Transport of Catecholamines by Resealed Chromaffin-Granule 'Ghosts'

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(Received 29 May 1974)

A method is described for the preparation of resealed chromaffin-granule 'ghosts'. The lysis and rapid purification procedures provide 'ghosts' in approximately 70% yield from crude granules; the preparation contains 0.1 μ mol of catecholamine/mg of protein (as compared with 2.8 μ mol/mg in unlysed granules), of which about one third is inside the 'ghosts'. The 'ghosts' retain their ability to accumulate catecholamines, a process dependent on Mg-ATP and inhibited by reserpine, and a simple assay for this transport is described.

Kirshner (1962) and Carlsson *et al.* (1963) demonstrated that chromaffin granules, the secretory vesicles of the adrenal medulla, contain an ATP-requiring catecholamine pump. Catecholamines and ATP are stored in high concentrations in the granules, estimated at 0.5 and 0.125 M respectively (Hillarp, 1959). Isolated granules are easily purified and provide a useful system for studying the mechanism of ion pumping. However, a major disadvantage is the high concentration of stored compounds, since these inevitably leak from the granules during an incubation, so that extragranular concentrations are unknown.

In this paper I show that 'resealed ghosts' may be prepared from granules and obtained free of mitochondria and relatively free of catecholamines and ATP. A simple assay is described for the pumping of catecholamines across the membranes of the 'ghosts', and their performance is compared with that of intact granules. In the following paper (Phillips, 1974) I present a kinetic analysis of the pump. A somewhat similar approach has been investigated by Taugner (1971).

Experimental

Materials

Radiochemicals were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Hepes† was from Hopkin and Williams, Chadwell Heath, Essex, U.K. Sephadex was from Pharmacia, Ealing, London, U.K. $^2\text{H}_2\text{O}$ was from Norsk Hydro-Elektrisk Kraestofaktieselskab, Oslo, Norway. Other chemicals were from BDH Chemicals Ltd., Poole, Dorset, U.K.,

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† Abbreviations: Hepes, 2-(*N*-2-hydroxyethyl)piperazin-*N'*-yl)ethanesulphonic acid; ATPase, adenosine triphosphatase.

and biochemicals were from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey, U.K.

'Buffered sucrose' contained 10 mM-Hepes. The pH of 1 M-Hepes was adjusted to 7.2 with 1 M-NaOH. All pH values given were measured at room temperature and are those of solutions at the concentrations at which they were used. Reserpine was dissolved in 2 M-acetic acid to give a 10 mM solution. This was diluted tenfold with 0.1 M-sodium phosphate, pH 6.5, to give a stock solution which was stored frozen.

Methods

Preparation of chromaffin-granule 'ghosts'. Bovine adrenal medullae were cooled on ice and used within 1 h of slaughter. After mincing and homogenization in buffered 0.3 M-sucrose, pH 7.0, the extract was centrifuged at 1000g for 10 min at 4°C. The pellet was discarded, and a portion of the supernatant was retained for assay of certain components (this is referred to as the 'homogenate' in Table 1). The remainder of the supernatant was centrifuged at 27000g for 20 min at 4°C. The upper layer of the resulting pellet was removed by washing with buffered 0.3 M-sucrose (Kirshner, 1962) and discarded. The remaining pellet was resuspended and re-centrifuged. Washings from the pellet were again discarded, and the resultant pellet was suspended in buffered 0.3 M-sucrose (approx. 0.5 ml/g wet wt. of tissue) to give crude chromaffin granules.

Crude granules (2.0 ml) were then applied to a column (5.0 cm $\times$ 1.6 cm diam.) of Sephadex G-50 equilibrated with 10 mM-Hepes, pH 7.0, at approx. 10°C. They were washed down the column with 3 ml of 10 mM-Hepes and the void volume was then collected in a further 5 ml. Any trailing material was discarded. This crude 'ghost' preparation was added dropwise to 5 ml of buffered 0.6 M-sucrose at 0°C and the resultant suspension applied in six portions to

sucrose gradients: each portion was layered carefully above 2.5 ml of buffered 0.4 M-sucrose, which was itself above 1.0 ml of 0.3 M-sucrose in $^2\text{H}_2\text{O}$ in a cellulose nitrate Spinco tube (5.1 cm $\times$ 1.3 cm). The gradients were centrifuged at 200000g for 30 min at 4°C in a Spinco SW 50.1 rotor. 'Ghosts' appeared as a cloudy band at the interface between the 0.4 M-sucrose and the 0.3 M-sucrose in $^2\text{H}_2\text{O}$. They were recovered with a Pasteur pipette. 'Ghosts' were stored at 0°C and were used within a few hours of preparation.

