Dibutylchloromethyltin Chloride, a Covalent Inhibitor of the Adenosine Triphosphate Synthase Complex

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1. The synthesis of dibutylchloromethyltin chloride, a new covalent inhibitor of the mitochondrial ATP synthase [oligomycin-sensitive ATPase (adenosine triphosphatase)] complex is described, together with a method for preparing dibutylchloro[³H]methyltin chloride. 2. Studies with the yeast mitochondrial oligomycin-sensitive ATPase complex show that dibutylchloromethyltin chloride inhibits both the membrane-bound enzyme and also the purified Triton X-100-dispersed preparation. 3. F₁-ATPase is not inhibited even at 500 nmol of dibutylchloromethyltin chloride/mg of protein, and the general inhibitory properties are similar to those of triethyltin, oligomycin and dicyclohexylcarbodiimide, known energy-transfer inhibitors of oxidative phosphorylation. 4. Binding studies with yeast submitochondrial particles show that dibutylchloromethyltin chloride antagonizes the binding of triethyl[¹¹³Sn]tin, indicating that there is an interaction between the two inhibitor-binding sites. 5. Unlike triethyltin, inhibition by dibutylchloromethyltin chloride is due to a covalent interaction which titrates a component of the inner mitochondrial membrane present at a concentration of 8–9 nmol/mg of protein. 6. All of the labelled component can be extracted with chloroform/methanol (2:1, v/v), and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the chloroform/methanol extract indicates that the labelled component has an apparent mol.wt. of 6000–8000. However, t.l.c. reveals the presence of only one labelled component which is lipophilic and non-protein and is distinct from the free inhibitor, mitochondrial phospholipids and the dicyclohexylcarbodiimide-binding protein (subunit 9). 7. Inhibition of mitochondrial ATPase and oxidative phosphorylation is correlated with specific interaction with a non-protein lipophilic component of the mitochondrial inner membrane which is proposed to be a co-factor or intermediate of oxidative phosphorylation.

Trialkyltin compounds are potent inhibitors of oxidative phosphorylation (Aldridge & Street, 1964; Sone & Hagihara, 1964; Rose & Aldridge, 1972) and inhibit dinitrophenol-stimulated ATPase* activity as well as P_i-ATP-exchange activity, indicating a mode of action in the ATP synthase complex similar to that of oligomycin and dicyclohexylcarbodiimide. Binding studies by Aldridge & Street (1970) showed that the inhibitory effects of triethyltin in rat liver mitochondria can be correlated with the presence of a high-affinity binding site.

Studies have shown that triethyltin is a potent inhibitor of the yeast oligomycin-sensitive ATPase complex in both the membrane-bound (submitochondrial particles) and purified Triton X-100-dispersed preparations (Cain & Griffiths, 1977). Moreover, binding studies with triethyl[¹¹³Sn]tin have shown that the inhibitory properties are correlated with a high-affinity binding site that is present in submito-

chondrial particles ($K_D = 0.6 \mu\text{M}$; $1.2 \pm 0.3 \text{ nmol/mg}$ of protein) and the purified oligomycin-sensitive ATPase complex ($K_D = 6.9 \mu\text{M}$; 11.9 nmol/mg of protein). Binding is reversible, and the triethyltin can be removed by washing, showing that triethyltin is not covalently bound. Studies on the distribution of the triethyltin-binding sites have shown that the binding sites were associated with membrane-bound components (F₀ component) of the oligomycin-sensitive ATPase complex (Cain & Griffiths, 1977). The triethyltin-binding site is not competed for by oligomycin and venturicidin, indicating separate binding sites for these inhibitors, a finding that is in agreement with biochemical-genetic studies (Lancashire & Griffiths, 1975; Griffiths *et al.*, 1975), which indicate that the oligomycin- and venturicidin-interaction sites involve different protein components from that involved in the trialkyltin-interaction site.

The present paper describes the synthesis and inhibitory properties of a trialkyltin derivative, dibutylchloromethyltin chloride, which has a potentially reactive functional group. Dibutylchloromethyltin chloride, in contrast with triethyltin, binds in an ap-

* Abbreviations: ATPase, adenosine triphosphatase; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; n.m.r., nuclear magnetic resonance; dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.

parently irreversible manner and behaves as a specific covalent inhibitor of mitochondrial ATPase and oxidative phosphorylation (Griffiths *et al.*, 1977a), which can be used as a specific affinity label for a component of the ATP synthase complex. This component is a non-protein lipophilic component of the mitochondrial inner membrane and the ATP synthase complex and has the properties of a cofactor or intermediate of oxidative phosphorylation.

