Biochimica et Biophysica Acta, 464 (1977) 65—81

BBA 77555

CORRELATION OF PHYSICAL AND MORPHOLOGICAL PARAMETERS WITH RELEASE OF CATECHOLAMINES, ATP, AND PROTEIN FROM ADRENAL MEDULLA CHROMAFFIN GRANULES

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(Received May 21st, 1976)

Summary

Exchanging 0.3 M sucrose for 0.3 M glycerol as the bathing medium for isolated bovine adrenal chromaffin granules at 0° C or raising the temperature of granules suspended in 0.3 M sucrose to 37° C causes the release of catecholamines, ATP and soluble protein stored in the granules. These treatments also reduce the turbidity (absorbance at a non-absorbing wavelength) of the same suspensions.

The glycerol-induced release is shown by morphology and morphometry to be due to lysis of the granules.

Advantage is taken of the difference in density of the intact granules and the granule ghosts to confirm this result by centrifugation of glycerol-incubated samples on continuous gradients of sucrose and metrizamide.

Similar centrifugation experiments performed on the granules incubated in sucrose at 37° C show that lysis also accounts for the fast phase of release of granule contents, and not graded selective release of material from otherwise intact granules.

Introduction

Chromaffin granules, the secretory granules of the adrenal medulla, store catecholamines, ATP and protein. There is strong evidence that these stores of material are released by exocytosis when adrenal glands are stimulated either in vivo or in vitro (for reviews see refs 1—3).

Chromaffin granules are easily isolated and purified [2]. When isolated bovine chromaffin granules are resuspended iso-osmotically in sucrose at

* HEPES, 2-(*N*-2-hydroxyethylpiperazin-*N'*-yl)ethanesulphonic acid.

0—4° C, they show little loss of their stores of catecholamines (adrenaline and noradrenaline), ATP and soluble proteins [4—6]. However, granules resuspended at higher temperatures show time-dependent loss of catecholamines [4—10]. Oka et al. [6] gives results that noradrenaline loss at room temperature was rapid and complete within 5 min while adrenaline was released more slowly over more than 30 min. A slow release of adrenaline and dopamine was seen in sheep granules by Lishajko [8] while noradrenaline showed both a fast component and a second slow component of release. Biphasic release of ^{14}C-labelled adrenaline at 37° C was shown by Slotkin et al. [5] and at 31° C by Taugner and Hasselbach [10] using granules which had first been labelled with radioactive noradrenaline.

Data on temperature-dependent release of protein is less well characterized. Poisner and Trifaró [7] note that incubation of isolated granules for 10 min at 30° C released protein as well as catecholamine and ATP. Slotkin et al. [5] found no release of soluble dopamine-β-hydroxylase over the course of 60 min at 37° C. However, these granules had been first incubated at 30° C for 20 min to stimulate radiolabelled adrenaline uptake. Unfortunately protein release was not followed during the first incubation.

Lishajko notes temperature-dependent release of protein from isolated sheep [9] and bovine [11] chromaffin granules which parallel fluxes of catecholamine and ATP. However, these protein determinations may be in error, since the Lowry method [12] used in these studies gives a false positive color in the presence of catecholamines.

These results have been interpreted in terms of two "pools" or "compartments" of materials which reside in the granules and from which the catecholamines are released in a graded fashion [2,5,13]. The suggestion has been made that each "pool" resides in a separate type of granule [2,5]. However, it is possible to explain these observations by an alternate hypothesis. Granules subjected to temperature pertubations could be lysing (breaking open and releasing their entire contents). Therefore what appears to be partial release of material from all granules could be caused by selective lysis. Since lysis would be expected to release protein as well as small molecules, a necessary (but not sufficient) finding in favor of such a model would be protein release which paralleled catecholamine and ATP release. Such a finding would, moreover, argue against selective release of materials sequestered in "pools" within the granules.

As the granules lose their stored materials as a result of environmental changes in vitro, one would reasonably expect to see resulting changes in the physical properties of the granules, e.g. turbidity, refractive index and density. In vivo drug-dependent changes induced in the density of granules which correlate with reduction in granule catecholamine content have been reported [14,15]. Temperature-dependent changes in the turbidity (absorbance (A) at a non-absorbing wavelength) have been seen when isolated granules have been incubated in vitro [16—18]. Hillarp and Nilson [16] were able to correlate loss of absorbance with release of catecholamine and protein when granules were subjected to hypo-osmotic shock at 3° C. Oka et al. [17] and Trifaró and Poisner [18] note that the granules show little decline in absorbance when incubated in 0.3 M sucrose at 0—4° C, but register a rapid loss of absorbance

when incubated at temperatures greater than 20° C, which parallel the losses of stored material from the granules.

While screening suitable media for the measurement of the refractive index of the granules, it was noted that the turbidity of chromaffin granules resuspended in 0.3—0.5 M glycerol, 20 mM HEPES, pH 7.2, at 0° C showed a time-dependent decline similar to that seen when granules were incubated at elevated temperatures. Granules treated in this manner also showed changes in density and release of catecholamine, ATP and protein. These changes provide a model system for studying the efflux problem.

Therefore it was decided to investigate both glycerol and temperature-dependent release of materials from isolated granules. Results of these studies suggested that release of granule contents by both treatments can be attributed to lysis phenomena.

In a separate communication, we discuss the accidental findings of electron-dense spots in the glycerol-treated granules which are apparently di- and trivalent ion binding sites, similar to those seen in other storage granules when fixed in media containing high concentrations of Ca^{2+} [19].

Materials and Methods

All chemicals were of reagent grade and were used without further purification. All solutions were buffered with 20 mM sodium HEPES, pH 7.2, except where noted.

Preparation of the bovine adrenal medulla chromaffin granules has been described in detail previously [20,21]. The twice washed crude granule (P_3) pellet was used as the starting material for these experiments.