Electron microscopy. A preparation of purified 'ghosts' (5 ml; 220 μg of protein) was incubated for 40 min at room temperature in 0.3 M-sucrose containing 20 mM-Hepes, pH 7.0. Glutaraldehyde was then added to a concentration of 1%. After a further 15 min at room temperature the 'ghosts' were collected by centrifugation (2 h at 4°C at 200000g). The pellet was washed with phosphate-buffered sucrose and treated with 2% (w/v) OsO_4 before dehydration and embedding in Epon. Sections were double-stained with 10% (w/v) uranyl acetate in methanol followed by alkaline lead citrate (Reynolds, 1963). Sections covering the whole depth of the pellet were examined.

Incorporation of catecholamines. Incubations generally contained (in 100 μl of buffered 0.3 M-sucrose, pH 7.0): 6 mM-ATP, 2 mM- MgCl_2 and (–)-[^{14}C]noradrenaline of the specific radioactivity indicated. 'Ghosts' were added to a concentration of approx. 100 μg of protein/ml. The incubation (37°C) was terminated by the addition of 2 ml of ice-cold buffered 0.3 M-sucrose (or cold 10 mM-Hepes for samples subjected to osmotic shock). The samples were then passed through 0.45 μm Millipore filters under suction, and these were washed with 6 ml of buffered 0.3 M-sucrose. Filters were dried and counted for radioactivity in a liquid-scintillation counter, by using toluene scintillation fluid containing 2,5-diphenyloxazole (5 g/l) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (0.3 g/l). Standards were prepared on Millipore filters and counted for radioactivity in the same way.

To determine the proportion of the radioactivity incorporated into the 'ghosts' that was retained on a filter in this assay, a portion of an incubation was passed down a column (10.5 cm $\times$ 0.7 cm) of Sephadex G-50 equilibrated with buffered 0.3 M-sucrose. Fractions (0.4 ml) were collected and the radioactivity of portions of these was determined after drying on filters. The radioactivity eluted in the void volume was compared with that which was retained by direct filtration of a portion of the incorporation mixture.

Included volume of 'ghosts'. 'Ghosts' (0.3–0.4 mg/ml) were incubated for 22 h at 4°C in buffered 0.3 M-sucrose containing: 1 mM-[^{14}C]leucine (2000 c.p.m./nmol), 10 mM-[^{14}C]choline chloride (350 c.p.m./nmol), 10 mM-[^{14}C]glycerol (370 c.p.m./nmol), 1 mM-(–)-[^{14}C]noradrenaline bitartrate (2800 c.p.m./nmol),

[^{14}C]sucrose (12 c.p.m./nmol), 10 mM-[$N\text{-Me-}^{14}\text{C}$]acetylcholine (250 c.p.m./nmol) or 10 mM-sodium [^{35}S]sulphate (400 c.p.m./nmol). Four different volumes of 'ghosts' were then transferred to 2 ml of ice-cold, non-radioactive incubation medium, or to a similar medium lacking sucrose. Samples were filtered through 0.45 μm Millipore filters, which were washed with 6 ml of cold incubation medium. They were dried and their radioactivity was determined. Values for the included volume of several different preparations of 'ghosts' agreed within a range of $\pm 25\%$.