Experimental

Materials

The sources of many of the materials and reagents used have been described previously (Cain & Griffiths, 1977). *n*-Dibutyltin dichloride was purchased from BDH Chemicals, Poole, Dorset, U.K., and redistilled before use [b.p. 140–143°C, 1.33 kPa (10 mmHg)]. $^3\text{H}_2\text{O}$ was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. All inhibitors were added as ethanolic solutions. DL-Dihydrolipoic acid, lipamide, dithiothreitol, 2,3-dimercaptopropanol and reduced glutathione were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Any other reagents were of A.R. grade and all solvents were redistilled before use.

General methods

Growth and isolation of yeast, isolation of yeast mitochondria, preparation and isolation of yeast sub-mitochondrial particles, the assay of mitochondrial protein, ATPase activity and inhibitor-sensitivity (I_{50} values) were essentially as described by Griffiths & Houghton (1974). A partially purified oligomycin-sensitive ATPase preparation (Triton X-100 extract) and a purified oligomycin-sensitive ATPase preparation were prepared from yeast submitochondrial particles by the method of Tzagoloff & Meagher (1971) as described by Cain & Griffiths (1977). F_1 -ATPase was prepared from yeast mitochondria by the method of Tzagoloff *et al.* (1968). Oxidative phosphorylation was assayed as described by Griffiths (1976a).

Radioactive labelling of the peptides of the oligomycin-sensitive ATPase with [^3H]leucine was performed as described by Tzagoloff & Meagher (1971). Polyacrylamide-gel electrophoresis in sodium dodecyl sulphate-containing gels was carried out as described by Weber & Osborn (1969). Radioactivity in polyacrylamide gels was determined by dissolving 1 mm slices in 1 ml of H_2O_2 (6%, v/v) and subsequent scintillation counting in 9 ml of Triton X-100 scintillant (Cain & Griffiths, 1977).

Binding experiments with dibutylchloro[^3H]methyltin chloride and triethyl[^{113}Sn]tin were carried out as described by Cain & Griffiths (1977) in a binding medium containing 0.5 M-sorbitol, 10 mM-

Hepes/KOH, pH 7.5, and 1–2 mg of yeast submitochondrial particles. Any other conditions or procedures are described in the Results section. Liquid-scintillation counting was carried out as described by Cain & Griffiths (1977). ^3H quenching was accounted for by running standards containing known amounts of dibutylchloro[^3H]methyltin chloride and the appropriate quantities of aqueous material being assayed.

Extraction of mitochondrial proteolipid from submitochondrial particles with chloroform/methanol (2:1, v/v) was carried out essentially as described by Cattell *et al.* (1971). Routinely, submitochondrial particles were labelled with dibutylchloro[^3H]methyltin chloride as described below in the Results section and resuspended to 20 mg of protein/ml before extraction for 18 h with 20 vol. of chloroform/methanol at room temperature (20°C). The extracted membranes were removed by centrifugation (5000g for 20 min) and filtering through glass wool. Subsequent procedures, such as washing with water and the ether-precipitation steps, were exactly as described by Cattell *et al.* (1971). Analysis and purification of the chloroform/methanol extract by t.l.c. were carried out as described in the Results section. Liquid-scintillation counting of organic-solvent extracts was carried out by adding 50 μl portions to 1 ml of 2% (w/v) Triton X-100 and heating until the organic solvents had evaporated. The aqueous Triton X-100 solution was then assayed for radioactivity as described previously (Cain & Griffiths, 1977). Protein determinations in organic solvents were carried out by the Lowry method as described by Hess & Lewin (1965).

Preparation of dibutylchloromethyltin chloride, $\text{Bu}_2(\text{CH}_2\text{Cl})\text{SnCl}$

The preparation of dibutylchloromethyltin chloride was essentially as described by Seyferth & Rochow (1955), by reaction of dibutyltin dichloride with an ethereal diazomethane solution. A solution of ethanol-free diazomethane in ether was prepared and assayed as described by Vogel (1956), dried over Na_2CO_3 and stored at -70°C . Redistilled dibutyltin chloride [m.p. 40–42°C; cf. 41–42°C (Poller, 1970)] had an i.r. spectrum identical with that published by Noel *et al.* (1956). A 90 MHz proton n.m.r. spectrum is shown in Fig. 1(a) and the two butyl groups appear as three multiplets of six protons, which is unusual, as one would expect four multiplets corresponding to six, four, four and four protons. However, several tin isotopes have a nuclear magnetic moment, so consequently the unusual features of the spectrum may reflect spin-spin interactions between protons and tin nuclei.