Release experiments. The release of total catecholamine, ATP and protein from the granules were assayed all on the same sample by the following scheme (Fig. 1). Samples (50 μl) of P_3 granules resuspended in 0.3 M sucrose at 0° C (containing 5—6 mg protein/ml) were resuspended in 1.5 ml of various media which had been preequilibrated to 0 or 37° C at time 0. The reaction was stopped by the addition of 4.5 ml ice-cold 0.3 M sucrose (0.6 M sucrose was used for the sucrose/glycerol experiments) and centrifuged at 0—2° C for 10 min at 16 000 rev./min in the Sorvall SS1 fixed angle rotor (RCF $\approx$ 30 000 $\times$ g_{max}). The supernatant (supt) was decanted and the tube wiped clear. 6 ml of

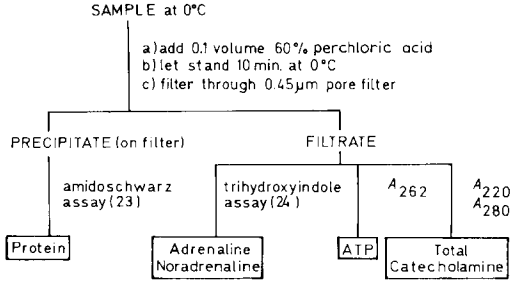

Fig. 1. Scheme for the analysis of protein, ATP and catecholamine released from chromaffin granules. See Materials and Methods for details. Numbers in brackets refer to references for the analyses.

of ice-cold water was added to the pellet and sonicated for 10—25 s to aid lysis, using a long tapered probe fitted to an MSE sonicator. Both the ice-cold supernatant and sonicated pellet samples were made up to 6% $HClO_4$ by the addition of 600 μl of 60% $HClO_4$ to precipitate protein. Samples were filtered through 45 μm Sartorius cellulose acetate filters. Removal of the protein precipitate was quantitative [22].

Protein was assayed on the filters using a modification [22] of the Amido Schwarz method of Schaffner and Weissmann [23] and results normalized to: % released = $([\text{protein}]_{supt}/([\text{protein}]_{supt} + [\text{protein}]_{pellet}) \times 100$. ATP was assayed by absorption at 262 nm and catecholamine was assayed by absorption at 280 and 220 nm, all three wavelengths yielding qualitatively similar results when expressed as: % released = $A_{supt}/A_{supt} + A_{pellet}) \times 100$. These normalizations reduce the need for extremely accurate pipetting of the original samples used for the incubations.

In some experiments, the individual catecholamines, adrenaline and noradrenaline, in the perchloric acid supernatants were assayed by a modification [24] of the method of von Euler and Lishajko [25] using 0.5 M potassium phosphate buffer containing 1 mM EDTA, pH 6.5.

Absorbance measurements were made in a Zeiss PMQ III spectrophotometer using 10-mm path length cells in a jacketed cell holder thermostatted with a LAUDA TUK30D water bath. Samples containing 25—50 μl of P_3 granules resuspended in either 0.3 or 0.5 M sucrose at 0°C (5—6 mg protein/ml) were rapidly resuspended in 2.0 ml ice-cold solvent in the cuvette at an initial $A_{380nm} \approx 1.0$ and absorbance was recorded continuously with occasional adjustment of the base line.

Density measurements. The methods for the preparation of the iso-osmotic 360 and 700 mosM sucrose/metrizamide density gradients have been described previously [21]. The density of the granule ghost membrane was measured by a modification of the method of Wallach et al. [26] using CsCl gradients containing constant concentrations of dimethyl sulfoxide (Me_2SO) and glycerol in 50 mM HEPES, pH 7.2. To check the possibility that the glycerol and Me_2SO were selectively partitioned in the membranes, their concentrations were varied between 10 and 20% (v/v). Within the limits of experimental error, no difference in membrane density was detected.

The density of sealed granule ghosts prepared by hypotonic lysis [27] was measured on gradients of Ficoll (Batch No. 6580) in 50 mM HEPES, pH 7.2. The densities of ghosts resealed in the presence of 0.3 M sucrose also was measured on 0.360 osM sucrose/metrizamide gradients as described in Results and Discussion.

Morphology and morphometry. Chromaffin granule pellets were prepared for electron microscopy as described previously [20] and examined in a Siemens Ia electron microscope.

Morphometric determination of the pellet volume of a time series of granules subjected to glycerol lysis were performed as follows: The pellet diameter was determined by photographing the osmicated pellets in their centrifuge tubes from above. The negative was placed in a Nikon model 6C Profile Projector and the vertical, horizontal and two diagonal diameters were measured, averaged and converted to millimeters using the mm scale which had been photographed

with the pellets. Then after dehydrating the pellets and cutting them in half across their diameter, the pellets were embedded in Epon and the thickness at the pellet center was carefully measured from 1 μm thick sections stained with Toluidine Blue. These values were used in the models of pellet volume developed below.

The time course of glycerol-induced lysis of the granules as seen by electron microscopy was quantitated as follows: Thin sections were prepared from the pellets described above and examined by electron microscopy. Each of the sections was photographed at 12 roughly equally spaced intervals between the top and the bottom of the pellet. A Quantimet model 720 was set to distinguish black/white and the threshold for black set to just exclude most ghosts but include most granule profiles. The ratio ((black area)/(total area)) was taken to be proportional to the number of whole granules present in the volume represented by the photo. This number was multiplied by the appropriate volume for the pellet depth of the photo, using the equations for model pellets.