Biochemical assay methods

Dopamine β -hydroxylase was assayed as described previously (Phillips, 1973). Incubations (15 min at 37°C) contained 10 μM -sodium *p*-hydroxymercuribenzoate. Monoamine oxidase was assayed by the method of Wurtman & Axelrod (1963): incubations contained 0.1 M-potassium phosphate, pH 7.8, and 48 μM -[^{14}C]tyramine hydrochloride (42 mCi/mmol) in 100 μl and were terminated after 15 min at 37°C. Protein was assayed as follows: samples were subjected to precipitation with 5% (w/v) trichloroacetic acid for 10 min at 0°C. After centrifugation, supernatants were carefully removed and the pellets were dissolved in 3% (w/v) NaOH containing 2% (w/v) sodium deoxycholate (Winkler *et al.*, 1970). The protein content was then determined by the method of Hartree (1972) with crystalline bovine serum albumin as a standard. Catecholamines were measured by the fluorimetric method of von Euler & Lishajko (1961), with adrenaline and noradrenaline bitartrate standards. Results in Table 1 are expressed as the sum of these two catecholamines. Free catecholamines in 'ghost' preparations were determined by using 5 mM- CaCl_2 to aggregate the 'ghosts' (15–30 min at room temperature). They were then centrifuged for 1 h at 4°C and 150000g; the catecholamine contents of supernatant and pellet were then determined. Measurements of protein concentration confirmed that the 'ghosts' were quantitatively recovered in the pellet. ATP was assayed by the method of Stanley & Williams (1969).

Results and Discussion

Preparation of resealed 'ghosts'

Experiments on the uptake of catecholamines by intact chromaffin granules utilize granules purified by centrifugation at forces of 15000–25000g, followed by washing of the pellet (Kirshner, 1962). These preparations are heavily contaminated by mitochondria and lysosomes, but purification by centrifugation through high sucrose concentrations (Smith & Winkler, 1967a) or through iso-osmotic Ficoll-sucrose- $^2\text{H}_2\text{O}$ solutions (Trifaro & Dworkind, 1970)

Table 1. *Analysis of subcellular fractions from the adrenal medulla*

The first part of the Table contains specific activities; units are mg derived from 1 g wet wt. of adrenal medulla (protein), $\mu\text{mol}/\text{mg}$ of protein (catecholamines, total of adrenaline and noradrenaline assays) and pmol of substrate converted/15min per μg of protein at 37°C (dopamine β -hydroxylase and monoamine oxidase). Values are means $\pm$ s.d., with numbers of preparations assayed in parentheses. In the second part of the Table total activities are given in the same units, but are calculated amounts from 1 g of tissue (1 mg for enzyme activities).

Specific activities	Protein	Catecholamines	Dopamine β -hydroxylase	Monoamine oxidase
Homogenate	47 $\pm$ 9 (8)	1.05 $\pm$ 0.20 (8)	9.2 $\pm$ 3.0 (6)	2.24 $\pm$ 0.48 (5)
Crude chromaffin granules	5.8 $\pm$ 1.5 (10)	2.77 $\pm$ 0.57 (10)	18.8 $\pm$ 3.2 (6)	2.14 $\pm$ 0.49 (5)
'Ghosts'	0.37 $\pm$ 0.10 (10)	0.11 $\pm$ 0.04 (10)	156 $\pm$ 39 (6)	0.87 $\pm$ 0.04 (4)
Total activities				
Homogenate	47 $\pm$ 9	48 $\pm$ 6	410 $\pm$ 150	114 $\pm$ 13
Crude chromaffin granules	5.8 $\pm$ 1.5	16.0 $\pm$ 4.3	150 $\pm$ 73	18.6 $\pm$ 16
'Ghosts'	0.37 $\pm$ 0.10	0.042 $\pm$ 0.020	53 $\pm$ 15	0.32 $\pm$ 0.09

severely impairs the catecholamine-transporting ability of the granules. In making 'ghosts', the following strategy was therefore adopted. A preparation of crude granules is lysed to release chromogranins, catecholamines and ATP; the resulting membranes are then separated on a density gradient from mitochondria and other cell membranes by virtue of their extremely low density (hydrated density = 1.12 g/cm³; Pollard *et al.*, 1973; Phillips, 1973). The gradient is maintained essentially iso-osmotic by using ²H₂O to provide the required high-density step, rather than increasing the sucrose concentration. The membranes are thus collected as a band above a cushion of 0.3M-sucrose in ²H₂O (density = 1.15 g/cm³). This method has the additional advantage of avoiding pelleting the 'ghosts', another process that damages them.

