (1.5mg of protein) were suspended at 20°C in: (*a*) 150mM-NH_4Cl, (*b*) 150mM-NH_4Br or (*c*) 150mM-NH_4SCN. All solutions contained 0.5mM-EDTA, adjusted to pH7.4 with tris, rotenone (1μg/ml) and antimycin (1μg/ml). Diphenyleneiodonium (DPI^+) was added where indicated. Swelling of mitochondria was followed by the rate of increase of E_{540}. The time-course of swelling in 150mM-NH_4SCN induced by diphenyleneiodonium was biphasic, showing an initial slower rate (*A*) and a subsequent faster rate (*B*). Spontaneous swelling of mitochondria occurs when mitochondria are added to 150mM-NH_4SCN (*c*), slowly at first and accelerating after 3min.

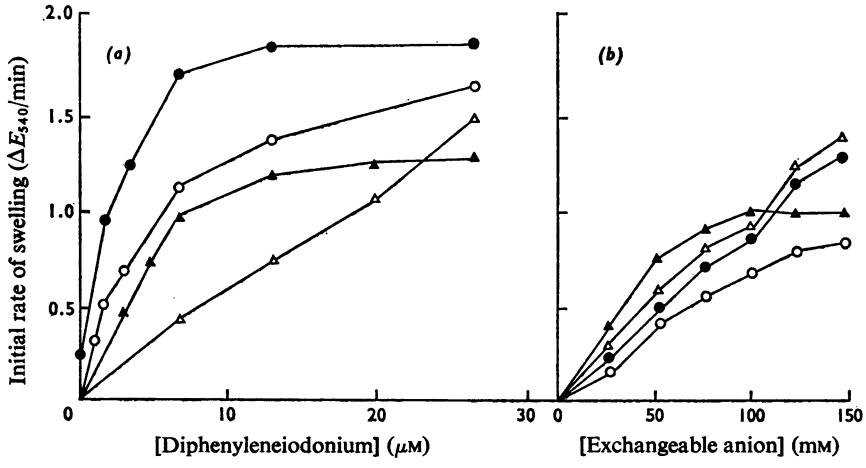
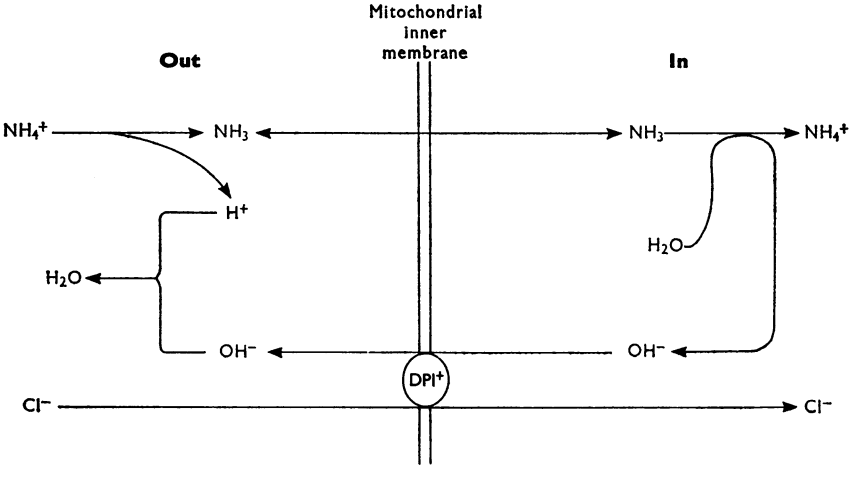


Fig. 3. *Variation of the initial rate of mitochondrial swelling with the concentration of diphenyleneiodonium and of exchangeable anion*

(*a*) Initial rate of swelling of mitochondria (1.1 mg of protein) suspended at 20°C in 3.0 ml of: 150 mM-NH_4Cl (△); 150 mM-NH_4Br (▲); 150 mM-NH_4SCN (○, ●), initial rate (○; *A*, Fig. 2*c*) and subsequent fast rate (●; *B*, Fig. 2*c*), induced by appropriate concentrations of diphenyleneiodonium. (*b*) Initial rate of swelling of mitochondria (0.9 mg of protein) suspended at 20°C in 3.0 ml of appropriate mixtures of 150 mM-NH_4NO_3 and 150 mM-NH_4Cl (△), induced by 20 μM-diphenyleneiodonium; or 15 mM-NH_4Br (▲), induced by 20 μM-diphenyleneiodonium; or 150 mM-NH_4SCN (○, ●), initial rate (○) and subsequent rate (●), induced by 3.3 μM-diphenyleneiodonium. All solutions also contained 0.5 mM-EDTA, rotenone (1 μg/ml) and antimycin (1 μg/ml) adjusted to pH 7.4 with tris. Swelling was followed by the increase in E_{540}.



Scheme 1. *Mechanism of mitochondrial swelling in* 150 mM-*ammonium chloride induced by diphenyleneiodonium*

The circle represents the coupled Cl^-–OH^- exchange catalysed across the inner mitochondrial membrane by diphenyleneiodonium (DPI^+).

mitochondria suspended in 150 mM-NH_4Cl (Fig. 2), and this is not affected by the addition of antimycin (1 μg/ml) and rotenone (1 μg/ml) to prevent all energy-generating reactions. The initial rate of swelling increases linearly with increasing concentration of diphenyleneiodonium (Fig. 3) and approximately

linearly with increasing concentration of Cl^-. The final extent of swelling (after 10min) was greater with higher concentrations of diphenyleneiodonium.

Mechanism of diphenyleneiodonium-induced swelling of mitochondria

Swelling of mitochondria in NH_4Cl solutions can be explained by postulating that diphenyleneiodonium forms undissociated lipid-soluble complexes with Cl^- and with OH^-, which can shuttle across the mitochondrial inner membrane and exchange with OH^- and with Cl^- respectively, according to Scheme 1, diphenyleneiodonium acting as a Cl^-–OH^- antiporter. This permits transfer of Cl^- into the inner space in exchange for OH^-. External NH_4^+ dissociates into H^+ and NH_3; the lipid-soluble NH_3 diffuses directly into the inner space, where it reacts with water to give NH_4^+ and OH^-. This OH^- formed internally then exchanges for external Cl^- and reacts with H^+ formed externally to give water. There is therefore a net transfer of NH_4Cl into the inner space and an osmotic equivalent of water follows, causing swelling of the mitochondria. The thermodynamic driving force for this transfer is the greater initial activities of NH_4^+ and Cl^- in the suspending medium than in the mitochondrial inner space.

Evidence that diphenyleneiodonium acts as a Cl^-–OH^- antiporter as in reaction (1):

$$\text{Base–Cl} + OH^- \rightleftharpoons \text{Base–OH} + Cl^- \qquad (1)$$

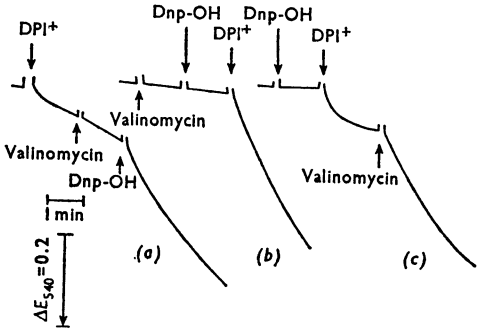


Fig. 4. *Requirement for diphenyleneiodonium, valinomycin and 2,4-dinitrophenol for rapid mitochondrial swelling*

Mitochondria (1.6mg of protein) were suspended in 3.0ml of 150mM-KCl containing 0.5mM-EDTA, 5mM-tris–HCl buffer, pH7.4, rotenone (1μg/ml) and antimycin (1μg/ml) at 30°C. Swelling was induced by the addition of 20μM-diphenyleneiodonium (DPI^+), 0.5μg of valinomycin and 100μM-2,4-dinitrophenol (Dnp-OH) in the orders indicated (*a*, *b*, *c*), and was followed by the increase in E_{540}.

