Inhibition of adenosine triphosphatase, 5-hydroxytryptamine transport and proton-translocation activities of resealed chromaffin-granule 'ghosts'

David K. APPS, James G. PRYDE, Raul SUTTON and John H. PHILLIPS
*Department of Biochemistry, University of Edinburgh Medical School, Teviot Place,
Edinburgh EH8 9AG, Scotland, U.K.*

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1. Highly purified resealed chromaffin-granule 'ghosts' were assayed for ATPase and ATP-driven H^+ -translocation and 5-hydroxytryptamine-uptake activities, and for 5-hydroxytryptamine uptake driven by an imposed transmembrane H^+ -gradient. The effects of several inhibitors on these activities were studied. 2. Dicyclohexylcarbodi-imide inhibits all of these activities, but not in parallel; at low concentrations it decreases the permeability of the membrane to protons. 3. 4-Chloro-7-nitrobenzofuran (Nbf-Cl) and silicotungstate inhibit ATP-dependent activities, without effect on 5-hydroxytryptamine uptake driven by an imposed H^+ -gradient. 4. Tributyltin chloride inhibits all of the activities. 5. Treatment of the 'ghosts' with low concentrations of urea inhibits 5-hydroxytryptamine uptake and ATP-dependent generation of a transmembrane H^+ -gradient, without inhibiting ATPase activity. 6. Nbf-Cl and silicotungstate are without effect on the rate of leakage of 5-hydroxytryptamine from preloaded 'ghosts', whereas dicyclohexylcarbodi-imide and tributyltin chloride accelerate the rate of leakage. 7. Treatment of the membranes with ^{14}C -labelled Nbf-Cl labels several proteins; membranes treated with dicyclohexyl[^{14}C]carbodi-imide are labelled predominantly in a protein of low molecular weight, which may be analogous to the mitochondrial H^+ -conducting proteolipid. 8. It is concluded that Nbf-Cl and silicotungstate inhibit the H^+ -translocating ATPase of the granule membrane; that dicyclohexylcarbodi-imide inhibits the ATPase, and inhibits 5-hydroxytryptamine accumulation by accelerating leakage of the amine; and that the effects of tributyltin chloride are due to inhibition of the ATPase, and collapse of the transmembrane H^+ -gradient through OH^- -anion exchange.

Like the secretory vesicles of nerve terminals, the catecholamine storage vesicles of the adrenal medulla, known as chromaffin granules, contain high concentrations of catecholamines and ATP, as well as proteins and other components (for a review, see Winkler, 1976). Accumulation of catecholamines and nucleotides occurs via specific translocases, uptake being driven by an ATPase (EC 3.6.1.3) in the granule membrane, which electrogenically translocates protons to the interior of the granule, creating a transmembrane H^+ -gradient (ΔpH) and membrane potential ($\Delta\psi$) (Njus & Radda, 1978). The mechanism by which transport is coupled to ATP hydrolysis is still unclear, although it appears that both ΔpH and $\Delta\psi$ may be important in driving

uptake of catecholamines (Njus & Radda, 1979; Johnson & Scarpa, 1979; Apps *et al.*, 1980), whereas uptake of ATP may be driven by $\Delta\psi$ alone (Aberer *et al.*, 1978).

The H^+ -translocating ATPase of the chromaffin-granule membrane has not yet been isolated, but it bears some resemblance to the mitochondrial ATPase complex in its sensitivity to dicyclohexylcarbodi-imide (Bashford *et al.*, 1976) and trialkyltin (Apps & Glover, 1978), and its failure to catalyse isotope exchange between ADP and ATP (Apps & Reid, 1977). Dichloromethane treatment of purified bovine adrenal chromaffin-granule membranes solubilizes an ATPase that is structurally and immunologically similar to mitochondrial F_1 -ATPase (Apps & Schatz, 1979). We now report a study of the effects of several ATPase inhibitors on the ATPase and catecholamine-transport activities of resealed chromaffin-granule 'ghosts'. The aim of this work

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonate; Mes, 4-morpholine-ethanesulphonate; Nbf-Cl, 4-chloro-7-nitrobenzofuran.

was to achieve further characterization of the H^+ -translocating ATPase and its coupling to catecholamine transport.

