The Identification of the Site of Action of *NN'*-Dicyclohexylcarbodi-imide as a Proteolipid in Mitochondrial Membranes

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Mitochondrial membranes were incubated with *NN'*-dicyclohexyl[¹⁴C]carbodi-imide, which irreversibly inhibited the partial reactions of oxidative phosphorylation by 95–100%. Solutions of the membranes were analysed on polyacrylamide gels. Of the radioactivity recovered from the gels 90% was shown to be associated with a single protein of molecular weight about 10 000. The radioactive protein and associated phospholipid was solubilized from the membrane by extraction with chloroform-methanol mixtures and was concentrated 50-fold by solvent fractionation and adsorption chromatography on Sephadex LH-20. Several protein-radioactivity peaks were obtained by Sephadex LH-20 chromatography. However, 90–100% of the radioactivity in each peak was shown to be associated with a single protein similar to the major radioactive protein observed in electrophoretograms of the membrane solutions. It is concluded that dicyclohexylcarbodi-imide inhibits mitochondrial oxidative phosphorylation by reacting covalently with a group on this chloroform-methanol-soluble protein. The possible role of this protein in oxidative phosphorylation is discussed.

We have previously presented evidence which suggests that *NN'*-dicyclohexylcarbodi-imide irreversibly inhibits oxidative phosphorylation by reacting covalently either with an intermediate in oxidative phosphorylation or with a membrane component essential for the functioning of an intermediate (Beechey, Robertson, Holloway & Knight, 1967; Beechey, Cattell, Holloway, Knight & Lindop, 1971; Robertson, Holloway, Knight & Beechey, 1968; Holloway, Robertson, Knight & Beechey, 1966). Consequently a series of experiments was started in which dicyclohexyl[¹⁴C]carbodi-imide was used as a means of labelling the membrane component with which it reacts to facilitate its isolation. It was shown that about 75% of the radioactivity was removed by chloroform-methanol extraction of mitochondrial membranes (Knight, Holloway, Robertson & Beechey, 1968). Preliminary studies indicated that the radioactivity in these extracts was associated with the proteolipid fraction. [Proteolipids are defined by Folch & Lees (1951) as being lipid-protein complexes that are soluble in organic solvents but insoluble in water.]

In this paper we present further evidence which suggests that the carbodi-imide acts as an inhibitor of oxidative phosphorylation by reacting covalently with the protein moiety of a single proteolipid species. A fractionation procedure is described by which a 50-fold concentration of this protein is obtained. Some properties of the protein are

described. Brief details of some of these results have been presented elsewhere (Cattell, Knight, Lindop & Beechey, 1970; Beechey *et al.* 1971).

METHODS

Preparation of mitochondria, mitochondrial membranes and dicyclohexyl[¹⁴C]carbodi-imide-treated membranes. Ox heart mitochondria and mitochondrial membranes (sub-mitochondrial particles) were prepared as described previously by Cattell *et al.* (1970). Mitochondrial membranes were suspended at a protein concentration of 40 mg/ml in a medium containing (final concentrations) 250 mM-sucrose, 1 mM-ATP, 1 mM-succinate, 5 mM-MgSO₄ and 10 mM-tris-sulphate buffer, pH 7.6, and incubated for 16–20 h at 4°C with the required amount of dicyclohexyl[¹⁴C]carbodi-imide. The ability of the carbodi-imide-treated membranes to catalyse the ATP-driven reduction of NAD⁺ by succinate was at least 95% inhibited when compared with control membranes. The membranes were sedimented by centrifuging at 105 000*g* for 30 min and were washed twice with 250 mM-sucrose–10 mM-tris-sulphate buffer, pH 7.6 at 4°C. They were then resuspended in this solution and stored at –20°C.

Chromatography on Sephadex LH-20. Crude mitochondrial proteolipids were chromatographed on a column (2.5 cm × 30 cm) of Sephadex LH-20 which was prepared by the method of Soto, Pasquini, Plácido & La Torre (1969). The column was equilibrated with chloroform and a sample of the crude proteolipid fraction was applied in a small volume of chloroform-methanol (2:1, v/v). It was eluted with the following sequence of solvents: 120 ml of chloroform, 60 ml of chloroform-methanol (15:1, v/v),

60 ml of chloroform-methanol (10:1, v/v), 60 ml of chloroform-methanol (8:1, v/v), 60 ml of chloroform-methanol (6:1, v/v), 60 ml of chloroform-methanol (4:1, v/v), 60 ml of chloroform-methanol (2:1, v/v) and 60 ml of chloroform-methanol (1:1, v/v). Fractions (8–10 ml) of eluant were collected in graduated test-tubes.

