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CHARACTERISTICS AND DETERMINANTS OF OSMOTIC LYSIS IN CHROMAFFIN GRANULES

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(1) Using isolated bovine chromaffin granules, we demonstrate that osmotic lysis is not a random process and establish the osmotic pressure dependence of osmotic lysis in chromaffin granules, the so-called osmotic fragility curve. (2) We show by measuring the release of constituents of the granule core and correlating these with changes in spectroscopic parameters (turbidity and endogenous catecholamine fluorescence), that the latter can be safely used to measure lysis. (3) Within a particular granule population, noradrenaline granules lyse at higher osmolarities than adrenaline granules, suggesting a higher core osmolarity of the noradrenaline granules. (4) The size distribution of chromaffin granules as a function of lysis was determined by the use of whole mount electron microscopy. It is shown that the mean size of chromaffin granules decreases as a function of lysis. (5) On the basis of theoretical considerations three alternative models of the sequence of osmotic lysis in chromaffin granules are proposed. The experimental results best support a model which postulates that during partial osmotic lysis, granule membranes reseal into smaller vesicles after graded release of contents. The osmotic fragility would represent several cycles of lysis and resealing and would not be a reflection of the distribution of osmotic pressures in the granule population.

Introduction

Catecholamines are released from the adrenal medulla by exocytosis [1,2], an extremely common secretory mechanism which involves fusion of the membranes of the secretory vesicles with the cell plasma membrane. In some model systems, fusion between lipid vesicles and between lipid vesicles and a planar bilayer is greatly facilitated by a transmembranous osmotic pressure gradient [3–6]. Exocytosis can be suppressed by high osmotic strength media in isolated parathyroid cells and platelets [7,8]. These findings suggest that fusion requires osmotic disruption and resealing of granule and plasma membranes after they have been brought into close contact, a view reinforced by recent work demonstrating membrane holes in phospholipid vesicles during fusion [9]. Thus it may be important to determine the forces required to rupture membranes and their resealing characteristics in secretory vesicle membranes.

Chromaffin granules, the secretory vesicles of the adrenal medulla, are easily isolated on a large scale and have been extensively characterized biochemically (for recent reviews, see Refs. 10 and 11). Chromaffin granules contain catecholamines, nucleotides and soluble proteins in high concentra-

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Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

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tions, which are probably all in a solution phase, their apparent osmotic pressure being lowered by non-specific ionic interactions [12–14]. Innumerable in vitro experiments have examined conditions under which chromaffin granules release some or all of their core, some with the intention of defining an in vitro model for exocytosis [15–21]. However, the characteristics and osmotic pressure dependence of lysis have yet to be investigated. Although it is well recognized that after complete lysis of chromaffin granules, ghosts form with perfectly resealed membranes [22,23], the time of resealing or the properties of partially lysed granule populations are unknown. These basic questions concerning the biophysical behavior of chromaffin granules may have relevance to their physiological functions as well as the interpretation of experiments involving osmotic pertubation of chromaffin granules, which may lead to lysis.

Materials and Methods

General. All chemicals were of reagent grade and were used without further purification. All solutions were buffered with 10 mM Hepes (pH 7.4) adjusted with NaOH at room temperature. Osmolarities of sucrose solutions were calculated from Ref. 24. All preparative procedures were carried out at 0–4°C; all experiments were performed at 6°C. Spectroscopic measurements were made in triplicate, while each biochemical measurement was done in duplicate; each experiment being repeated several times.

Preparation of chromaffin granules. Chromaffin granules were prepared as described previously [14].

