Biochem. J. (1976) **158**, 307–315
Printed in Great Britain

The Effects of Diphenyleneiodonium on Mitochondrial Reactions

RELATION OF BINDING OF DIPHENYLENE[^{125}I]IODONIUM TO MITOCHONDRIA TO THE EXTENT OF INHIBITION OF OXYGEN UPTAKE

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(*Received* 8 *March* 1976)

1. Several ring-substituted derivatives of diphenyleneiodonium catalyse the exchange of Cl^- and OH^- ions across the inner membrane of rat liver mitochondria. They also inhibit state 3 and state 3u oxidations of glutamate plus malate in the presence of Cl^- more than in its absence. Most have activities similar to diphenyleneiodonium, although 2,4-dichlorodiphenyleneiodonium is up to 50 times more active. 2. Diphenyleneiodonium inhibits soluble rat liver NADH dehydrogenase and NADH oxidation by rat liver submitochondrial particles directly; 2,4-dichlorodiphenyleneiodonium is only about twice as inhibitory. 3. Liver mitochondria contain two classes of binding sites for diphenylene-[^{125}I]iodonium, namely high-affinity sites with an affinity constant of $3 \times 10^5 M^{-1}$ (1–2 nmol/mg of protein), and low-affinity sites with an affinity constant of $1.3 \times 10^3 M^{-1}$ (80 nmol/mg of protein). Both sites occur in hepatocytes with a relative enrichment of the low-affinity site. NADH dehydrogenase preparations only apparently contain high-affinity binding sites. Only low-affinity sites occur in erythrocytes. 4. 2,4-Dichlorodiphenyleneiodonium competes with diphenylene[^{125}I]iodonium for both low- and high-affinity sites, whereas tri-*n*-propyltin only competes for the low-affinity sites. 5. The high-affinity sites are apparently associated with NADH dehydrogenase and the low-affinity sites probably represent electrostatic binding of diphenylene[^{125}I]iodonium to phospholipids. The high-affinity site does not appear to be associated with a rate-limiting stage of NADH oxidation.

Diphenyleneiodonium inhibits the ADP- or uncoupler-stimulated oxidation of succinate or of glutamate in Cl^--containing media by a mechanism dependent on the catalysis of a linked Cl^-–OH^- exchange across the inner mitochondrial membrane (Holland & Sherratt, 1972). In this respect diphenyleneiodonium resembles the trialkyltin compounds which also mediate this exchange (Selwyn *et al.*, 1970). However, these latter compounds also inhibit mitochondrial ATPase† similarly to oligomycin, whereas, by contrast, diphenyleneiodonium inhibits the mitochondrial oxidation of NADH-linked substrates in the absence of Cl^- by a direct effect on NADH dehydrogenase (EC 1.6.99.3) with a time-dependent onset (Holland *et al.*, 1973). The powerful hypoglycaemic action of diphenyleneiodonium [T. Hanley, R. W. J. Neville, G. A. Stewart & F. W. Webb (unpublished work quoted by Stewart & Hanley, 1969)] was attributed by Holland *et al.* (1973) to impaired gluconeogenesis as a result of this inhibition at site I of the respiratory chain. This conclusion was supported by a study of a series of ring-substituted diphenyleneiodonium derivatives on gluconeogenesis in isolated rat hepatocytes, where impairment of glucose synthesis by these compounds was not proportional to their ability to promote Cl^-–OH^- exchange (Gatley *et al.*, 1975).

As part of an investigation of the mechanism of inhibition of mitochondrial oxidations by diphenyleneiodonium and by 2,4-dichlorodiphenyleneiodonium, we compared the binding of diphenylene-[^{125}I]iodonium to mitochondria with the extent of the inhibition of the oxidation of various substrates by diphenyleneiodonium. Evidence was obtained that occupation of a class of high-affinity binding sites is related to inhibition of NADH dehydrogenase, whereas low-affinity binding represents interaction with membrane phospholipids. The extent of inhibition of respiration in Cl^--containing media is not, however, simply proportional to the occupancy of either class of binding site; the mechanism of Cl^--dependent inhibition is discussed in the following paper (Gatley & Sherratt, 1976).

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† Abbreviations: ATPase, adenosine triphosphatase (EC 3.6.1.3); EGTA, ethanedioxybis(ethylamine) tetra-acetic acid; Hepes, 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)-ethanesulphonic acid.

