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Specific Labelling of a Constituent Polypeptide of Bovine Heart Mitochondrial Reduced Nicotinamide–Adenine Dinucleotide–Ubiquinone Reductase by the Inhibitor Diphenyleneiodonium

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1. NADH–ubiquinone-1 and NADH–menadione reductase activities of Complex I were inhibited by diphenyleneiodonium (apparent K_i 23 and 30 nmol/mg of protein respectively). Reduction of $K_3Fe(CN)_6$ and juglone was relatively unaffected. 2. Iodoniumdiphenyl and derivatives were much less effective inhibitors. Compounds with similar ring structures to diphenyleneiodonium, in particular dibenzofuran, were inhibitors of NADH–ubiquinone-1 oxidoreductase. 3. Diphenylene[^{125}I]iodonium specifically labelled a polypeptide of mol.wt. 23500. Maximum incorporation was 1 mol/mol of Complex-I flavin or 1 mol/mol of the 23500-mol.wt. polypeptide. 4. The label associated with this polypeptide was of limited stability, especially at lower pH. 5. Complete inhibition of ubiquinone reduction was achieved when 1 mol of inhibitor was incorporated/mol of Complex-I flavin, but the relationship between inhibition and labelling was not linear. 6. No evidence for covalent interaction between diphenyleneiodonium and the phospholipids of Complex I was obtained. 7. Rotenone increased the apparent affinity of diphenyleneiodonium for the 23500-mol.wt. polypeptide without affecting the maximum incorporation. 8. The 23500-mol.wt. polypeptide was not solubilized by chaotropic agents. Prior treatment of Complex I with chaotropic agents or sodium dodecyl sulphate prevented incorporation of diphenyleneiodonium into this polypeptide.

The reduction of ubiquinone by NADH in the mitochondrial respiratory chain is specifically inhibited by a wide variety of compounds (see, e.g., Garland *et al.*, 1969). Of these, rotenone (Lindahl & Öberg, 1961) and piericidin (Hall *et al.*, 1966) inhibit this reaction at concentrations stoicheiometric with NADH dehydrogenase (Hatefi & Rieske, 1967; Gutman *et al.*, 1970). The site of action of these compounds has been localized to the junction of the NADH dehydrogenase and ubiquinone (Singer & Gutman, 1971), but since neither reacts covalently with the enzyme, details of the binding site are unknown. Of the compounds that inhibit NADH oxidation, but at rather higher concentrations, diphenyleneiodonium (Holland *et al.*, 1973; Gatley & Sherratt, 1976) was of interest because of its specificity, its moderately high potency, its simple structure compared with rotenone or piericidin, and most importantly, its potential chemical reactivity (Banks, 1966). The latter property suggested that diphenyleneiodonium might exert its inhibitory effect by covalent modification of the NADH dehydrogenase. In the present report, we describe the effects of diphenyleneiodonium and structurally related compounds on the activity of the particulate NADH–ubiquinone oxidoreductase (EC 1.6.99.3) or Complex I (Hatefi *et al.*, 1962) from bovine heart mitochondria.

Materials and Methods

Chemicals

Diphenyleneiodonium nitrate was a generous gift from Dr. H. S. A. Sherratt, Department of Pharmacological Sciences, University of Newcastle. Diphenyleneiodonium sulphate was synthesized as described by Collette *et al.* (1956). Diphenylene-[^{125}I]iodonium sulphate was synthesized as described by Gatley & Sherratt (1976). Na^{125}I was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Other iodonium compounds were kindly given by ICI, Pharmaceuticals Division, Macclesfield, Cheshire, U.K. Dibenzofuran, dibenzothiophen, dibenzothiophen sulphone and fluorene were obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K. Ubiquinone-1 was generously given by Hoffman–La Roche, Basle, Switzerland.

Biological materials

Submitochondrial particles from bovine heart (Racker, 1962) and Complex I (Hatefi *et al.*, 1962) were prepared as indicated.

Assays

NADH–ubiquinone-1 oxidoreductase and NADH–$K_3Fe(CN)_6$ oxidoreductase were measured at 30°C as described by Ragan (1976*a*). NADH–juglone (5-hydroxy-1,4-naphthaquinone) oxidoreductase and NADH–menadione oxidoreductase were measured by the decrease in A_{340} of a solution containing 20μmol of potassium phosphate, pH8, 0.1μmol of NADH, 0.2μmol of juglone or menadione and 20μg of Complex-I protein in a final volume of 1ml and at 30°C. NADH oxidase and succinate oxidase were measured polarographically in a solution containing 20μmol of potassium phosphate, pH8, 0.5μmol of NADH or 5μmol of potassium succinate and 1mg of submitochondrial-particle protein in a final volume of 1ml at 30°C. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin [fraction V from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.] as a standard. Flavin was extracted by precipitation of protein with 10% (w/v) trichloroacetic acid, and determined in the neutralized supernatant from the dithionite-reduced-minus-oxidized difference spectrum. An absorption coefficient of 9800litre·mol^{-1}·cm^{-1} at 450nm was used (Massey & Swoboda, 1963).

Inhibition studies

Inhibition by diphenyleneiodonium was performed by incubating Complex I (1–2mg of protein/ml) in 0.1M-Tris/H_2SO_4, pH8.2, with various amounts of diphenyleneiodonium for 1h at 21°C. Samples were then taken for enzyme assays or electrophoresis. Diphenyleneiodonium was dissolved in 50% (v/v) ethanol. Solutions of diphenylene[^{125}I]iodonium were standardized by using $\varepsilon = 15100$litre·mol^{-1}·cm^{-1} at 264nm.