Diazomethane (33 mmol in 120 ml of anhydrous ether) was added dropwise to dibutyltin dichloride (5 g) in anhydrous ether (75 ml) in a 250 ml three-

Fig. 1. 90 MHz proton n.m.r. spectra of dibutyltin dichloride (a) and dibutylchloromethyltin chloride (b)

Dibutyltin dichloride and dibutylchloromethyltin chloride were prepared as described in the Experimental section. Spectra were run at a concentration of 10 mg/ml in C^2HCl_3 on a 90 MHz Bruker n.m.r. spectrophotometer with tetramethylsilane (Me_4Si) as a reference source. Numbers in parentheses refer to the number of protons obtained by integration of the spectrum.

necked flask equipped with a reflux condenser and drying tube. The reaction was carried out at $-70^\circ C$ with vigorous stirring provided by means of a Teflon stirrer bar and magnetic stirrer. A dry N_2 atmosphere was maintained throughout the procedure. After the addition of the diazomethane solution, the bright-yellow mixture was stirred at $-70^\circ C$ for 1 h and then allowed to reach room temperature, stirring being continued until the reaction mixture was colourless. The resultant solution was stored overnight at $-70^\circ C$, and the ether was removed on a rotary evaporator to give a viscous liquid which was fractionally distilled. Two fractions were collected, dibutyltin dichloride [b.p. $78^\circ C$, 40 Pa (0.3 mmHg)] and a mixture of dibutyltin dichloride and dibutylchloromethyltin chloride [b.p. $48-68^\circ C$, 13.3 Pa (0.1 mmHg)]. The latter fraction yielded pure dibutylchloromethyltin chloride after a second distillation {b.p. $102-106^\circ C$ [50.7 Pa (0.38 mmHg)], cf. b.p. $106-110^\circ C$ (50.7 Pa); Seyferth & Rochow, 1955}. The n.m.r. spectrum shown in Fig. 1(b) demonstrates that there is no contamination by the precursor dibutyltin dichloride. The latter point is important, as the dibutyltin dichloride is also a potent inhibitor of the oligomycin-sensitive ATPase (Fig. 2a).

Toxic hazard

It should be noted that trialkyltin compounds are toxic (Barnes & Stoner, 1959). As dibutylchloromethyltin chloride is volatile and a powerful vesicant even in dilute solutions (100 $\mu g/ml$) it should be handled with care, in a fume cupboard, and solutions should not be pipetted by mouth.

Preparation of dibutylchloro[3H]methyltin chloride

Preparation of the labelled derivative was carried out as described for the unlabelled compound, by using diazo[3H]methane, which was prepared by adapting a method for preparing diazo[2H]methane (Gassman & Greenlee, 1973). Diazomethane (40 mmol) in anhydrous ether (100 ml) was stirred for 3 h at $0^\circ C$ with NaOH (40 mg) and 0.8 ml (4 Ci) of 3H_2O . The ethereal solution was dried (over Na_2CO_3) at $-70^\circ C$. The resultant solution was used to prepare dibutylchloro[3H]methyltin chloride as described above. The dibutylchloro[3H]methyltin chloride [b.p. $100^\circ C$, 33.33 Pa (0.25 mmHg)] from the second distillation was shown to be pure by n.m.r. spectroscopy and had a specific radioactivity of 19000 d.p.m./nmol.

Results

Inhibition and binding properties

Dibutylchloromethyltin chloride is a good inhibitor of the oligomycin-sensitive ATPase activity of submitochondrial particles. A comparison with triethyltin, tributyltin, trimethyltin and dibutyltin shows that triethyltin, tributyltin and dibutylchloromethyltin have similar inhibitory potencies on the ATPase activity (Fig. 2a). In contrast, trimethyltin has very little effect even at high concentrations. Surprisingly, the dibutyltin compound is also a good inhibitor, being one-third as potent as dibutylchloromethyltin chloride, a result which underlies the necessity of rigorously purifying the dibutylchloromethyltin chloride.

Dibutylchloromethyltin chloride is also a potent inhibitor of mitochondrial oxidative phosphorylation, with an I_{50} value of 6 nmol/mg of protein and maximal inhibition at 15–20 nmol/mg of protein.

The yeast mitochondrial oligomycin-sensitive ATPase complex can be readily solubilized from the mitochondrial inner membrane by Triton X-100 extraction, as described by Tzagoloff & Meagher (1971). Fig. 2(b) demonstrates that dibutylchloromethyltin chloride inhibits the partially purified Triton X-100 extract and the purified oligomycin-sensitive ATPase preparation, as was demonstrated for triethyltin (Cain & Griffiths, 1977). In contrast, dibutylchloromethyltin chloride does not inhibit the F_1 -ATPase, even at a concentration of 500 nmol/mg

Fig. 2. Inhibition of detergent-solubilized oligomycin-sensitive ATPase and membrane-bound oligomycin-sensitive ATPase by dibutylchloromethyltin chloride