Materials and Methods

Materials

The sources of most chemicals are given by Holland & Sherratt (1972) and Holland *et al.* (1973). Collagenase (EC 3.4.24.3) and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone were obtained from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K. EGTA was obtained from Fluka A.G., Buchs, Switzerland. 2-Iodobiphenyl was obtained from K & K Laboratories, New York, NY, U.S.A., and 2-aminobiphenyl from the Aldrich Chemical Co., Milwaukee, WI, U.S.A. $Na^{125}I$ (100mCi/ml) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Diphenyleneiodonium sulphate was synthesized in 70% yield from 2-iodobiphenyl as described by Collette *et al.* (1956). Diphenylene[^{125}I]iodonium sulphate was prepared from 85mg of 2-aminobiphenyl which was added to 1.1ml of 1M-HCl at 0°C with magnetic stirring; then 40mg of $NaNO_2$ in 0.4ml of water was added dropwise over 10min. $Na^{125}I$ (5mCi) and 100mg of KI in 1.0ml of water were added. After a further 10min the mixture was heated at 100°C for 20min. Crude [^{125}I]iodobiphenyl separated as a black oil, which was converted into diphenylene[^{125}I]iodonium sulphate (1.67mCi/mmol) (Collette *et al.*, 1956). Diphenyleneiodonium and its derivatives were used as solutions in dimethyl sulphoxide or in 50% (v/v) ethanol. Potassium isethionate was prepared by passing 0.3M-sodium isethionate through a column (50cm × 5cm) of Dowex 50 (20–50 mesh; H^+ form) followed by neutralization with solid KOH, concentration and crystallization.

Methods

Preparation of mitochondria. Mitochondria were prepared from the livers of albino male rats (200–300g), from a local inbred strain, essentially as described by Senior & Sherratt (1968), except that 0.3M-mannitol/0.1mM-EGTA/5mM-Hepes, adjusted to pH7.2 with solid KOH, was used as isolation medium. Rat heart and kidney mitochondria were prepared similarly. Protein was determined as described by Gornall *et al.* (1949) and by using Boehringer 'precimat' standard.

Preparation of submitochondrial particles. Mitochondria (20mg of protein/ml) suspended in 50ml of isolation medium at 0–4°C were sonicated for five 2min periods with 1min intervals at an amplitude of 12μm at full power in an MSE sonicator, in a vessel surrounded by ice. Unbroken mitochondria were removed by centrifugation at 10000$g_{av.}$ for 10min at 2°C, and the submitochondrial particles were sedimented at 100000$g_{av.}$ for 45min at 0°C. The pellet was suspended in isolation medium (15–25mg of protein/ml).

Measurement of oxygen uptake by mitochondria. Oxygen concentration was recorded polarographically at pH7.2 and 30°C in a final volume of 3.0ml, with 1–3mg of mitochondrial protein/ml in either 120mM-KCl or 120mM-potassium isethionate, also containing 2.5mM-P_i and 5mM-Hepes, adjusted to pH7.2 with solid KOH. Respiration in the absence of a phosphate acceptor is termed state 4, and in the presence of ADP or uncoupler as state 3 or state 3u respectively (Holland & Sherratt, 1972), and the rates are expressed as ng-atom of oxygen consumed/min per mg of protein.

Measurement of mitochondrial swelling. Mitochondria (0.4–0.6mg of protein/ml) were suspended in 3.0ml of 150mM-ammonium chloride, bromide, iodide, thiocyanate or nitrate and also containing 5mM-Hepes, pH7.4, antimycin (1μg/ml) and rotenone (1μg/ml) at 20°C; the initial rate of swelling induced by the addition of diphenyleneiodonium derivatives was assessed by recording the decrease in E_{540} (Holland & Sherratt, 1972).

Preparation of soluble low-molecular-weight NADH dehydrogenase from rat liver. The procedure of Pharo *et al.* (1966) was used. Submitochondrial particles were suspended in water at 0°C (about 25mg of protein/ml), and the pH was adjusted to 5.3 with 1M-acetic acid. Ethanol was added to a concentration of 10% (v/v), and the suspension was heated at 45°C in a shaking water bath for 15min. The suspension was then cooled to 0°C and the pH was adjusted to pH7.0 with 1M-NaOH; the suspension was then centrifuged at 40000$g_{av.}$ for 30min. The pale-yellow supernatant, which contained 1.86mg of protein/ml, with a specific activity of 170 units/mg of protein, was used.

Assay of NADH dehydrogenase. NADH dehydrogenase (NADH menadione reductase) activity was measured at 30°C and pH8.0 in 25mM-Tris sulphate, 135μM-menadione and 170μM-NADH in a final volume of 1.85ml. The reaction was started as a routine by adding NADH, and the initial rate of decrease in E_{340} was recorded. A unit of activity was defined as 1nmol of NADH oxidized/min per mg of protein.

Preparation of isolated rat hepatocytes These were prepared essentially as described by Berry & Friend (1969) after perfusion of livers with collagenase; usually 95% of the cells appeared viable and these were able to synthesize glucose from 10mM-alanine at rates of about 150μmol/h per g dry wt. (Gatley *et al.*, 1975).

Preparation of erythrocytes. Fresh heparinized human blood (20ml) was diluted with 30ml of 0.14M-NaCl and centrifuged at 1000$g_{av.}$ for 5min at 0–4°C. The pellet was washed three times with 50ml of 0.14M-NaCl by centrifugation and the erythrocytes were finally resuspended in 5ml of 0.14M-NaCl.