Inhibition by other iodonium compounds or structural analogues of diphenyleneiodonium was performed as follows. All constituents of the enzyme assay, except NADH, were incubated at 30°C with various amounts of the inhibitor for 5min before initiation of the reaction with NADH. This procedure applied to diphenyleneiodonium produced the same results as the above method. Stock solutions of the inhibitors were prepared in the following solvents: iodoniumdiphenyl nitrate (10mM) in 25% (v/v) ethanol; 2,2′,4,4′-tetrachloroiodoniumdiphenyl chloride (10mM) in hot 50% (v/v) acetonitrile; 3,3′-dinitroiodoniumdiphenyl bisulphate (10mM) in 25% (v/v) ethanol; fluorene, dibenzofuran and dibenzothiophen (100mM) in ethanol; dibenzothiophen sulphone (25mM) in hot ethanol. Ethanol and acetonitrile in the amounts introduced with the inhibitors had no effect on enzyme rates.

Gel electrophoresis

After treatment with diphenylene[^{125}I]iodonium as described above, 0.15ml samples were treated with 20μl of 10% (w/v) sodium dodecyl sulphate, 20μl of 10% (v/v) 2-mercaptoethanol, 20μl of 1M-sucrose and 2μl of 0.4% Bromophenol Blue, and incubated at 100°C for 2min. For analysis of labelled polypeptides, samples contaning 150μg of protein in a volume of less than 150μl were electrophoresed on 12cm×6mm (internal diam.) gels containing 12.5% (w/v) acrylamide and 0.34% bisacrylamide at 5.5mA/gel for 16h (Ragan, 1976*a*), by the procedure of Weber & Osborn (1969). Alternatively, samples were electrophoresed through a stacking gel into a running gel of the above composition, by using the buffer system J4179 of Jovin *et al.* (1971) as described by Neville & Glossmann (1974). For measurements of the incorporation of radioactivity into the major component as a function of diphenylene[^{125}I]-iodonium, an abbreviated procedure was used. Dissociated samples containing 75–100μg of protein were electrophoresed on 7cm×4mm (internal diam.) gels of the same composition by using the Weber & Osborn (1969) procedure at 8mA/gel for 4h. A migration of the dye front of 4cm provided adequate separation.

Radioactivity determinations

After electrophoresis, gels were removed from their tubes and sliced in the semi-frozen state into 1.8mm slices. Slices were then counted for radioactivity in a Beckman Biogamma counter for 10min.

Determination of end points in diphenylene[^{125}I]-iodonium titrations

The extent of covalent modification by diphenylene[^{125}I]iodonium in titration experiments was analysed by using a hyperbolic equation of the type:

$$n = \frac{N \cdot D}{K + D}$$

where n is the observed diphenyleneiodonium binding, N is the binding at saturation, D is the diphenyleneiodonium concentration and K is a constant required to fit the data to a hyperbolic form. K has dimensions of molarity, but its meaning is more complex than a simple dissociation constant associated with a binding phenomenon. The data were fitted by using an unweighted least-squares regression analysis (Wilkinson, 1961).

Extraction of phospholipids, and thin-layer chromatography

Complex I (20–30mg of protein/ml of 0.67M-sucrose/50mM-Tris/HCl, pH8.0 at 0°C) was treated with

1mM-diphenylene[^{125}I]iodonium for 20min at 21°C. Then 20vol. of cold chloroform/methanol (2:1, v/v) was added and protein removed by centrifugation (5000*g*, 5min). The supernatant was evaporated to dryness under reduced pressure, and the residue re-extracted with 4vol. of chloroform/methanol. After centrifugation as above, the supernatant was evaporated to dryness and dissolved in 0.4vol. of chloroform/methanol. The extract (5μl) was chromatographed on silica-gel thin-layer plates (DC Fertigfolien F1500 LS254 Kieselgel; Schleicher und Schüll, Dassel, W. Germany) together with the following markers: diphenylene[^{125}I]iodonium (12.5nmol), soya-bean phosphatidylcholine (Ragan & Racker, 1973) (100nmol), soya-bean phosphatidylethanolamine (Ragan & Racker, 1973) (100nmol) and bovine heart cardiolipin (Sigma; 60nmol). Chromatograms were developed with solvents described in the text. After drying, spots were detected with iodine vapour. Radioactive spots were located with a radiochromatogram scanner.

Results

Inhibition of NADH–acceptor oxidoreductase activities of Complex I by diphenyleneiodonium

The inhibition by diphenyleneiodonium of NADH oxidation with a variety of electron acceptors is shown in Fig. 1. NADH–ubiquinone-1 oxidoreductase activity was the most susceptible to inhibition by diphenyleneiodonium. Oxidoreduction with $K_3Fe(CN)_6$ or juglone as acceptor was only slightly inhibited. Unexpectedly, menadione reduction was inhibited at diphenyleneiodonium concentrations similar to those required to inhibit ubiquinone reduction. This result contrasts with previous observations (Holland *et al.*, 1973) in rat liver mitochondria, where menadione reduction was unaffected by diphenyleneiodonium. Further, this result illustrates that the mode of inhibition by diphenyleneiodonium differs from that of rotenone, which does not affect NADH–menadione oxidoreductase (Schatz & Racker, 1966). With four different preparations of Complex I, 50% inhibition of NADH–ubiquinone-1 oxidoreductase was obtained with 20–26nmol of diphenyleneiodonium/mg of protein. This range of values was independent of whether diphenyleneiodonium nitrate or sulphate was used. Inhibition of ubiquinone reduction by diphenyleneiodonium does not follow a simple hyperbolic curve. Rather, inhibition of the last 20–30% of enzyme activity requires a disproportionate increase in inhibitor concentration. The next experiment (Fig. 2) points out that the reaction of diphenyleneiodonium with the enzyme appears to be a bimolecular process. In this case, for Fig. 1, a linear relation between inhibitor concentration and percentage inhibition would be expected up to 100% inhibition. The absence of such a result could be explained by a number of possibilities, including the fact that the reaction had not gone to completion or that there were alternative reactions for diphenyleneiodonium with other anions in the reaction mixture.