To facilitate the application of the 'ghosts' to the gradients, it is desirable to keep volumes as low as possible. The granules are therefore lysed, not by dilution, but by gel filtration through a small column of Sephadex G-50, equilibrated with dilute buffer. The void volume is collected and trailing material discarded. The 'ghosts' are resealed by readjusting the void-volume effluent to 0.3M-sucrose. The mechanism of resealing is unknown, but the method is similar to that used for resealing erythrocyte 'ghosts', except that in the latter case salt solutions are used (Schwoch & Passow, 1973). By contrast with these, however, incubation of the 'ghost' preparation at 37°C does not promote further resealing (as measured by catecholamine uptake).

Properties of the resealed 'ghost' preparation

An electron micrograph of a thin section of a pellet of 'ghosts' prepared in this way is presented in Plate 1. The material consists mainly of more-or-

less circular profiles of a size corresponding to that of intact chromaffin granules (which have a mean diameter of about 0.3 μm). In addition, there are smaller membranous vesicles, which are probably derived from chromaffin-granule membranes by spontaneous vesiculation during lysis. Although there is little sign of contamination by mitochondria or microsomal fractions, some 'ghosts' contain residues of granular core material, presumably chromogranin that has not been completely released by the lysis procedure (see below).

Some properties of the 'ghosts' are summarized in Table 1. The method of preparation is designed to provide highly purified 'ghosts' rapidly: the yield of purified 'ghosts' from crude granules is about 70% as judged by dopamine β -hydroxylase recovery, assuming that 50% of this enzyme is released by the lysis procedure (Winkler *et al.*, 1970). This implies that 'ghosts' contain only 11% of the protein of the crude intact granules (data from Table 1). Winkler *et al.* (1970) found that 22% of the protein of purified granules was present in their membranes; the recovery in the present work (in which only that membrane material found in the most compact part of the band on the gradient is utilized) may therefore be somewhat less than 70%, since the dopamine β -hydroxylase assays are rather variable from one preparation to another. In the procedure 99.7% of the catecholamines of the original crude granule fraction are removed. Approx. 70% of the catecholamine remaining in the 'ghost' preparation appears to be free in solution, rather than inside the 'ghosts': this value is deduced by centrifuging the 'ghosts' in the presence of 5mM-CaCl₂ (see under 'Methods') and assaying the pellet and the supernatant. Although accurate measurements of ATP were not made (owing to its very low concentration), luciferase assays of several preparations suggested that the concentration of

Fig. 1. Sucrose density gradients of chromaffin granules and resealed 'ghosts' after incorporation of [^{14}C]noradrenaline

Crude chromaffin granules (0.2 ml; 140 μg of protein) or resealed 'ghosts' (0.2 ml; 28 μg of protein) were incubated for 15 min at 37°C in the presence of 28 μM -DL-[^{14}C]noradrenaline (54 mCi/mmol), 5 mM-ATP and 2 mM-MgCl₂. After cooling, the mixtures were passed down columns (6 cm $\times$ 0.5 cm) of Sephadex G-50 equilibrated with buffered 0.3 M-sucrose. The void volumes (0.3 ml) were collected in buffered sucrose and applied to sucrose gradients of the density shown (Δ). After centrifugation (3 h at 4°C at 200000g), fractions from the tubes were diluted with 2 ml of cold buffered sucrose and collected on Millipore filters: granules, gradient 1 ($\circ$); 'ghosts', gradient 2 ($\bullet$). A third identical gradient received unincubated 'ghosts'. Portions (10 μl) of the fractions from this third gradient were assayed for dopamine β -hydroxylase activity ($\square$). The arrows show the approximate position of lysosomal (L) and mitochondrial (M) markers on these gradients [acid ribonuclease and monoamine oxidase respectively (Phillips, 1973)].

ATP in the 'ghost' preparation was about one-tenth that of the catecholamines.

The 'ghost' preparation does not seem to be contaminated with intact granules. These have a high density (approx. 1.24 g/cm³ in high sucrose concentrations) and appear to be absent from electron micrographs of the bottom of 'ghost' pellets.