Measurement of binding of diphenylene[^{125}I]iodonium to mitochondria, hepatocytes and erythrocytes. Mitochondria (10mg of protein/ml) were incubated in 96mM-KCl/60mM-mannitol/5mM-Hepes, containing 1μg of antimycin and 1μg of oligomycin at pH7.2 and 20°C. Hepatocytes (5mg dry wt./ml) were incubated in Krebs–Ringer bicarbonate solution, pH7.4 (Umbreit *et al.*, 1964), at 20°C. Erythrocytes were incubated in 0.14M-NaCl (50μl packed cell volume). Incubations with appropriate concentrations of diphenylene[^{125}I]iodonium (indicated in the legends to Figs. 2, 3, 4 and 5) were conducted in a final volume of 1.0ml in Eppendorf microcentrifuge tubes. After addition of mitochondria, hepatocytes or erythrocytes, the tubes were immediately mixed with a vortex mixer, and after 2min they were centrifuged for 2min at 20000*g* in an Eppendorf microcentrifuge. The supernatant was removed with a hypodermic syringe, and the radioactivity in both the supernatant and the pellet was determined in a Nuclear–Chicago γ-counter, optimized to a counting efficiency of 50%, and at least 1000 c.p.m. above background were collected. The radioactivity in the pellet from the mitochondrial incubation was corrected for that in the outer mitochondrial space and adhering suspension medium by using the data given in the following paper (Gatley & Sherratt, 1976) and expressed as counts per mg of protein (Gatley & Sherratt, 1976). The data were analysed by the method of Scatchard (1949). The results for hepatocytes and erythrocytes were not corrected for adhering medium and were expressed as counts per mg dry wt. or per ml of packed cell volume respectively.

Measurement of binding of diphenylene[^{125}I]iodonium to submitochondrial particles and to NADH dehydrogenase. Submitochondrial particles suspended in 120mM-KCl/5mM-Hepes, pH7.2 (2ml), in Visking tubing were dialysed against 20ml of the same medium containing diphenylene[^{125}I]iodonium for 24h, at 0°C. Solutions of NADH dehydrogenase (1ml) were dialysed against 10ml of 50mM-Tris sulphate, pH8.0, containing diphenylene[^{125}I]iodonium for 24h at 0°C. The final extent of radioactivity and volume of the dialysis residue were determined, and the radioactivity of the diffusate was also measured. After appropriate corrections, the amounts of bound and free diphenylene[^{125}I]iodonium were calculated.

Results

Inhibition of mitochondrial respiration by diphenyleneiodonium and its ring-substituted derivatives

The preliminary observations that several substituted diphenyleneiodonium derivatives catalyse a linked Cl^-–OH^- exchange across membranes and inhibit the mitochondrial oxidation of succinate and glutamate (Gatley & Sherratt, 1974) have been confirmed and extended. The activities of diphenyleneiodonium and six of these derivatives in catalysing this exchange, as assessed by their ability to cause swelling of mitochondria in iso-osmotic ammonium salts (see Holland & Sherratt, 1972), are given in Table 1. These correlate reasonably well with the concentrations that cause 50% inhibition of uncoupler-stimulated oxidation of succinate or of glutamate-plus-malate in Cl^--containing media (Table 2) (Holland & Sherratt, 1972; Gatley & Sherratt, 1974). Of these derivatives, most have an order of activity similar to diphenyleneiodonium, although 2,4-dichlorodiphenyleneiodonium was about 20–50 times more active. Three further derivatives, namely 3-chlorodiphenyleneiodonium, 3-nitrodiphenyleneiodonium and 3,7-dichlorodiphenyleneiodonium, could only be shown to be qualitatively similar to diphenyleneiodonium, since it was difficult to assess the extent of their solubilities. Gatley & Sherratt (1974) give the concentrations of some diphenylene-

Table 1. *A comparison of the initial rates of swelling induced by diphenyleneiodonium and by six of its derivatives of mitochondria suspended in ammonium salts* (150mM)

Experimental details are given in the text; the rates are expressed as the initial rate of decrease in E_{540}/min. A maximum rate of swelling was reached in experiments marked*. Note that a 50-fold lower concentration of 2,4-dichlorodiphenyleneiodonium than of the other diphenyleneiodonium derivatives was used.