(where 'base' represents diphenyleneiodonium) rather than as a Cl^- uniporter, and also as an OH^- uniporter, acting together as in reactions (2) and (3):

$$\text{Base}^+ + Cl^- \rightleftharpoons \text{Base–Cl} \qquad (2)$$

$$\text{Base}^+ + OH^- \rightleftharpoons \text{Base–OH} \qquad (3)$$

is provided by the experiment illustrated in Fig. 4. Rapid swelling of mitochondria suspended in 150mM-KCl in the presence of 20μM-diphenyleneiodonium is only obtained when valinomycin (0.17μg/ml), a K^+-uniporter (Henderson *et al.*, 1969), and 100μM-2,4-dinitrophenol are also added (in the presence of antimycin and rotenone); all three reagents are necessary, the order of addition being unimportant. This requirement implies that the entry of Cl^- is compulsorily coupled to the exit of OH^- and that 2,4-dinitrophenol, a proton uniporter (Mitchell & Moyle, 1969), allows a rapid influx of Cl^- by permitting an efflux of H^+ equivalent to that of OH^- (see Mitchell & Moyle, 1969; Selwyn *et al.*, 1970). The addition of 2,4-dinitrophenol would be unnecessary for rapid swelling in the presence of valinomycin if diphenyleneiodonium were a simple Cl^- uniporter, since there would be no opposing pH gradient built up as is generated by the Cl^-–OH^- exchange. Gramicidin D was also used instead of valinomycin with similar results in preliminary experiments (Holland & Sherratt, 1971*b*).

Similarly, diphenyleneiodonium causes swelling of mitochondria suspended in 150mM-NaCl [containing antimycin (1μg/ml) and rotenone (1μg/ml)] since these possess a mechanism mediating a passive Na^+–H^+ exchange (Mitchell & Moyle, 1969) and the Cl^-–OH^- exchange enables a net uptake of NaCl into the mitochondrial inner space followed by swelling (Fig. 5).

These mechanisms for diphenyleneiodonium-induced swelling do not necessarily mean that the inner membrane is normally impermeable to Cl^-, since Cl^- could be largely excluded by the Donnan equilibrium, as it is by the plasma membrane of animal cells (see Blondin & Green, 1970). The slow swelling of mitochondria suspended in 150mM-NH_4Cl caused by the addition of 20μM-2,4-dinitrophenol (ΔE 0.025/min) illustrates a slow rate of penetration of the inner membrane by Cl^- with our experimental conditions (see Brierley *et al.*, 1970), as does the slow rate of swelling in 150mM-KCl in the presence of valinomycin alone (Fig. 4).

The requirements for an organic cation to have the ability to promote a Cl^-–OH^- exchange across a cellular membrane are that it can form partly dissociated lipid-soluble chloride and hydroxyl derivatives, which can react as indicated in reaction (1), at a rate that is fast compared with reactions (2) and (3). Reaction (2) cannot occur at a rate sufficient for diphenyleneiodonium and valinomycin alone to cause rapid swelling of mitochondria suspended in

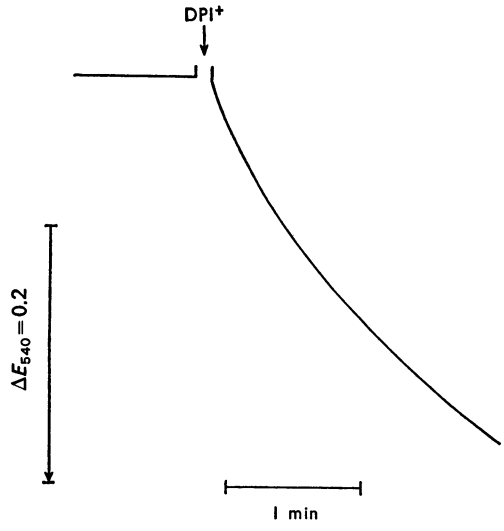


Fig. 5. *Swelling of mitochondria in* 150mM-*sodium chloride induced by diphenyleneiodonium*

Mitochondria (1.5mg of protein) were suspended in 3.0ml of 150mM-NH_4Cl containing 0.5mM-EDTA, 5mM-tris–HCl buffer, pH7.4, rotenone (1 μg/ml) and antimycin (1 μg/ml) at 30°C. Swelling was induced by the addition of 10mM-diphenyleneiodonium (DPI^+), and was followed by the increase in E_{540}.

150mM-KCl (Fig. 4), although reactions (2) and (3) must occur readily enough to form a sufficient concentration of the covalent chloride and hydroxyl derivatives to prime a fast Cl^-–OH^- exchange rate in 150mM-NH_4Cl by the mechanism indicated in Scheme 1, since the rate of swelling is not influenced by the anion associated with the organic cation (P. C. Holland & H. S. A. Sherratt, unpublished work). In the absence of data about the pK of diphenyleneiodonium it is not possible to calculate the proportion of its ionized and un-ionized forms. With high concentrations of Cl^- (130–150mM) and low concentrations of diphenyleneiodonium (5–100 μM) it is likely that diphenyleneiodonium is present mainly as its covalent chloride, whereas in the absence of Cl^- at pH7.0 or 7.4 it is likely to be present mainly as the cation.

Several cyclic polypeptides (ionphores) are uniporters for univalent cations and nigericin is a K^+–H^+ antiporter (Pressman, 1969). The only known anion uniporters appear to be some organic mercury compounds, particularly di(pentafluorophenyl)mercury, which transport I^- across the mitochondrial inner membrane (Liberman & Skulachev, 1970).

Anion specificity of diphenyleneiodonium-induced anion–hydroxyl ion exchange

Diphenyleneiodonium causes rapid swelling of mitochondria suspended in 150mM-NH_4Br (Figs. 2 and 3), and also in 150mM-KBr (containing antimycin and rotenone) in the presence of valinomycin (0.17 μg/ml) and 100 μM-2,4-dinitrophenol, indicating that it also mediates a linked Br^-–OH^- exchange. Mitochondria swell slowly at first when suspended in 150mM-NH_4SCN, the rate accelerating rapidly after 3min; diphenyleneiodonium when added immediately after addition of the mitochondria causes an exceedingly rapid swelling with a characteristic time-course (Figs. 2 and 3), and this is not influenced by antimycin and rotenone.

Diphenyleneiodonium does not cause swelling of mitochondria suspended in iso-osmotic ammonium iodide, nitrate, malate, succinate, oxalate, tartrate, glycerol 2-phosphate or citrate, or accelerate their spontaneous swelling in ammonium phosphate, sulphate, fluoride or borate. It causes a limited swelling of mitochondria suspended in 150mM-tris chloride, pH7.4, confirming that undissociated tris penetrates into the inner space to a limited extent (Brierley *et al.*, 1970).