Incubation experiments. Incubations for measuring the lysis of chromaffin granules as a function of osmotic pressure (the osmotic fragility curve) were started by adding a 50-μl sample of a fresh granule suspension at 0°C to 2.0 ml of the appropriate solution preequilibrated at 6°C. After 60 min incubation, the suspensions were either centrifuged ($40000 \times g_{max}$ for 20 min) and the amount of protein and catecholamine release was determined, or their turbidity of fluorescence were measured; the former at 320 nm in a PMQ 3 Zeiss spectrophotometer, the latter at 285 nm excitation and 317 nm emission wavelengths with a 310 nm emission cutoff filter and slits of 2 nm in a Perkin-Elmer MPF-4 spectrofluorometer. All spectroscopic measurements were made in thermostatically controlled cuvette holders at 6°C. Control experiments established that lysis was complete within 60 min and that the buffering capacity of the 10 mM Hepes (pH 7.4) was sufficient even for total lysis.

The degree of lysis was calculated from the turbidity and fluorescence measurements as the respective percent decrease or increase of the total changes in these parameters associated with resuspending chromaffin granules in 0.26 M sucrose, 10 mM Hepes or in 10 mM Hepes only. With protein and catecholamine release, the percent soluble protein and the free catecholamines in the samples incubated in 0.26 M sucrose at 6°C were first subtracted as background lysis, before the percent releasable protein and percent releasable catecholamines were calculated. The background lysis usually corresponded to approximately 10% of releasable catecholamines and proteins.

Electron microscopy. Chromaffin granules incubated as detailed above were centrifuged, washed and resuspended in small volumes of either the sucrose solution in which they had been lysed or in 0.26 M sucrose. Whole mount preparations of these suspensions were prepared for electron microscopy by adding 10 μl of suspension to a copper grid coated with collodion and carbon and rapidly blotting with filter paper [25,26]. The samples were examined immediately after preparation in a JOEL 100 B electron microscope. Representative areas of the fields were photographed and the size distribution measured with a Zeiss TGZ-3 particle size analyzer.

Chemical assays. Protein concentrations were determined according to Bradford [27] with bovine serum albumin as a standard. Catecholamines were determined by a trihydroxyindole fluorescence assay modified from Anton and Sayre [28]; a 0.2 ml sample was mixed with 0.1 ml of 0.5 M phosphate buffer (pH 7.0) containing 5 mM EDTA. 25 μl of 0.25% (w/v) $K_3Fe(CN)_6$ was added and after exactly 1 min, 0.2 ml 2% (w/v) ascorbate mixed with 7.5 M NaOH in a ratio of 1 : 9 was added. Again after exactly 1 min, 1 ml of water was added and the sample was transferred to a cuvette. Samples were thoroughly vortexed between addi-

tions. The fluorescence was read within 5 min at excitation/emission wavelength pairs of 380/485 and 436/530 nm. Adrenaline and noradrenaline concentrations were calculated from these readings by means of a set of simultaneous equations [29] using the hydrochlorides of the bases as standards.

Results

Characteristics of osmotic lysis in chromaffin granules

A curve which shows the osmotic pressure dependence of osmotic lysis in a subcellular particle is commonly called its osmotic fragility curve [30]. Fig. 1A depicts the osmotic fragility of chromaffin granules and demonstrates that the measurements are quite reproducible among preparations. It has been known for a long time that the turbidity of a granule suspension decreases and its catecholamine fluorescence increases as the particles lyse [19,31–34]. In Fig. 1 the fractional turbidity decrease and fluorescence increase also has been correlated with biochemical determinations of the released proteins and catecholamines, demonstrating that the spectroscopic parameters accurately measure lysis in chromaffin granules.

The amount of the spectroscopic changes can be appreciated from Table I which gives the specific turbidities and of intact and lysed granules and the fluorescence enhancement factor during lysis. Our determination of the percentage of releasable proteins from chromaffin granules as 83.9 ± 1.1% is slightly larger than the average value of 77% calculated by Winkler [36] from published results.

Osmotic lysis of chromaffin granules at 6°C which begins at osmolarities not very much lower than the isolation osmolarity, is half maximal at approx. 190 mM sucrose and almost complete in 150 mM sucrose. As we recently demonstrated [14], Fig. 1B shows that lysis occurs in a log-normal distribution as a function of the deviation of the osmotic pressure from the internal osmotic pressure of the granules. Our osmotic fagility curve differs slightly from those of Creutz and Pollard [20]. This, however, is not surprising in view of the difference in the incubation procedures. While all of our measurements were made at 6°C, Creutz and Pollard's experiments involved a temperature jump to 37°C for 5 min, a procedure which in itself lyses chromaffin granules [34,37].