Materials and methods

NN' -Dicyclohexyl $[^{14}C]$ carbodi-imide and 4-chloro-7-nitro $[U-^{14}C]$ benzofuran ($[^{14}C]$ Nbf-Cl) were obtained from CEA, Gif-sur-Yvette, France. $[^{14}C]$ -Methylamine hydrochloride and 5-hydroxy[side chain 2- ^{14}C]tryptamine were from The Radiochemical Centre, Amersham, Bucks., U.K. Triphenyltin chloride was from Cambrian Chemicals. Trimethyltin chloride, tributyltin chloride, NN' -dicyclohexylcarbodi-imide and dodeca-silicotungstic acid were obtained from BDH. Ouabain, phlorizin, 4-chloro-7-nitrobenzofuran and ATP were from Sigma. Triethyltin bromide was the gift of Dr. A. P. Dawson, School of Biology, University of East Anglia, Norwich, Norfolk, U.K.

Resealed chromaffin-granule 'ghosts' were prepared by a modification of the method previously described (Phillips, 1974). Freshly prepared bovine chromaffin granules were lysed at $0^\circ C$ by approx. 50-fold dilution into 10 mM-Hepes/NaOH, pH 7.0; the sucrose concentration was re-adjusted to 0.3 M, the membranes collected by centrifugation (30 min, 23000 rev./min, $4^\circ C$, in a Beckman type 35 rotor, g_{av} . 41000), resuspended in 0.3 M-sucrose to a concentration of approx. 2 mg of protein/ml, and 5 ml portions were layered on discontinuous gradients of 4.5 ml of 0.4 M-sucrose over 2.5 ml of 0.4 M-sucrose in 2H_2O . All solutions were buffered with 10 mM-Hepes/NaOH, pH 7.0. The gradients were centrifuged (30 min, 40000 rev./min, $4^\circ C$ in a Beckman SW41 rotor, g_{av} . 196000) and the 'ghosts' were collected from the interface between sucrose and sucrose/ 2H_2O ; the final protein concentration was 2.5–4.5 mg/ml, and associated catecholamine approx. 150 nmol/mg of protein. Specific ATP-hydrolytic and 5-hydroxytryptamine-uptake activities did not decline significantly on storage of the 'ghosts' for 2 weeks at $-20^\circ C$; in all experiments reported, 'ghosts' had been frozen and thawed once only.

Purified chromaffin-granule membranes were prepared by a procedure published elsewhere (Apps & Schatz, 1979). Mitochondrial contamination was assessed by comparing the specific cytochrome *c* oxidase (EC 1.9.3.1) activity (Mason *et al.*, 1973) of each preparation with that of mitochondria; it was approx. 1.3% for purified membranes (Apps & Schatz, 1979) and 1.7% for resealed 'ghosts'. Bovine heart submitochondrial particles were prepared by the method of Hansen & Smith (1964). Protein was determined by the method of Bradford (1976).

ATP hydrolysis and ATP-dependent uptake of

5-hydroxy $[^{14}C]$ tryptamine were determined in chromaffin-granule 'ghosts' (approx. 0.1 mg of protein/ml) incubated at $37^\circ C$ with 6 mM-ATP, 3 mM-MgSO₄, 59 μ M-5-hydroxy $[^{14}C]$ tryptamine (sp. radioactivity 8.5 Ci/mol), 0.3 M-sucrose, 10 mM-Hepes/NaOH, pH 7.0; 100 μ l samples were withdrawn at intervals for determination of accumulated 5-hydroxytryptamine by membrane filtration and counting for radioactivity (Phillips, 1974) or colorimetric determination of P_i (Muszbek *et al.*, 1977). Uptake of 5-hydroxytryptamine driven by an imposed H^+ -gradient was assayed as described previously (Phillips, 1978); 'ghosts' (0.2–0.4 mg of protein/ml) were preincubated for 15 min at $37^\circ C$ in 0.3 M-sucrose/10 mM-Mes/NaOH (pH 5.6), then diluted with an equal volume of 88 mM-Hepes/NaOH, pH 8.8, in 0.3 M-sucrose, containing 18 μ M-5-hydroxy $[^{14}C]$ tryptamine (sp. radioactivity 58 Ci/mol). The final pH of the incubation medium was 8.0, and the transmembrane ΔpH , measured by $[^{14}C]$ methylamine distribution, was approx. 2.1. Samples (100 μ l) were removed at intervals, diluted in 2.5 ml of 0.3 M-sucrose/10 mM-Hepes/NaOH (pH 7.0) ($0^\circ C$) and rapidly filtered through 25 mm cellulose nitrate filters (Sartorius; 0.45 μ m pore size). The filters were washed with two 2.5 ml portions of the same buffer, dried and counted for radioactivity in toluene-based scintillator.