	Swelling rate				
Ammonium salt (150mM) ...	NH_4Cl	NH_4Br	NH_4I	NH_4SCN	NH_4NO_3
Derivative					
10μM-Diphenyleneiodonium	0.3	1.0	1.1*	0.6	0.1
10μM-2-Chlorodiphenyleneiodonium	0.7	3.1	0.7*	2.8	0.1
10μM-3,7-Dimethoxydiphenyleneiodonium	0.5	0.6	0.1*	0.6	0
10μM-3,7-Dimethyldiphenyleneiodonium	1.9	2.7	0.1	1.1*	<0.1
10μM-2-Nitrodiphenyleneiodonium	0.2	0.7	0.4*	0.4	0
10μM-4-Nitrodiphenyleneiodonium	1.5	1.2	0.9	0.3	<0.1
0.2μM-2,4-Dichlorodiphenyleneiodonium	1.1	0.7	0.7	0.9	0

Table 2. *Concentrations (μM) of diphenyleneiodonium and five of its derivatives causing 50% inhibition of 2,4-dinitrophenol-stimulated respiration*

Mitochondria were incubated as described in the text with appropriate concentrations of diphenyleneiodonium and its derivatives, except that mannitol medium contained 250mM-mannitol instead of Cl^- or isethionate. Glutamate (10mM) and malate (1mM), or succinate (10mM), were then added, followed after 2min by 20μM-2,4-dinitrophenol, and the subsequent rate of oxidation was recorded for 2min.

	Concentration of compound (μM)		
	Isethionate medium		Chloride medium
Substrate	10mM-Glutamate and 1mM-malate	10mM-Succinate	10mM-Succinate
Diphenyleneiodonium	18.0	—	1.8
2,4-Dichlorodiphenyleneiodonium	8.0	15.5	0.1
	Mannitol medium		
2-Chlorodiphenyleneiodonium	26.0	—	10.0
3,7-Dimethyldiphenyleneiodonium	33.0	—	8.0
3,7-Dimethoxydiphenyleneiodonium	45.0	—	3.5
2-Nitrodiphenyleneiodonium	32.0	—	12.0

iodonium derivatives that inhibit ADP-stimulated respiration by 50%.

The mechanism of inhibition of respiration in Cl^--containing media is analysed in detail in the following paper (Gatley & Sherratt, 1976). However, Table 2 shows that diphenyleneiodonium also inhibits the oxidation of glutamate plus malate, but not of succinate, in a Cl^--free medium. Of these derivatives, only 2,4-dichlorodiphenyleneiodonium inhibits the oxidation of succinate in a Cl^--free medium at concentrations less than 50μM.

Inhibition of rat liver soluble low-molecular-weight NADH dehydrogenase by diphenyleneiodonium

The initial rate of oxidation of NADH was inhibited by 50% by 50μM-diphenyleneiodonium, and this inhibition increased progressively (Fig. 1). The development of inhibition was not increased by preincubation with diphenyleneiodonium for up to 10 min. This suggests that the presence of NADH or electron flow 'through' the enzyme is necessary to get maximum inhibition. A double-reciprocal plot of initial rate against NADH concentration indicates that 16μM-diphenyleneiodonium changed the apparent K_m value and $V_{max.}$ values from 80μM and 170nmol/min per mg of protein to 60μM and 90μmol/min per mg of protein respectively.

Inhibition of NADH oxidase activity of rat liver submitochondrial particles by diphenyleneiodonium and by 2,4-dichlorodiphenyleneiodonium

NADH was oxidized by submitochondrial particles with O_2 as electron acceptor with a $V_{max.}$ of 147nmol/min per mg of protein and an apparent K_m of 4μM. The initial rate of oxidation of 100μM-NADH was inhibited by 35% by 20μM-diphenyleneiodonium, and the extent of inhibition progressively increased to 85% after 2min. 2,4-Dichlorodiphenyleneiodonium was about twice as inhibitory as diphenyleneiodonium, and double-reciprocal plots of initial rate against concentration were linear in the presence of

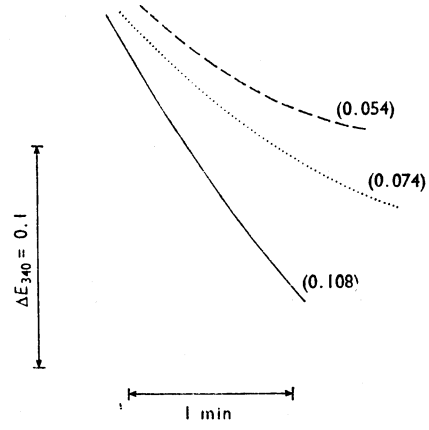

Fig. 1. *Inhibition of low-molecular-weight NADH dehydrogenase by diphenyleneiodonium*

NADH menadione reductase activity was measured as described in the text; ——, control; ····, with 16μM-diphenyleneiodonium; ----, with 50μM-diphenyleneiodonium. The numbers in parentheses are the initial rates of the reactions (μmol/min per mg of protein).