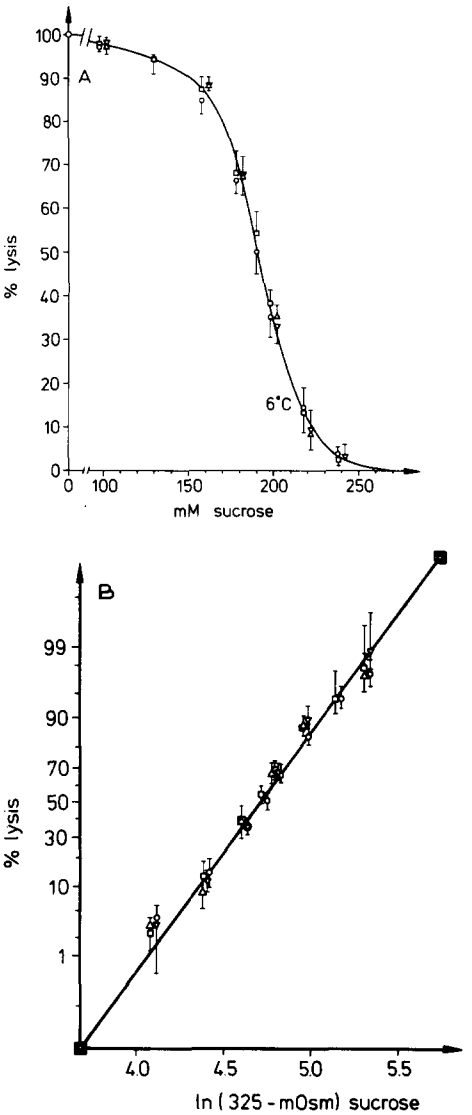


Fig. 1. (A). The osmotic fragility curve of chromaffin granules suspended in sucrose at 6°C determined by protein (▽) and catecholamine (△) release, fluorescence (□) and turbidity (○). Points are means and bars are S.D. of 11 (protein release), 3 (catecholamine release) and 7 (turbidity and fluorescence) experiments. Some points are slightly offset for clarity. (B). The osmotic fragility curve can be represented as a Gaussian distribution of the logarithm of the deviation from the internal osmotic pressure of the chromaffin granules.

Differential lysis of adrenaline and noradrenaline containing granules

Although total catecholamine release follows

TABLE I

PARAMETERS OF TOTAL LYSIS IN CHROMAFFIN GRANULES

Values are expressed as the mean ±S.E. (number of experiments). Specific turbidity is defined as the turbidity at 320 nm per total protein concentration [35] and expressed in 1000× A_{320} per g per ml protein.

Specific turbidity of intact chromaffin granules in 0.26 M sucrose	12.58±0.36 (15)
Specific turbidity of lysed chromaffin granules in 10 mM Hepes	0.70±0.04 (15)
Relative enhancement of catecholamine fluorescence upon lysis (not corrected for scattering)	3.71±0.10 (20)
Maximal protein release (percent of total)	83.9 ±1.07 (14)

protein release, differential catecholamine determinations show that, in the high osmolarity range, noradrenaline is preferentially released. This is demonstrated in Fig. 2 which depicts the relationship of released adrenaline to noradrenaline as a function of sucrose concentration. The free catecholamines present in granule suspensions in 0.26 M sucrose (the isolation osmolarity defined as 0% lysis) already preferentially contain noradrenaline, suggesting that the background lysis inherent in manipulating the granules also preferentially affects noradrenaline granules.