We have chosen two model shapes for the pellet produced when sub-micron particles are pelleted at high gravitational forces in swing-out rotors. Model I assumes that the bottom of the centrifuge tube is hemispherical and that the upper boundary of the pellet is a flat surface perpendicular to the gravitational field (Fig. 2A). Choosing coordinates (0,0) at the center of the sphere, the pellet thickness t is defined in this case by trigonometry: Defining $y_1 = (R_T^2 - x_1^2)^{1/2}$

$$t = R_T - \sqrt{R_T^2 - x_1^2} = R_T - y_1 \tag{1}$$

Therefore

$$x_1 = \sqrt{2R_T t - t^2} = r \tag{2}$$

The volume of the pellet can be integrated as a series of infinitesimal rings of thickness dx and height Δy.

$$dV = 2\pi x \, dx \, \Delta y = 2\pi x \{\sqrt{R_T^2 - x^2} - y_1\} \, dx \tag{3}$$

$$V = \int_0^{x_1} 2\pi x \{\sqrt{R_T^2 - x^2} - y_1\} \, dx \tag{4}$$

$$= \frac{2\pi}{3}\{R_T^3 - (R_T^2 - x_1^2)^{3/2}\} - \pi y_1 x_1^2 \tag{5}$$

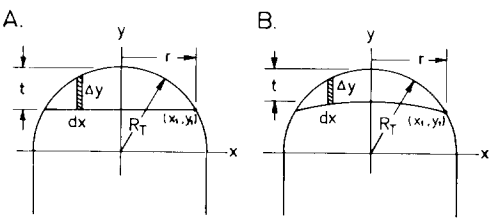

Fig. 2. Models for the shapes of pellets produced by high speed centrifugation in swing out rotors. (A) "Sliced sphere" model. (B) "Crescent moon" model. See Materials and Methods for details.

Model II was suggested by the shape of the osmicated, dehydrated pellets prepared for electron microscopy, which appear more like crescent moons than sliced spheres (Fig. 2B). Here we approximate the pellet cross-section as a parabola intersecting a sphere of radius R_T = inner radius of the centrifuge tube (Fig. 2A). Setting the center of the sphere at (0,0), these figures intersect at points $(-x_1, y_1)$ and (x_1, y_1) where $x_1 = r$, the diameter of the pellet of thickness t.

$$y_1 = \sqrt{R_T^2 - r^2} \tag{6}$$

for the parabola

$$y_1 = a_0 + a_1 r + a_2 r^2 \tag{7}$$

where $a_0 = R_T - t$ and $a_1 = 0$ (by symmetry). Therefore

$$a_2 = \frac{\sqrt{R_T^2 - r^2} - (R_T - t)}{r^2} \tag{8}$$

We integrate by summing the volumes of infinitesimal rings of thickness dx and height Δy, using the cylindrical symmetry of the pellet about the y-axis:

$$\Delta y = \sqrt{R_T^2 - x^2} - (a_0 + a_2 x^2) \tag{9}$$

The volume of one infinitesimal ring:

$$dv = 2\pi x \, dx \, \Delta y \tag{10}$$

therefore

$$V_P = \int_0^r 2\pi x \{\sqrt{R_T^2 - x^2} - (a_0 + a_2 x^2)\} \, dx \tag{11}$$

$$= 2\pi \left\{ \tfrac{1}{3}[R_T^3 - (R_T^2 - r^2)^{3/2}] - \left(\frac{a_0 r^2}{2} + \frac{a_2 r^4}{4} \right) \right\} \tag{12}$$

We found that this approximation gave better results for pellets of small thickness (t) than a similar model which considered the upper surface of the pellet also to be spherical.

Assuming that our pellets contained 12 lamina of thickness = $t/12$, we calculated the volume of the i-th lamina as

$$\Delta V_i = V_i - V_{i-1} \tag{13}$$

where the first lamina is at the bottom of the pellet, etc.

Results and Discussion

Correlation of release of granule core material with absorbance changes

Fig. 2 shows the time dependence of absorbance changes for P_3 chromaffin granules which have been resuspended in 0.3 M sucrose at 0° C, when they are diluted into various media. Granules resuspended in 0.3 M sucrose at 0° C show no change (Fig. 3A) while granules resuspended in 10 mM HEPES, pH 7.2 (a