Leakage of 5-hydroxytryptamine from the 'ghosts' was assayed in 'ghosts' pre-loaded (30 min at $30^\circ C$) in the incubation mixture used to measure ATP-dependent uptake of 5-hydroxytryptamine. These were diluted 50-fold into 0.3 M-sucrose/10 mM-Hepes/NaOH (pH 7.0) ($30^\circ C$) containing the required inhibitor. Samples (5 ml) were removed at intervals and filtered, and the filters were washed, dried and counted for radioactivity. A correction was made for the rate of re-accumulation of 5-hydroxytryptamine after dilution, with 'ghosts' pre-loaded in 50 μ M-5-hydroxy $[^{12}C]$ tryptamine and diluted into 5-hydroxy $[^{14}C]$ tryptamine (0.14 μ M; sp. radioactivity 58 Ci/mol). Uptake of 5-hydroxy $[^{14}C]$ -tryptamine was then measured as before: the concentration and specific radioactivity of 5-hydroxy $[^{14}C]$ tryptamine in the medium after dilution should be very close to the values occurring when ^{14}C -labelled substrate was also present during the loading phase of the experiment.

ATP-dependent formation of a transmembrane H^+ -gradient (ΔpH) was determined by measuring accumulation of $[^{14}C]$ methylamine by the 'ghosts' (Phillips, 1978) incubated (protein concentration, 0.5 mg/ml) at $37^\circ C$ in 6 mM-ATP, 3 mM-MgSO₄, 50 μ M-5-hydroxytryptamine, 40 mM-KI, 2.5 μ M- $[^{14}C]$ methylamine (sp. radioactivity 40 Ci/mol), 30 mM-Hepes/NaOH, pH 7.0. At intervals, 200 μ l samples were removed and filtered without dilution through 13 mm membrane filters, which were

counted for radioactivity, without washing or drying, in scintillator containing 33% (v/v) Triton X-100. In calculating the size of the H^+ -gradient, an internal volume of $4 \mu\text{mol}/\text{mg}$ of protein was assumed (Phillips & Allison, 1978).

Inhibition of 5-hydroxytryptamine uptake, H^+ -translocation and ATP hydrolysis was studied in 'ghosts' that had been preincubated with inhibitor for 5 min at 37°C , at a protein concentration of $0.5\text{--}0.8 \text{ mg}$ of protein/ml. Assays were performed immediately after preincubation, and initial rates were determined from plots of the time course of each reaction, constructed with at least five points. Since most of the inhibitors used were hydrophobic and would be expected to partition into the membrane, relative activities were plotted against the ratio of inhibitor to membrane protein. Where inhibitors were added as ethanolic solutions, the final ethanol concentration did not exceed 1% (v/v), and was the same in all samples.

The effects of urea were studied by incubating 'ghosts' (0.6 mg of protein/ml) with various concentrations of freshly dissolved urea (BDH; Aristar grade) in 0.3 M -sucrose/ 25 mM -Hepes/NaOH (pH 7.8)/ 3 mM -EDTA. After 30 min at 0°C , the membranes were centrifuged (40 min, g_{av} 98 000) at 0°C , the pellets rinsed with 1 ml of 0.3 M -sucrose/ 10 mM -Hepes/NaOH, pH 7.0, and then resuspended in the same medium.