The inhibition of Complex I by diphenyleneiodonium was time-dependent (Fig. 2). In this experiment, concentrations of diphenyleneiodonium sufficient to give eventual maximum inhibition were used (1μM-diphenyleneiodonium was equivalent to 100nmol of diphenyleneiodonium/mg of Complex-I protein). A double-reciprocal plot of the apparent rate constants for inhibition versus diphenyleneiodonium concentration gave a straight line which intercepted at the origin (inset, Fig. 2). This is apparently consistent with a bimolecular reaction process (Kitz & Wilson, 1962) and the second-order rate constant was estimated to be 4.5×10^5 M$^{-1}\cdot$min^{-1}. The observation that such a clear-cut result was obtained may be deceptively simple and limited to the range of diphenyleneiodonium concentrations (1–4μM) which had to be used to obtain substantial inhibition within the 2min duration of the assay, which were the conditions used in Fig. 2.

Inhibition of NADH–ubiquinone-1 oxidoreductase activity by other iodonium compounds

Table 1 shows the inibitory effect of a number of iodonium compounds. All were considerably less potent than diphenyleneiodonium. In particular,

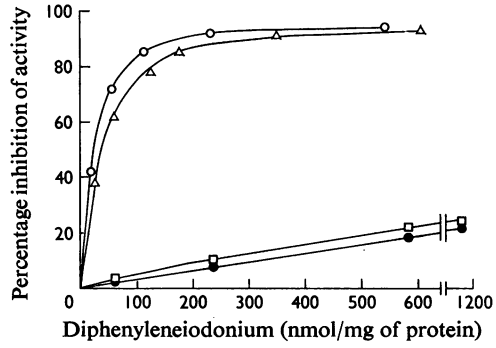


Fig. 1. *Inhibition of NADH–acceptor oxidoreductase activities of Complex I by diphenyleneiodonium*
Assays were performed as described in the Materials and Methods section. Uninhibited activities were as follows: NADH–ubiquinone-1 oxidoreductase (○), 2.94μmol of NADH/min per mg of protein; NADH–menadione oxidoreductase (△), 0.37μmol of NADH/min per mg of protein; NADH–$K_3Fe(CN)_6$ oxidoreductase (□), 54μmol of NADH/min per mg of protein; NADH–juglone oxidoreductase (●), 1.92μmol of NADH/min per mg of protein.

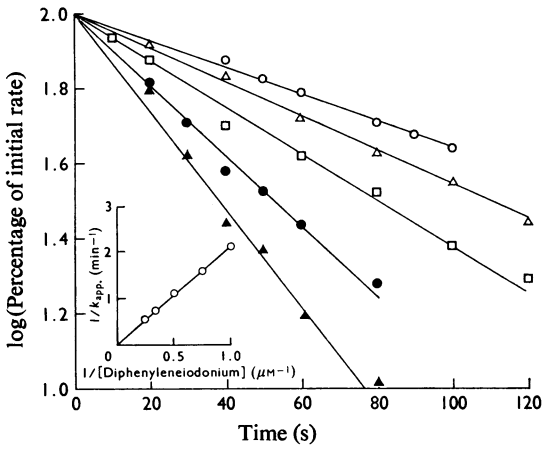
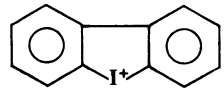
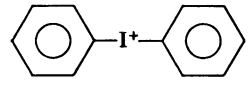
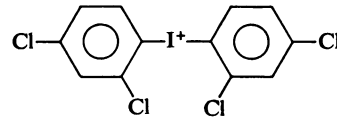
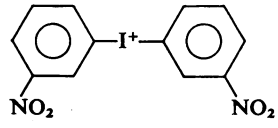
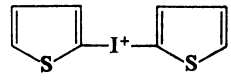


Fig. 2. *Kinetics of inhibition of NADH–ubiquinone-1 oxidoreductase activity by diphenyleneiodonium*

Complex I (9.6μg of protein) was incubated at 25°C in 1ml final volume containing 20μmol of potassium phosphate, pH8.0, 0.4μmol of soya-bean phosphatidylcholine and 0.1μmol of NADH. Reaction was initiated by the addition of 5μl of 16mM-ubiquinone-1 (dissolved in methanol) and 5–20μl of 20mM-diphenyleneiodonium (to give 1–4μM final concentration), and the reaction followed at 340nm. Corrections were made for the non-linearity of the rate in the absence of diphenyleneiodonium. The maximum correction applied to any of the points in the Figure was 24%. Apparent first-order rate constants were determined from the slopes. The insert shows a double-reciprocal plot of the pseudo-first-order rate constants versus diphenyleneiodonium concentration. The following diphenyleneiodonium concentrations were used: 1μM (○); 1.33μM (△); 2μM (□); 3μM (●); 4μM (▲).

Table 1. *Inhibition of NADH–ubiquinone-1 oxidoreductase activity by iodonium compounds*

Complex I (10μg of protein in 1ml final volume) was treated with various concentrations of the indicated compounds as described in the Materials and Methods section.

	Compound		Concentration of inhibitor required for 50% inhibition (μM)
(I)		NO_3^-	0.23
(II)		NO_3^-	88
(III)		Cl^-	1.8
(IV)		HSO_4^-	6.5
(V)		Cl^-	2.5

iodoniumdiphenyl (II), which differs from diphenyleneiodonium (I) only in that the central ring is absent, was a very poor inhibitor. Introduction of electron-withdrawing groups into the benzene rings [as in the tetrachloro (III) and dinitro (IV) derivatives] considerably enhanced the potency of these open-ring compounds.