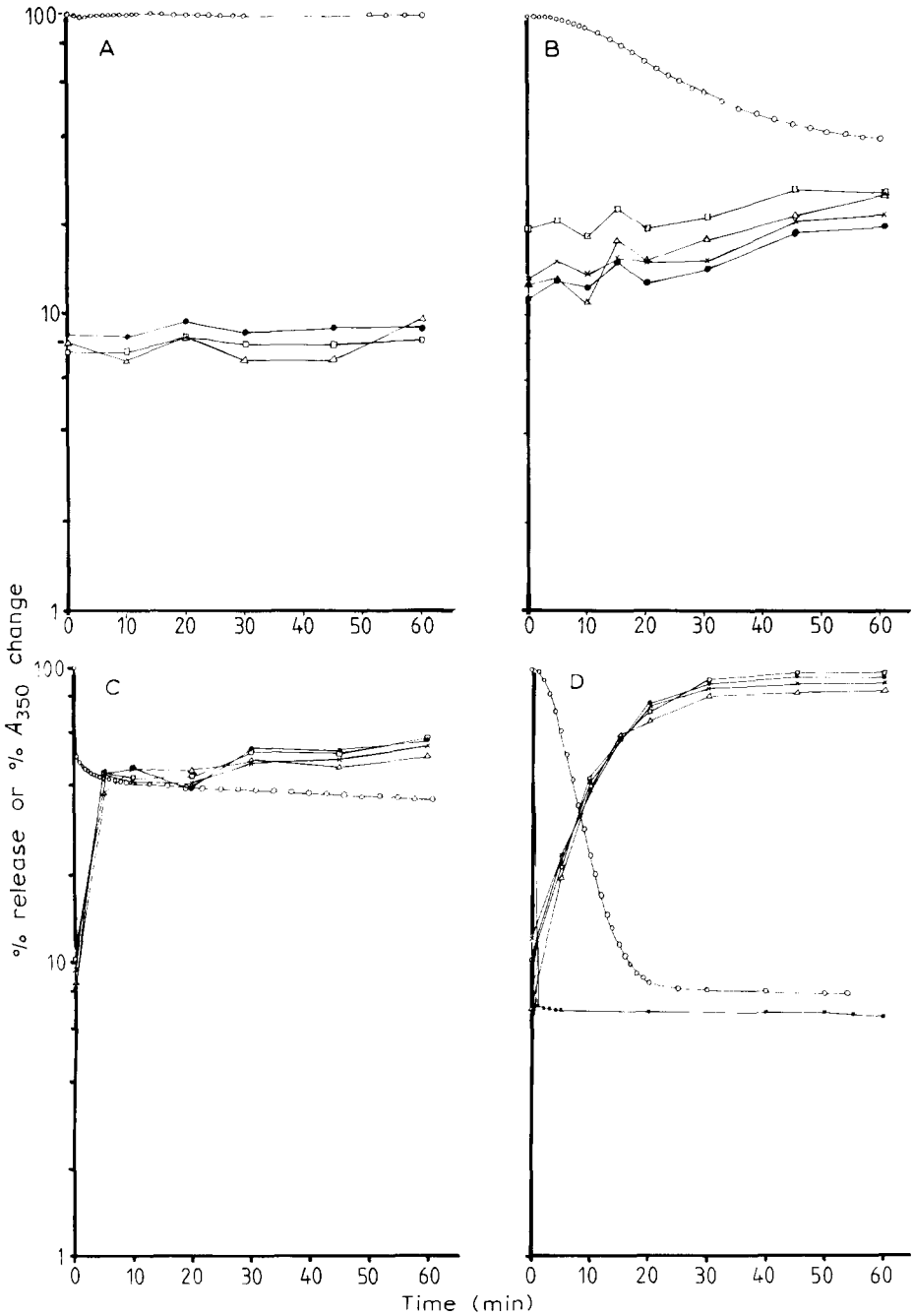

Fig. 3. Time dependence of changes in absorbance and release of protein, ATP and catecholamines from P_3 chromaffin granules. (A) Granules resuspended in 0.3 M sucrose, 0°C. (B) 0.3 M sucrose, 0.3 M glycerol, 0°C. (C) 0.3 M sucrose, 37°C. (D) 0.3 M glycerol, 0°C. Data normalized to percent released. △———△, protein; X———X, A_{220nm} catecholamine; ●———●, A_{280nm} catecholamine; □———□, A_{262nm} (ATP); ○———○, A_{350nm} (turbidity); ■———■, A_{350nm} of granules resuspended in 10 mM HEPES at 0°C. (This treatment produces almost complete lysis of the granules.)

treatment which lyses most of the granules) show a precipitous decline in absorbance followed by no change (Fig. 3D). Granules resuspended in 0.3 M glycerol show a slow log-linear decline to approximately the level of the HEPES-treated material (Fig. 3D). Granules resuspended in 0.3 M glycerol/0.3 M sucrose at 0° C undergo a very slow log-linear decline (Fig. 3B). Qualitatively similar results are obtained when 0.5 M solutions are used (data not shown). The granules resuspended in sucrose at 37° C show a very rapid initial drop in absorbance within the first 5 min followed by a long, very slow decline (Fig. 3C).

The time course of release of total catecholamines, ATP and protein from these same granule samples was assessed using the scheme outlined above (Fig. 1). The results varied quantitatively from one experiment to the next, but showed the same overall pattern. The older the preparation the greater the amounts of material released at 0 time. The rates of release were quite similar, however. The experiments presented in Fig. 3 are representative of these results. They are the mean values of duplicate determinations, and are presented along with the tracings of the absorbance changes from the same samples which were done simultaneously with the incubations thus allowing for direct comparison.

The granules incubated in 0.3 M sucrose at 0° C show little release of their soluble contents, whereas incubation at 37° C produces a rapid release within the first 5 min followed by a slow steady release. Incubation in glycerol at 0° C produces a sustained log-linear rate of release which is similar in rate and duration to the absorbance change. Granules resuspended in sucrose/glycerol show qualitatively similar patterns to that of the 0.3 M glycerol incubation, but the rate of change is slower.

In all cases the release curves for the protein and small molecules had similar patterns. In all experiments the release curves were mirror images of the changes in absorbance. In some experiments the fluxes of adrenaline and noradrenaline from granules incubated in 0.3 M glycerol at 0° C and 0.3 M sucrose at 37° C were measured separately. No differential fluxes of the two amines was observed.

Morphology

Effects of incubation. Electron micrographs of P_3 granules incubated for 1 h in various media at 0 or 37° C are presented in Fig. 4. The changes in morphology observed upon incubation in sucrose at elevated temperatures (Figs. 4a and 4c) has been discussed previously [28,29]. Briefly, one sees on the average a lightening of the stain in the cores of the granules and an increase in the diameter of the subunit "grains" of the core material of many granules. The average diameter of the granules becomes smaller, the numbers of hollow core vesicles in the diameter range 500—1500 Å increases and many granules, especially those with the large subunit cores, have outfoldings of membranes. The number of large ghosts is increased over the control. Since these preparations were not purified on gradients, one sees contamination by mitochondria and less frequently large cell debris.

The granules incubated in sucrose/glycerol are mostly intact, but the number of ghosts is greatly increased compared to the control (Fig. 4b).