To identify membrane proteins covalently labelled with dicyclohexylcarbodi-imide or Nbf-Cl, purified chromaffin-granule membranes (2.0 mg of protein/ml) were incubated for 18 h at 22°C in 10 mM -Hepes/NaOH/ 0.1 mM -EDTA plus either $50 \mu\text{M}$ -dicyclohexyl ^{14}C carbodi-imide (sp. radioactivity 50 Ci/mol) and 10 mM -ATP, or $25 \mu\text{M}$ - ^{14}C Nbf-Cl (sp. radioactivity 109 Ci/mol). The membranes were diluted with 5 vol. of 10 mM -Hepes/NaOH, pH 7.0, and centrifuged (40 min, g_{av} 235 000). They were re-suspended in the same buffer (2 mg of protein/ml), diluted with 4 vol. of acetone at 0°C , and centrifuged (30 min, g_{av} 27 000) in Corex tubes. Finally the pellets were dissolved in 0.125 M -Tris/HCl, pH 6.8, 2% (w/v) sodium dodecyl sulphate, and proteins were separated by electrophoresis on 15 cm polyacrylamide slab gels, in which the acrylamide concentration increased exponentially from 10% at the top to 15% (w/v) at the bottom (Douglas & Butow, 1976). After electrophoresis, the gels were stained with Coomassie Blue R250, dried on to paper, and autoradiographed with Curix RP-1 film (Agfa-Gevaert).

Results

Activities of resealed chromaffin-granule 'ghosts'

To test the effects of inhibitors on various energy-linked functions of the chromaffin-granule

membrane, we assayed four different activities: (i) ATP hydrolysis, measured by the release of P_i from ATP; (ii) ATP-dependent uptake of 5-hydroxytryptamine; (iii) ATP-dependent H^+ -translocation and establishment of ΔpH , indicated by the distribution of ^{14}C methylamine; and (iv) uptake of 5-hydroxytryptamine driven by an imposed ΔpH , produced by a pH-jump in the absence of ATP. Assays (i) and (ii) were performed on samples of 'ghosts' taken from the same incubation mixture; the activities of preparations used in the experiments reported in the present paper, $\pm\text{s.d.}$, were respectively $116 \pm 30 \text{ nmol/mg per min}$ ($n = 6$) and $12.1 \pm 1.2 \text{ nmol/mg per min}$ ($n = 5$). This ATPase activity of resealed 'ghosts' is somewhat lower than that reported for purified membranes, assayed under different conditions (Apps & Schatz, 1979). Typical time courses are shown in Fig. 1(a), from which it can be seen that, under the conditions of our experiments, the initial rate of 5-hydroxytryptamine uptake was maintained for at least 5 min and that of ATP hydrolysis for even longer.

When measuring the establishment of ΔpH , we included 40 mM -KI in the incubation medium. High concentrations of permeant anion are inhibitory to 5-hydroxytryptamine accumulation (Phillips, 1978), but were necessary to maximize the ΔpH , and to enable H^+ -translocation to be measured by the filtration method. In contrast with the hydrolysis of ATP and uptake of the amine, ΔpH was established very rapidly (Fig. 1a) and the rate of methylamine uptake [$0.27 \pm 0.06 \text{ nmol/mg per min}$ ($n = 4$)], with $2.5 \mu\text{M}$ -methylamine in the medium, started to decline within 30 s. The methylamine concentration ratio (C_{in}/C_{out}) is plotted in Fig. 1(a); the logarithm of this is equal to the ΔpH generated.

In addition to uptake in the presence of ATP, accumulation of 5-hydroxytryptamine may be driven by a transmembrane H^+ -gradient produced by a pH-jump in the absence of ATP. Fig. 1(b) shows a typical time course of such an experiment; uptake ceased after about 1.5 min, the initial rate of uptake [$4.6 \pm 1.8 \text{ nmol/mg per min}$ ($n = 8$)] being maintained for about 30 s.

In the experiments described below, we report the effect of a variety of inhibitors on these values, initial rates being obtained from plots of the type shown in Fig. 1. Data for percentage inhibition refer to decreases of these initial rates; this includes measurements of the rate of H^+ -translocation (as measured by methylamine distribution), so that the data in this case do not refer to the size of the maximal H^+ -gradient achieved.

Inhibition of chromaffin granule membrane ATPase

A number of compounds were screened as inhibitors of ATP hydrolysis and ATP-dependent uptake of 5-hydroxytryptamine (Table 1). Azide,

Fig. 1. ATPase and transport activities of chromaffin-granule 'ghosts'

'Ghosts' were assayed as described (see the Materials and methods section); time courses of (a) ATP hydrolysis (Δ), ATP-dependent 5-hydroxytryptamine uptake (○) and ATP-dependent H⁺ uptake (▲) and (b) 5-hydroxytryptamine uptake, driven by an H⁺-gradient produced by a pH-jump in the absence of ATP (●) are shown.

phlorizin, ouabain and vanadate had only slight effects, and were not studied further, whereas triethyl-, trimethyl- and triphenyl-tin were similar in action to tributyltin.