Inhibition of NADH–ubiquinone-1 oxidoreductase activity by structural analogues of diphenyleneiodonium

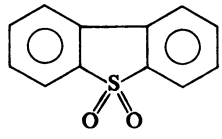
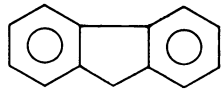
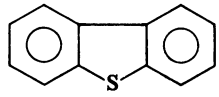
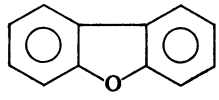
Some compounds containing similar ring structure to diphenyleneiodonium were tested for inhibitory activity (Table 2). Dibenzothiophen sulphone (I) was not inhibitory to NADH–ubiquinone-1 oxidoreductase at a concentration of 250μM. Higher concentrations were not used because of the extremely poor water solubility of this compound and the others in Table 2. In order of increasing potency, fluorene (II), dibenzothiopen (III) and dibenzofuran (IV) all showed some inhibitory activity. Dibenzofuran at a concentration of 500μM caused 86% inhibition of NADH–ubiquinone-1 oxidoreductase activity, but had little (10%) or no inhibitory effect on NADH–$K_3Fe(CN)_6$ and NADH–juglone oxidoreductase activities respectively. Dibenzofuran (100μM) also inhibited the NADH oxidase activity of submitochondrial particles by 50%. Succinate oxidase activity was inhibited, but required 4–5 times as much dibenzofuran for comparable inhibition. The inhibitory effects of dibenzofuran are therefore broadly similar to those of diphenyleneiodonium.

Binding of diphenyleneiodonium to the constituent polypeptides of Complex I

The superior potency of diphenyleneiodonium as an inhibitor of Complex I compared with structurally similar, but chemically unreactive, compounds

Table 2. *Inhibition of NADH–ubiquinone-1 oxidoreductase by analogues of diphenyleneiodonium*

Complex I (10 μg of protein in 1 ml final volume) was treated as in Table 1. N.D., not detected. No inhibition was observed with 250 μM-dibenzothiophen-sulphone (I).

Compound		Concentration required for 50% inhibition (μM)
(I)		N.D.
(II)		650
(III)		320
(IV)		170

such as dibenzofuran suggested the possibility that diphenyleneiodonium might be reacting covalently with some group on Complex I. This would be consistent with the susceptibility of this compound to nucleophilic attack, particularly in a non-polar environment (Banks, 1966).

After treatment of Complex I with diphenylene-[^{125}I]iodonium, significant amounts of radioactivity were associated with some of the constituent polypeptides after separation by gel electrophoresis in the presence of sodium dodecyl sulphate. Most of the radioactivity was associated with a polypeptide of mol.wt. 23500 (Fig. 3). Lesser labelling of polypeptides of mol.wts. 75000, 53000, 42000, 33000 and 15500 was also evident, particularly at higher concentrations of diphenylene[^{125}I]iodonium. As a routine, radioactivity determinations were performed on unstained gels, and radioactive peaks were identified with particular polypeptides by comparing electrophoretic mobilities with a parallel gel which had been stained. Stained gels had lost much of the radioactive label, but sufficient remained to confirm more directly the above identifications. Fig. 3 also shows that treatment of Complex I with diphenylene-[^{125}I]iodonium after solubilization of the protein with sodium dodecyl sulphate resulted in negligible incorporation of radioactivity into any polypeptides.

In the Weber & Osborn (1969) electrophoretic system, the 23500-mol.wt. polypeptide was not readily resolved from adjacent polypeptides (Ragan, 1976*a*, and Fig. 4*a*). A discontinuous system, running at pH9.5 (Jovin *et al.*, 1971), produced superior separations in this molecular-weight range, and the 23500-mol.wt. polypeptide was clearly resolved (Fig. 4*b*). Electrophoresis of labelled Complex I in this system confirmed that the 23500-mol.wt. polypeptide was the major labelled component. From the intensity of Coomassie Blue staining, the 23500-mol.wt. polypeptide accounted for approx. 2.4% of the total protein, which, on the basis of 850000 for the protein molecular weight of Complex I (Ragan, 1976*a*), is equivalent to 0.87 mol of this polypeptide/mol of Complex I (i.e. in all probability 1 mol of this polypeptide/mol of Complex I).

Fig. 5 shows the incorporation of radioactivity into the 23500-mol.wt. polypeptide as a function of diphenylene[^{125}I]iodonium concentration. In this experiment, the incorporation, extrapolated to infinite diphenyleneiodonium concentration, was 1.17 ± 0.10 (S.E.M., $n = 6$) nmol/mg of protein. This preparation of Complex I had an acid-extractable flavin content of 1.10 nmol/mg. Thus the maximum incorporation of label into the polypeptide was 1.06 ± 0.09 (S.E.M., $n = 6$) mol/mol of flavin. Six separate determinations of the stoicheiometry on two preparations of Complex I gave a value of 1.04 ± 0.05 (S.E.M.) mol/mol of flavin. This stoicheiometry is readily explained by the assumption of 1 mol of flavin/mol of Complex I (Ragan, 1976*b*) and 1 mol of the 23500-mol.wt. polypeptide/mol of Complex I. The extent of covalent modification was also confirmed by using diphenylene[^{125}I]iodonium of different specific radioactivities. If radioactive diphenyleneiodonium was diluted 4-fold with non-radioactive diphenyleneiodonium, the incorporation of label into Complex I was decreased by an equivalent amount. This confirms that the incorporation is due to diphenyleneiodonium and not an undetected contaminant.

The relationship between inhibition of ubiquinone reduction and radiochemical labelling of the 23500-mol.wt. polypeptide is shown in Fig. 6. 100% inhibition of the activity corresponded closely to the incorporation of 1 mol of inhibitor/mol of Complex I. However, it is clear that there is not a linear relationship between inhibition and labelling. This observation is a major objection to the proposal that inhibition of enzyme activity is directly correlated with polypeptide labelling.