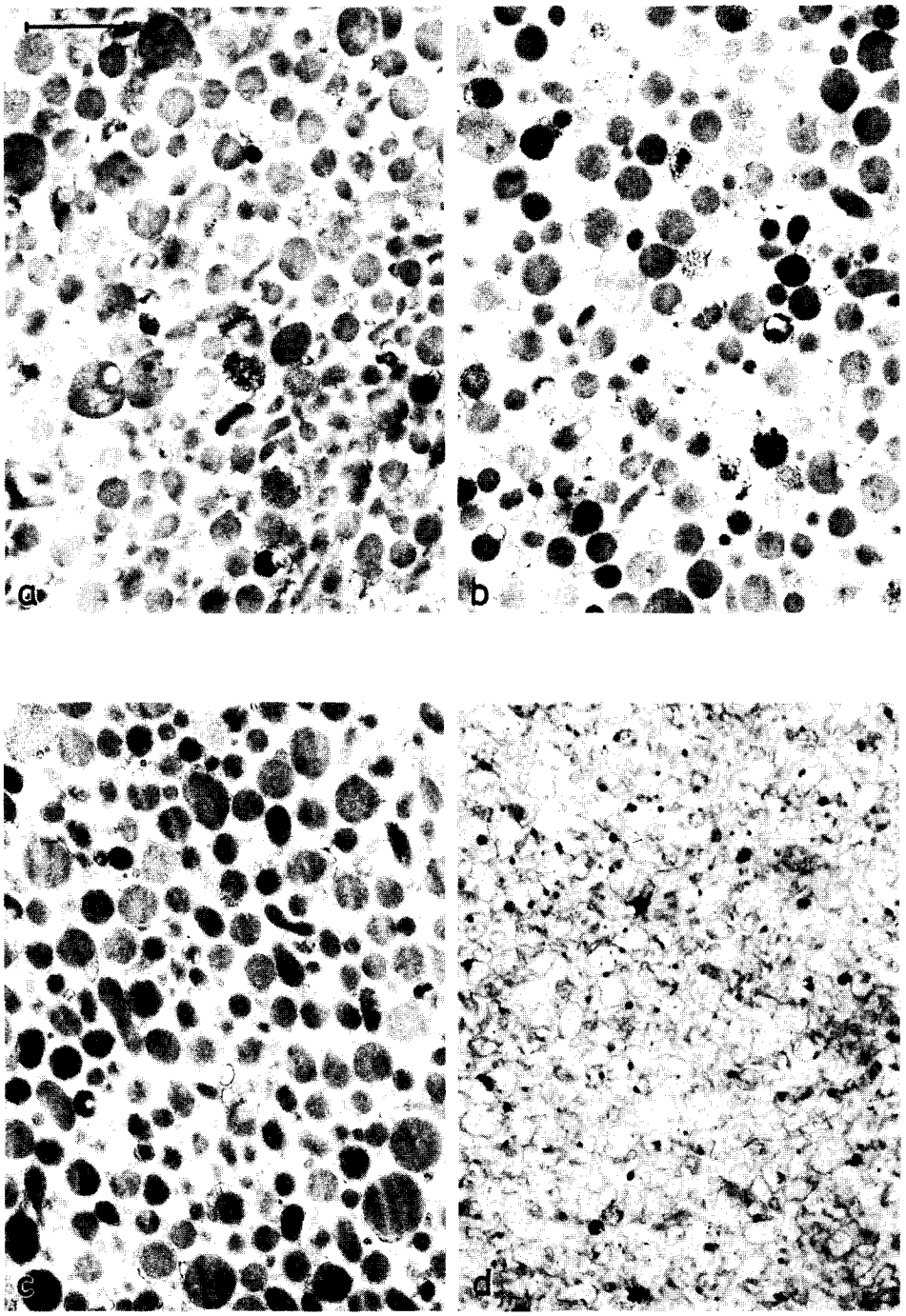

Fig. 4. Morphology of P_3 granules incubated for 1 h under various conditions. The P_3 pellet was resuspended in 0.5 M sucrose and about 50 µl of this suspension was added to 2 ml of the solvent pre-equilibrated to either 0 or 37°C. Incubation was stopped by addition of an equal volume of ice-cold 0.5 M sucrose containing 2% glutaraldehyde. Black bar represents 1 µm. a, P_3 granules incubated in 0.5 M sucrose, 0°C; b, P_3 granules incubated in 0.5 M sucrose, 0.5 M glycerol, 0°C; c, P_3 granules incubated in 0.5 M sucrose, 37°C; d, P_3 granules incubated in 0.5 M glycerol, 0°C.

By contrast the glycerine-incubated granules (Fig. 4d) are empty ghosts almost completely devoid of recognisable core material. Many of the profiles contain a single very electron dense irregular spot of variable size which almost always appears to be adhering to the inner membrane at one point. This finding is discussed separately [19].

Morphology of the time course of glycerol-induced lysis. The results of the changes in morphology coupled with the absorbance and release measurements of the glycerol-treated granules strongly suggested that this treatment was producing lysis of the granules. To investigate this effect further, the morphology of the time course of the glycerol treatment was studied as follows. Equal volume samples of P_3 granule suspensions incubated in 0.3 M glycerol at 0°C for 0, 10, 20, 30, 45, 90 and 120 min were fixed by the addition of glutaraldehyde and centrifuged into well-packed pellets which were prepared for examination by electron microscopy. A higher concentration of granules (approx. 25 mg protein/ml) was chosen for these experiments to lengthen the time required to complete the change and provide pellets large enough for easy removal from the tube and subsequent embedding.

The pellets were embedded, semithin sectioned, trimmed and thin sectioned to be examined from "top to bottom", i.e. parallel to the centrifugal field. To control for stratification in the pellet due to denser particles sedimenting at the bottom of the pellet, photographs were taken at 12 roughly equally spaced intervals from the top of the pellet to the bottom. The results are presented in a photomontage (Fig. 5) in which representative fields taken from the top, upper one-third, lower one-third and bottom of the 0-, 20-, 45- and 120-min pellets are shown. As can be seen, the relative number of granule ghosts quickly increases as a function of time. As would be expected by the densities of the granules and ghosts (Table I) in any given time series, the relative number of whole granules increases with the distance from the top of the pellet. However, those granules which remain appear to have normal morphology.

Morphometry of glycerol-induced lysis. The effect of glycerol on the granule morphology clearly shows that the granules lyse with time. A direct assessment of the time course of the disappearance of whole granules was made using a Quantimet Model 720. Each of the 12 electron photomicrographs was examined on the Quantimet and the number of granules per photograph was modeled as described in Materials and Methods. To correct for the change of pellet volume with depth, the estimate of granule number was multiplied by the volume of the stratum, using the models developed in Fig. 2. These results are presented in Fig. 6. Excellent agreement between the two estimates was seen.