(a) Inhibition of oligomycin-sensitive ATPase activity (specific activity, $5 \mu\text{mol/min per mg}$ of protein) in submitochondrial particles by dibutylchloromethyltin chloride (○), triethyltin sulphate (●), dibutyltin dichloride (□), trimethyltin chloride (▲), tributyltin chloride (■) and tripropyltin chloride (△). (b) Inhibition of membrane-bound and soluble oligomycin-sensitive ATPase by dibutylchloromethyltin chloride: ○, in submitochondrial particles (specific activity $5 \mu\text{mol/min per mg}$ of protein); △, in Triton X-100 extract (specific activity $9.0 \mu\text{mol/min per mg}$ of protein); □, in purified oligomycin-sensitive ATPase (specific activity $27 \mu\text{mol/min per mg}$ of protein). ATPase assays were carried out at pH 9.5 at 30°C , and P_i release was measured as described by Cain & Griffiths (1977). The enzyme preparations used are described in the Experimental section and were preincubated with inhibitor for 10 min before initiating the reaction with ATP.

of protein, demonstrating the requirement for the F_0 components of the oligomycin-sensitive ATPase for its inhibitory actions. These inhibitory characteristics

of dibutylchloromethyltin chloride are therefore similar to those of triethyltin (Cain & Griffiths, 1977) and other classical energy-transfer inhibitors such as oligomycin and dicyclohexylcarbodi-imide.

If the interaction sites for dibutylchloromethyltin chloride and triethyltin are identical, there should be evidence of competition between the two ligands for the site, according to eqn. (1):

where B is the binding site, L is triethyltin and L^* is dibutylchloromethyltin chloride (the interaction between B and L^* is shown as an irreversible reaction).

These possibilities were tested by preincubating submitochondrial particles with dibutylchloromethyltin chloride and other trialkyltin compounds before adding a low concentration of triethyl[^{113}Sn]tin chloride. As discussed by Cain & Griffiths (1977), this will predominantly titrate the high-affinity binding site, which is responsible for the inhibitory actions. Only dibutylchloromethyltin chloride, tributyltin chloride and tripropyltin chloride inhibit the binding of triethyl[^{113}Sn]tin chloride to submitochondrial particles (Table 1). Oligomycin and venturicidin do not inhibit the binding (Cain & Griffiths, 1977). Trimethyltin chloride, which was markedly less potent than the other trialkyltin compounds as an inhibitor of the ATPase activity, is also a very poor inhibitor of triethyltin binding. In contrast, neither triethyltin, tripropyltin nor tributyltin have any effect on dibutylchloro[^3H]methyltin chloride binding (Table 2). It therefore appears that triethyltin and dibutylchloromethyltin chloride do not bind to the same interaction site. However, the antagonism of triethyltin binding by dibutylchloromethyltin chloride shows that there is obviously an interaction between these two binding sites.

Fig. 3 shows the binding of dibutylchloro[^3H]methyltin chloride to submitochondrial particles. It is apparent that there are at least two different binding sites, a high-affinity binding site which is apparently saturated at around 8–10 nmol/mg of protein and a low-affinity binding site which is present at a much greater concentration. The concentration of this component was determined by incubating particles (2 mg/ml) with dibutylchloro[^3H]methyltin chloride (20 nmol/mg of protein) for 18 h at 0°C (to ensure complete interaction between ligand and binding sites). The particles were then washed by resuspension and re-centrifugation ($100000g$ for 35 min) until all the reversibly bound inhibitor was removed. The remaining tightly bound inhibitor cannot be removed, even with a further five washes with buffer containing 1 mg of bovine serum albumin/ml (control experiments show that the bovine serum albumin will sequester any free or loosely bound inhibitor).

Triethyltin bound to submitochondrial particles and triethyltin inhibition can be removed by washing

Table 1. *Inhibition of triethyl[¹¹³Sn]tin binding to yeast submitochondrial particles by various trialkyltin compounds*
Binding experiments were carried out as described in the Experimental section, except that the submitochondrial particles (2mg of protein) were preincubated with the indicated trialkyltin compounds for 30 min at 0°C before addition of triethyl[¹¹³Sn]tin to a final concentration of 0.16 μM and incubating for a further 30 min.

Inhibitor	Concn. (μM)	Triethyl[¹¹³ Sn]tin bound (nmol/mg of protein)	Inhibition of binding (%)
No inhibitor	—	0.36	0
Tripropyltin chloride	3.7	0.2	45
	11.0	0.16	55
	18.4	0.09	74
	0.5	0.31	14
Tributyltin chloride	2.35	0.29	20
	4.7	0.29	20
	1.6	0.27	26
	3.2	0.21	42
Dibutylchloromethyltin chloride	6.4	0.18	50
	9.6	0.17	53
	16.0	0.16	56
	1.4	0.36	0
Trimethyltin chloride	6.8	0.32	11

Table 2. *Effect of trialkyltin compounds on dibutylchloro-[³H]methyltin chloride binding to yeast submitochondrial particles*

Binding experiments were carried out as described in the Experimental section, except that the submitochondrial particles (2mg of protein) were preincubated with the indicated trialkyltin compounds for 30 min at 0°C before addition of dibutylchloro[³H]-methyltin chloride to a final concentration of 0.25 μM and incubating for a further 30 min.