A contributing factor to this deviation from linearity may be instability of the covalent complex after dissociation of Complex I with sodium dodecyl sulphate. In the experiment of Fig. 7, labelling and

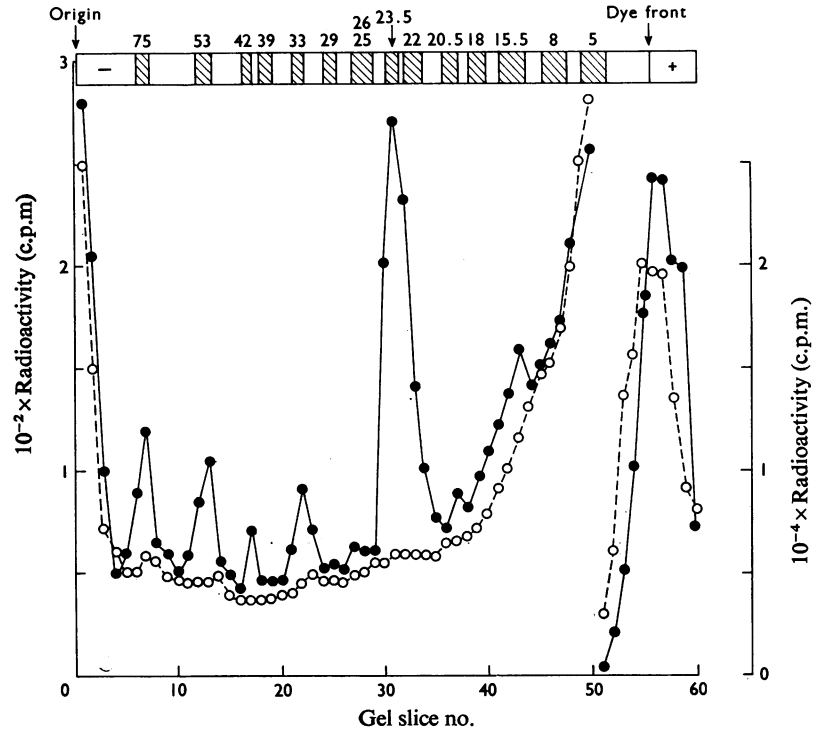


Fig. 3. *Labelling of the polypeptides of Complex I by diphenylene[^{125}I]iodonium*

Complex I was treated with diphenylene[^{125}I]iodonium (271 nmol/mg of protein) before (●) or after (○) dissociation with sodium dodecyl sulphate, and samples containing 135 μg of protein were electrophoresed on 12 cm gels in the Weber & Osborn (1969) system. The specific radioactivity of the diphenylene[^{125}I]iodonium was 9140 c.p.m./mol. Incorporation into the 23 500-mol.wt. polypeptide was 0.072 nmol, or 0.53 nmol/mg of protein. The positions of the various polypeptides of Complex I are indicated together with their molecular weights (in thousands) (from Ragan, 1976*a*).

dissociation of Complex I were performed at different pH values, and after 6h the material was electrophoresed in the Weber & Osborn (1969) system at pH 7.2. Despite the identical degree of inhibition of oxidoreduction in each case, the extent of labelling of the polypeptide was much less at pH 7 than at pH 8 or 9, indicating dissociation of the label at more acid pH. Even at the optimum pH of 8, increasing the time delay between dissociation with sodium dodecyl sulphate and electrophoresis considerably decreased the extent of labelling of the 23 500-mol.wt. polypeptide (Table 3). In all cases, changes in the degree of labelling of the 23 500-mol.wt. polypeptide were paralleled by changes in the labelling of the other polypeptides (Fig. 7).

Despite the failure to show good correlation between the extent of inhibition of NADH–ubiquinone-1 oxidoreductase and the extent of labelling of the 23 500-mol.wt. polypeptide, it was clearly the only polypeptide to take up stoicheiometric quantities of inhibitor in the concentration range used. As shown in Fig. 1, diphenyleneiodonium inhibited other activities of Complex I at higher concentrations of the inhibitor, and this may be related to the low labelling of other polypeptides, which was relatively more pronounced at higher concentrations of diphenylene[^{125}I]iodonium.

Binding of diphenyleneiodonium to the phospholipids of Complex I

In view of the higher than stoicheiometric concentrations of diphenyleneiodonium required to inhibit Complex I, it is possible that the excess was binding to some other constituent, such as the phospholipids, which are present in large amounts relative to protein (Hatefi & Rieske, 1967). Both Holland *et al.* (1973) and Gatley & Sherratt (1976)

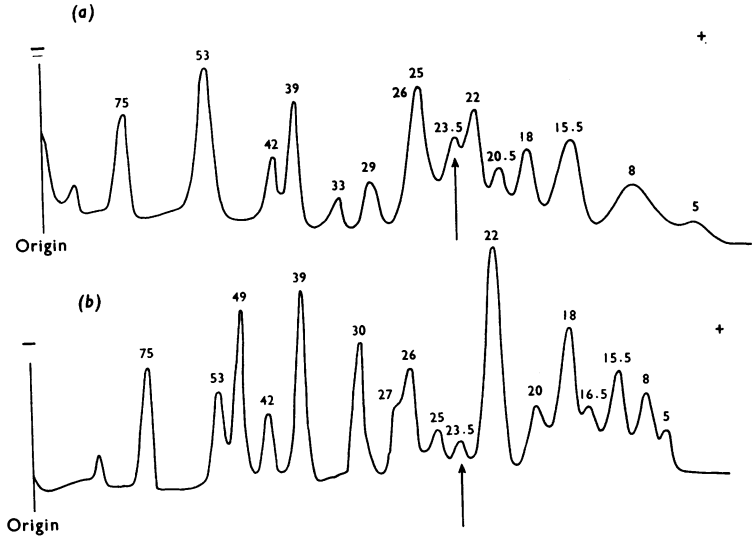


Fig. 4. *Electrophoretic separation of the constituent polypeptides of Complex I*

Complex I (100 μg of protein) was dissociated with sodium dodecyl sulphate and electrophoresed (*a*) as described by Weber & Osborn (1969) or (*b*) as described by Neville & Glossman (1974). Molecular weights (in thousands) in (*a*) were from Ragan (1976*a*), or in (*b*) were redetermined by the same procedure. The two polypeptides of apparent mol.wt. 53000 in (*a*) separate in (*b*) into a 53000- and 49000-mol.wt. polypeptide. The 33000- and 29000-mol.wt. polypeptides in (*a*) co-migrate with an apparent mol.wt. of 30000 in (*b*). The 26000-mol.wt. polypeptide in (*a*), which is scarcely resolved, is better separated in (*b*), with an apparent mol.wt. of 27000. Additional polypeptides in (*b*) which are not resolved in (*a*) are the 25000- and 16500-mol.wt. polypeptides. The arrows indicate the major labelled polypeptide in each system.