Thin-layer chromatography. Phospholipids were chromatographed on pre-coated silica gel t.l.c. plates (Kieselgel F₂₅₄, E. Merck, A.-G., Darmstadt, Germany) with either chloroform-methanol-aq. 32.5% (w/v) NH₃ (17:7:1, by vol.; Cuzner & Davison, 1967) or chloroform-methanol-acetic acid-water (25:15:4:2, by vol.; Skipski, Peterson & Barclay, 1964) as the developing solvent. Molybdenum Blue reagent (Dittmer & Lester, 1964) was used to detect phospholipids.

Dicyclohexyl[¹⁴C]carbodi-imide and *NN'*-dicyclohexyl-[¹⁴C]urea were chromatographed on pre-coated basic alumina t.l.c. plates (Aluminium oxide F₂₅₄, type E; E. Merck, A.-G.) with benzene-ethyl acetate (9:1, v/v) as the developing solvent. A radiochromatogram scanner (Berthold series 600) was used to detect radioactivity on t.l.c. plates.

Polyacrylamide-gel electrophoresis. A modification of the method of Lenard (1970) was used for electrophoresis of mitochondrial membranes and proteolipid fractions in polyacrylamide gels containing sodium dodecyl sulphate. Samples of membranes and proteolipid were dissolved at a protein concentration of approx. 1 mg/ml by incubating at 40°C for 14 h in a solution containing (final concentrations) 3% (w/v) of sodium dodecyl sulphate, 5 mM-EDTA, 5 mM-dithiothreitol and 0.1 M-sodium phosphate buffer, pH 7.0. Suspensions of membranes containing 60 mg of protein/ml were diluted directly into this solution. Solutions of proteolipid were evaporated to near-dryness on a rotary evaporator before addition of the solubilizing solution. Residual organic solvents were then removed under reduced pressure. Glycerol (0.1 vol.) was added to each solution to facilitate layering of samples on top of the gels. Between 50 and 100 µg of protein was loaded on to the gels.

Polyacrylamide gels (6 mm × 100 mm) were prepared from a solution containing acrylamide solution, reservoir buffer solution, 1.5% (w/v) ammonium persulphate and *NNN'*-tetramethyl-1,2-diaminoethane (15:13:5:1.5:0.04, by vol.). The acrylamide solution contained 20% (w/v) acrylamide, 0.6% (w/v) *NN'*-methylene diacrylamide and 1% (w/v) sodium dodecyl sulphate in 0.1 M-sodium phosphate buffer, pH 7.0. The reservoir buffer solution contained 1% (w/v) sodium dodecyl sulphate in 0.1 M-sodium phosphate buffer, pH 7.0. The gels were prerun at 4 mA/gel. After 1 h the upper reservoir buffer solution was removed and 2 µl of 0.5% (w/v) Bromophenol Blue in water and up to 100 µl of sample solution were layered on to the surface of the gels. The gel tubes and the reservoir were carefully filled with buffer solution. Electrophoresis was carried out at 4 mA/gel until the Bromophenol Blue had migrated about 6 cm. The gels were washed in two changes of 50% (w/v) trichloroacetic acid over 20 h to fix the protein and to remove residual sodium dodecyl sulphate, which was found to inhibit staining. The gels were stained for 2 h in 0.25% Coomassie Brilliant Blue in 20% (w/v) trichloroacetic acid. The gels were destained by washing in several changes of 7% (v/v) acetic acid for 4–5 days. Permanent records of

gel patterns were recorded with a Joyce Loebel densitometer.

The distribution of radioactivity in gels was determined by slicing them into 1 mm discs with a gel slicer (The Mickle Laboratory Engineering Co., Gomshall, Surrey, U.K.), dissolving each disc in 1 ml of 28% (w/v) hydrogen peroxide by heating at 80°C for 14 h and measuring the radioactivity in each solution.

Analytical methods. Protein was determined in samples of mitochondrial membranes by the biuret method of Gornall, Bardawill & David (1949) and in samples from organic solvents by the Lowry method as modified by Hess & Lewin (1965). Phosphorus was measured by the method of Chen, Toribara & Warner (1956). Radioactivity was measured in a Packard Tri-Carb liquid-scintillation spectrometer, with Bray's (1960) solution containing Cab-O-Sil (Packard Instrument Co. Inc., La Grange, Ill., U.S.A.) to keep solid material in suspension.