Other compounds that mediate anion–hydroxyl ion exchanges across membranes

Selwyn *et al.* (1970) and Watling & Selwyn (1970) have shown that trimethyltin, tri-*n*-propyltin, tri-*n*-butyltin, triphenyltin chlorides and phenylmercuric acetate mediate a linked Cl^-–OH^- exchange across biological and artificial lipid membranes, and have concluded that these compounds are not H^+–Cl^- symporters (formally equivalent to Cl^-–OH^- antiporters) and that they do not activate a carrier present in biological membranes.

0.1 μM-Tri-*n*-propyl-lead acetate is about as effective as 0.1 μM-tripropyltin in causing a rapid swelling of mitochondria suspended in 150mM-NH_4Cl; valinomycin and 2,4-dinitrophenol are both also required for rapid swelling in 150mM-KCl, indicating that tri-*n*-propyl-lead acetate mediates a compulsory Cl^-–OH^- exchange. Similarly, 1.0 μM-methylmercuric iodide causes rapid swelling in these media. Tripropyl-lead acetate and phenylmercuric acetate also mediate bromide–, iodide– and thiocyanate–hydroxyl ion exchanges, and tripropyltin mediates fluoride, bromide, iodide and thiocyanate exchanges (see also Stockdale *et al.*, 1970). These compounds were apparently up to about 100 times as active in mediating Cl^-–OH^- exchanges as diphenyleneiodonium, although by contrast they have different pharmacological effects.

Diphenyliodonium, $(C_6H_5)_2I^+$, is less than 10 times as effective as diphenyleneiodonium in causing swelling of mitochondria suspended in 150mM-NH_4Cl, NH_4Br or NH_4SCN; it caused no swelling in solutions of other ammonium salts tested. This agrees with the fact that diphenyliodonium is a stronger base

than diphenyleneiodonium. Diphenyleneiodonium forms a stable covalent hydroxide (Greidanaus *et al.*, 1962), which is presumably because the positive charge of the diphenyleneiodonium cation is delocalized since its iodine atom is part of an heterocyclic ring, in contrast with the iodine atom in diphenyliodonium.

It is expected that many other organometal or organometal-like cations will be found to mediate anion–hydroxyl ion exchanges; for example, a large number of heterocyclic iodonium compounds are known (Banks, 1966). Indeed, nine substituted diphenyleneiodonium derivatives briefly examined all mediated a linked Cl^-–OH^- exchange and of these 2,4-dichlorodiphenyleneiodonium was at least 10 times as active on a molar basis as the parent compound. Compounds that do not cause swelling of mitochondria in 150mM-NH_4Cl include tetrabutylphosphonium chloride and 2,2-dimethylisoarsindoline iodide.

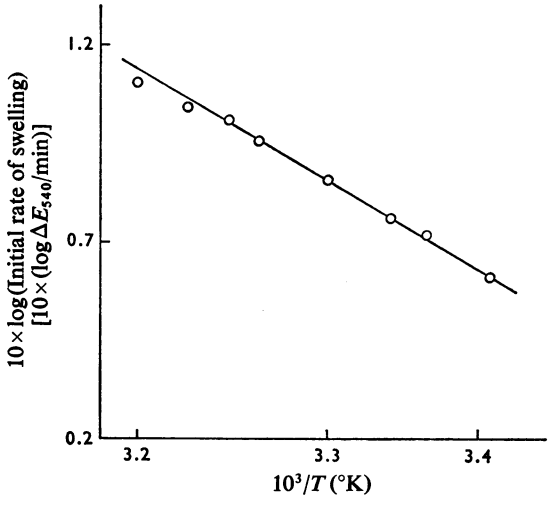


Fig. 6. *Effect of temperature on the initial rate of mitochondrial swelling in* 150*mM-ammonium chloride induced by diphenyleneiodonium*

Mitochondria (1.3mg of protein) were suspended in 3.0ml of 150mM-NH_4Cl containing 0.5mM-EDTA and 5mM-tris–HCl buffer, pH7.4, rotenone (1μg/ml) and antimycin (1μg/ml), at various temperatures between 20° and 40°C. The temperature in the cuvette was measured with a thermistor probe and a digital thermometer (United Systems Corp., Dayton, Ohio, U.S.A.). Swelling was induced by the addition of 10μM-diphenyleneiodonium and was followed by the increase in E_{540}.

Effect of temperature on diphenyleneiodonium-induced swelling of mitochondria

The initial rate of diphenyleneiodonium-induced swelling of mitochondria suspended in 150mM-NH_4Cl increased with increasing temperature from 20° to 40°C and a straight line was obtained in an Arrhenius plot (Fig. 6). This suggests that one process is rate-limiting over this temperature range, although it was not possible to determine an energy of activation.

Diphenyleneiodonium-induced swelling of erythrocytes

The rate of spontaneous swelling of human erythrocytes suspended in 200mM-NH_4Cl containing 10μM-acetazolamide is slow (Selwyn *et al.*, 1970). This rate is accelerated by diphenyleneiodonium (Fig. 7), although higher concentrations are necessary than are required with mitochondria. Selwyn *et al.* (1970) also found that higher concentrations of trialkyltin compounds were necessary to cause rapid swelling of erythrocytes than of mitochondria. The rate of spontaneous swelling is also increased by tripropyl-lead acetate and methylmercuric iodide. These results indicate that the catalysis of Cl^-–OH^- exchange across membranes by these compounds is not confined to those of mitochondria.

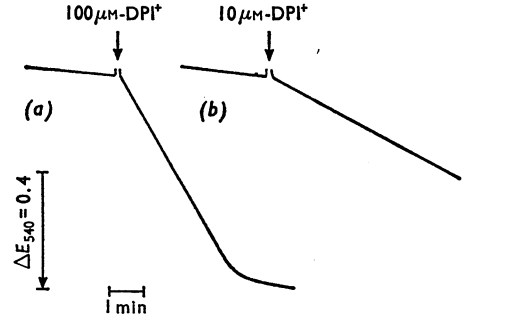


Fig. 7. *Effect of diphenyleneiodonium on the swelling of human erythrocytes in* 200*mM-ammonium chloride*

Erythrocytes were obtained by centrifugation of 50ml of outdated human bank blood at 1000*g* for 10min at 0°C. The cells were washed six times with 0.14M-NaCl at 0°C and finally suspended at 20°C in 10ml of 0.14M-NaCl; 0.2ml of this suspension was added to 3.0ml of 200mM-NH_4Cl containing 0.5mM-EDTA and 5mM-tris–HCl buffer, pH7.4, and 10μM-acetazolamide to inhibit carbonic anhydrase (EC 4.2.1.1) to slow the rate of spontaneous swelling, which was recorded as the increase in E_{540}. Diphenyleneiodonium (DPI^+) was then added: (*a*) 100μM; (*b*) 10μM.

Media used for measurement of oxygen uptake and adenosine triphosphatase activities of mitochondria

Media used for incubating mitochondria usually contain high concentrations of Cl^-. Two Cl^--free media were also used because of the Cl^-–OH^- exchange mediated by diphenyleneiodonium. In one medium [based on one used by Aldridge (1957)] glycerol 2-phosphate was substituted for Cl^- and used because diphenyleneiodonium did not cause swelling of mitochondria in 100mM-ammonium glycerol 2-phosphate. The other contained sucrose and was similar to that used by Selwyn *et al.* (1970). The rates of oxidation in state 3 with succinate as substrate in glycerol 2-phosphate- and sucrose-containing medium were about 30% less than in Cl^--containing medium (Figs. 8 and 9). Both these media contained 5mM-$MgSO_4$, which was used as a convenient magnesium salt since diphenyleneiodonium does not increase the slow rate of swelling of mitochondria suspended in 100mM-$(NH_4)_2SO_4$. Mitchell & Moyle (1969) inferred that rat-liver mitochondria contain a natural SO_4^{2-}–OH^- antiporter since they swell slowly in K_2SO_4 solutions in the presence of valinomycin and an uncoupler, and we have confirmed these observations. However, omission of $MgSO_4$ made no detectable difference to the pattern of results obtained in sucrose-containing medium (Fig. 9).