Inhibitor	Concn. (μM)	Dibutylchloro[³ H]-methyltin chloride bound (nmol/mg of protein)	Inhibition of binding (%)
No inhibitor	—	0.44	0
Triethyltin sulphate	0.8	0.44	0
	1.6	0.46	-6
	2.4	0.44	0
	3.2	0.45	2
	4.0	0.44	0
	40.0	0.43	7
	120.0	0.42	9
	200.0	0.40	13
Tributyltin chloride	0.7	0.44	0
	0.0	0.44	0
	3.3	0.44	0
Tripropyltin chloride	14.4	0.40	13
	45.0	0.39	15
	72.0	0.39	15

the particles with buffer (Cain & Griffiths, 1977), demonstrating that the two types of binding site for triethyltin interact with the inhibitor in a reversible manner. This is not the case with dibutylchloromethyltin chloride. Table 3 shows that the inhibitor

Fig. 3. *Binding of dibutylchloro[³H]methyltin chloride to submitochondrial particles*

The amount bound is plotted against free-ligand concentration. Binding assays were carried out as described in the Experimental section. The inset shows the binding over a wider range of ligand concentration.

dibutylchloromethyltin chloride binds to submitochondrial particles and that the binding can be differentiated into two distinct classes: (1) a dissociable type of binding, where inhibitor can be removed by washing, and presumably corresponds to low-affinity binding; (2) an apparently irreversible binding phenomenon, where inhibitor cannot be removed by washing, owing to a particularly high-affinity binding or covalent reaction with a membrane

Table 3. *Effect of washing on dibutylchloro[^3H]methyltin chloride binding to yeast submitochondrial particles*
Binding assays were carried out as described in the Experimental section. The inhibitor was incubated with the submitochondrial particles for 30 min. The sedimented particles were washed by repeated resuspension in binding buffer and sedimentation at 100000g for 35 min. Two concentrations of the inhibitor were used: (A) a sub- I_{50} concentration of 0.31 nmol/mg; (B) an excess concentration of 31 nmol/mg, i.e. fifteen times the I_{50} concentration.

Number of washes	(A) Amount bound		(B) Amount bound	
	(nmol/mg)	(% of initial)	(nmol/mg)	(% of initial)
0	0.23	100	21.0	100
1	0.20	89	16.8	80
2	0.19	82	13.4	63
3	0.15	65	8.4	40
4	0.16	67	8.2	39

component. A similar finding to that described in Table 3 is shown in Table 4, except that the particles are precipitated by trichloroacetic acid and then washed. The results in Tables 3 and 4 both show that the proportion of applied inhibitor that is irreversibly bound decreases with increasing amounts of inhibitor. It is apparent that the inhibitor is titrating covalently a component in the inner mitochondrial membrane; consequently at low inhibitor concentrations virtually all of the bound inhibitor is bound to this membrane component, whereas at higher concentrations all of the components are titrated and excess of inhibitor is bound to the low-affinity sites, and consequently can be easily removed by washing.

The concentration of this component in five preparations was 8.6 ± 1.3 (s.d.) nmol/mg of protein, and the ATPase activity was maximally inhibited. Dibutylchloromethyltin chloride therefore titrates in an apparently covalent reaction a component(s) of the inner mitochondrial membrane which is (are) present at a concentration of 8–10 nmol/mg of protein. The concentration of dibutylchloromethyltin chloride-binding components is approx. five to six times the concentration of triethyltin-inhibitory sites (Cain & Griffiths, 1977). The inhibition data (Fig. 2b) show that at a concentration of $1 \mu\text{M}$ -inhibitor (10–12 nmol/mg of protein) the ATPase is maximally inhibited. The binding curve (Fig. 3) shows that at this concentration virtually all of the inhibitor would be bound, and, moreover, 60–70% (6–8 nmol/mg of protein) of the bound inhibitor would have reacted with the covalent interaction site. Therefore the concentration of the covalently bound inhibitor would account for the inhibitory properties.