Materials. All chemicals were analytical reagent grade. Organic solvents were distilled before use. Dicyclohexyl-[¹⁴C]carbodi-imide, specific radioactivity 2.9 Ci/mol, and dicyclohexyl[¹⁴C]urea, specific radioactivity 1.4 Ci/mol, were synthesized in these laboratories (Haarhorn, 1968).

RESULTS

Extraction of radioactivity from dicyclohexyl[¹⁴C]-carbodi-imide-treated membranes

Because of the initial lack of success with attempts to isolate proteolipids from chloroform-methanol extracts of dicyclohexyl[¹⁴C]carbodi-imide-treated membranes, we tried a variety of aqueous and organic solvent systems in the hope of developing an alternative extraction and fractionation procedure. These included extractions of dicyclohexyl[¹⁴C]carbodi-imide-treated mitochondrial membranes with solutions at different pH, solutions of urea, ionic detergents, solutions of Triton X-100 containing ammonium acetate and aqueous solutions of 2-chloroethanol, butan-1-ol, pentan-1-ol, acetone, acetic acid and pyridine. Although some of these reagents solubilized a large proportion of the radioactivity they were also effective in solubilizing the bulk of the membrane protein and lipid. Attempts to isolate and purify the components associated with the radioactivity from such solutions were, to varying degrees, unsuccessful. Consequently we decided to return to chloroform-methanol extracts of mitochondria and to investigate the possibility of fractionating mitochondrial membrane proteolipid by using methods introduced by Soto *et al.* (1969) for the fractionation of cat white and grey matter proteolipids.

Determination of extraction conditions. As a first step in this investigation the conditions for extracting radioactivity from dicyclohexyl[¹⁴C]-carbodi-imide-treated membranes were optimized with respect to the volume of chloroform-methanol required, the temperature and the time of extrac-

tion. It was found that the radioactivity extracted was independent of the volume of chloroform-methanol (2:1, v/v) used, provided the volume was greater than the minimum required to give a single phase system with the aqueous membrane suspension (19 vol. of chloroform-methanol:1 vol. of membrane suspension): 6% less radioactivity was extracted at 0°C than at room temperature. Overnight extraction at room temperature resulted in the solubilization of 8% more radioactivity than was obtained during 3 h.

The following procedure was used as a routine. Dicyclohexyl [^{14}C]carbodi-imide-treated membranes were thawed and washed with 250 mM-sucrose-10 mM-tris-sulphate buffer, pH 7.6, and resuspended in this solution at a protein concentration of 60 mg/ml. This suspension was added to 25 vol. of chloroform-methanol (2:1, v/v) at room temperature. After being stirred for 14 h the suspension was filtered through a sintered-glass funnel. This fraction is subsequently referred to as the chloroform-methanol extract. The average percentage of the total radioactivity extracted by 25 vol. of chloroform-methanol at room temperature overnight in four experiments was 76% (values ranged between 73–78%). Less than 1% of the remaining radioactivity was solubilized by a second extraction.

Preparation of a crude mitochondrial proteolipid fraction. A crude mitochondrial proteolipid fraction was prepared by using a modification of the method described by Soto *et al.* (1969). The chloroform-methanol extract was washed by mixing it with one-fifth of its volume of water. The resultant emulsion was separated by centrifuging at 1000g for 1 h at 30°C and the upper aqueous phase removed by suction. A few drops of methanol were added to

clarify the chloroform-methanol phase which was then concentrated under reduced pressure at 35°C to one-fifth of its volume. After cooling this concentrated solution to -20°C 5 vol. of diethyl ether, also at -20°C, was added and the solution was kept at this temperature for 16 h. The resultant precipitate was collected by centrifuging in closed glass centrifuge tubes at 2000g for 30 min at -10°C. The white residue readily dissolved in chloroform-methanol (2:1, v/v) at room temperature to give the crude proteolipid fraction. The average proportions of radioactivity and protein precipitated from washed and concentrated chloroform-methanol extracts by the addition of diethyl ether in four experiments was respectively 74% (values ranged between 63–91%) and 74% (values ranged between 58–95%). Since there was significant variation between experiments several alternative precipitation procedures were tried. When the volume of diethyl ether was increased or the temperature was kept at 4°C the proportion of radioactivity recovered in the precipitate was greatly decreased. When ethanol-ether (1:1, v/v), trichloroacetic acid in ethanol (5%, w/v) or sodium acetate in ethanol (10%, w/v) were used in place of diethyl ether neither radioactivity nor protein was precipitated. Consequently the original precipitation procedure was retained.