Osmotic lysis as a nonrandom process

Partial osmotic lysis of a chromaffin granule population results in the survival of a granule subpopulation that is more resistent to osmotic stress. This is demonstrated in experiments depicted in Fig. 3 in which partially lysed chromaffin granules were reisolated after centrifugation, resuspended in 0.26 M sucrose and resubjected to osmotic fragility measurements. Fig. 3 shows the osmotic fragility curves for the original population and the partially lysed population. If osmotic lysis were a random event, the osmotic fragility curve of the partially lysed population should duplicate the curve for the original population, which is evidently not the case. In Fig. 3 the osmotic fragility curve is also given which would be expected if partial osmotic lysis was a process that selectively and completely lysed a susceptible part of the original population but left the remainder unchanged (dotted line). The behaviour of our resistent population was slightly more fragile than the original at higher osmolarities but considerably more resistent at low osmolarities, suggesting that

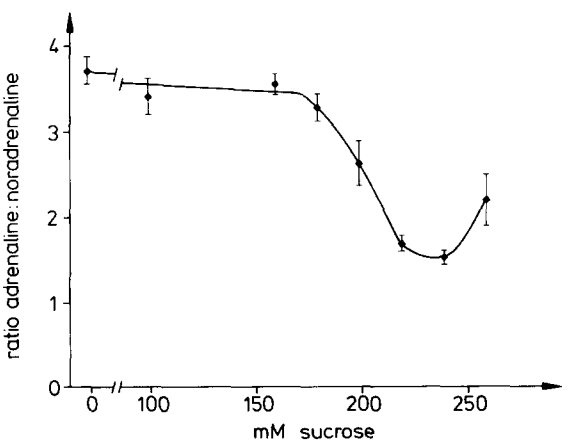


Fig. 2. The molecular ratio of the released adrenaline to noradrenaline as a function of the sucrose concentration. Complete (100%) lysis in 10 mM Hepes (pH 7.4) gave a ratio of adrenaline to noradrenaline of 3.72±0.16 ($n = 3$), which is in good agreement with the literature [2]. However, background (control) lysis and partial lysis gave much lower ratios, showing that at the higher osmolarities noradrenaline-containing granules lyse preferentially.

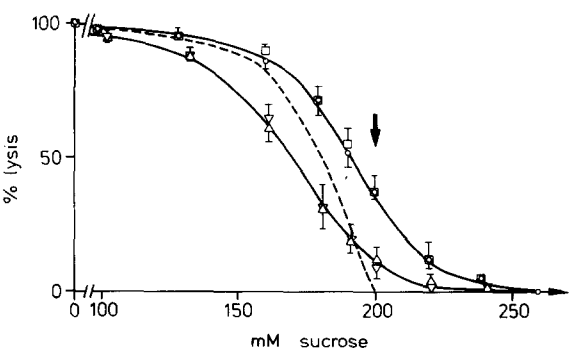


Fig. 3. The osmotic fragility curves of untreated (upper curve) and partially lysed granule populations (lower curve). Granules were partially lysed in 0.20 M sucrose (see arrow), reisolated in 0.26 M sucrose and again subjected to lysis. The dotted line gives the osmotic fragility curve predicted for the partially lysed population if partial lysis led to the removal of a susceptible part of the granule population but left the remainder unchanged. Points and bars represent means and standard deviations of three experiments in which lysis was determined by turbidity (○, △) and fluorescence (□, ▽) measurements.

the surviving population or any point during lysis was not identical with the original one.

Size distribution of chromaffin granules as function of lysis

In whole mount electron micrographs [14], chromaffin granules appear as highly electron dense, round structures with well demarcated borders and can be easily distinguished from ghosts which are seen as irregular electron-lucent shadows (Fig. 4). Occasionally indented, elongated or fragmented forms are seen. Partially lysed granule populations were centrifuged after incubation and resuspended either in 0.26 M sucrose or in buffered sucrose solutions of the concentration in which they have been lysed. Representative areas from grid were photographed and their size distributions determined [14].