No measurements have been made of the concentration of chromogranins in the 'ghost' preparation. Chromogranins are acidic proteins found in high concentration inside chromaffin granules; they are assumed to be involved in binding the catecholamines. However, it is known that these proteins are released with the small molecules by osmotic lysis and they presumably emerge in the void volume of the Sephadex column [chromogranin A, the major component, has a molecular weight of 77000, but has a strong tendency to form aggregates (Smith & Winkler, 1967b)]. It is assumed that most free protein is retained at the top of the preparative density gradient, and, indeed, the recoveries in Table 1 and assays of the supernatant (not shown) confirm that this is so. However, traces of chromogranins are found on

sodium dodecyl sulphate-containing polyacrylamide gels of 'ghost' preparations, albeit in very low concentrations compared with intact granules; this is to be expected, since residual core material can be seen inside a few of the 'ghosts' (Plate 1).

Uptake of catecholamines by resealed 'ghosts'

Incubation of intact granules or of 'ghosts' with radioactive catecholamines in the presence of Mg-ATP (Kirshner, 1962) leads to incorporation of radioactivity into particulate material. Density-gradient centrifugation (Fig. 1) shows that in the 'ghost' preparation this particulate material is of low density and sediments with dopamine β -hydroxylase activity (a marker for chromaffin-granule membranes); any catecholamines incorporated into intact granules would be found in the most dense regions of the gradient. In the experiment shown in Fig. 1, 8% of the particulate radioactivity from the 'ghosts' has sedimented faster than the main band, and this may result from the presence of 'ghosts' containing some core protein.

EXPLANATION OF PLATE I

Electron micrograph of a pellet of chromaffin-granule 'ghosts'

The bar represents 1 μ m.

Table 2. Incorporation of (–)-[¹⁴C]noradrenaline by chromaffin granules and by resealed 'ghosts'

Crude chromaffin granules (100 μ l; 7 μ g of protein) or resealed 'ghosts' (100 μ l; 3 μ g of protein) were incubated with 116 μ M (–)-[¹⁴C]noradrenaline (14.4 $\times$ 10³ c.p.m./nmol) for 10 min at 37°C under standard conditions, or with the alterations shown. Both granules and 'ghosts' were from the same preparation. Several other preparations of material gave qualitatively similar results, although the complete experiment (variation of all conditions on both 'ghosts' and granules from the same preparation) was not performed in other cases. Specific activities vary with the preparation, and combined values from several preparations obscure the comparison between both components from the same original material.

	Incorporation of (–)-[¹⁴ C]noradrenaline (pmol/10 min per μ g of protein)	
	Granules	'Ghosts'
Complete mixture	8.9	91.0
Incubation followed by osmotic shock	0.3	8.3
Replace MgCl ₂ by EDTA	0.7	9.3
Omit ATP	2.8	22.9
Replace ATP by ADP	1.0	5.8
Replace ATP by GTP	0	3.7
Add reserpine (10 μ M)	0.7	3.1
Add <i>N</i> -ethylmaleimide (0.4 mM)	3.4	11.2
Add excess of (–)-noradrenaline (2 mM)	0.2*	5.3*

* This value does not include the incorporation of non-radioactive noradrenaline.

Assay for catecholamine transport

Assays of catecholamine uptake by intact granules (Kirshner, 1962; Carlsson *et al.*, 1963; Hasselbach & Taugner, 1970) or by 'ghosts' (Taugner, 1971) have previously utilized centrifugation to remove the incubation medium. However, not only is this method very time-consuming, but it is difficult to remove 'ghosts' quantitatively even at very high speeds. I have therefore used filtration through cellulose nitrate filters as the method of choice for assaying uptake. Both 'ghosts' and granules are retained nearly quantitatively on Millipore filters with 0.45 μ m pores: I present evidence for this below, after outlining criteria for uptake.

In general, I have based assays on the method proposed by Kirshner (1962). The following criteria are used to indicate that radioactive catecholamine in the incubation medium is being transported inside the granule or 'ghost': (1) the uptake should be dependent on the presence of Mg²⁺ and ATP; (2) the uptake should be inhibited by reserpine, a *Rauwolfia* alkaloid which potently inhibits transport by chromaffin granules; (3) the uptake should be saturable; and (4) radioactive catecholamine incorporated inside granules or ghosts should be released into the medium again by sudden osmotic shock. These criteria are illustrated for the uptake of (–)-noradrenaline in Table 2.