Fig. 2. Effects of Nbf-Cl on activities of chromaffin-granule 'ghosts'

'Ghosts' were preincubated with various concentrations of Nbf-Cl, and assayed for ATPase activity (Δ), ATP-dependent uptake of 5-hydroxytryptamine (○), ATP-dependent H⁺-translocation (▲) and 5-hydroxytryptamine uptake, driven by a pH-jump (●).

Effects of Nbf-Cl

Fig. 2 shows the effects of Nbf-Cl on ATP-dependent and transport functions of chromaffin-granule 'ghosts'. All of the ATP-dependent functions are strongly inhibited by preincubation for 5 min with low concentrations of the reagent: in contrast, uptake of 5-hydroxytryptamine is insensitive to Nbf-Cl if driven by a pH-jump in the absence of ATP, being only 10% inhibited when ATP-dependent uptake is inhibited 95%.

Effects of dicyclohexylcarbodi-imide

The effects of dicyclohexylcarbodi-imide on chromaffin-granule 'ghosts' (Fig. 3) are complex. ATPase activity is inhibited, in agreement with the report of Bashford *et al.* (1976). ATP-dependent uptake of 5-hydroxytryptamine appears more sensitive to inhibition by dicyclohexylcarbodi-imide than is ATP hydrolysis, being 90% inhibited under conditions

Table 1. Inhibition of ATPase and ATP-dependent 5-hydroxytryptamine-uptake activities of resealed chromaffin-granule ‘ghosts’

‘Ghosts’ were assayed as described in the Materials and methods section, with the inclusion of the inhibitors in the final incubation at the concentrations shown. Percentage activities are expressed relative to controls without inhibitor, but with ethanol where appropriate.

Inhibitor	Concentration (mM)	ATPase	5-Hydroxytryptamine uptake
Azide	3.5	100	68
Dicyclohexylcarbodi-imide	0.10	42	12
Nbf-Cl	0.01	23	13
Ouabain	1.0	98	90
Phlorizin	2.0	96	100
Tributyltin chloride	0.01	31	6
Triethyltin chloride	0.01	29	17
Trimethyltin chloride	0.01	38	27
Triphenyltin chloride	0.01	38	15
Sodium orthovanadate	0.01	87	86

Fig. 3. Effects of dicyclohexylcarbodi-imide on activities of chromaffin-granule ‘ghosts’
For explanation of symbols see the legend to Fig. 2.

cyclohexylcarbodi-imide stimulate the initial rate of [¹⁴C]methylamine accumulation, and only at inhibitor concentrations above 100 nmol/mg of protein is the rate of establishment of the H⁺-gradient inhibited.

Effects of alkyltins

Of the various alkyltin derivatives found to inhibit chromaffin-granule membrane ATPase and transport activities (Table 1), tributyltin was selected for study in detail: its effects are shown in Fig. 4, and are in some ways comparable with those of dicyclohexylcarbodi-imide. ATP hydrolysis, ATP-dependent uptake of 5-hydroxytryptamine and ATP-dependent H⁺-translocation are inhibited, though not in parallel, and transport driven by a pH-jump is also inhibited.

Urea and silicotungstate

Treatment of submitochondrial particles with urea or silicotungstate has been used to selectively remove the F₁-component of the H⁺-translocating ATPase from the membrane (Schatz *et al.*, 1967; Racker *et al.*, 1969), and we investigated the effects of similar treatment of chromaffin-granule ‘ghosts’.

Chromaffin-granule membrane ATPase was found to be fairly resistant to urea, inactivation by 2 M-urea being insignificant (Fig. 5a); however, this concentration of urea produced over 50% inactivation of 5-hydroxytryptamine uptake and of methylamine accumulation. Silicotungstate is a potent inhibitor of the ATPase and of the ATP-dependent functions 5-hydroxytryptamine uptake and H⁺-translocation, whereas uptake driven by an imposed H⁺-gradient is insensitive to this inhibitor (Fig. 5b).

that produce only 50% inhibition of the ATPase; this result is in contrast with that of Bashford *et al.* (1976), who found similar inhibition of both activities. Significantly, uptake driven by an imposed H⁺-gradient is also inhibited (see also Schuldiner *et al.*, 1978). However, low concentrations of di-

Fig. 4. Effects of tributyltin chloride on activities of chromaffin-granule 'ghosts'.
For explanation of symbols see the legend to Fig. 2.