The size distributions for unlysed and two partially lysed populations from a representative experiment are shown in Fig. 5, demonstrating an almost Gaussian distribution of radii in the unlysed granule population and a reduction in mean sizes occurring with lysis. The change in mean radius of granules surviving lysis at various osmotic pressures determined for 28 different populations in nine experiments are graphed in Fig. 6. In all experiments there was a statistically significant decrease in mean size as a function of lysis. The size reduction was observed regardless of whether the size distributions were determined in granules resuspended in 0.26 M sucrose after lysis or in granules resuspended in the same sucrose solutions in which they had been lysed. (The sizes were determined under both conditions in order to rule out osmometer effects on the granule sizes [14].) As can be seen from Fig. 6, the general size reduction is quite reproducible between experiments. There was some variation in the absolute mean sizes of the granules determined in different preparations. This is not surprising since no internal standards were used and the microscope was not recalibrated between experiments. This does not affect interpretation since each experimental set contains its own unlysed control to which all other data points were normalized.

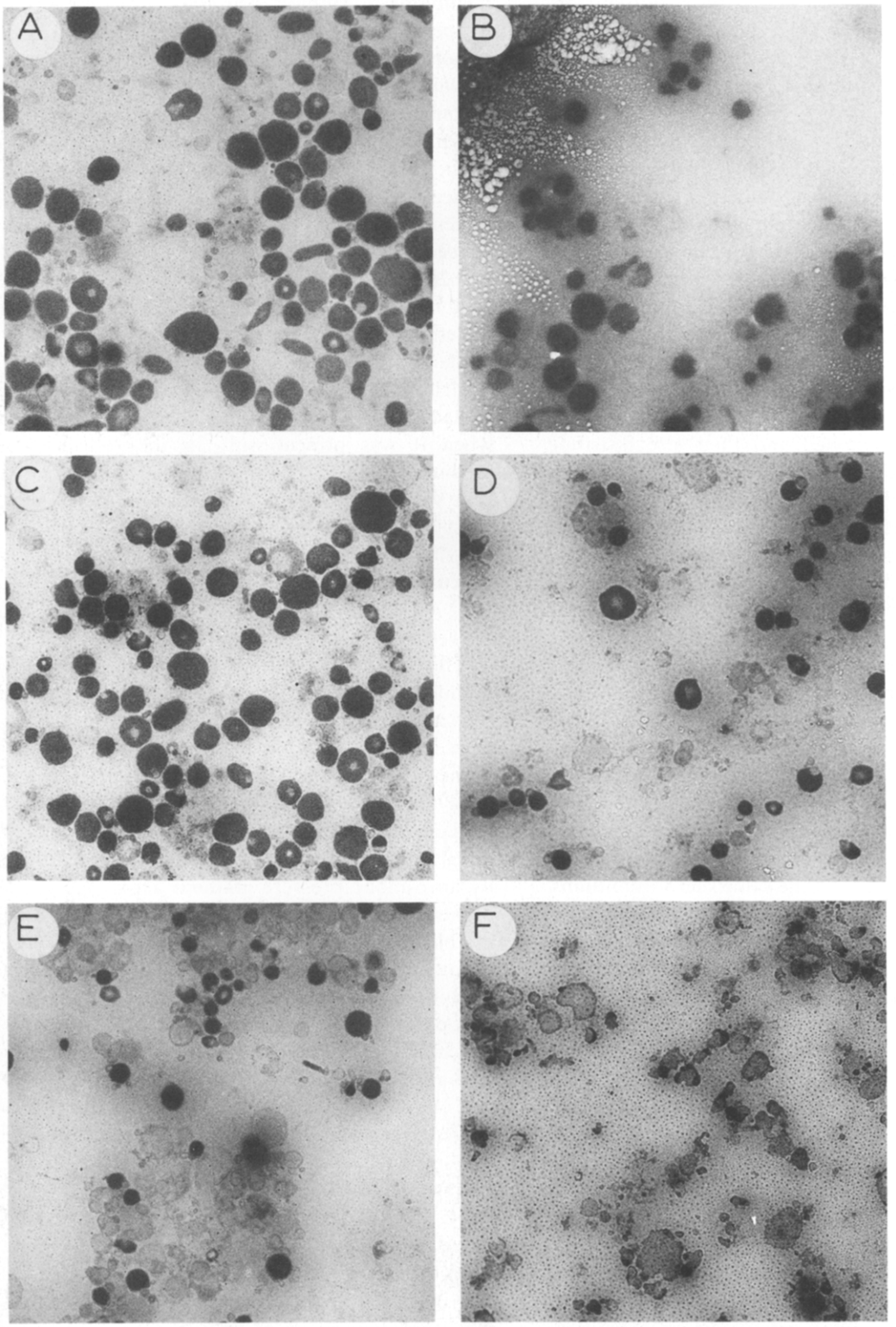
Membrane resealing after total lysis

Since it has been shown for erythrocytes that membrane resealing is a prolonged process at low temperatures [38,39] and since this question is of importance for the interpretation of our results, the approximate time of membrane resealing in chromaffin granules after lysis was investigated. Chromaffin granules were lysed in 10 mM Hepes in the presence or absence of 1 mM 6-carboxyfluorescein, a soluble, membrane-impermeable fluorescent probe. The membranes were then pelleted by centrifugation, washed, and then resuspended in 0.26 M sucrose either in the presence or absence of 1 mM 6-carboxyfluorescein. Thereafer, the resealed