It can be seen from the results presented in Table 1 that the chloroform-methanol extraction procedure results in an efficient extraction of the radioactivity from the membrane fraction, but relatively little of the membrane protein is solubilized. The net result is a 27-fold increase in the radioactivity/protein ratio. This ratio is increased again by the washing procedure but falls slightly after the precipitation and concentration of the

Table 1. *Summary of the concentration of the dicyclohexyl [^{14}C]carbodi-imide-binding proteolipid during the preparation of the crude proteolipid fraction*

A crude proteolipid fraction was prepared from a suspension of dicyclohexyl [^{14}C]carbodi-imide-treated membranes (2.08 g of protein) by using the methods described in the text. The values refer to a single experiment; the values in parentheses give the range of values obtained in four experiments.

	Fraction			
	[^{14}C]Carbodi-imide-treated membranes	Chloroform-methanol extracts	Washed chloroform-methanol extracts	Crude proteolipid fraction
% of total radioactivity	100	78 (73–78)	68 (67–70)	43 (43–55)
% of total protein	100	2.9 (2.9–7.2)	1.9 (1.9–4.7)	1.3 (1.3–4.2)
Dicyclohexyl [^{14}C]carbodi-imide (nmol/mg of protein)	0.9 (0.68–1.36)	24.7 (11.8–24.7)	31.7 (10.15–31.7)	29.4 (9.2–29.4)
Concentration factor	1	27.4 (9.0–27.4)	35.2 (11.7–35.2)	32.6 (10.4–32.6)

Fig. 1. Chromatography of a crude proteolipid fraction on Sephadex LH-20. A crude proteolipid fraction containing 21.0 mg of protein was loaded on to the column and eluted with the sequence of solvents shown (the solvent change-over points indicated are approximate). Fractions (8–10 ml) were collected and each fraction was analysed for protein (—), phosphorus (----) and radioactivity (.....). Peaks A–G are referred to in the text. The numbers indicate fractions that were used for further study.

dicyclohexyl[^{14}C]carbodi-imide binding proteolipid by the ether precipitation step. The ether precipitation represents a true concentration step in that a fraction of the radioactivity which is not precipitated is in the form of dicyclohexyl[^{14}C]urea and dicyclohexyl[^{14}C]carbodi-imide which had been extracted along with the carbodi-imide-binding proteolipid. As will be demonstrated later all the radioactivity in the crude proteolipid fraction appears to be bound to a single proteolipid.

Chromatography of crude proteolipid fractions on Sephadex LH-20. A typical resolution of the components of the crude proteolipid fraction obtained by chromatography on Sephadex LH-20 is shown in Fig. 1. The major portions of the radioactivity and protein and a small amount of phosphorus-containing material were eluted as two major peaks (F and G) by mixtures of chloroform–methanol (6:1 and 4:1, v/v). A smaller peak (E) was eluted with chloroform–methanol (8:1, v/v). The bulk of the phosphorus-containing compounds and a small proportion of the protein and radioactivity were eluted in four peaks (A, B, C and D) by chloroform alone and chloroform–methanol (15:1, v/v). The recoveries of the protein and radioactivity from the column were 90% and 74% respectively. Of the radioactivity and the protein recovered from the column 81% and 48% respectively were present in peaks E, F and G.

The radioactivity and phosphorus contents of the principal fractions contained in peaks A, E, F and G are shown in Table 2. The overall concentration of the [^{14}C]carbodi-imide-binding component from the [^{14}C]carbodi-imide-treated mem-

Table 2. Concentration of the dicyclohexyl[^{14}C]carbodi-imide-binding proteolipid by chromatography of a crude proteolipid fraction on Sephadex LH-20

The fractions and peaks are those indicated in Fig. 1.

Fraction	[^{14}C]Carbodi-imide (nmol/mg of protein)	Phosphorus ($\mu\text{g-atom/mg}$ of protein)
Fraction 6 (peak A)	11.3	0.00
Fraction 34 (peak E)	16.1	0.46
Fraction 39 (peak F)	38.1	0.31
Fraction 43 (peak G)	45.1	0.55

branes in fractions 6, 34, 39 and 43 was 12.5-, 16.8-, 42.3- and 50.1-fold respectively.

The elution patterns obtained in four separate experiments consistently showed the major features demonstrated in Fig. 1. A similar elution pattern for protein and phosphorus was also obtained in a control experiment in which a crude proteolipid fraction prepared from mitochondrial membranes that had not been treated with dicyclohexylcarbodi-imide was chromatographed on Sephadex LH-20.