Leakage of 5-hydroxytryptamine from pre-loaded 'ghosts'

If chromaffin-granule 'ghosts' are pre-loaded by incubating with 5-hydroxytryptamine in the presence of ATP, then diluted into iso-osmotic buffer so that the concentrations of 5-hydroxytryptamine and ATP are much less than the K_m values of the carrier and the ATPase, the concentration gradient is dissipated by efflux. The effects on leakage of various inhibitors, included in the dilution medium, are shown in Fig. 6. The experiments were performed at 30°C, to decrease the rate of 5-hydroxytryptamine efflux in the absence of inhibitors, and each trace is corrected for re-accumulation occurring after dilution (see the Materials and methods section). For comparison, the effects of two uncouplers, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone and K^+ plus nigericin, were also investigated.

Fig. 5. Effects of (a) urea and (b) silicotungstate on activities of chromaffin-granule 'ghosts'.
For explanation of symbols see the legend to Fig. 2. The silicotungstate concentration shown is that used in preincubation of the 'ghosts'; it was diluted 10-fold in the assay media. The urea concentration shown is that used in preincubation of the 'ghosts', which were washed by centrifugation before assay.

Covalent modification of membrane proteins

Two of the inhibitors used, Nbf-Cl and dicyclohexylcarbodi-imide, probably exert their effects through covalent modification of proteins in the

Fig. 6. Effects of inhibitors on leakage of 5-hydroxytryptamine from preloaded 'ghosts'

After loading with 5-hydroxytryptamine, 'ghosts' (0.7 mg of protein/ml) were diluted 50-fold into iso-osmotic buffered sucrose, containing the following additions: ○, none; ●, Nbf-Cl (5 μ M); △, carbonyl cyanide *p*-trifluoromethoxyphenylhydraz-one (10 μ M); ▽, silicotungstate (200 μ g/ml); ▲, dicyclohexylcarbodi-imide (50 μ M); ▼, tributyltin chloride (2 μ M); □, nigericin (50 ng/ml) + K₂SO₄ (20 mM).

chromaffin-granule membrane. Both are non-specific reagents, likely to modify more than one protein. The number of proteins reacting was investigated by preincubation of purified chromaffin-granule membranes with dicyclohexyl[¹⁴C]carbodi-imide or [¹⁴C]Nbf-Cl followed by separation of membrane proteins by electrophoresis in the presence of dodecyl sulphate, and identification of radioactive proteins by autoradiography of the dried gel. The results are shown in Fig. 7. Labelling with dicyclohexyl[¹⁴C]carbodi-imide was carried out in the presence of 10 mM-ATP, as this has been found to increase the incorporation of radioactivity (R. Sutton, unpublished work). For comparison, samples of bovine heart submitochondrial particles were also labelled, under the same conditions.

Discussion

The chromaffin-granule ATPase

How similar are the H⁺-translocating ATPases of mitochondria and chromaffin granules? This question is prompted by the observation (Apps & Glover, 1978; Appa & Schatz, 1979) that dichloromethane treatment of purified chromaffin-

Fig. 7. Reaction of [¹⁴C]Nbf-Cl and dicyclohexyl[¹⁴C]carbodi-imide with purified chromaffin-granule membranes

Autoradiograph of ¹⁴C-labelled proteins after separation by dodecyl sulphate/polyacrylamide-gel electrophoresis. (1), Chromaffin-granule membranes labelled with [¹⁴C]Nbf-Cl; (2), chromaffin-granule membranes and (3) submitochondrial particles, labelled with dicyclohexyl[¹⁴C]carbodi-imide. The arrow indicates the top of the separating gel.