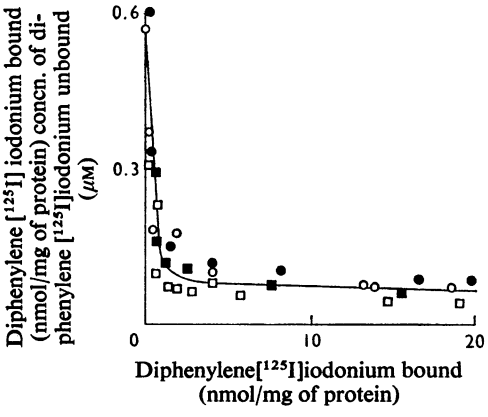

Fig. 2. *Binding of diphenylene[^{125}I]iodonium to liver mitochondria*

Mitochondria were incubated with diphenylene[^{125}I]-iodonium as described in the text. Four different preparations of mitochondria (○, ●, □ and ■) were used.

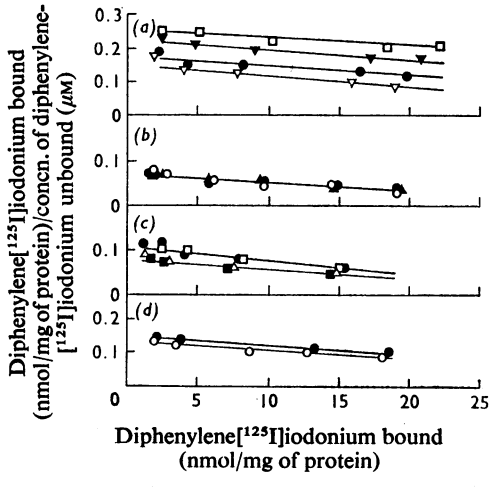

Fig. 3. *Effects of carbonyl cyanide p-trifluoromethoxyphenylhydrazone, tri-n-propyltin, EDTA, Mg^{2+} and isethionate on the low-affinity binding of diphenylene[^{125}I]-iodonium to liver mitochondria*

Mitochondria were incubated with diphenylene[^{125}I]-iodonium as described in the text. Different preparations of mitochondria were used in (*a*) (*b*) (*c*) or (*d*). Other additions, 5mM-$MgCl_2$, 1mM-EDTA, 0.5μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, 10μM-tri-*n*-propyltin (TPT) or 10μM - 2,4 - dichlorodiphenyleneiodonium (DCDPI$^+$), or the substitution of isethionate for Cl^-, or pH changes are marked where appropriate: (*a*) ●, control; □, pH8.0; ▼, pH7.6; ▽, pH6.8; (*b*) ●, control; ▲, Mg^{2+}; ○, EDTA; (*c*) ●, control; □, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; ■, TPT; △, DCDPI$^+$: (*d*) ●, control; ○, isethionate.

either inhibitor (results not shown). However, replots of $K_m/V_{max.}$ against inhibitor concentration curved upwards, suggesting that these iodonium compounds inhibit at more than one site.

Binding of diphenylene[^{125}I]iodonium to mitochondria

The binding of diphenylene[^{125}I]iodonium to rat liver mitochondria indicated the occurrence of two classes of binding site; one with an affinity constant of $3 \times 10^5 M^{-1}$ present at 1–2nmol/mg of protein ('high-affinity site') and one with an affinity constant of $1.3 \times 10^3 M^{-1}$ at a concentration of 80nmol/mg of protein ('low-affinity site') (Fig. 2).

Low-affinity binding sites

Tri-*n*-propyltin and 2,4-dichlorodiphenyleneiodonium competed with diphenylene[^{125}I]iodonium at this site in mitochondria (Fig. 3*c*), indicating that the low-affinity sites for these compounds are probably identical. Aldridge & Street (1970) give a value for the low-affinity binding constant for tripropyltin of $1.4 \times 10^3 M^{-1}$ with a concentration of 66nmol/mg of protein.

EDTA increased the Cl^--dependent inhibition of succinate oxidation by diphenyleneiodonium, whereas Mg^{2+} diminished this (Gatley & Sherratt, 1976). However, neither EDTA nor Mg^{2+} influenced binding at the low-affinity site (Fig. 3*b*).

If diphenyleneiodonium exists as a cation in solution, binding by energized mitochondria might represent accumulation at the expense of the membrane potential. However, antimycin and oligomycin were also added in these experiments so that such accumulation could not be very large. Further, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, which releases accumulated Ca^{2+} from mitochondria (Lehninger, 1970) did not influence binding of diphenylene[^{125}I]iodonium.

Replacing Cl^- in the incubation medium with isethionate did not change the affinity of binding of diphenyleneiodonium, but slightly decreased the amount bound. This suggests that binding in itself does not cause the effects of diphenyleneiodonium in Cl^--containing medium. That binding of diphenyleneiodonium was greater at pH7.6 than at pH7.2 (Fig. 3*a*), although diphenyleneiodonium does not inhibit oxidation of succinate strongly at pH 7.6 (Gatley & Sherratt, 1976), supports this view.