Properties of the fractions obtained by chromatography of the crude proteolipid fraction on Sephadex LH-20

Chromatographic properties of radioactive material in the proteolipid fractions. Although the results of the fractionation experiments indicated that there was a close association of radioactivity with the proteolipid fraction from the [^{14}C]carbodi-imide-

treated membranes, the following experiments were done to show that this radioactivity was neither dicyclohexyl[^{14}C]carbodi-imide nor dicyclohexyl[^{14}C]urea. A control experiment showed that these compounds were eluted by chloroform alone through the Sephadex LH-20 column in contrast with the radioactive material extracted from mitochondrial membranes (see Fig. 1). Samples from each of the radioactive peaks obtained during chromatography on Sephadex LH-20 were also subjected to t.l.c. in a system designed to separate dicyclohexylcarbodi-imide (R_F 0.9) and dicyclohexylurea (R_F 0.4). It was found that the radioactivity remained at the origin in all fractions except the chloroform-methanol extracts and the supernatant remaining after precipitation of the crude proteolipid fraction. In these two fractions small amounts of dicyclohexyl[^{14}C]carbodi-imide and dicyclohexyl[^{14}C]urea were separated from the bulk of the radioactivity which remained at the origin. Thus it was concluded that all of the radioactive material present in the crude proteolipid fraction and the Sephadex LH-20 column fractions was neither dicyclohexyl[^{14}C]carbodi-imide nor dicyclohexyl[^{14}C]urea.

Nature of the phosphate-containing compounds associated with the proteolipid fractions. It was thought that the phosphate-containing compounds in the proteolipid fractions were probably phospholipids because they originate from mitochondrial membranes, which contain a high concentration of phospholipid (Rouser, Nelson, Fleischer & Simon, 1968), and they were extracted by a method originally developed for the separation of lipids from biological materials (Folch, Lees & Sloane-Stanley,

1957). This assumption was supported by the results of t.l.c. of material from peaks B-G.

The R_F values of the phospholipids detected by the Molybdenum Blue reagent after chromatography on silica gel plates with chloroform-methanol-ammonia as developing solvent are given in Table 3. Four phosphate-containing components were detected in the chloroform-methanol extracts and the crude proteolipid fraction and they had R_F values similar to the authentic marker phospholipids, phosphatidylinositol, phosphatidylcholine, phosphatidylethanolamine and diphosphatidylglycerol. Table 3 shows that peaks B, C and D represent a partial separation of the phospholipid components of the crude proteolipid fraction. It is also apparent that diphosphatidylglycerol was the only phospholipid detected in peaks E, F and G. This finding agrees with that of Eichberg (1969) who found that diphosphatidylglycerol was the major phospholipid component of a proteolipid fraction isolated from whole heart tissue.

After spraying with Molybdenum Blue the t.l.c. plates of the crude proteolipid fraction and the fractions from the Sephadex LH-20 column were scanned for radioactivity. It was found that all of the radioactivity applied to the plates remained at the origin. No Molybdenum Blue-staining material was detected at the origin even when the plates were grossly overloaded with respect to phosphorus-containing materials. The results obtained with chloroform-methanol-acetic acid-water as developing solvent were similar to those reported above. In this solvent system the radioactivity was found to streak a short way along the t.l.c. plate. However, no Molybdenum Blue-staining material was detected

Table 3. R_F values of phospholipids in proteolipid fractions from dicyclohexyl[^{14}C]carbodi-imide-treated mitochondrial membranes.

Samples of the indicated fractions eluted from the Sephadex LH-20 column (see Fig. 1) were spotted on to silica gel t.l.c. plates and eluted with chloroform-methanol-aq. 32.5% (w/v) NH_3 (17:7:1, by vol.). After evaporation of the eluting solvents the plates were sprayed with Molybdenum Blue reagent. The plates were also scanned for radioactivity.