Table 2 illustrates several features of the assay. First, after osmotic shock a greater proportion of the incorporated radioactivity is released from intact granules than from 'ghosts'. This is presumably due to 'dilution' of the radioactive catecholamine inside

the granule by the high concentration of unlabelled material there; less labelled catecholamine is therefore retained by intragranular binding sites. No 'blank' values have been subtracted from the data in Table 2 (apart from the radioactivity remaining on the filters when medium lacking granules or 'ghosts' is filtered); the value after osmotic shock for granules approximately equals that for incubation in the presence of reserpine. In general, I define catecholamine transport by 'ghosts' as the reserpine-sensitive incorporation. Subtraction of the uptake into 'ghosts' in the presence of 10 μ M-reserpine allows one to eliminate artifacts arising from continued sealing of 'ghosts' during the incubation, and also from non-specific uptake in transport studies.

Secondly, there is some uptake in the absence of ATP, although this is small compared with that in its presence. This component of transport is inhibited by replacing the ATP with ADP or GTP, and is not found when the Mg²⁺- and ATP-induced transport is inhibited by reserpine.

Third, addition of excess of unlabelled (–)-noradrenaline abolishes the incorporation of radioactive (–)-noradrenaline. This is because the transport shows saturation kinetics (Phillips, 1974); the incorporation is not due to a passive 'space-filling' phenomenon. The dependence of uptake velocity on substrate concentration is discussed in the accompanying paper (Phillips, 1974).

The dependence of catecholamine uptake on 'ghost' or granule concentration is shown in Fig. 2. Uptake into intact granules appears to decrease at high granule concentrations; this is presumably due to leakage of unlabelled adrenaline and noradrenaline

Fig. 2. Incorporation of $(-)-[^{14}\text{C}]$ noradrenaline by chromaffin granules and resealed 'ghosts'

Incubations (under standard conditions) contained the amount of protein shown in $100\ \mu\text{l}$. $(-)-[^{14}\text{C}]$ Noradrenaline was $95\ \mu\text{M}$ (9.2×10^3 c.p.m./nmol). Crude chromaffin granules (○); resealed 'ghosts' (●).

into the incubation medium, and competition of these with the radioactive noradrenaline used for the measurements. The specific activities of the preparations shown in Fig. 2 are $25\ \text{pmol}/10\text{min}$ per μg (crude granules) and $62\ \text{pmol}/10\text{min}$ per μg ('ghosts'). If 80% of the protein of the granule preparation were lost on lysis, one might expect a specific activity of about $125\ \text{pmol}/10\text{min}$ per μg for the 'ghosts' if these resealed completely. However, specific activities of both granules and 'ghosts' vary considerably from one preparation to another.

Efficiency of the filter assay

Incubations are terminated by diluting the 'ghost' suspension with 20 vol. of ice-cold iso-osmotic sucrose (0.3M). The processes of dilution and rapid temperature change result in leakage of 20% of the incorporated radioactivity: this may be shown by comparing the radioactivity retained on a filter before and after dilution (with identical washing procedures).

However, after dilution the incorporated radioactivity does not leak at all over at least 15 min at 0°C .

What proportion of particle-bound catecholamine is retained by the filter? The total particle-bound catecholamine may be measured independently by passing a complete incubation mixture of 'ghosts' with radioactive catecholamine down a column of Sephadex G-50 equilibrated with iso-osmotic buffered sucrose: this method was introduced by Marchbanks (1968) for measuring the acetylcholine within synaptic vesicles. Free catecholamines are retained by the column. In one such experiment 15.8×10^4 c.p.m. of $[^{14}\text{C}]$ noradrenaline was collected in the void volume. Direct filtration of another portion of the incubation through a Millipore filter ($0.45\ \mu\text{m}$) showed that 15.4×10^4 c.p.m. could be retained from the same volume (after correction for loss on dilution before filtration). That is, retention by the filter is essentially complete. Identical results are obtained when filters of $0.22\ \mu\text{m}$ pore size replace those with $0.45\ \mu\text{m}$ pores. The latter were used as a routine, in view of their more rapid filtration rate.

Apparent volume of 'ghosts'

The method of Kasai & Changeux (1971) was used to determine the included volume of the 'ghost' preparation. 'Ghosts' were incubated overnight at 4°C in a solution containing a radioactive solute. Samples were then treated in the same way as 'ghost' suspensions used for transport experiments: they were diluted either into buffered sucrose or buffered water, and passed through a Millipore filter. The radioactivity on the second filter provides a measure of the solute bound to membranes. The difference in the radioactivities is a measure of the solute that is free inside the 'ghosts'.