Inhibition by diphenyleneiodonium of mitochondrial oxygen uptake in Cl^--containing medium

Diphenyleneiodonium strongly inhibits state 3 respiration with 3.3mM-succinate or 10mM-glutamate as substrate and causes a small stimulation of state 4 respiration in a conventional Cl^--containing medium (Figs. 8 and 9). The inhibition was stronger with 5μM-diphenyleneiodonium in state 3 if this was stimulated by 2,4-dinitrophenol rather than with ADP, and this difference was significant at the 1% level (Fig. 9). The inhibition is partly reversed by increasing the concentration of substrate (Fig. 10). The onset of inhibition is time-dependent if diphenyleneiodonium is added after ADP or 2,4-dinitrophenol, maximal inhibition with 5μM-diphenyleneiodonium being obtained 1–2min after its addition (Fig. 8) and more rapidly with higher concentrations, similarly to inhibition caused by trialkyltin compounds in media containing Cl^- (Sone & Hagihara, 1964; Stockdale *et al.*, 1970).

Effects of diphenyleneiodonium on mitochondrial oxygen uptake in Cl^--free media

Diphenyleneiodonium does not inhibit state 3 respiration significantly at 0.1mM concentration in these Cl^--free media, in marked contrast with Cl^--containing medium (Figs. 8 and 9), although there is a greater stimulation of state 4 (in the presence of 2.5mM-P_i; Fig. 9). The stimulation of state 4 was less if P_i was omitted from the glycerol 2-phosphate-containing medium (Fig. 11).

The effects of diphenyleneiodonium differ from those of the trialkyltin compounds, which inhibit state 3 respiration stimulated by ADP or by 2,4-dinitrophenol in Cl^--containing media (Aldridge, 1958) and which also inhibit state 3 stimulated by

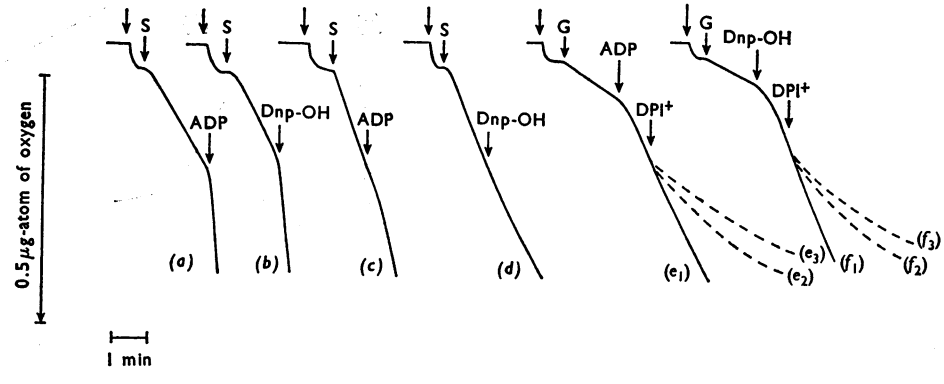


Fig. 8. *Effect of diphenyleneiodonium on the oxidation of succinate and of glutamate by mitochondria in Cl^--containing medium*

Mitochondria [5.7mg (*a*, *b*, *c* and *d*) or 4.2mg (*e* and *f*) of protein] were added where indicated (by unlabelled arrows) to 6.0ml of Cl^--containing medium at 30°C. 3.3mM-Potassium succinate (S), pH 7.0, or 10mM-potassium glutamate (G), pH 7.0, were added where indicated. (*a*) and (*b*), Controls; (*c*) and (*d*), 10μM-diphenyleneiodonium (DPI^+) was added to the medium before addition of mitochondria; (e_1) and (f_1), solid lines, controls; (e_2) and (f_2), broken lines, 5μM-diphenyleneiodonium was added where indicated; (e_3) and (f_3), broken lines, 10μM-diphenyleneiodonium was added where indicated. Other details are given in the text. Dnp-OH, 2,4-Dinitrophenol.

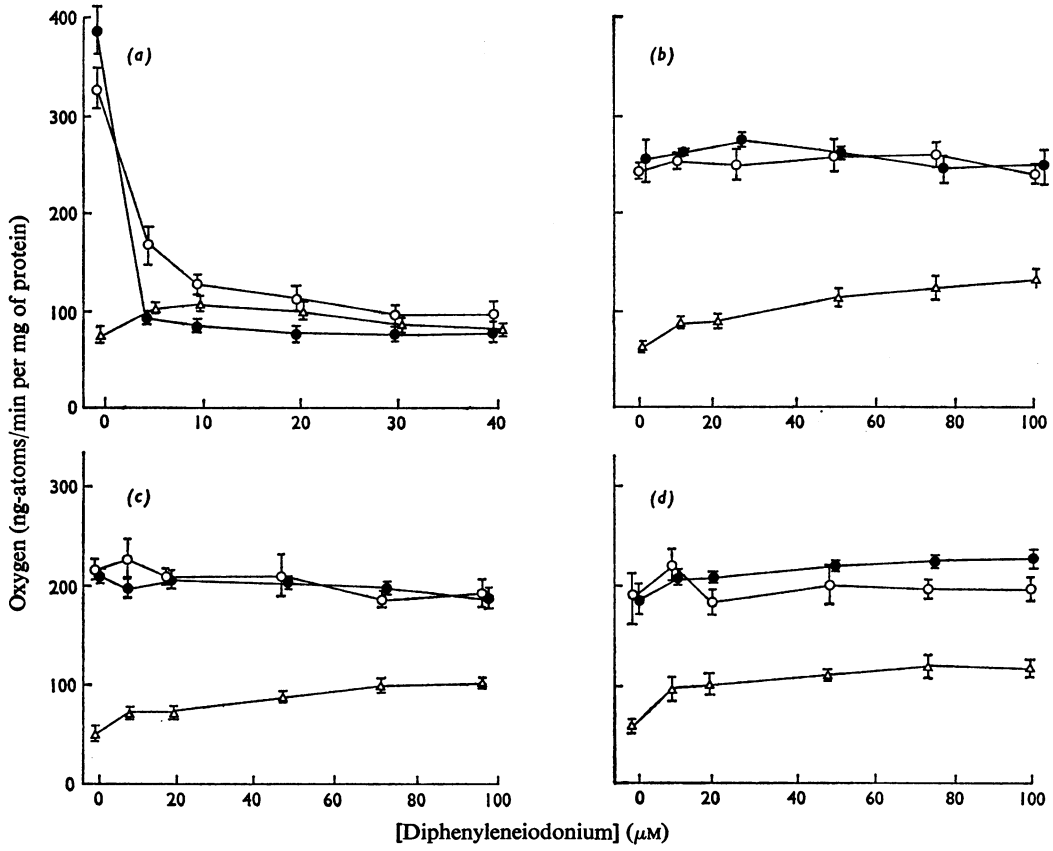


Fig. 9. *Effect of diphenyleneiodonium concentration on the oxidation of succinate by mitochondria in Cl^--, glycerol 2-phosphate- and sucrose-containing media*

Mitochondria (1.2–2.5 mg of protein) were added to 3.0 ml of Cl^--containing medium (*a*) or glycerol 2-phosphate-containing medium (*b*) or sucrose-containing medium (*c*) or sucrose-containing medium with $MgSO_4$ omitted (*d*), and were incubated with diphenyleneiodonium at 30°C for 2 min. 3.3 mM-Succinate was then added and the rate of state 4 oxidation (△) recorded for 2 min, and the subsequent rate of oxidation on adding 0.4 mM-ADP (○) or 20 μM-2,4-dinitrophenol (●) was recorded for a further 2 min. Each point is the mean ± S.E.M. of four experiments with different preparations of mitochondria. Other details are given in the text.