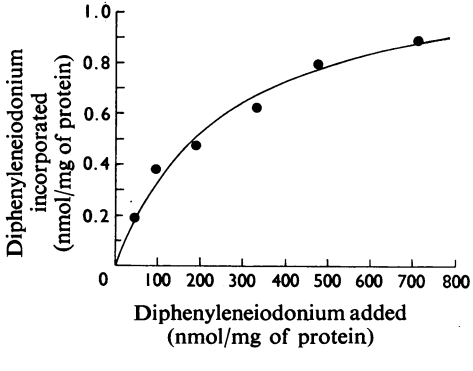


Fig. 5. *Labelling of the* 23500-*mol.wt. polypeptide by diphenylene*[^{125}*I*]*iodonium*

Complex I was treated with the indicated amounts of diphenylene[^{125}I]iodonium (8980c.p.m./mol) and samples containing 82 μg of protein were electrophoresed on 7cm gels as described in the Materials and Methods section. The solid line indicates the computed line of best fit.

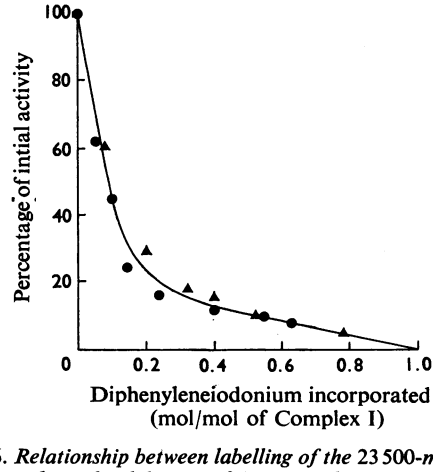


Fig. 6. *Relationship between labelling of the* 23500-*mol.wt. polypeptide and inhibition of NADH–ubiquinone oxidoreductase*

Data were taken from experiments of the type shown in Figs. 1 and 5. Results from two experiments were combined.

concluded that diphenyleneiodonium interacted with membrane lipids. In view of the dependence of NADH–ubiquinone-1 oxidoreductase activity on the presence of phospholipids (Ragan & Racker, 1973), it also possible that the inhibitory effect of diphenyleneiodonium is due to some specific interaction with phospholipid, although the concentration of phospholipid in Complex I (250nmol/mg of protein; Ragan & Racker, 1973) is considerably greater than the concentration of diphenyleneiodonium for 50% inhibition (20–26nmol/mg of protein). Interaction of diphenyleneiodonium with phospholipid might also provide an explanation for the observation that all the radioactivity in experiments such as that of Fig. 3 was either associated with polypeptides or migrated with the dye front to the anode, whereas free diphenyleneiodonium would be expected to migrate to the cathode.

After incubation of Complex I with diphenylene-[^{125}I]iodonium, the phospholipids were extracted with chloroform/methanol (2:1, v/v) and subjected to t.l.c. on silica. No radioactivity was associated with the three principal phospholipids of Complex I, and the single radioactive spot had the same R_F value as free diphenyleneiodonium (Table 4) in three different solvent systems. It appears therefore that no stable complex of diphenyleneiodonium and phospholipid was produced. The migration of diphenyleneiodonium to the anode during electrophoresis is probably due to interaction with dodecyl sulphate anions. This explanation was supported by the observation that diphenyleneiodonium migrated quantitatively to the anode in the absence of Complex I.

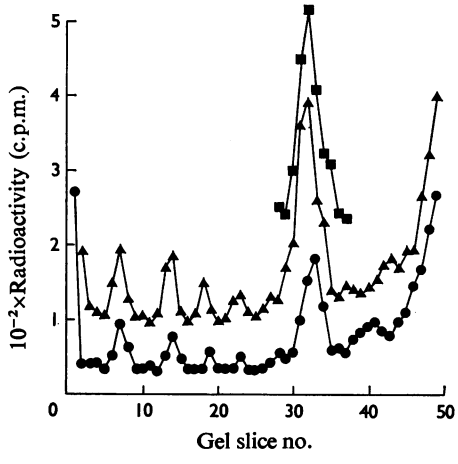


Fig. 7. *Effect of pH on the labelling of the* 23500-*mol.wt. polypeptide by diphenylene*[^{125}*I*]*iodonium*

Complex I was labelled with diphenylene[^{125}I]-iodonium (240nmol/mg of protein, specific radioactivity 11460c.p.m./nmol) as described in the Materials and Methods section, except that the buffer was 0.1M-sodium phosphate, pH7.0 (●), 0.1M-Tris/H_2SO_4, pH8.0 (▲), or 0.1M-Tris/H_2SO_4, pH9.0 (■), After assay of NADH–ubiquinone-1 oxidoreductase activity, samples containing 150μg of protein were electrophoresed on 12cm gels, starting 4h after addition of sodium dodecyl sulphate. Only that portion of the gel containing the 23500-mol.wt. polypeptide was counted for radioactivity for the pH9.0 sample. For clarity, the graphs for the pH8 (▲) and pH9 (■) samples are displaced vertically by 50 and 200c.p.m. respectively.

Table 3. *Time-dependence of the dissociation of label from the* 23500-*mol.wt. polypeptide*

Complex I was treated with diphenylene[^{125}I]-iodonium (300nmol/mg of protein), and dissociated with sodium dodecyl sulphate. The times include the time for electrophoresis.