granule membranes solubilizes a protein that is in many ways similar to mitochondrial F₁-ATPase. The inhibitors dicyclohexylcarbodi-imide, Nbf-Cl and tributyltin, found in the present work to inhibit chromaffin-granule membrane ATPase, also inhibit mitochondrial ATPase, but since all three are potentially non-specific, this does not necessarily reflect structural similarity between the two enzymes. Indeed, some or all of the inhibitors studied have been reported to inhibit the ATPases of synaptic vesicles (Toll & Howard, 1978), gastric mucosa (Sachs *et al.*, 1976) and the plasma membranes of *Neurospora crassa* (Bowman *et al.*, 1978) and *Schizosaccharomyces pombe* (Delhez *et*

al., 1977), all of which may be H^+ -translocating enzymes. Silicotungstate inhibits the ATPase activity of submitochondrial particles, high concentrations causing dissociation of F_1 -ATPase from the membrane (Racker *et al.*, 1969), but its effects on chromaffin-granule 'ghosts' seem to be due to inhibition of the ATPase, without its dissociation; dodecyl sulphate/polyacrylamide-gel electrophoresis of silicotungstate-treated chromaffin-granule membranes reveals no depletion of the α - and β -subunits of the ATPase, and the membranes are still agglutinated by antibodies against mitochondrial F_1 -ATPase (D. K. Apps & J. G. Pryde, unpublished work). Attempts to reconstitute the ATPase activity of these membranes by addition of mitochondrial F_1 -ATPase have been unsuccessful. Urea (3 M) fails to remove or inactivate the ATPase, suggesting that it may be more firmly attached to the membrane than is the mitochondrial enzyme.

In mitochondria, bacteria and chloroplasts, dicyclohexylcarbodi-imide inhibits ATP-dependent H^+ -translocation by covalent modification of the proteolipid that constitutes the proton-conducting channel (Cattell *et al.*, 1971; Fillingame, 1976; Sigrist-Nelson *et al.*, 1978); alkyltins may also act by binding to the membrane segment of the enzyme, rather than to the F_1 -ATPase itself (Cain & Griffiths, 1977). Does chromaffin-granule ATPase contain a similar proton-conducting proteolipid subunit? Two pieces of evidence suggest that it does. First, treatment of chromaffin-granule membranes with dicyclohexyl[^{14}C]carbodi-imide reveals a major covalently-reacting protein of low molecular weight, with greater mobility in dodecyl sulphate/polyacrylamide-gel electrophoresis than the mitochondrial proteolipid. Reaction of dicyclohexyl[^{14}C]carbodi-imide with a mixture of mitochondrial and chromaffin-granule membranes labelled two electrophoretically distinct proteins; the difference in mobility observed (Fig. 7) is therefore not an artefact of the gel system, and the protein labelled in chromaffin granules cannot be a mitochondrial contaminant (R. Sutton, unpublished work). Secondly, low concentrations of dicyclohexylcarbodi-imide increase the rate of establishment of the ATP-generated transmembrane H^+ -gradient; this could be due to the inhibitor preferentially blocking H^+ -channels with non-functional or detached ATPase, and thus decreasing the H^+ -permeability of the membrane without much inhibition of the H^+ -pump: only at high dicyclohexylcarbodi-imide concentrations, when active H^+ -translocation is extensively inhibited, is development of the H^+ -gradient affected. The stimulatory effects of low concentrations of dicyclohexylcarbodi-imide on photophosphorylation in chloroplasts has been explained similarly (McCarty & Racker, 1967).

Even high concentrations of dicyclohexylcarbodi-imide or tributyltin fail to totally inhibit the chromaffin-granule membrane ATPase, suggesting either that a fraction of the enzyme is dislocated from the H^+ -conducting channel, and hence is unaffected by its blockage, or that the membrane contains more than one ATPase.

Catecholamine translocase

Catecholamine uptake by chromaffin granules is catalysed by a specific permease, as indicated by its stereospecificity and inhibitor sensitivity (Phillips, 1974). A variety of biogenic amines is transported, including 5-hydroxytryptamine as well as catecholamines (Kanner *et al.*, 1979).