ADP in sucrose-containing media (Stockdale *et al.*, 1970). These effects of tripropyltin chloride were confirmed, and it was also shown to inhibit respiration stimulated by ADP but not by 2,4-dinitrophenol in glycerol 2-phosphate-containing medium.

The extent of inhibition of state 3 respiration by 20 μM-diphenyleneiodonium increased approximately linearly with the concentration of Cl^- when this was progressively substituted for glycerol 2-phosphate, although little inhibition was found below 15 mM-Cl^- (Fig. 12).

Effects of diphenyleneiodonium on swelling of mitochondria in media used for measurement of oxygen uptake

Swelling of mitochondrial suspensions was followed by using the same media and conditions as during the polarographic measurement of O_2 uptake. There was some swelling in all media on addition of succinate.

In Cl^--containing medium diphenyleneiodonium at low concentrations (1–10 μM) caused only slight

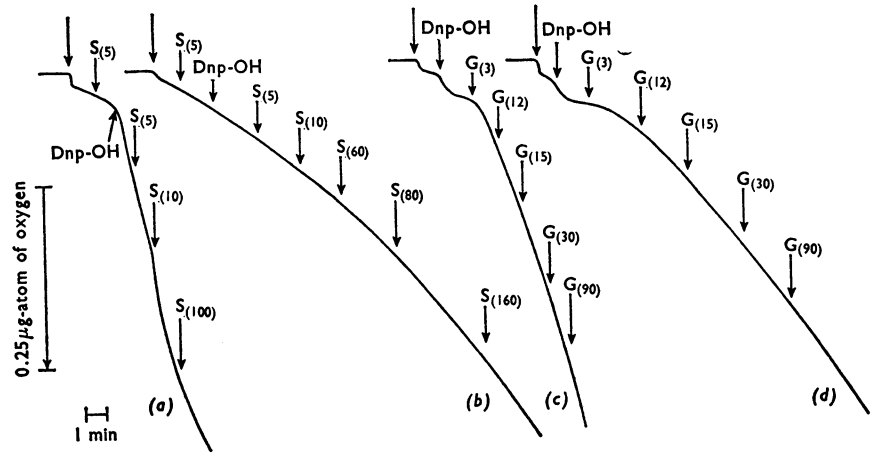


Fig. 10. *Effect of substrate concentration on the inhibition of* 2,4-*dinitrophenol-stimulated respiration of mitochondria in* Cl^-*-containing medium*

Mitochondria [4.2mg (*a* and *b*) or 5.7mg (*c* and *d*) of protein] were added where indicated (by unlabelled arrows) to 6.0ml of Cl^--containing medium at 30°C; in (*b*) and (*d*) they were preincubated with 10μM-diphenyleneiodonium; (*a*) and (*c*) were controls. 20μM-2,4-Dinitrophenol (Dnp-OH) and potassium succinate (S), pH7.0, or potassium glutamate (G), pH7.0, were added where indicated; the numbers in parentheses give the number of μmol of substrate. Other details are given in the text.

swelling. Subsequent addition of succinate resulted in marked swelling, the rate of which was proportional to the concentration of diphenyleneiodonium (Fig. 13). At very low concentrations of diphenyleneiodonium (about 1 μM) the final extent of swelling was also dependent on the amount added.

Swelling of mitochondria in Cl^--containing medium on addition of diphenyleneiodonium and succinate was inhibited by antimycin (Fig. 13). This result is consistent with the finding of Harris *et al.* (1966) that mitochondria possess a respiration-dependent K^+–H^+ exchange carrier. Simultaneous operation of this exchange and the Cl^-–OH^- exchange catalysed by diphenyleneiodonium will result in a net influx of KCl and osmotic swelling.

In Cl^--free media high concentrations of diphenyleneiodonium (0.1mM) induced slight mitochondrial swelling in the presence of succinate. The extent of this was increased by P_i and swelling was not obtained in the absence of succinate (Fig. 14). With these conditions limited swelling may be due, at least in part, to accumulation of the diphenyleneiodonium cation in the matrix space if this cation is sufficiently lipid-soluble to cross the inner membrane (cf. Bakeeva *et al.*, 1970).

Effect of diphenyleneiodonium on the reduction of intramitochondrial nicotinamide nucleotides by succinate or glutamate

20μM-Diphenyleneiodonium markedly inhibited the rate and extent of reduction of mitochondrial nicotinamide nucleotides by succinate (Fig. 15) or by glutamate in Cl^--containing medium but it had no significant effect in glycerol 2-phosphate-containing medium. More data are necessary to exclude the possibility that in Cl^--containing medium diphenyleneiodonium inhibits succinate dehydrogenase (EC 1.3.99.1) or the reduction of nicotinamide nucleotides by oxidation of malate formed from succinate or by reversed electron transport. Diphenyleneiodonium (20μM) does not inhibit glutamate dehydrogenase (EC 1.4.1.2) in the direction of glutamate oxidation.

Effects of diphenyleneiodonium on mitochondrial adenosine triphosphatase activity

In Cl^--containing media diphenyleneiodonium at high concentrations inhibited the 2,4-dinitrophenol-stimulated adenosine triphosphatase activity (up to 45% at 0.1mM) (Fig. 16). The inhibition was very much less than the inhibition of state 3 respiration by

similar concentrations of diphenyleneiodonium (Fig. 9). There was no significant inhibition of the 2,4-dinitrophenol-stimulated adenosine triphosphatase and no stimulation in the absence of 2,4-dinitrophenol in glycerol 2-phosphate-containing medium or in sucrose-containing medium (Fig. 16). In marked contrast with diphenyleneiodonium, tripropyltin strongly inhibited the 2,4-dinitrophenol-stimulated adenosine triphosphate in all three media (Fig. 16).

Mechanism of inhibition by diphenyleneiodonium of oxygen uptake by mitochondria in state 3

The inhibition of state 3 respiration by diphenyleneiodonium depends on the presence of Cl^- and is proportional to its concentration in the medium (Fig. 12). It appears therefore that inhibition is a consequence of the Cl^-–OH^- exchange catalysed by these compounds. This conclusion is strongly supported by preliminary results with 2,4-dichlorodiphenyleneiodonium, which is at least 10 times as active as diphenyleneiodonium, both in causing swelling of mitochondria suspended in 150mM-NH_4Cl and in inhibiting state 3 respiration in Cl^--containing media. It is unlikely that Cl^- transported into the mitochondrial inner space inhibits oxidative phosphorylation directly, since Cl^- is usually included in the medium when this is assayed in submitochondrial particles (Gregg, 1967), where the polarity of the membrane is usually reversed (Bakeeva *et al.*, 1970). Stockdale *et al.* (1970) pointed out that although Cl^-–OH^- exchange will collapse any pH gradient across the inner membrane it will not directly collapse any transmembrane potential necessary for oxidative phosphorylation and for respiratory control according to the chemiosmotic hypothesis (Mitchell, 1966). Two possible mechanisms for inhibition of respiration, depending on the Cl^-–OH^- exchange catalysed by diphenyleneiodonium, merit consideration.