Buoyant density of chromaffin granules and ghosts. Density determinations of P_3 granules incubated for 1 h in the various media used for the absorbance measurements were performed on iso-osmotic 0.360 osM sucrose/metrizamide gradients by determining the density of the ATP peak. Results are presented in Table I. Incubation in 0.3 M sucrose at 0 or 37°C or in 0.3 M sucrose/0.3 M glycerol at 0°C gave quite similar values of approx. 1.123. Particle-bound ATP was absent from the granules incubated in 0.3 M glycerol at 0°C. Therefore the density of the putative ghost peak was determined by absorbance measurements as approx. 1.085. By contrast, the ghosts seen when hypotonic lysed granules are run on this type of gradient is approx. 1058 or just slightly

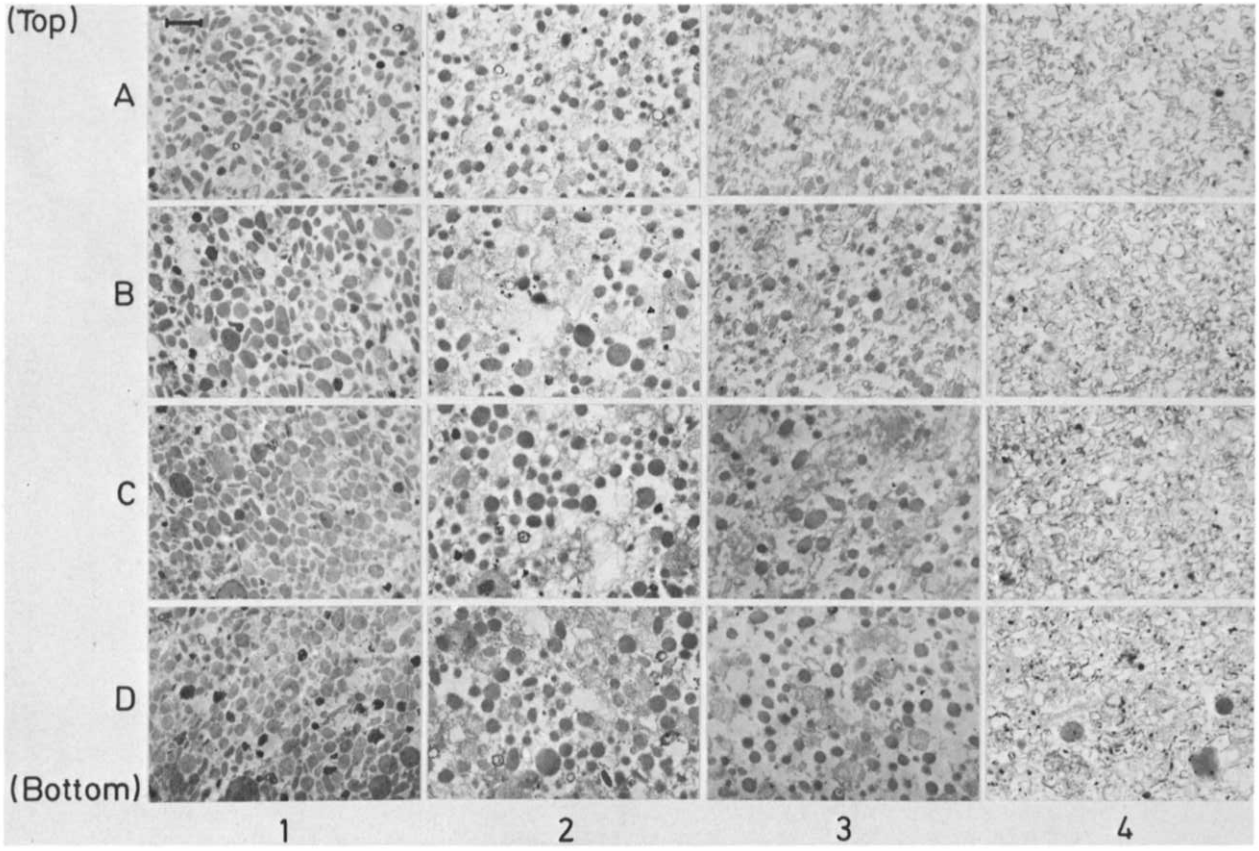

Fig. 5. Morphology of P_3 granules incubated in 0.3 M glycerol at 0°C (cf. text for details). bar = 1 μm.

TABLE I

ISOPYCNIC BUOYANT DENSITIES OF VARIOUS CHROMAFFIN GRANULE PREPARATIONS

Samples were layered on the top of preformed, continuous gradients and centrifuged 2 h at 32 500 rev./min (129 600 × g_{av}) in a Beckman SW41 Ti rotor at 1.5°C. Densities are reported ± S.D. (number of determinations).

Sample	Treatment	Gradient type	Density
P_3 chromaffin granules	Incubation for 1 h in 0.3 M sucrose at 0°C	sucrose/metrizamide 360 mosM	1.125 ± 0.0038 (6)
	Incubation for 1 h in 0.3 M sucrose at 37°C	360 mosM	1.124 ± 0.0016 (3)
	Incubation for 1 h in 0.3 M sucrose, 0.3 M glycerol at 0°C	700 mosM	1.120 ± 0.0007 (2)
	Incubation for 1 h in 0.3 M glycerol at 0°C	360 mosM	1.085 ± 0.0010 (3)
Chromaffin granule ghosts	Lysed and resealed in 0.3 M sucrose, 37°C	360 mosM	1.067 ± 0.0001 (2)
	Hypotonic lysis, resealed in 10 mM KCl, 10 mM HEPES, pH 7, 0°C	Ficoll	1.039 ± 0.0052 (3)
	Hypotonic lysis, resealed in 10 mM KCl, 10 mM HEPES, pH 7, 0°C	CsCl, 10% * Me_2SO, 10% glycerol	1.162 ± 0.0023 (5) *

* Assuming that all the constituents can diffuse into the core space of the lysed ghosts, this value represents the density of the membrane. See ref. 26.

denser than the 0.3 M sucrose. However, both types of ghosts produce values of approx. 1.04 when measured on Ficoll gradients.