It is apparent from Figs. 3 and 4 that dicyclohexylcarbodi-imide and tributyltin have effects on 5-hydroxytryptamine uptake that are not due solely to inhibition of the ATPase, since uptake produced by an imposed H^+ -gradient is also inhibited. Possible explanations for this include direct inhibition of the translocase, dissipation of the transmembrane H^+ -gradient, or acceleration of catecholamine leakage. Measurements of the rate of 5-hydroxytryptamine leakage (Fig. 6) indicate that both dicyclohexylcarbodi-imide and tributyltin increase the rate of leakage above that observed in controls, or in Nbf-Cl- or silicotungstate-treated 'ghosts'.

The mechanism by which 5-hydroxytryptamine leaks from 'ghosts' is unknown. Evidence has been presented that catecholamine uptake occurs by electrogenic exchange of protonated amine with protons (Johnson & Scarpa, 1979); under the conditions used in leakage experiments it is possible that 5-hydroxytryptamine leaves, via the translocase, by reversal of this process, in which case agents that increase the H^+ -permeability of the membrane would promote leakage. However, uncouplers such as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, although they inhibit ATP-driven uptake of catecholamines by chromaffin-granule 'ghosts' (Bashford *et al.*, 1975), do not accelerate leakage. Since uncouplers of this type catalyse electrogenic H^+ -uniport, this suggests that electroneutral dissipation of the H^+ -gradient may be required; it is therefore particularly noteworthy that a combination of K^+ and nigericin, which permits K^+/H^+ exchange, does indeed promote rapid leakage of 5-hydroxytryptamine from the 'ghosts', although nigericin alone has only slight effects on the rate of leakage. It seems likely, therefore, that passage of unprotonated amine through the membrane may take place. This presumably also occurs in H^+ -gradient-driven uptake of catecholamines by liposomes (Nichols & Deamer, 1976). It should be noted, however, that it is not possible to exclude a

specific internal effect of the K^+ that is taken up by the 'ghosts' in the presence of nigericin. The alkyl tins have been shown to catalyse rapid OH^- -anion antiport in membranes (Selwyn *et al.*, 1970) and tributyltin might therefore dissipate the transmembrane H^+ -gradient electroneutrally, by transporting anions from the interior of the 'ghosts'. It is not clear whether high concentrations of anions are accumulated during loading with 5-hydroxytryptamine, since the only anions known to be present are SO_4^{2-} , Hepes ATP^{4-} ; but tributyltin strongly inhibited the generation of the transmembrane H^+ -gradient, as measured by methylamine distribution, since 40 mM-KI was present in these experiments. We tentatively conclude that the effects of this reagent on 5-hydroxytryptamine uptake by the 'ghosts' are due to its effects on the H^+ -gradient.

In contrast, dicyclohexylcarbodi-imide accelerates leakage, although apparently decreasing the H^+ -permeability of the membranes; this is confirmed by ΔpH measurements. This high leakage rate, the mechanism of which is at present obscure, presumably accounts for the effects of dicyclohexylcarbodi-imide on 5-hydroxytryptamine uptake seen in Fig. 3.

Although it is difficult to explain the effects of some of these inhibitors in detail, it is clear that the data are fully consistent with the idea that the ATPase and the catecholamine translocase are separate entities. Thus, Nbf-Cl and silicotungstate abolish ATPase activity, ATP-dependent H^+ -translocation and ATP-dependent uptake of 5-hydroxytryptamine, without affecting the translocase. In contrast, low concentrations of urea inhibit translocation without affecting the ATPase, apparently by increasing the H^+ -permeability of the membrane. This duality of action is strong evidence in favour of the indirect coupling of transport to ATPase activity via the H^+ -gradient. Recently, Maron *et al.* (1979) have reported the differential solubilization of the translocase and the ATPase by cholate treatment of membranes.

The insensitivity of the translocase to Nbf-Cl is interesting, in view of the demonstration (Fig. 7) that this reagent reacts covalently with many proteins in the chromaffin-granule membrane. Although dicyclohexylcarbodi-imide appears to react predominantly with a single membrane protein, as judged by electrophoresis, it has a complicated effect on the membrane, as shown by its acceleration of amine leakage (Fig. 6). Both dicyclohexylcarbodi-imide and tributyltin are also noteworthy for their incomplete inhibition of the ATPase, suggesting either that some of the membrane ATPase is not coupled to H^+ -translocation, or that there is an additional inhibitor-insensitive ATPase present. This clearly merits further investigation, as does the question of the mechanism of 5-hydroxytryptamine leakage.

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