Fraction	R_F values of components detected with Molybdenum Blue reagent			
Chloroform-methanol extracts	0.11	0.21	0.33	0.40
Crude proteolipid fractions	0.11	0.23	0.34	0.42
Sephadex LH-20 column fractions				
14 (peak B)		0.24		
17 (peak C)	0.09	0.22	0.35	0.42
20 (peak D)			0.36	
35 (peak E)				0.40
37 (peak F)				0.43
43 (peak G)				0.41
Phosphatidylinositol	0.10			
Phosphatidylcholine		0.21		
Phosphatidylethanolamine			0.34	
Diphosphatidylglycerol				0.41

Fig. 2. The distribution of protein and radioactivity in polyacrylamide gels used for electrophoresis of (a) dicyclohexyl[^{14}C]carbodi-imide-treated mitochondrial membranes, (b) crude proteolipid fraction and fractions obtained by chromatography of a crude proteolipid fraction on Sephadex LH-20, (c) fraction 6 (peak A), (d) fraction 33 (peak E), (e) fraction 39 (peak F), (f) fraction 44 (peak G). Electrophoresis and fractionation of the gels is described in the Methods section. —, Densitometric trace; ----, radioactivity.

in the region containing radioactivity. From these observations it was concluded that the radioactivity in the crude proteolipid fraction and the fractions obtained by chromatography of the crude proteolipid fraction was not associated with the phospholipids present in these fractions. Consequently the radioactivity was assumed to be associated with the proteolipid-protein. The protein nature of the proteolipid fractions from the Sephadex LH-20 column was emphasized by their u.v. and i.r. spectra, which showed characteristic protein absorption bands.

Solubility of the proteolipid fractions. To test the solubility of the material present in peaks E, F, and G, samples of fractions from these peaks were mixed with 5 vol. of diethyl ether at -20°C . The precipitates obtained, which contained over 90% of the radioactivity from each fraction, were collected by centrifugation. Small volumes of different solutions including buffer solutions at pH 1.8, 8.0 and 11.7, sodium deoxycholate (1.5%, w/v, in 0.1M-sodium phosphate buffer, pH 7.0), Triton X-100 (1%, w/v, in 0.1M-sodium phosphate buffer, pH 7.0) and 8M-urea were added to the moist pellets. It was found that the only solutions that were effective in solubilizing all of the radioactivity and particulate material present in the pellets were aqueous sodium dodecyl sulphate (1%, w/v),

chloroform-methanol (2:1, v/v) and phenol-acetic acid-water (2:1:1, w/v/v).

Analysis of radioactively labelled fractions by polyacrylamide-gel electrophoresis. The crude proteolipid fractions and the different protein-radioactivity peaks separated by chromatography on Sephadex LH-20 were investigated by electrophoresis in polyacrylamide gels containing the dissociating agent sodium dodecyl sulphate (Lenard, 1970). In this system the electrophoretic mobility of proteins is related to their molecular weight. After electrophoresis the buffer solutions from above the gels were removed and shown to contain no radioactivity. Since, in addition, no Coomassie Blue-staining material remained on the surface of the gels, it is assumed that all of the protein and radioactivity in the samples penetrated the gels. The position of radioactively labelled material was detected by slicing the gels at 1mm intervals and measuring the radioactivity in each disc. The recovery of radioactivity from the gels was 60–85%. This discrepancy is probably due to the observed diffusion of the protein bands from the gels during destaining. Fig. 2 shows densitometric traces and the distribution of radioactivity in gels obtained after electrophoresis of dicyclohexyl[^{14}C]carbodi-imide-treated membranes and different proteolipid fractions. More than 20 protein bands

were visible in the gels used for electrophoresis of dicyclohexyl[^{14}C]carbodi-imide-treated membranes (Fig. 2a). However, about 90% of the radioactivity in these gels was associated with a single protein band which had a similar electrophoretic mobility to cytochrome *c*. The remaining radioactivity was associated with another protein of higher molecular weight. It can be seen from Fig. 2(b) that there was a trailing of the protein bands in gels of the crude proteolipid fraction but the bulk of the radioactivity was associated with a protein band having the same apparent molecular weight as the principal protein-radioactivity band seen in gels of the dicyclohexyl[^{14}C]carbodi-imide-treated membranes. This protein was the major component of fractions 6, 33, 39 and 44 from peaks A, E, F and G (Fig. 1) respectively (Figs. 2c, 2d, 2e and 2f). Virtually all of the radioactivity recovered from the gels was associated with this protein. Other minor protein bands were observed in the gels but were present in various amounts in the different fractions.

DISCUSSION

Dicyclohexylcarbodi-imide is unique as an inhibitor of mitochondrial ATP synthesis because it has a known structure and a well-documented chemistry (Kurzer & Douraghi-Zadeh, 1967). Previous work has shown that it acts irreversibly at the level of a phosphorylated intermediate (Robertson *et al.* 1968). Thus it has potential as a tool for identifying and elucidating the role of an intermediate in oxidative phosphorylation. Ideally, for this purpose to be achieved it must first be demonstrated that the inhibitor acts at a specific site. The molecule on which this site is located must then be isolated and the chemical nature of the binding site identified.