chromaffin were separated from contaminants and unlysed granules as described by Apps et al. [40]. The dye was taken up into ghosts only if it had been present in the lysing medium, not when it was present only in the resuspension medium (data not shown). Since the centrifugation time was kept short (10 min), membrane resealing must have taken place rapidly.

Discussion

Properties of osmotic lysis in chromaffin granules

This study demonstrates that in chromafin granules, as opposed to erythrocytes [30], osmotic lysis occurs at relatively high osmotic pressures, with comparatively rapid resealing of the membranes after rupture. These results are relevant to exocytosis since membrane fusion can be considered as a sequence of rupture and resealing of membranes which has been shown to be facilitated by an osmotic pressure gradient across the membranes involved [3–6]. Furthermore, we have established that the turbidity and fluorescence changes observed during lysis accurately reflect the biochemical release of the vesicle contents; and that partial osmotic lysis is a nonrandom process, which results in the emergence of a new population with a considerably smaller mean diameter and changed adrenaline/noradrenaline ratio. Prolonged stimulation of the adrenal medulla in vivo and in vitro also results in a decrease in the average size of the granules in the chromaffin cell [41,42]. It has been speculated that this is merely an adaptive phenomenon and that the remaining granules are more fragile than the original ones. The in vitro results presented here would suggest that these observations may be profitably reinvestigated.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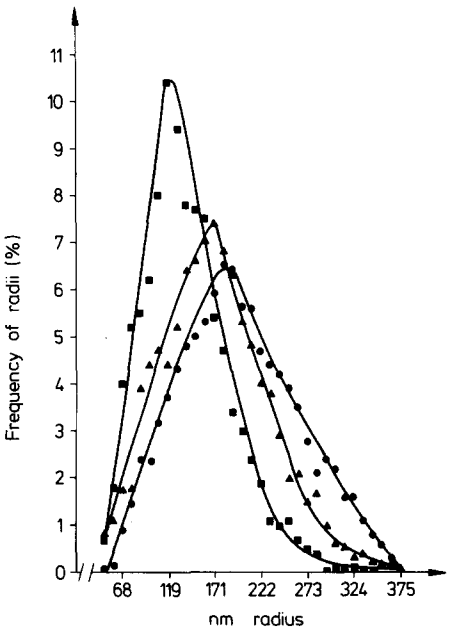

Fig. 5. Frequency distribution of the sizes of chromaffin granules in the intact population (●), the same population lysed 42% (▲) and the same population lysed 75% (■) in a representative experiment. The radii appear normally distributed with a narrowing of the distributions and a shift to lower sizes after partial lysis.