First, it may be postulated that in the presence of diphenyleneiodonium respiration is inhibited as a result of gross structural damage of mitochondria caused by respiration-dependent swelling. However, although marked swelling occurred with conditions where state 3 respiration was strongly inhibited (Fig. 14), it may not be enough to explain this inhibition, since it is partly reversed by increasing the substrate concentration (Fig. 10) when the mitochondria

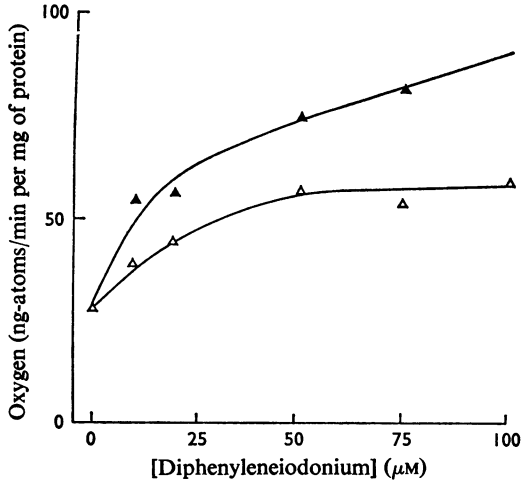


Fig. 11. *Effect of P_i on the state 4 mitochondrial oxidation of succinate in glycerol 2-phosphate-containing medium*

Mitochondria were added to 3.0ml of glycerol 2-phosphate-containing medium with (▲) or without (△) 2.5mM-P_i and were incubated with diphenyleneiodonium at 30°C for 2min. 3.3mM-Succinate was then added and the rate of state 4 oxidation recorded for 2min. Other details are given in the text.

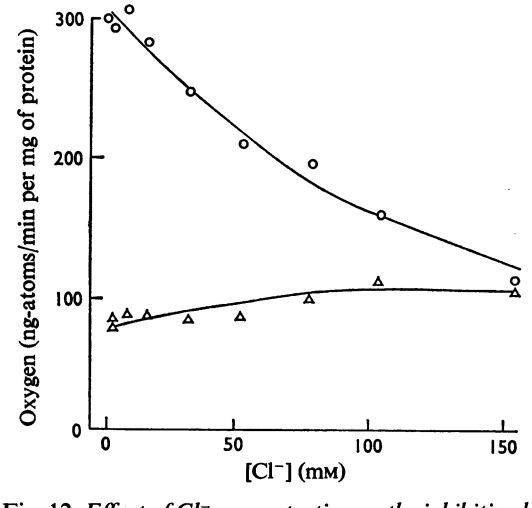


Fig. 12. *Effect of Cl^- concentration on the inhibition by diphenyleneiodonium of mitochondrial oxidation of succinate*

Mitochondria were incubated at 30°C for 2min with 20μM-diphenyleneiodonium in 3.0ml of solutions containing various amounts of Cl^-, made by mixing appropriate volumes of Cl^--containing and glycerol 2-phosphate-containing media. 3.3mM-Succinate was then added and the rate of state 4 oxidation (△) was recorded for 2min; the subsequent rate of oxidation on addition of 20μM-2,4-dinitrophenol (○) was recorded for a further 2min. Other details are given in the text.

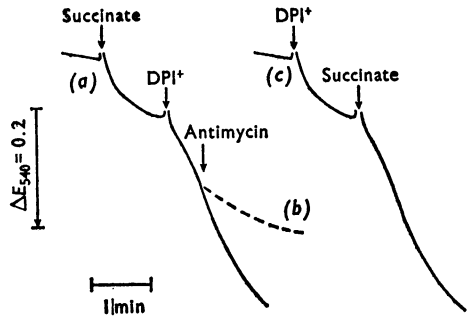


Fig. 13. *Swelling of mitochondria in Cl^--containing medium used for the measurement of oxygen uptake*

Mitochondria (1.4mg of protein) were added to 3.0ml of Cl^--containing medium at 30°C, containing rotenone (1μg/ml): (*a*) and (*c*), succinate (10μmol) and 10μM-diphenyleneiodonium (DPI^+) were added where indicated; (*b*) antimycin (6μg) was added where indicated (the broken line represents swelling in its presence). Swelling was followed by the increase in E_{540}. Other details are given in the text.

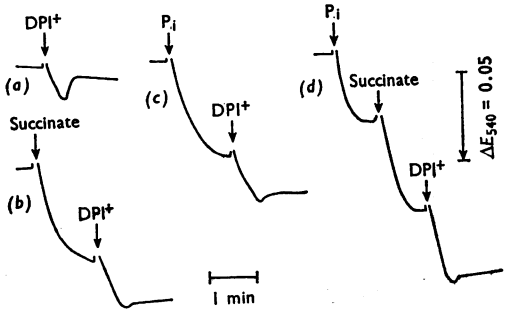


Fig. 14. *Swelling of mitochondria in glycerol 2-phosphate-containing medium used for measurement of oxygen uptake*

Mitochondria (1.4mg of protein) were added to 3.0ml of 2-glycerophosphate-containing medium at 30°C, containing rotenone (1μg/ml). (*a*) Effect of 10μM-diphenyleneiodonium (DPI^+); (*b*) effect of 10μM-diphenyleneiodonium after addition of succinate (10μmol); (*c*) effect of 10μM-diphenyleneiodonium after P_i (7.5μmol); (*d*) effect of 10μM-diphenyleneiodonium after succinate (10μmol) and P_i (7.5μmol). Swelling was followed by the increase in E_{540}. Other details are given in the text.

are already swollen. Similarly, Stockdale *et al.* (1970) suggested that the inhibition of 2,4-dinitrophenol-stimulated respiration in Cl^--containing medium by

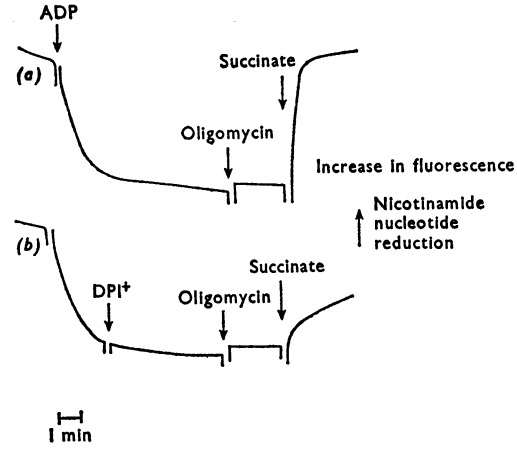


Fig. 15. *Effect of diphenyleneiodonium on the reduction of mitochondrial nicotinamide nucleotides by succinate in Cl^--containing medium*

Mitochondria (0.7mg of protein) were suspended in 2.0ml of Cl^--containing medium at 30°C and 3.3mM-ADP was added to stimulate utilization of endogenous substrate. When nicotinamide nucleotide oxidation was maximum oligomycin (10μg/ml) was added to slow electron transport and therefore to facilitate reduction by exogenous 3.3mM-succinate. (*a*) Control; (*b*) 20μM-diphenyleneiodonium (DPI^+) added where indicated. Fluorescence of reduced nicotinamide nucleotides was excited at 366nm and recorded at 468nm. Other details are given in the text.

trialkyltin compounds was a consequence of structural damage after swelling. This proposal for the mechanism of action of trialkyltin compounds has been recently challenged by Aldridge & Street (1971) with the finding that inhibition is not necessarily associated with gross swelling of mitochondria.