High-affinity binding sites in rat liver mitochondria

Scatchard plots of binding over the range 1–2nmol/mg of protein are convex-upwards. The amount of diphenylene[^{125}I]iodonium bound was lowered by 2,4-dichlorodiphenyleneiodonium but not by tripropyltin or by rotenone (Fig. 4). The apparent concen-

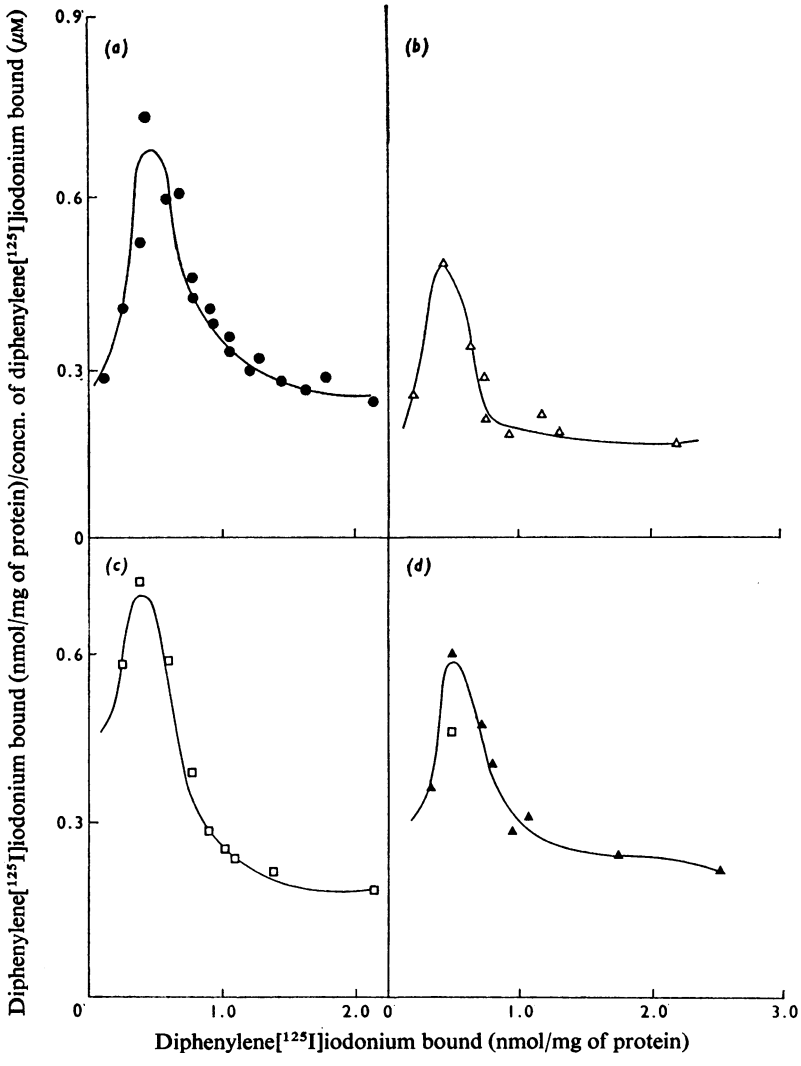

Fig. 4. *Effects of* 2,4-*dichlorodiphenyleneiodonium, tri-n-propyltin and rotenone on the high-affinity binding of diphenylene-*[^{125}*I]iodonium to liver mitochondria*

Mitochondria were incubated with diphenylene[^{125}I]iodonium as described in the text. The same preparation of mitochondria was used for all incubations. Other additions: (*b*) 20μM-2,4-dichlorodiphenyleneiodonium; (*c*) 20μM-tri-*n*-propyltin; (*d*) rotenone (1μg/ml).

tration of binding sites was not changed by 0.2μM-carbonylcyanide*p*-trifluoromethoxyphenylhydrazone plus valinomycin (0.2mg/ml) (Fig. 5), which together collapse any remaining membrane potential or pH gradient across the inner membrane, thus preventing possible electrogenic import of diphenyleneiodonium. However, in this experiment the binding curve was more symmetrical and with a higher maximum value of bound/free diphenyleneiodonium (Fig. 5) than in the absence of carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and valinomycin. These data suggest a model with co-operative binding sites (see the Discussion section).

Binding of diphenylene[^{125}*I*]*iodonium to rat hepatocytes*

The concentration of the high-affinity sites was enriched in particles (5nmol/mg of protein) compared with intact mitochondria. The affinity constant was $3 \times 10^5 M^{-1}$.