The reasons for believing that the carbodi-imide acts at a single site in the mitochondrial membrane are as follows. Low concentrations of the carbodi-imide are required to give 95% inhibition of the reactions involving the synthesis or utilization of ATP. This concentration range, 0.6–1.0 nmol/mg of mitochondrial protein, is similar to that of the cytochrome components of the electron transport chain in ox heart mitochondria and hence the concentration at which intermediates in ATP synthesis might be expected to exist (Beechey *et al.* 1967). Also the results of polyacrylamide-gel electrophoresis (Fig. 2a) show that about 90% of the radioactivity bound to dicyclohexyl[^{14}C]carbodi-imide-treated membranes is associated with a single protein or protein subunit. The molecular weight of this protein is about 10000. Further, polyacrylamide-gel electrophoresis of the fractions in which the radioactivity is concentrated demonstrates that the radioactivity is always associated

with a single protein having a similar electrophoretic mobility to the major radioactively labelled protein observed in intact membranes (see Fig. 2).

The observed concentration of the protein and associated radioactivity by procedures designed for the fractionation of proteolipid-protein confirms the previous preliminary observation that the site of action of the carbodi-imide is on the proteolipid-protein fraction of mitochondrial membranes (Knight *et al.* 1968).

Although several protein-radioactivity peaks were obtained by chromatography of a crude proteolipid fraction on Sephadex LH-20, over 90% of the radioactivity in each peak was shown to be associated with a protein of molecular weight about 10000. The reasons for the separation of several protein-radioactivity peaks are not clear. It is possible that the separation is due to the association of a single, common dicyclohexyl[^{14}C]carbodi-imide-labelled protein with different amounts of other proteins or phospholipids. Alternatively, the separation may be an artifact arising from the elution procedure. It is obvious from the volume and nature of the solvents used that the separation is not due to gel-filtration effects, although this phenomenon may account for the partial separation of the phospholipids into peaks B, C and D (Fig. 1). It is more likely that the protein peaks are separated by adsorption chromatography (Soto *et al.* 1969). Whatever the reasons for the separation, chromatography on Sephadex LH-20 is extremely useful as a means of further concentrating the proteolipid-protein, while separating it from the bulk of the phospholipid.

At present we cannot eliminate the possibility that the carbodi-imide may react with high-affinity groups on several different membrane proteins of similar molecular weight. However, because of its complexity we feel that this interpretation is less probable.

The carbodi-imide may interact with its site of action in the following ways: (1) covalent bonding resulting in the formation of a stable adduct; (2) reaction with functional groups resulting in the formation of an anhydride bond and *NN'*-dicyclohexylurea; (3) hydrophobic bonding; (4) hydrogen bonding. It is improbable that hydrogen bonding plays a significant role because the treatment of radioactively labelled proteolipid with urea or extremes of pH does not dissociate the radioactivity from the protein. The part played by hydrophobic bonding is probably unimportant. There is no separation of radioactivity and protein on treatment of either dicyclohexyl[^{14}C]carbodi-imide-treated membranes or proteolipid with organic solvents or non-ionic and ionic detergents. In addition, chromatography and electrophoresis of proteolipid derived from dicyclohexyl[^{14}C]carbodi-

imide-treated membranes gave no separation of protein and radioactivity. The interaction of the carbodi-imide and its site of action with the concomitant formation of an anhydride bond and dicyclohexylurea is unlikely because the greater proportion of the radioactivity extracted from dicyclohexyl[^{14}C]carbodi-imide-treated membranes does not behave as dicyclohexylurea during chromatography on Sephadex LH-20 and t.l.c. Thus there is much circumstantial evidence for the formation of a stable adduct between the carbodi-imide and its site of action. This concept is supported by results presented in this and previous papers (Holloway *et al.* 1966; Knight *et al.* 1968) which are summarized as follows. There is a stable association between the radioactivity from the carbodi-imide and the mitochondrial membrane proteolipid. The onset of inhibition of energy-conserving reactions by the carbodi-imide varies with time, pH, temperature and concentration of the carbodi-imide in a way consistent with the formation of a covalent bond (Beechey *et al.* 1967). Also the observation that other hydrophobic carbodi-imides including *NN'*-di-*n*-propylcarbodi-imide, *NN'*-di-*n*-butylcarbodi-imide, *NN'*-di-*n*-pentylcarbodi-imide, *NN'*-di-*n*-octylcarbodi-imide, *NN'*-di-*n*-hexylcarbodi-imide, *NN'*-dibenzylcarbodi-imide and *NN'*-dibenzhydrylcarbodi-imide also inhibit oxidative phosphorylation shows that it is the chemically reactive carbodi-imide moiety of these molecules that is essential for their activity as inhibitors (I. G. Knight, unpublished work). This information, considered with the fact that carbodi-imides are known to form stable adducts with several of the functional groups found on proteins (Carraway & Koshland, 1968; Carraway & Triplett, 1970; Hoare & Koshland, 1966), suggests that dicyclohexylcarbodi-imide acts by combining with a functional group essential for oxidative phosphorylation.