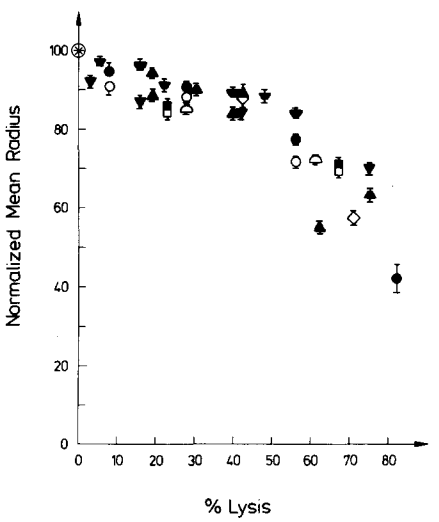

Fig. 6. The mean sizes of chromaffin granules as a function of lysis. The results of nine experiments are shown as the means ±S.E. as percentages of the mean size of the unlysed population. Each symbol represents a different experiment, with the closed symbols representing measurements of diameters in after resuspension in 0.26 M sucrose medium after partial lysis, and the open symbols in resuspension sucrose solutions of the concentration used for the partial lysis. An unequivocal decrease in mean size proportional to lysis can be observed which is highly significant and independent of the resuspension medium of the partially lysed populations.

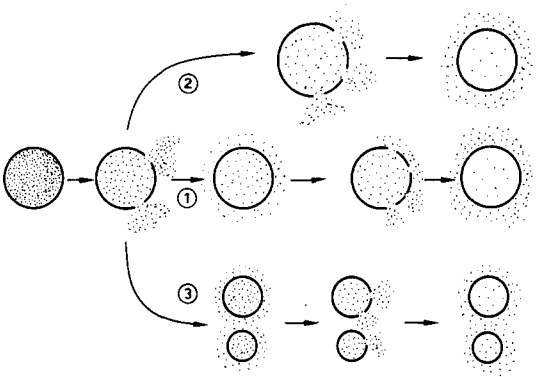

Fig. 7. A schematic drawing of the three proposed models of osmotic lysis in membranous particles. In Model 1, pores of restricted size are thought to be created by the osmotic stress, which spontaneously reseal after enough core material has escaped to lower the internal osmotic pressure substantially. In Model 2, it is thought that once a membrane discontinuity has been created through osmotic stress, the whole of the core of that particular granule is released into the medium and the empty membrane ghost reseals. Partial lysis would be the selective and complete lysis of a susceptible part of the granule population without a change in the other granules. In Model 3, the membrane holes created by the osmotic pressure are thought to be too large and unstable to allow outward diffusion of core materials with consecutive resealing in the location of their creation. Instead, in a compromise between the resealing tendency of the membrane and the core osmotic pressure, the holes are envisioned to grow rapidly, giving the membrane the opportunity to reseal into smaller vesicles which contain smaller volumes of the original core solution. Both Model 1 and Model 3 predict that partial osmotic lysis creates a granule subpopulation with new osmotic properties. However, with Model 1, osmotic resistence is achieved by core dilution while with Model 3 it is due to a decrease in particle diameter.

Fig. 4. Electron microscopy plate. Whole mount electron micrographs of chromaffin granules lysed to different degrees and resuspended in 0.26 M buffered sucrose (final magnification 9000×). Granules are easily discernible as well demarcated, electron dense structures, while ghosts are electrolucent shadows. The assigned percent lysis is biochemically measured lysis. A, 0% lysis; B, 29% lysis; C, 33% lysis; D, 46% lysis; E, 75% lysis; F, 100% lysis. Note the decrease in the size of the intact granules and the increase in 'ghosts' as the lysis proceeds.