Secondly, the slowing of the rate of reduction of intramitochondrial nicotinamide nucleotides by succinate or glutamate in the presence of diphenyleneiodonium (Fig. 15) suggests that entry of these substrates into the inner space is impaired. Several recent papers have reported that the intramitochondrial concentration of succinate and some other anions is governed by the transmembrane pH difference (Palmieri *et al.*, 1971; Papa *et al.*, 1971; Quagliariello *et al.*, 1971; McGivan & Klingenberg, 1971). Manger (1969) has shown that triethyltin lowers the concentration of substrate anions in rat liver mitochondria in Cl^--containing medium. Stockdale *et al.* (1970) explained this lowering by invoking competition of external Cl^- for intramitochondrial OH^- necessary for substrate entry, although apparently they did not consider this effect as a mechanism of inhibition of

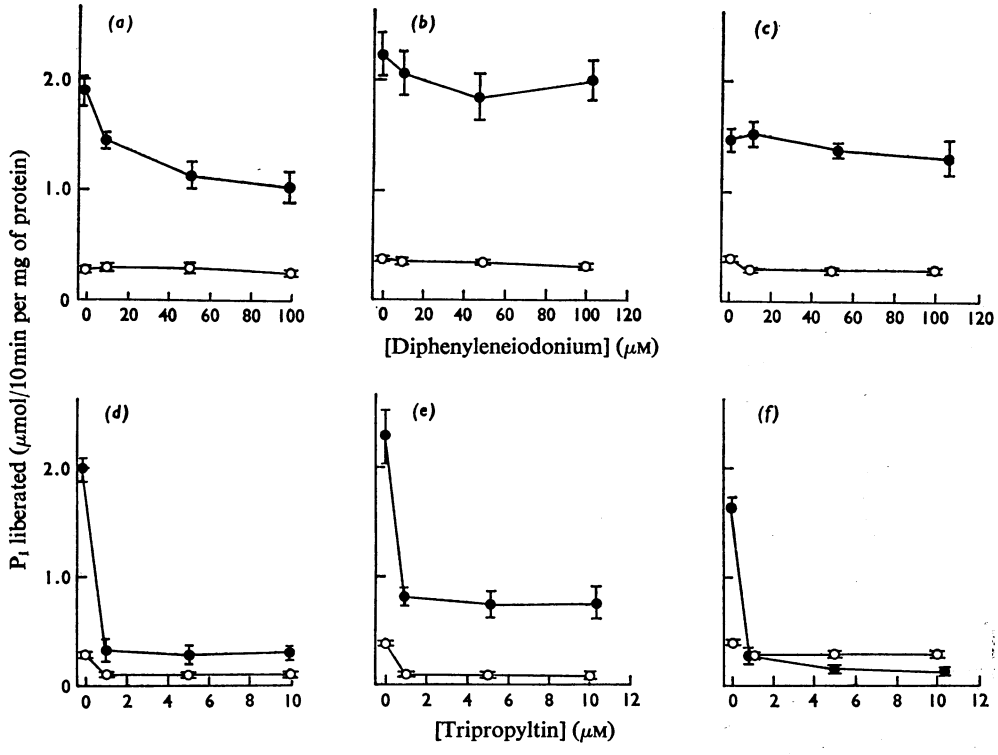


Fig. 16. *Effects of diphenyleneiodonium and of tripropyltin on the latent and 2,4-dinitrophenol-stimulated mitochondrial adenosine triphosphatase activities*

(*a*) and (*d*), Cl^--containing medium; (*b*) and (*e*), glycerol 2-phosphate-containing medium; (*c*) and (*f*), sucrose-containing medium. o, Latent adenosine triphosphatase activity; ●, 2,4-dinitrophenol-stimulated adenosine triphosphatase activity. Each point represents the mean of three experiments ± S.E.M. with different preparations of mitochondria. Other details are given in the text.

respiration. Similarly, it may be proposed that diphenyleneiodonium decreases the rate of succinate uptake by catalysing competition of Cl^- for intramitochondrial OH^-. External P_i is thought to exchange for intramitochondrial OH^- followed by exchange of intramitochondrial P_i for external succinate (Chappell & Haarhoff, 1966; Chappell, 1968). The partial reversal of inhibition of succinate oxidation by increasing the substrate concentration (Fig. 10) is in agreement with this interpretation, although binding of diphenyleneiodonium by high concentrations of succinate or glutamate cannot be excluded. This reversal has not been examined further because of the complexity of the system. Further, no significant inhibition by diphenyleneiodonium of the rate of P_i-induced swelling of mitochondria suspended in 100mM-ammonium succinate containing 5mM-tris–HCl, pH7.4, was found (Fig. 1), where the succinate/Cl^- ratio was about 20, compared with a ratio of 0.025 in experiments where succinate oxidation in state 3 was strongly inhibited by similar concentrations of diphenyleneiodonium (Fig. 9). No conclusions can yet be drawn concerning the effects of diphenyleneiodonium on glutamate uptake, since there is disagreement about the dependence of glutamate transport on the transmembrane pH difference (Quagliariello *et al.*, 1971; McGivan & Klingenberg, 1971).

Finally, it is not known whether covalent diphenyleneiodonium chloride at high concentrations has any direct effects on the respiratory chain.

Mechanism of stimulation of state 4 oxygen uptake of mitochondria by diphenyleneiodonium

In the Cl^--containing medium diphenyleneiodonium may partly uncouple mitochondria by the mechanism proposed by Stockdale *et al.* (1970), in which electrogenic leakage of Cl^-, accumulated by the Cl^-–OH^- exchange, from the inner space tends to

discharge the membrane potential, believed to be negative on the inside (Mitchell, 1966; Liberman & Skulachev, 1970), thus stimulating respiration (at least up to the limit set by any imposed substrate limitation or by any other cause). This explanation could apply whether or not the membrane potential is directly coupled to ATP synthesis (Mitchell, 1966; Slater, 1967). The greater stimulation of state 4 respiration by diphenyleneiodonium in a thiocyanate-containing medium (Fig. 17) than in Cl^--containing medium (Fig. 9), similar to the effects of trimethyltin (Stockdale *et al.*, 1970), supports this explanation, since SCN^- crosses the inner membrane much more readily than Cl^- (Mitchell & Moyle, 1969).

The anion–hydroxyl ion exchange cannot be invoked to explain stimulation of state 4 respiration in glycerol 2-phosphate- and sucrose-containing media (Fig. 9). Uncoupling may occur by the mechanism proposed by Bakeeva *et al.* (1970) for lipid-soluble cations, if the diphenyleneiodonium cation is sufficiently lipid-soluble to cross the mitochondrial inner membrane to a significant extent as a result of the membrane potential. Dibenzyldimethylammonium, tetrabutylammonium and triphenylmethylphosphonium at relatively high concentrations (approx. 0.1 mM), and in the presence of a penetrating anion, cause extensive swelling in energized mitochondria when these cations accumulate in the inner space (Bakeeva *et al.*, 1970). It is extremely unlikely on chemical grounds that these organic cations can act as Cl^-–OH^- antiporters. Diphenyleneiodonium causes less stimulation of state 4 respiration in the glycerol 2-phosphate-containing medium if P_i is omitted (Fig. 11), suggesting that this stimulation is partly caused by swelling after accumulation of diphenyleneiodonium and phosphate (Fig. 13). Some SO_4^{2-} (from $MgSO_4$) and some NO_3^- (Brierley *et al.*, 1970) may also accompany diphenyleneiodonium (which was added as its nitrate salt).