Beechey *et al.* (1967) and Cattell *et al.* (1970, 1971) have shown that the inhibitory actions of dicyclohexylcarbodi-imide in ox heart mitochondria are due to the inhibitor binding covalently to a small proteolipid of approx. mol.wt. 10000 which can be extracted from the inner mitochondrial membrane with chloroform/methanol (2:1, v/v). The following series

Table 4. *Effect of trichloroacetic acid precipitation on the binding of dibutylchloro[^3H]methyltin to yeast submitochondrial particles*

Submitochondrial particles (1 mg) were incubated with dibutylchloro[^3H]methyltin chloride for 30 min at 0°C in 3 ml of binding buffer; the protein was then precipitated with 2 ml of 10% (w/v) trichloroacetic acid. The precipitate was separated from the supernatant by filtration through a Whatmans GF/F glass fibre filter. The filter was then washed with 5 ml of water and dried before assaying for radioactivity by liquid-scintillation counting. Control experiments showed that free inhibitor did not bind to the filter.

Dibutylchloro[^3H]methyltin chloride applied (nmol/mg of protein)	Dibutylchloro[^3H]methyltin chloride precipitated (nmol)
3.1	2.2
15.6	3.24

of experiments shows that the dibutylchloromethyltin chloride-binding component can also be extracted with chloroform/methanol (2:1, v/v). The following experiments have all been carried out on a preparation which will be termed 'labelled particles'. This preparation is produced as described above by incubating submitochondrial particles with dibutylchloro[^3H]methyltin chloride (20–30 nmol/mg of protein) for 18 h at 0°C . The loosely bound inhibitor is then washed free by repeated resuspension and centrifugation in buffer. After five washes, only the covalently bound inhibitor remains.

Table 5 shows that the inhibitor-labelled binding component(s) can be readily extracted from the 'labelled particles' with chloroform/methanol (2:1, v/v). In this experiment, 84% of the radioactivity was extracted. The removal of extracted membranes from the organic-solvent phase involves filtering through glass wool and this, coupled with obvious phase-partition effects, invariably leads to some loss of the extract. However, in other experiments over 95% of

Table 5. *Chloroform/methanol extraction of labelled particles*

Labelled particles (20mg/ml) were prepared as described in the Results section and extracted for 18h with 20vol. of chloroform/methanol (2:1, v/v). Washing of the extract and the ether precipitation step were carried out as described by Cattell *et al.* (1971).

Preparation	Protein		$10^{-3} \times$ Radioactivity (c.p.m.)	Inhibitor		Recovery (%)
	(mg)	(%)		(nmol)	(nmol/mg)	
Labelled particles	56.7	100	5926	454	8.0	100
Chloroform/methanol extract	9.4	16.6	4914	378	40.2	84†
Washed chloroform/methanol extract	1.6	3.0	4407	339	211.8	75
Ether supernatant	0.44	0.8	3094	238	540.0	53
Ether precipitate*	1.2	2.1	132	10.2	8.5	2

* Proteolipid material is lost during this step owing to incomplete solubilization of the precipitated material.

† In other experiments a recovery of over 95% has been obtained at this stage.

the radioactivity can be extracted and in fact all of the covalent interaction sites are probably extracted. Washing of the extract with water to remove all non-proteolipid protein removes a very small proportion of the radioactivity. The amount of protein removed by the washing procedure is variable, whereas the radioactivity removed appears to be constant. There does not appear to be a correlation between protein removed and radioactivity lost.

The ether precipitation step shown in Table 5 is known to precipitate only protein (Kuntzel *et al.*, 1975), and Cattell *et al.* (1971) have shown that this procedure precipitates the dicyclohexylcarbodi-imide-binding protein. Table 5 shows that 80% of the protein is precipitated, but only 3% of the radioactivity. Other preparations have yielded similar results for the amount of radioactivity precipitated, but variable amounts of protein precipitated. Thus there is no correlation between the amount of protein and radioactivity precipitated, as would be expected if there was specific covalent labelling of a protein subunit, and, moreover, the binding component has different solubility characteristics from that of the dicyclohexylcarbodi-imide-binding protein (Cattell *et al.*, 1970). Nevertheless, polyacrylamide-gel co-electrophoresis of the washed extract with radioactively labelled oligomycin-sensitive ATPase subunits as molecular-weight markers showed one labelled component which moves just in front of the Bromophenol Blue dye marker and has an apparent mol.wt. of 6000–8000 (Fig. 4), which is usually associated with the presence of subunit 9, the dicyclohexylcarbodi-imide-binding component in the yeast ATP synthase complex. However, analysis of the washed chloroform/methanol extract by t.l.c. (Fig. 5) shows that the radioactivity is associated with a single spot (R_F approx. 0.8). Moreover, the dibutylchloromethyltin chloride-binding component (R_F 0.8) is

Fig. 4. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of dibutylchloro[3H]methyltin chloride-labelled washed chloroform/methanol extract

(a) Dibutylchloro[3H]methyltin chloride - labelled washed chloroform/methanol extract co-electrophoresed with an antisera precipitate of Triton X-100 extract prepared from submitochondrial particles radioactively labelled with [3H]leucine. (b) As (a) but without the labelled chloroform/methanol extract. Gel electrophoresis in 10% (w/v) polyacrylamide gels was performed in sodium dodecyl sulphate as described by Weber & Osborn (1969). Preparation of the antisera precipitate from a radioactively labelled Triton X-100 extract was as described by Tzagoloff & Meagher (1971), except that the alkali treatment was omitted; consequently subunit 9 appears as a 45000-mol.wt. component. Preparation of the labelled chloroform/methanol extract is described in the Results section.