The Laplace relationship predicts that the membrane tension excerted by a given osmotic pressure gradient is directly proportional to the particle's radius. This is in excellent agreement with the size reduction after partial lysis which suggests that the bigger granules may be lysed first. Noradrenaline and adrenaline are stored in and secreted from separate chromaffin granules in separate cells in the adrenal medulla [43]. Our results suggest that noradrenaline granules are more fragile than adrenaline granules. Since noradrenaline granules are smaller, on the average, than adrenaline granules [44], this result suggests a considerably greater core osmolarity of the noradrenaline granules. In view of the differences in chemical structures between the two catecholamines and of the reported differences in composition of the two types of granule [45] this result is not altogether surprising. The greater osmotic fragility of the noradrenaline granules may offer an explanation for the selective lysis of noradrenaline-containing granules by low concentrations of the Ca^{2+}-ionophore Br X537A [46]. However, the ionophore X-14547 depletes granules of both catecholamines without lysing either population [47].

Modelling osmotic lysis: rupture and resealing of the membranes

The membrane rupture during osmotic lysis, is, by definition and experimental design, due to a transmembranous osmotic pressure gradient that builds up in media of low osmolarity. However, the fate of a given particle after its membrane has been ruptured and the meaning of the osmotic fragility curve are completely undetermined. In the past osmotic lysis has been modelled as one of two alternative concepts. It was proposed that after partial release of core contents, granules reseal rapidly into vesicles of the same size with diluted core solutions (Fig. 7, Model 1) [37]. In this case the osmotic fragility curve would be an expression of successive cycles of lysis of the same particles. Alternatively, Morris et al. [34] suggested that lysis is an all-or-none event for each granule with the membranes of an individual granule resealing only after all of its core contents have escaped (Model 2). In this case the osmotic fragility curve would accurately reflect the distribution of osmotic fragility in the particle population.

The results of the experiments in which we found a reproducible reduction in granule size as a function of lysis are incompatible with Model 1. However, two of our experiments are also incompatible with a lytic process according to Model 2. The experimental work of Fig. 3 demonstrated that after partial lysis the osmotic fragility curve does not reflect a complete lysis of a fragile granule subpopulation with no change in the remaining granules but rather a change in fragility of the entire population. Second, we calculated expected size distributions of lysed granules on the basis of Model 2 by assuming a spherical or uniform lenticular shape and further assuming that the percent lysis could be equated to the percent volume lost from the population of intact granules. Such calculations led to negative size distributions at small radii for all experiments, implying the existence of a considerably larger population of small granules after partial lysis than can be accounted for by Model 2 (data not shown).

In the light of these results we propose a new concept for the sequence of osmotic lysis in chromaffin granules (Model 3 of Fig. 7 and legend thereto): some osmotically stressed granules resealed rapidly after membrane rupture and partial core release. This class of resealed vesicles would be smaller due to loss of membrane material and contain practically undiluted core solutions. The lost membrane material could either form many small empty vesicles or fragments, either of which would be hard to sediment. Resealing would have to be very rapid to retain granule core material. Our 6-carboxyfluorescein experiments showed that after total lysis, resealing was much faster than the time required for isolating ghosts. Apps et al. [40] have established that chromaffin granules reseal much faster than do erythrocytes. However, rapid kinetic measurements will be required to validate this point. If true, Model 3 offers an explanation for some of the conflicting results concerning all-or-none versus graded release of granule contents [34,37,48–50].

Our proposed model for the sequence of lysis in chromaffin granules might also serve to resolve the controversy about the presence of lipids in the supernatant after centrifugation of totally lysed granules. This has been reported frequently [51–53], and a high molecular weight lipoprotein

complex has been isolated from the chromaffin granule lysate [54]. Winkler, however, has pointed out that the lipid content of the lysate extremely variable [36] and that most of it can be pelleted by centrifugation at $180000 \times g$ for 1 h [55]. It is possible that the lipid content of the lysate is due to membrane fragmentation inherent to lysis according to Model 3, producing small membrane vesicles that are not pelleted. Reinvestigation of the lysate of such experiments using fixation followed by filtration to prepare samples for electron microscopy [56] might experimentally resolve this point.

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