The calculated included volume of a given preparation (Table 3) clearly varies with the radioactive solute used. This was found by Kasai & Changeux (1971) for electroplax 'microsacs', and indeed the results for chromaffin 'ghosts' resemble their results qualitatively. The main difference is that, for a given solute, the included volume of chromaffin 'ghosts' (per mg of protein) is two to three times greater than that for 'microsacs'.

The values of Table 3 cannot be interpreted in detail. It was found that incorporation of the solute had not reached a plateau after 22 h incubation at 4°C , and increased by 35–40% during a further 24 h. Incorporation at room temperature led to values about twice as high, but also failed to reach plateaus. Kasai & Changeux (1971) found that microsacs were impermeable to $[^{35}\text{S}]$ sulphate, and it is possible that the chromaffin 'ghosts' are somewhat leaky. The high value for $(-)-$ noradrenaline may reflect binding to residual chromogranin within 'ghosts'; on the other hand it may reflect a realistic included volume, since

Table 3. *Included volume of 'ghost' preparations*

Resealed 'ghosts' were incubated with the radioactive solute shown in buffered 0.3 M-sucrose for 22 h at 4°C. All values shown were obtained with the same 'ghost' preparation, except those for sulphate and acetylcholine.

Radioactive permeant	Concn. in incubation medium (mM)	Included volume (μ l/mg of protein)
[¹⁴ C]Leucine	1.0	1.7
[¹⁴ C]Choline chloride	10.0	4.1
[¹⁴ C]Glycerol	10.0	2.0
(-)-[¹⁴ C]Noradrenaline bitartrate	1.0	21.6
[¹⁴ C]Sucrose	300	1.7
[¹⁴ C]Acetylcholine chloride	10.0	0.6
Sodium [³⁵ S]sulphate	10.0	1.1

rough calculations based on the catecholamine content of intact granules suggest that complete resealing should give an included volume of about 20 μ l/mg.

Conclusion

The best-studied 'ghost' preparation is that from human erythrocytes (Schwoch & Passow, 1973), in which case, as with the 'microsacs' of Kasai & Changeux (1971), one is dealing with a resealed plasma membrane. The present preparation, from an intracellular organelle, though not studied in the detail of erythrocyte 'ghosts', clearly shares many features with them as far as both preparation method and properties are concerned. Above all, one must remember that the preparation is heterogeneous, containing resealed 'ghosts', with or without traces of chromogranin, leaky 'ghosts' and membrane fragments. No attempt has been made to separate these.

It is not clear how much membrane reorganization occurs during the processes of lysis and resealing, although the membrane is able not only to transport catecholamines but to retain them, at concentrations greater than those in the surrounding medium (Phillips, 1974). The mechanism of resealing is unknown, and no experiments have yet been performed to investigate this. For erythrocyte-'ghost' formation, haemolysis is usually performed in the presence of Mg²⁺ ions, and resealing is performed by incubation at 37°C after raising of the osmolarity; the role of bivalent cations is unclear, and their inclusion during haemolysis at 0°C in fact has rather little effect on resealing (Schwoch & Passow, 1973). Mg²⁺ ions aggregate chromaffin granules and lead to changes in their membranes (Edwards *et al.*, 1974); they were therefore excluded. Incubation at 37°C was also eliminated; it fails to increase the specific activity of preparations in transport assays.

Resealed chromaffin-granule 'ghosts', as described in the present paper, closely resemble those prepared by a somewhat different method by Taugner (1971).

They show, however, considerably higher rates of catecholamine incorporation. This may be because Taugner (1971) has followed a strategy of purifying the intact granules before lysing them with 15 mM-KCl; purification by various methods appears to damage the granule membranes. Taugner's (1971) method, although more laborious than that described here, yields a 'ghost' preparation with a lower catecholamine content, which is particularly valuable in detailed flux measurements (Taugner, 1971). The resealed 'ghosts' are likely to prove useful in the study of secretory granules in view of their ease of preparation and the convenience with which they can be used in transport studies.

I am most grateful to Dr. W. Edwards for providing the electron micrograph.

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