The difference in density between granule ghosts produced by hypotonic lysis and those which were lysed in the presence of sucrose can be explained by

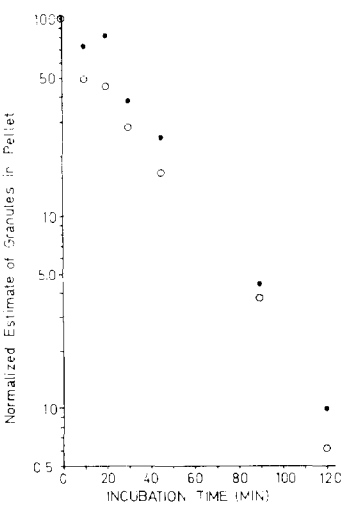

Fig. 6. Morphometry of P_3 granules incubated in 0.3 M glycerol at 0°C. •, cresent model; ○, sliced sphere model. Data was normalized by dividing all points by the 0-time estimate and multiplying by 100%.

the presence of sucrose in the core of the latter particles. If we apply the density mixture rule proposed by Wallach et al. [26] for such empty ghosts, for a granule of density ρ_g containing a core of ρ_c surrounded by a membrane ρ_m,

$$\rho_g = \rho_m v_m + \rho_c v_c \tag{14}$$

where the volume fractions of the membrane and core

$$v_m + v_c = 1 \tag{15}$$

For the hypotonic lysed ghosts (Table I)

$$1.039 = 1.162\,(1 - v_c) + 1.000\,(v_c) \tag{16}$$

giving a value for v_c of 0.76. Assuming that the volume fractions do not change appreciably for ghosts lysed in the presence of 0.3 M sucrose and taking $\rho_{sucrose} \approx 1.036$, the predicted density of such a ghost would be

$$\rho_{g\,sucrose} = (1.162)(0.24) + (1.036)(0.76) = 1.066 \tag{17}$$

which is in good agreement with the measured 1.067. The greater disparity between the densities of glycerol-lysed ghosts measured on Ficoll and sucrose gradients is discussed separately [19].

When ghosts are fixed prior to pelleting they maintain their spherical shape (Figs. 4 and 5). On the other hand, Pollard et al. [16] report that when unfixed ghosts are centrifuged into a firm pellet, they can be reduced to completely flattened sacks. In published experiments with J.H. Phillips, it was impossible to demonstrate a significant water space in granule ghosts using a method involving centrifugation of the ghosts after incubation in [^{14}C]sucrose/$^{3}H_2O$, although these same ghosts were able to store transported catecholamines against a concentration gradient [27] and had a water space of 76% of the total volume. This would be consistent with the picture of the granule ghost as a good osmometer with a flexible, inelastic membrane. External pressure created by the gravitational field would force water out of the pelleted ghosts leading to their collapse. Resuspension of the pellet would allow iso-osmotic rehydration as the ghost resumed its former spherical shape.

Glycerol-induced changes in granule density. Chromaffin granules are good osmometers when immersed in sucrose solutions of >250 mosM but rapidly lyse at lower osmolalities [21]. Since the absorbance for the glycerol-treated granules eventually drops to that of the lysed granules (Fig. 3D) and was accompanied by loss of protein at the same rate as ATP and catecholamine, it was reasoned that the glycerol treatment represented slow lysis. This would be effected by the ability of glycerol to diffuse into the granules. Thus the osmotic pressure of the glycerol is only apparent and decreases as the diffusion proceeds. A similar ability of glycerol to lyse red blood cells has recently been noted [30]. This interpretation is consistent with the changes in density produced by incubation in glycerol and the morphology and morphometry of glycerol-treated granules.

Granules immersed in 0.3 M sucrose/0.3 M glycerol at 0°C show log-linear declines in absorbance qualitatively similar to those seen for granules immersed in glycerol alone although the $t_{1/2}$ is larger. In this case the granules experience an initial hypertonic change (0.3—0.6 M) since water exits faster than glycerol

can enter the granules. This accounts for the slight increase in absorbance often seen in the first few minutes of incubation. Then, as for the case of immersion in 0.3 M glycerol, the glycerol enters the granules, the apparent osmotic pressure falls, water enters the granules and they lyse open. However, many granules can withstand the osmotic pressure drop from approx. 0.6 to 0.3 M, as has been noted for dilution of the sucrose concentration [16]. Hence the absorbance of sucrose/glycerol-treated granules never falls to the level of the water- or glycerol-lysed granules.

It appears from the data presented above that the glycerol-promoted lysis and release of granule contents are first-order processes. Two models suggest themselves which could correspond to the first-order changes observed: (1) The granules leak catecholamine, ATP and protein at constant rates until these stores are exhausted and only ghosts remain. The efflux would be proportional to the concentration of material left in the granules. (2) The granules lyse completely; the lysis occurring with a given half-life similar to radioactive decay.

The release data argues against model 1 since all fluxes from a given experiment have about the same rate and leakage would be expected to favor the small molecules over protein, but this is not conclusive.

A second line of evidence favoring model 2 is derived from the morphology of granules incubated in glycerol for various times and then fixed in glutaraldehyde and centrifuged into pellets (Fig. 5). The appearance of ghosts increases as a function of time and the remaining granules have well preserved intact cores. Morphometric observations on this material shows that this lysis preceeds with the same first-order kinetics as the absorbance and release data, but does not rule out loss of material before the lysis occurs (Fig. 6).