Fig. 5. T.l.c. analysis of dibutylchloro[^3H]methyltin chloride-labelled washed chloroform/methanol extract

T.l.c. analysis was carried out on Merck pre-prepared silica-gel 60 t.l.c. plates ($20\text{ cm} \times 20\text{ cm} \times 0.2\text{ mm}$ thick) with a chloroform/methanol/HCl/water (6500:2500:31:360, by vol.) solvent system. The spots were located with I_2 vapour. Radioactivity was determined by slicing the plates into 0.5 cm strips, deactivating the silica gel with 1 ml of 2% Triton X-100 overnight, then subsequent liquid-scintillation counting with 9 ml of scintillant. (a) Dibutylchloro[^3H]methyltin chloride-labelled washed chloroform/methanol extract. (b) Free dibutylchloro[^3H]methyltin chloride. The migrations of some common phospholipids and subunit 9 are indicated on the Figure: 1, subunit 9; 2, phosphatidylserine; 3, phosphatidylcholine; 4, phosphatidic acid; 5, cardiolipin; 6, phosphatidylethanolamine.

completely separable from the dicyclohexylcarbodi-imide-binding protein or subunit 9 (R_F 0.16) and common phospholipids (Fig. 5).

T.l.c. analysis of the washed extract in two other solvent systems, propan-1-ol or light petroleum (b.p. $60\text{--}80^\circ\text{C}$)/diethyl ether/acetic acid (90:10:1, by vol.), also showed only one radioactive spot, which was separable from the free inhibitor and subunit 9.

The labelled component has been purified by preparative t.l.c. with two solvent systems (chloroform/methanol/HCl/water, 6500:2500:31:360, by vol.; chloroform/methanol/17% (v/v) NH_3 , 2:2:1, by vol.). The purified component did not exhibit a Folin or ninhydrin reaction. Further, treatment of the purified component with 6M-HCl for 18 h *in vacuo* at 105°C , a treatment that has been shown to hydrolyse yeast subunit 9 into its component amino acids, did not yield any amino acids detectable by treatment with dansyl chloride. *N*-Terminal amino acid analysis of the dansylated component as described by Hartley

(1963) does not produce any detectable *N*-terminal amino acids. It appears that the dibutylchloromethyltin chloride binds covalently to a lipophilic non-protein component of the inner mitochondrial membrane which is distinct from the dicyclohexylcarbodi-imide-binding protein (subunit 9).

Preliminary identification of the labelled component

The dibutylchloro[^3H]methyltin chloride-labelled component has not been fully identified, but the following investigations indicate that the reactive component is either a dithiol residue of lipoic acid or a conjugated non-protein form of lipoic acid.

(a) The normally irreversible inhibition of ATPase activity can be specifically relieved by dihydrolipoate (Table 6; Griffiths (1976a,b)). Lipoate, lipoamide, dihydrolipoamide, 2,3-dimercaptopropanol and dithiothreitol are ineffective. Investigations of the reactivity of dibutylchloromethyltin chloride towards dithiols with the 2,6-dichlorophenol-indophenol technique described by Aldridge & Cremer (1955) have shown that all of the dithiols used in the present study will react as rapidly with the inhibitor as does dihydrolipoate. Therefore it is unlikely that the reversal of inhibition is due to a specific 'scavenging' action of dihydrolipoate. Specific titration of a co-factor which is relieved by the addition of the co-factor or a metabolically transformed derivative of the cofactor is indicated.

(b) Dibutylchloromethyltin chloride rapidly reacts with a thiol group of dihydrolipoic acid to give a 1:1 covalent complex, which is indistinguishable from the labelled component in three t.l.c. systems [chloroform/methanol/HCl/water (6500:2500:31:350, by vol.); light petroleum/diethyl ether/acetic acid (90:10:1, by vol.); propan-1-ol] or by descending paper chromatography in butanol saturated with 0.5 M-NH_3 .

(c) Bioassay of lipoic acid (Herbert & Guest, 1975) in submitochondrial particles and the purified oligomycin-sensitive ATPase preparation show that the concentration of lipoic acid is equivalent, within experimental error, to the concentration of dibutylchloro[^3H]methyltin chloride-interaction sites. Detection of the lipoic acid requires prior hydrolysis with HCl, indicating that it is possibly in a conjugated form, although the possibility that the lipoic acid is in a protected non-conjugated form has not yet been ruled out.