The specificity of the interaction of dicyclohexylcarbodi-imide with a group on the proteolipid rather than similar groups on other membrane proteins may be explained, in part, by the lipophilic nature of both the carbodi-imide and the proteolipid and by the possibility that the proteolipid functional group may be more reactive due to its environment. There is no direct evidence that the isolated radioactively labelled proteolipid is the primary site of action of dicyclohexylcarbodi-imide. However, there is nothing in the well-documented chemistry of this carbodi-imide that would support the hypothesis that the initial adduct is unstable and that the isolated radioactive material is the product of a subsequent rearrangement.

The protein group that reacts with dicyclohexylcarbodi-imide may be involved in oxidative phosphorylation as a phosphate-activating group or as part of a system involved in maintaining the mitochondrial membrane in a conformation necessary for oxidative phosphorylation to occur.

Our current work is aimed at the characterization of this protein group and the elucidation of the role of proteolipid protein in mitochondrial oxidative phosphorylation.

REFERENCES

- Beechey, R. B., Cattell, K. J., Holloway, C. T., Knight, I. G. & Lindop, C. R. (1971). In *Energy Transduction in Respiration and Photosynthesis*. Ed. by Slater, E. C. & Quagliariello, E. Bari: Adriatica Editrice. (in the Press).
- Beechey, R. B., Robertson, A. M., Holloway, C. T. & Knight, I. G. (1967). *Biochemistry, Easton*, **6**, 3867.
- Bray, G. A. (1960). *Analyt. Biochem.* **1**, 279.
- Carraway, K. L. & Koshland, D. E. (1968). *Biochim. biophys. Acta*, **160**, 272.
- Carraway, K. L. & Triplett, R. B. (1970). *Biochim. biophys. Acta* **200**, 564.
- Cattell, K. J., Knight, I. G., Lindop, C. R. & Beechey, R. B. (1970). *Biochem. J.* **117**, 1011.
- Chen, P. S., Toribara, T. Y. & Warner, H. (1956). *Analyt. Chem.* **28**, 1756.
- Cuzner, M. L. & Davison, A. N. (1967). *J. Chromat.* **27**, 388.
- Dittmer, J. C. & Lester, R. L. (1964). *J. Lipid Res.* **5**, 126.
- Eichberg, J. (1969). *Biochim. biophys. Acta*, **187**, 533.
- Folch, J. & Lees, M. (1951). *J. biol. Chem.* **191**, 807.
- Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). *J. biol. Chem.* **226**, 497.
- Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
- Haarhorn, P. A. (1968). *Proc. 2nd int. Conf. on Methods of Preparing and Storing Labelled Compounds, Euratom, Brussels*, p. 123.
- Hess, H. H. & Lewin, E. (1965). *J. Neurochem.* **12**, 205.
- Hoare, D. G. & Koshland, D. E. (1966). *J. Am. chem. Soc.* **88**, 2057.
- Holloway, C. T., Robertson, A. M., Knight, I. G. & Beechey, R. B. (1966). *Biochem. J.* **100**, 79P.
- Knight, I. G., Holloway, C. T., Robertson, A. M. & Beechey, R. B. (1968). *Biochem. J.* **109**, 27.
- Kurzer, F. & Douraghi-Zadeh, K. (1967). *Chem. Rev.* **67**, 107.
- Lenard, J. (1970). *Biochemistry, Easton*, **9**, 1129.
- Robertson, A. M., Holloway, C. T., Knight, I. G. & Beechey, R. B. (1968). *Biochem. J.* **108**, 445.
- Rouser, G., Nelson, G. J., Fleischer, S. & Simon, G. (1968). In *Biological Membranes*, p. 5. Ed. by Chapman, D. New York and London: Academic Press.
- Skipiski, V. P., Peterson, R. F. & Barclay, M. (1964). *Biochem. J.* **90**, 374.
- Soto, E. F., Pasquini, J. M., Plácido, R. & La Torre, J. L. (1969). *J. Chromat.* **41**, 400.