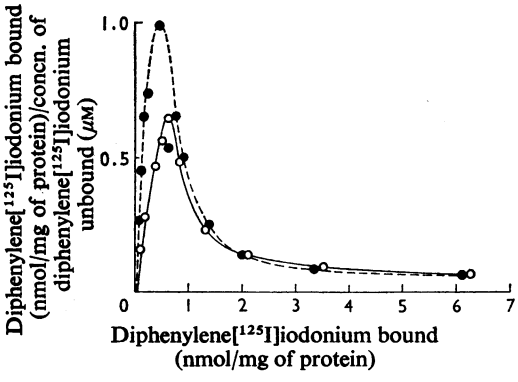

Fig. 5. *Effects of valinomycin and carbonyl cyanide p-trifluoromethoxyphenylhydrazone on the high-affinity binding of diphenylene[^{125}I]iodonium to rat liver mitochondria*

Mitochondria were incubated with diphenylene[^{125}I]-iodonium as described in the text; ○, control; ●, addition of valinomycin (1 μg/ml) and 1 μM-carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone.

Binding of diphenylene[^{125}I]iodonium to soluble NADH dehydrogenase

It was not possible to measure binding to soluble NADH dehydrogenase very accurately. However, if only one class of binding sites is assumed to be present, the affinity constant is between 2×10^5 and 3×10^6 M^{-1} and the concentration 0.5–1.0 nmol/mg of protein. Most of the high-affinity binding site was lost during isolation of the enzyme from submitochondrial particles.

Binding of diphenylene[^{125}I]iodonium to human erythrocytes

Only one class of binding sites was apparent in erythrocytes. Their affinity constant was 1.5×10^3 M^{-1} and their concentration 8 μmol/ml of packed cell volume. The similarity of the affinity constant to that found for low-affinity binding to mitochondria supports the contention that this is a general interaction with membranes. 2,4-Dichlorodiphenyleneiodonium competed with diphenylene[^{125}I]iodonium for this binding site.

Partition of diphenylene[^{125}I]iodonium between chloroform and an aqueous phase

The presence of halide ions increased the solubility of diphenylene[^{125}I]iodonium in chloroform in order of increasing effectiveness: $Cl^- < Br^- < SCN^-$, with partition coefficients of about 5, 30 and 40 respectively, compared with 1.3 when the anion was isethionate. This order was the same as that of the increasing rate of mitochondrial swelling in solutions of different ammonium halides catalysed by diphenyleneiodonium (Table 1).

Discussion

High-affinity binding of diphenyleneiodonium to mitochondria

The enrichment of the high-affinity binding site relative to the low-affinity site during the preparation of submitochondrial particles from mitochondria, the presence of high-affinity binding sites in hepatocytes and soluble enzyme preparations and their absence from erythrocytes are consistent with the interpretation that occupation of the high-affinity binding sites is associated with inhibition of NADH oxidation.

We have considered two possible explanations for the convex-upwards Scatchard plots of high-affinity binding to mitochondria. One is that an endogenous compound competes with diphenylene[^{125}I]iodonium for the sites. A more likely explanation is that occupation of one or more of the sites facilitates binding to other sites. At equilibrium, the proportion of sites occupied, x, is given by:

$$x = \frac{nKA}{1 + KA} \quad (1)$$

where A is the concentration of free diphenyleneiodonium, K is the affinity constant and n is the total concentration of sites. For a pair of binding sites with perfect co-operativity the expression:

$$A^2 = \frac{x^2}{K(n - x)} \quad (2)$$

can be derived (see Reed & Bygrave, 1974). This interpretation is consistent with the complex pattern of inhibition of NADH dehydrogenase by diphenyleneiodonium, which suggests that there may be more than one site of inhibition. We do not know of any established cases of co-operative binding of inhibitors to mitochondria. However, convex-upwards Scatchard plots suggest that there is co-operative binding of 17β-[^{3}H]oestradiol to a soluble cytosolic protein from calf or rat uterus (Puca *et al.*, 1971; Sanborn *et al.*, 1971).

Fig. 6 shows theoretical plots of saturation of low- and high-affinity binding sites against diphenyleneiodonium concentration. Curves derived from both eqns. (1) and (2) for the high-affinity site are given. The experimental curve for inhibition of glutamate-plus-malate oxidation clearly does not coincide with either theoretical curve. The simplest explanation for this is that the part of the respiratory chain that binds diphenyleneiodonium is not rate-limiting for the overall rate of the state-3u oxidation of glutamate plus malate. The rate-limiting step could be another

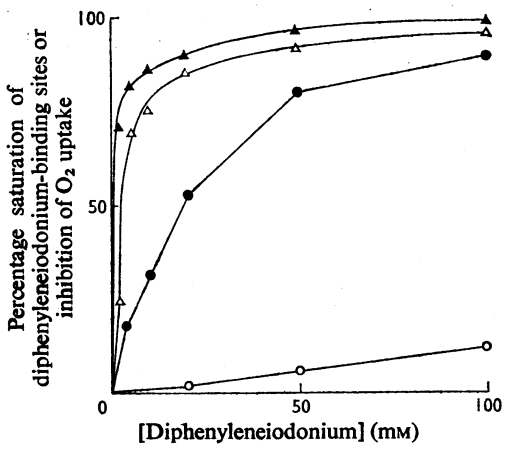

Fig. 6. *Extent of inhibition of the 2,4-dinitrophenol-stimulated oxidation of glutamate and malate by liver mitochondria in relation to the occupancy of the low- and high-affinity binding sites by diphenyleneiodonium*