Discussion

Dibutylchloromethyltin chloride is a potent specific inhibitor of the yeast oligomycin-sensitive ATPase complex and mitochondrial oxidative phosphorylation which also inhibits oxidative phosphorylation and ATP-driven energy-linked reactions in ox heart

Table 6. *Reversal of dibutylchloromethyltin chloride-inhibited oligomycin-sensitive ATPase activity by dihydrolipoate*

Enzyme preparations were preincubated at 1 mg/ml in 0.25 M-sucrose/20 mM-HCl/1 mM-EDTA, pH 7.5, at 4°C for 5 min with and without dihydrolipoate; 0.1 ml samples were then taken for the ATPase assay. Labelled particles refer to submitochondrial particles labelled with dibutylchloro[³H]methyltin chloride (8 nmol/mg of protein) as described in the Experimental section. Labelled oligomycin-sensitive ATPase refers to a purified oligomycin-sensitive ATPase preparation prepared from labelled particles.

Additions	ATPase activity (% of control)
1. Labelled particles	0
2. Labelled particles+100 nmol of dihydrolipoate	70
3. Labelled particles+100 nmol of dihydrolipoate+5 µg of oligomycin	0
4. Labelled oligomycin-sensitive ATPase	0
5. Labelled oligomycin-sensitive ATPase +100 nmol of dihydrolipoate	100
6. Labelled oligomycin-sensitive ATPase +100 nmol of dihydrolipoate+5 µg of oligomycin	0

mitochondria (Griffiths *et al.*, 1977a), demonstrating that it is a classical energy-transfer inhibitor similar to triethyltin, oligomycin and dicyclohexylcarbodiimide. Unlike its analogues, triethyltin and tributyltin, the inhibitory properties are the result of a covalent interaction with a component of the inner mitochondrial membrane. This labelled component is totally extractable by neutral chloroform/methanol (2:1, v/v), and t.l.c. analysis indicates the presence of only one labelled component, which does not contain amino acids and is thus distinct from the dicyclohexylcarbodiimide-binding protein (Cattell *et al.*, 1970; Partis *et al.*, 1976). Dibutylchloromethyltin thus inhibits mitochondrial energy-transfer reactions by covalent titration of a specific lipid component of the mitochondrial inner membrane. This component has not been finally identified, but is either free lipoic acid or a readily hydrolysable lipophilic non-protein lipoic acid conjugate. As identification rests on t.l.c. analysis and reaction in a bioassay, the possibility that we are dealing with a structurally related species cannot be ruled out at present. It should be noted that other reactive dithiol species such as asparagusic acid (4-carboxy-1,2-dithiolane) have been detected in mitochondria (Yanagawa & Egami, 1976), but we have no analytical data at present which would indicate that we are not dealing with lipoic acid, although some t.l.c. analyses of the crude chloroform/methanol extract and the necessity for hydrolysis before bioassay would indicate that a lipoate conjugate may be present.

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The specific titration by dibutylchloromethyltin chloride of a mitochondrial lipid containing lipoic acid residues, with concomitant inhibition of oligomycin-sensitive ATPase and oxidative phosphorylation, implicates lipoate residues in the mechanism of mitochondrial oxidative phosphorylation. The specific reversal by dihydrolipoate of dibutylchloromethyltin inhibition of oligomycin-sensitive ATPase activity (Table 6; Griffiths, 1976a,b), the inhibition of oxidative phosphorylation by 8-methyl-lipoic acid (Griffiths *et al.*, 1977b), a structural analogue of lipoic acid, and the demonstration of dihydrolipoate-dependent ATP synthesis (Griffiths, 1977b), all point to a specific cofactor role for lipoic acid in oxidative phosphorylation.

The finding that dibutyltin dichloride is also a potent inhibitor of oligomycin-sensitive ATPase (Fig. 2a) also provides supporting evidence for a role for lipoic acid. Previous work by Aldridge & Cremer (1955) showed that diethyltin dichloride reacted with dithiols and that it inhibits pyruvate oxidation in a manner similar to that shown by arsenite. Further studies in our laboratory (K. Cain, R. L. Hyams & D. E. Griffiths, unpublished work) have shown that dibutyltin dichloride has an arsenite-like mode of action on pyruvate and 2-oxoglutarate oxidation and, in addition, has an energy-transfer-inhibitory action with all substrates, which is similar to the classical energy-transfer inhibitors such as oligomycin and triethyltin.

The specific titration of mitochondrial inner-membrane lipoate by dibutylchloromethyltin chloride and the specific reversal of inhibition by dihydrolipoate indicates that this compound is a unique inhibitor probe of energy-transfer reactions in oxidative phosphorylation, which has been of key importance in identifying a lipoic acid moiety and its functional role in energy-conserving membranes.

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