Time after dissociation with sodium dodecyl sulphate (h)	Label associated with the 23500-mol.wt. polypeptide (mol/mol of Complex-I flavin)
6	0.54
30	0.32
54	0.18

Table 4. *T.l.c. of phospholipids from Complex I labelled with diphenylene*[^{125}*I*]*iodonium*

Lipid extracts and markers were prepared as described in the Materials and Methods section. Solvent systems were as described by Rouser & Fleischer (1967). Abbreviations: PE, phosphatidylethanolamine; PC, phosphatidylcholine; CL,cardiolipin; DPI, diphenyleneiodonium.

Solvent system	R_F values				
	PE	PC	CL	DPI	Radioactive spot
Butan-1-ol/acetic acid/water (3:1:1, by vol.)	0.61	0.41	0.68	0.56	0.57
Chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, by vol.)	0.53	0.34	0.54	0.63	0.63
Chloroform/methanol/aq. 28% (w/v) NH_3 (13:7:1, by vol.)	0.61	0.47	0.68	0.52	0.53

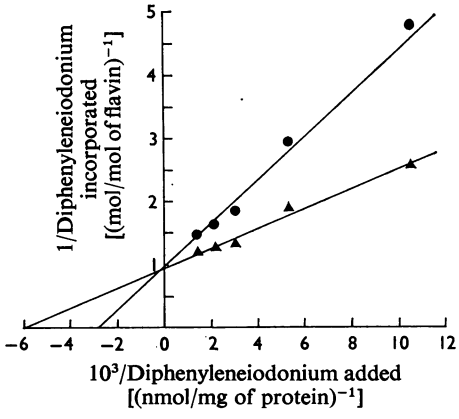


Fig. 8. *Effect of rotenone on the labelling of the* 23500-*mol.wt. polypeptide by diphenylene*[^{125}*I*]*iodonium*
Complex I, with (▲) or without (●) prior incubation for 30min at 20°C with 550nmol of rotenone/mg of protein, was treated with the indicated amounts of diphenylene[^{125}I]iodonium for 20min at 20°C. After dissociation with sodium dodecyl sulphate, samples containing 0.085nmol of acid-extractable flavin were electrophoresed on 7cm gels. Binding is expressed as mol/mol of flavin in this experiment. Specific radioactivity was 8650c.p.m./nmol. Solid lines indicate the computed lines of best fit.

Effect of rotenone on the labelling of the 23500-*mol. wt. polypeptide by diphenylene*[^{125}*I*]*iodonium*

Rotenone (80μM, or 550nmol/mg of Complex-I protein) increased the incorporation of diphenylene-[^{125}I]iodonium into the 23500-mol.wt. polypeptide. As shown in Fig. 8, the effect of rotenone was on the apparent affinity for diphenyleneiodonium and not on the maximum extent of labelling obtained by extrapolation to infinite concentration of diphenyleneiodonium. Thus the concentration of diphenyleneiodonium required to label this polypeptide to 50% of the maximum extent was lowered from 350 to 170nmol/mg of protein by inclusion of rotenone. In an experiment where rotenone was added after labelling Complex I with diphenylene[^{125}I]-iodonium and dissociation with sodium dodecyl sulphate, the amount of label associated with the 23500-mol.wt. polypeptide was unaffected. This established that rotenone was not exerting its effect by decreasing the rate of breakdown of the covalent complex after solubilization with sodium dodecyl sulphate. To investigate the specificity of the rotenone effect, we looked at the effect of a similar concentration of another inhibitor of NADH–ubiquinone oxidoreductase with lower affinity. In the presence of 200μM-dibenzofuran (apparent K_i 170μM; Table 2), the labelling of the 23500-mol.wt. polypeptide by diphenylene[^{125}I]iodonium (apparent K_i 0.23μM, Table 2) in the concentration range 40–700μM was very slightly decreased. This can be attributed to displacement of diphenyleneiodonium by dibenzofuran.

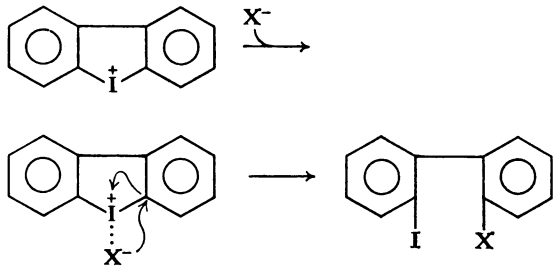
Effect of chaotropic agents on the labelling of the 23500-*mol.wt. polypeptide by diphenylene*[^{125}*I*]-*iodonium*

Chaotropic agents solubilize a low-molecular-weight NADH dehydrogenase and a non-haem-iron-containing fraction from Complex I (Hatefi & Stempel, 1967). Certain subunits of Complex I are quantitatively solubilized (Ragan, 1976*a*), but apparently not the 23500-mol.wt. polypeptide. Moreover, the high-affinity inhibition site for rotenone is destroyed by chaotropic agents (Hatefi *et al.*, 1969).

After labelling Complex I with diphenylene[^{125}I]-iodonium (305nmol/mg of protein), 0.58mol/mol of flavin was found to be incorporated into the 23500-mol.wt. polypeptide after analysis by gel electrophoresis. After treatment with 0.5M-$NaClO_4$ for 10min at 30°C, this was slightly decreased to 0.50mol/mol of flavin. After separation of the soluble and particulate fractions by centrifugation (100000*g*, 20min), the labelled polypeptide of mol.wt. 23500 was exclusively (48% of total recovery) located in the particulate fraction. The poor recovery can be attributed to dissociation of the label during centrifugation and resuspension of the particulate fraction. These results confirm that the major labelled polypeptide was not solubilized by chaotropic agents. In an experiment where diphenylene[^{125}I]iodonium was added after resolution of Complex I by 0.5M-$NaClO_4$, incorporation of label was only 22% of that found with unresolved Complex I.