The rate of the oxidation of 10mM-glutamate and 1mM-malate stimulated by 20μM-2,4-dinitrophenol in the presence of different concentrations of diphenyleneiodonium was measured as described in the text (●). The percentage saturation of the high-affinity binding sites for diphenyleneiodonium co-operative model (▲) or non-co-operative model (△) (see the text), or of the low-affinity binding sites (○), was calculated by using experimentally determined values for the affinity constants (Fig. 2) and a concentration of mitochondrial protein of 1mg of protein/ml.

part of the respiratory chain, the transport of glutamate or aspartate across the inner membrane, glutamate–oxaloacetate aminotransferase (EC 2.6.1.1) or one of the enzymes of the citric acid cycle between 2-oxoglutarate and oxaloacetate.

There is a better correlation between the inhibition of NADH oxidation in submitochondrial particles and the extent of binding of diphenyleneiodonium than in intact mitochondria. It can be calculated that when submitochondrial particles are incubated with 15μM-diphenyleneiodonium, 80% of the high-affinity binding sites are occupied (assuming an affinity constant of $3 \times 10^5 M^{-1}$) and this value is close to the maximum percentage inhibition of the control rate of O_2 uptake, which is observed 2min after addition of diphenyleneiodonium. Since NADH has direct access to NADH dehydrogenase in this preparation (Holland *et al.*, 1973), this result is consistent with a process other than electron transport normally being rate-limiting in the oxidation of glutamate plus malate in intact mitochondria.

The concentration of specific binding sites for rotenone, an inhibitor of NADH dehydrogenase, is about 0.02nmol/mg of protein (Horgan *et al.*, 1968), similar to that of FMN in mitochondria (Cremona & Kearney, 1964) and generally assumed to represent the concentration of NADH dehydrogenase (see Singer & Gutman, 1971). The high-affinity binding site for diphenyleneiodonium is present at a higher concentration than the rotenone-binding site. Neither this result, nor the fact that rotenone did not affect binding of diphenyleneiodonium, is an argument against diphenyleneiodonium binding to a component of the respiratory chain. The structure of the NADH dehydrogenase region is complex, and there is no requirement for stoicheiometry between the components of a reaction sequence provided that the flux through each component is equal. Further, according to Williams (1968), cytochromes *c*, c_1, *b* and aa_3 are present in rat liver mitochondria in amounts ranging from 0.17 to 0.28nmol/mg of protein.

Low-affinity binding of diphenyleneiodonium

The observation that the concentration of the low-affinity binding site increases by 80nmol/mg of protein over the pH range 6.8–8.0 (Fig. 3) suggests that this binding may be electrostatic. The main membrane component whose charge changes over this pH range is phosphatidylethanolamine, which has a p*K* of 8.5. This result is in reasonable agreement with the fact that phosphatidylethanolamine comprises 35% of the mitochondrial lipid (Ronser *et al.*, 1968) and the amount of this lipid that would provide an additional 80nequiv. of negative charge over the pH range from 6.8 to 8.0 is about 0.4g/g of protein.

Since tripropyltin competes with diphenylene[^{125}I]-iodonium for low-affinity binding sites, it is likely that their mechanism of binding is similar. The low-affinity binding constants are close to $10^3 M^{-1}$ for several trialkyltins, which further supports this view (Aldridge & Street, 1970). The high-affinity binding site of trialkyltins to mitochondria is on the respiratory-chain-linked ATPase (Aldridge & Street, 1971). Schafer (1974) and Schafer & Bojanowski (1972) concluded that the biguanides also bind to mitochondria by electrostatic interaction with membrane phospholipids.

Catalysis of Cl^-–OH^- exchange

Holland & Sherratt (1972) suggested that the mechanism of Cl^-–OH^- exchange involves passage of lipid-soluble covalent diphenyleneiodonium chloride and hydroxide species through the membrane, which subsequently dissociate at the membrane/water interfaces. This idea is consistent with the observation that the presence of halide ions makes diphenylene[^{125}I]iodonium more soluble in chloroform (Fig. 6). Presumably, both the concentrations of these lipid species and their 'binding constants' for dissolution in membranes are very low, and are obscured in Scatchard plots by the electrostatic binding.

This is supported by the plot of binding in isethionate-containing medium, which is virtually identical with those for Cl^--containing medium (Fig. 3), although isethionate (in contrast with Cl^-) did not increase the lipid solubility of diphenylene[^{125}I]iodonium.

We thank the British Diabetic Association for financial support and Eli Lily Ltd. for a gift of diphenyleneiodonium derivatives. S. J. G. was in receipt of a Medical Research Council training grant.

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