The large difference between the density of whole granules and ghosts when measured on sucrose/metrizamide gradients provides a method for choosing between the alternative models. Since the granules show a large change in density when treated with glycerol (Table I) one would expect to see the granule density of the entire population shift slowly if model 1 were operating while model 2 would produce a decline in the number of particles with the density of whole granules and a simultaneous increase in the numbers of less dense ghosts without producing particles of intermediate density. To choose between these two alternatives, samples of granules incubated for various times in 0.3 M glycerol at 0°C were rapidly loaded onto 360 mosM sucrose/metrizamide gradients and centrifuged (Fig. 7). The results are entirely consistent with model 2. The 0-time gradient contains a large peak of whole granules ($\rho \approx 1.123$) and a small number of sucrose filled ghosts ($\rho \approx 1.067$). As the lysis proceeds, the size but not the position of the whole granule peak is greatly reduced while a peak of glycerol-ghosts ($\rho \approx 1.085$) grows with time. Qualitatively similar results with a broader time base are seen when granules incubated in 0.3 M sucrose/0.3 M glycerol are centrifuged on 700 mosM gradients (data not shown).

Temperature-induced changes in granule density. Hillarp [31] and others [1,2] report that approx. 80% of the total protein of the granules is soluble which is in substantial agreement with the results of the glycerol-induced release (Fig. 2). In our experiments, incubation at 37°C releases about 50% of this soluble store within 5 min; the remaining material shows a slow decline for

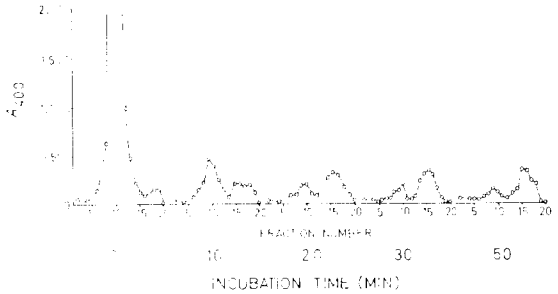

Fig. 7. Absorbance of fractionated gradients from glycerol incubation experiment. P_3 granules were incubated for various lengths of time in 0.3 M glycerol at 0°C. Incubations were timed so that all samples were completed at 50 min. The longer times were carefully loaded onto the gradients without mixing, while the incubation proceeded. The 0-time sample was loaded last, immediately after mixing and the centrifuge was started within 5 min. Tracing A is the 0-time sample; B, 10 min incubation; C, 20 min; D, 30 min; E, 50 min. The 40-min sample was similar to D and E.

the next hour. As was noted in the introduction, the current interpretation of these experiments is that the granules contain two pools of stored material which is released in a graded fashion with one pool showing faster efflux. However, since protein shows the same efflux as catecholamine and ATP, it is equally likely that about half the population of granules undergo a rapid thermal lysis while the remainder lyse and release material more slowly.

As in the case of the glycerol-induced release of granule contents, if the granules have two stores of catecholamine, ATP and protein, one of which is easily released, then their density should shift downward but not approach that of ghosts. The latter possibility should produce large numbers of ghosts but leave the density of the remaining granules unchanged. This was tested by incubating granules for various times, quickly cooling them to 0° C and then centrifuging on iso-osmotic sucrose/metrizamide gradients. Again, the data support lysis as the mechanism for the fast phase of release. The density of the whole granules remains unchanged but within 2 min a large number of ghosts appear at $\rho \approx 1.067$ (Fig. 8) and the relative numbers of granules are reduced in a reciprocal manner which resembles the fall of the absorbance.

Measurements of the fluxes of individual catecholamines suggest that the fast phase of the temperature-dependent amine release does not favor one type of amine-containing granule over another as has been reported previously [6,8]. The slow phase of amine release may also be due to lysis since ATP and protein release runs roughly parallel. However, since the slow increase in the number of ghosts in the slow phase could not be quantitated in the experiments reported here, the possibility remains that this represents true leakage.

From the foregoing discussion it is obvious that previous interpretation of work on the temperature dependence of catecholamine release from granule in terms of fluxes across the granule membrane will require fresh scrutiny, since appearance of ATP, catecholamine or protein in the suspension medium is not necessarily indicative of a process of diffusion out of otherwise intact particles.

The literature concerning the release of chromaffin granule storage contents abounds with reports of enhancement or retardation of release by various biochemical and pharmacological agents [4—11,13,16,17,32—35]. We hope that

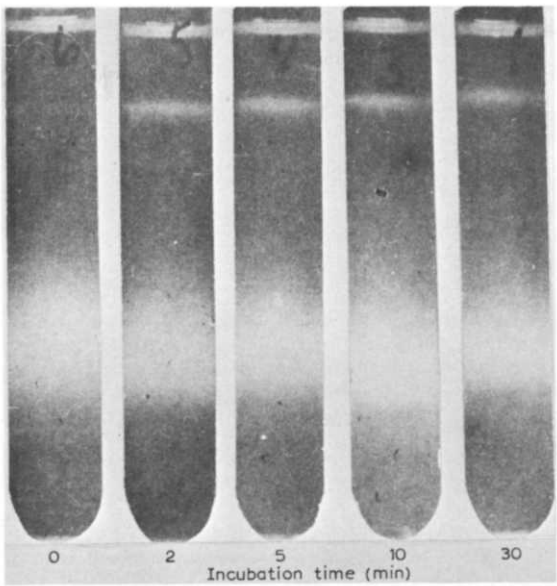

Fig. 8. Photograph of the gradients of P_3 granules incubated in 0.3 M sucrose at 37°C. The experiment was performed similarly to that described for Fig. 7, except that the incubations were performed in long thin tubes to aid heat transfer and were cooled 2 min at 0°C prior to loading onto the gradients. The 15-min point was similar to the 10 and 20 min points.

the methods reported in this study may be used to analyse the underlying physical basis of such phenomena and provide tools for the elucidation of their mechanism of action.

Acknowledgements

We wish to thank our colleagues Drs. J.H. Phillips, R.H. Michell and V.P. Whittaker for helpful discussion, Dr. S. Eins for the Quantimet measurements and Mr. I. Schovanka and Mrs. D. Schilling for their excellent technical assistance. H.A.S. gratefully acknowledges fellowship support from the Max-Planck-Gesellschaft.

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