Discussion

The original identification of the specific inhibitory action of diphenyleneiodonium on mitochondrial NADH oxidation showed that the inhibitor was very tightly bound to mitochondria (Holland *et al.*, 1973) and the inhibitor could not be completely removed by serum albumin. This contrasts with rotenone which, although a more potent inhibitor, is displaced by this treatment (Horgan *et al.*, 1968). These observations indicated that diphenyleneiodonium might form a covalent bond with a constitutive component of the NADH dehydrogenase complex. Consideration of the chemistry of iodonium compounds suggests that this is quite feasible, since in non-polar solvents (i.e. benzene, dimethylformamide) diphenyleneiodonium may react with nucleophiles according to the sequence of reactions shown in Scheme 1 (Banks, 1966). The stability of the final covalent complex will depend on the exact nature of the nucleophile (X^-). It may be predicted that both mercaptan and



Scheme 1. *Reaction of diphenyleneiodonium with nucleophile in non-polar solvents*

secondary-amine derivatives would be stable; however, derivatives with either carboxyl or imidazole groups will probably have only limited stability. The results of the present work indicate that diphenyleneiodonium does form a specific covalent complex with a polypeptide of mol.wt. 23500 in the NADH dehydrogenase complex, but the derivative formed is only of limited stability. The principal results on inhibition of NADH–ubiquinone reductase are summarized in the following paragraph.

Diphenyleneiodonium specifically labels a polypeptide of mol.wt. 23500 which is stable to electrophoresis, and this provides substantial evidence for the formation of a relatively stable covalent bond. Breakdown of the covalent complex can be demonstrated directly by incubating diphenyleneiodonium-labelled Complex I in sodium dodecylsulphate. Under these conditions there is no further specific binding of diphenyleneiodonium and the complex is observed to decompose (Table 3).

The inhibitory action of diphenyleneiodonium on NADH–ubiquinone oxidoreductase activity shows a reasonable degree of specificity, and this is equally matched by the labelling of Complex I. Estimation of the amount of Coomassie Blue stain in the labelled polypeptide indicated that there is 1 mol of the labelled polypeptide/mol of Complex I. In addition, at saturating diphenyleneiodonium concentration, 1 mol of inhibitor is incorporated/mol of Complex I. The labelling of the 23500-mol.wt. polypeptide is a highly specific reaction. Although other polypeptides are labelled by diphenyleneiodonium, they do not show equimolar stoicheiometry with the molar concentration of Complex I. In addition, the modification of the polypeptide is dependent on the structural integrity of the complex, since denaturation of Complex I with sodium dodecyl sulphate or resolution by chaotropic agents before treatment with diphenyleneiodonium both prevent incorporation into the 23500-mol.wt. polypeptide.

Despite the specificity of the alkylation reaction, it is important to question whether the labelling reaction is directly connected with the inhibition of NADH dehydrogenase. With the present experimental data it is necessary to be highly critical of this connexion. However, we consider that the demonstration that complete inhibition of enzyme activity coincides with the incorporation of 1 mol of diphenyleneiodonium/mol of Complex I is highly indicative of a relationship between these two events. The principal evidence against this proposal must be the non-linear relation between inhibition and binding (Fig. 6). If the two events are related, then the non-linearity can only be accounted for by assuming that modification by diphenyleneiodonium induces a co-operative effect in the activity of NADH–ubiquinone oxidoreductase. Co-operativity in the binding of covalent inhibitors has been reported previously for much simpler systems. Rasool *et al.* (1976) showed that the modification of hexameric bovine liver glutamate dehydrogenase by 4-amino-6-chloro-5-oxohexanoic acid was active-site-directed and highly co-operative. A key requirement for co-operativity within Complex I is that the enzyme should exist in an oligomeric state in which catalytic subunits are linked. The basis for interaction of Complex-I molecules could be their existence as membranous aggregates in the absence of high concentrations of detergents. A similar explanation has been proposed to explain the behaviour of the Ca^{2+}-dependent adenosine triphosphatase of sarcoplasmic reticulum, which exhibits negative co-operativity in the binding of either MgATP or MgADP (Yates & Duance, 1976).

These arguments still leave the possibility that the modification of the 23500-mol.wt. polypeptide is only indirectly connected with the inhibition of NADH–ubiquinone oxidoreductase activity. Thus diphenyleneiodonium might initially bind to a different region of Complex I which is responsible for inhibition. Subsequently, transfer to a hydrophobic region could result in covalent modification of the 23500-mol.wt. polypeptide. Experimentally it has been observed that a number of polypeptides are slightly labelled by diphenyleneiodonium, which may merely reflect the fact that they are further from the primary binding site for diphenyleneiodonium.

The experiment with rotenone points out the fallacy of considering that a single polypeptide contains the binding characteristics for all the inhibitors of NADH–ubiquinone oxidoreductase. Since rotenone and diphenyleneiodonium are similar in their mode of inhibition of NADH–ubiquinone oxidoreductase, it would be expected that if there was a single binding peptide, then rotenone should prevent the binding of diphenyleneiodonium. Since this was not the case (Fig. 8) is shows that rotenone and diphenyleneiodonium must be bound simultaneously. Further, the binding of rotenone must induce a conformational change in Complex I in order to result in enhanced binding of diphenyleneiodonium. At present, the structural studies with diphenylene-

iodonium and its analogues do not provide a clear picture of the potential structure of the receptor region. However, certain points are worth noting. Firstly, the presence of a cationic group substantially increases the inhibitory potency of the reagents, e.g. compare diphenyleneiodonium with fluorene. Secondly, the planar diphenyleneiodonium is a much more potent inhibitor than iodoniumbiphenyl, which will exist in the staggered configuration. It was this observation which initially prompted us to try dibenzofuran, which turned out to be a fairly effective and specific inhibitor of NADH–ubiquinone oxidoreductase. From a chemical viewpoint this structure provides a particularly useful point for the synthesis of new inhibitors, which may enable further probes of the structure of Complex I.

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