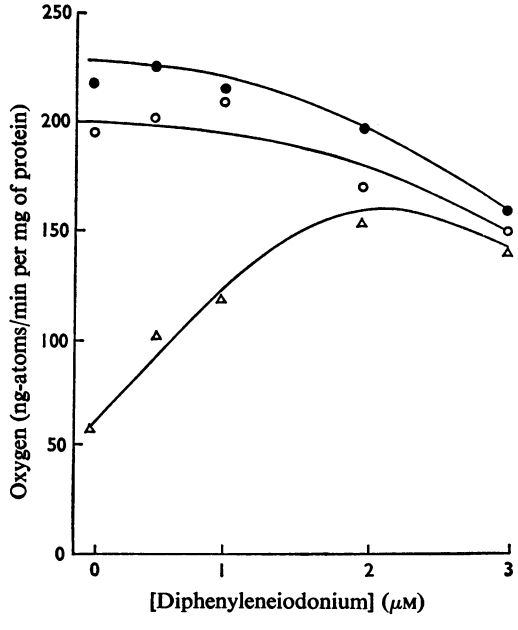


Fig. 17. *Effect of diphenyleneiodonium concentration on the mitochondrial oxidation of succinate in thiocyanate-containing medium*

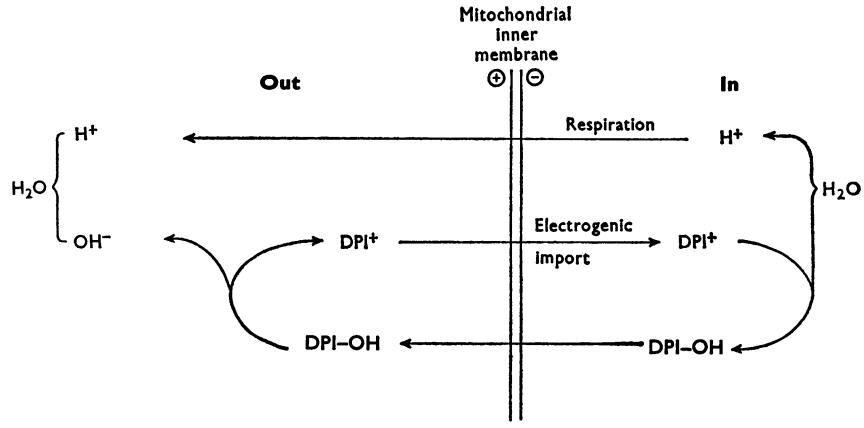
Mitochondria were incubated in thiocyanate-containing medium with diphenyleneiodonium for 2 min at 30°C. 3.3 mM-Succinate was then added and the rate of state 4 respiration recorded for 2 min (△); the subsequent rate of respiration after addition of 0.4 mM-ADP (○) or of 20 μM-2,4-dinitrophenol (●) was recorded for a further 2 min. Other details are given in the text.

Some limited uncoupling by diphenyleneiodonium may also occur by a mechanism that is not dependent on the presence of penetrating anions (Scheme 2). It is proposed that there is an electrogenic entry of diphenyleneiodonium into the inner space where undissociated diphenyleneiodonium hydroxide is formed and which then diffuses out, where it regenerates diphenyleneiodonium and OH^-. The reaction of this OH^- with H^+ also extruded from the mitochondria by respiration would uncouple by collapsing the membrane potential without necessarily causing swelling. This mechanism would presumably not apply to the Cl^--containing medium, since it is likely that diphenyleneiodonium will be present as the undissociated chloride. Both these proposed mechanisms might contribute to the stimulation of state 4 respiration by diphenyleneiodonium in Cl^--free media in the presence of a penetrating anion.

Mechanism of the hypoglycaemic effects of diphenyleneiodonium

Although diphenyleneiodonium is 10–100 times less active on a molar basis than some of the trialkyltin compounds in promoting Cl^-–OH^- exchange its toxicity appears to be of the same order or greater [T. Hanley, R. W. J. Neville, G. A. Stewart & F. W. Webb, unpublished work, quoted by Stewart & Hanley (1969); data collected by Poller (1970)].

The pharmacological effects of trialkyltin compounds are quite different from those of diphenyleneiodonium, since their main toxic effect is to cause cerebral oedema (Aldridge & Cremer, 1955) and they are mildly hyperglycaemic (Stoner *et al.*, 1955). Further, extrapolation from results *in vitro* to effects *in vivo* is complicated by the fact that trialkyltin compounds bind firmly to some cellular proteins containing adjacent histidine residues (Aldridge & Street, 1971; Rose & Lock, 1970). The mechanism of the



Scheme 2. *Proposed mechanism of uncoupling of mitochondria by diphenyleneiodonium in the absence of an exchangeable anion*

DPI^+ represents the diphenyleneiodonium cation and DPI–OH the covalent diphenyleneiodonium hydroxide. Although the rate of the reaction $DPI^+ + OH^- \rightleftharpoons$ DPI–OH is assumed to be slow (reaction 3, see the text) it may account for the extent of uncoupling in the absence of an exchangeable anion since the stimulation of state 4 respiration is small even with high concentrations of diphenyleneiodonium (Fig. 9).

hypoglycaemic action of diphenyleneiodonium is unknown. It may not inhibit mitochondrial reactions very much *in vivo* as a consequence of the Cl^-–OH^- exchange, since the intracellular concentration of Cl^- is low (1–5mM; cf. Fig. 12), unless it causes extensive Cl^- uptake into some tissues from the extracellular fluid. Uncoupling by diphenyleneiodonium (Scheme 2) may occur if high enough concentrations occur in some cells; a toxic dose of 4mg/kg body wt. would give a concentration of about 17μM if uniformly distributed throughout the body water. It is possible that diphenyleneiodonium directly modifies the permeability of cells, which may cause massive and lethal redistribution of electrolytes and of glucose between extracellular fluid and cell water. Any increase in the glucose space so caused might be due to specific activation of glucose transport similar to the increase caused by 2,4-dinitrophenol or other metabolic poisons (Randle & Smith, 1958; Morgan *et al.*, 1959). Diphenyleneiodonium may be expected to affect other physiological processes, for example secretion of HCl by the gastric mucosa and acid–base regulation by the renal tubules.

Conclusions

The ability to catalyse a Cl^-–OH^- exchange across cellular membranes is a recently recognized property of many non-nitrogenous organic bases. This is the only molecular effect of diphenyleneiodonium yet found and its known effects on mitochondrial reactions appear to be explicable in terms of ion transport. The effects of trialkyltin compounds are more complex, since they also have oligomycin-like effects (Aldridge & Rose, 1969; Selwyn *et al.*, 1970; Stockdale *et al.*, 1970). However, the use of diphenyleneiodonium enables effects due to the Cl^-–OH^- exchange to be dissociated from other inhibitions, and the results obtained in this study strongly support the general conclusions of Stockdale *et al.* (1970) that many of the effects of trialkyltin compounds on mitochondria depend on this exchange. It is likely therefore that diphenyleneiodonium will be a valuable reagent for the investigation of membrane-dependent systems.

We thank Mrs. Freda Hardy for excellent technical assistance. This work was generously supported by a grant from the British Diabetic Association.

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