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Two different types of lysis of chromaffin granules characterised by freeze-fracture electron microscopy and photon correlation spectroscopy

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When bovine chromaffin granules are incubated in hyperosmolar sucrose solutions and subsequently transferred back towards isoosmolarity they undergo lysis ('hyperosmotic relaxation lysis'). This type of lysis was compared with the common effect of hypotonic lysis by means of photon correlation spectroscopy (PCS) and freeze-fracture electron microscopy. Both methods revealed differences regarding mean sizes and size distributions of granules lysing under either hypotonic or hypertonic conditions. However, the results obtained by these two methods were not consistent. In the case of hypotonic lysis, a nonmonotonic behaviour of the mean diameter as a function of the sucrose concentration was observed by PCS, but not in the micrographs. From EM size determinations we obtained a decrease in the mean diameter and an increase of the width of the distribution due to the appearance of small (50–200 nm) vesicles. Probably these vesicles are intragranular vesicles released during lysis. The maximum in photon correlation spectroscopy (PCS) diameter being 140% of the isotonic diameter is shown to be caused by the changing size distribution and geometry of the lysing granules. In the case of hyperosmotic relaxation, micrographs revealed that originally shrunken, nonspherical granules regained their spherical shape and formed small (60 nm) vesicles upon lysis. In contrast, no difference was observed between the sizes of granules prior to and after hyperosmotic relaxation by means of PCS. The paper discusses the validity of intensity-weighted light scattering data of polydisperse particle suspensions with changing size distributions. The mechanism of hyperosmotic relaxation lysis is considered.

Introduction

Chromaffin granules, the storage vesicles for catecholamines and neuropeptides in adrenomedullary cells, have been frequently used as a model system for exocytosis. Many studies have indicated that osmotic forces are involved in exocytosis [1–4] though the role of osmotic swelling for the membrane fusion-fission reaction is still controversial [5,6]. Therefore osmotic properties and lysis behaviour of chromaffin granules have been investigated [7–11].

There are two types of osmotic lysis of chromaffin granules: Hypoosmotic lysis is a common effect to all cells and vesicles. It occurs when the external osmotic pressure is lowered leading to an influx of water into the granule. The membrane is stretched until a critical membrane tension is reached which causes membrane rupture and release of the contents of the granule. Hypoosmotic lysis of chromaffin granules is half-maximal at 200 mosM [7,11].

But chromaffin granules and a few other secretory vesicles [12] also undergo lysis under hyperosmotic conditions. This type is called hyperosmotic relaxation lysis [7]. It occurs when chromaffin granules are suspended in hypertonic sucrose or salt solutions and subsequently transferred back to lower osmolarities at which they had been stable before. Hyperosmolarity itself does not cause lysis of the granules and release of their contents but induces changes in their structure. Upon shrinkage in hyperosmotic sucrose solution the granule membrane becomes tightly attached to intragranular vesicles of unknown function [11,13]. This adhesion probably decreases the granule membrane area accessible for

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; osM, osmolar; PCS, photon correlation spectroscopy; IMP, intramembraneous particle; IGV, intragranular vesicle.

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osmotic reexpanding. Thus the granules lyse and release their contents at still hypertonic osmolarities.

We characterised these two types of lysis in more detail. Photon correlation spectroscopy (PCS) and freeze-fracture electron microscopy were used. Both methods revealed considerable differences regarding the size distributions and shapes of granules lysing under hypertonic or hypotonic conditions. However, the results of these two methods contradicted one another. The validity of the intensity-weighted light scattering data from a polydisperse particle suspension with a changing size distribution or geometry and the possible correction of these data with regard to number-average parameters are considered.

Materials and Methods

Preparation of chromaffin granules. Chromaffin granules were prepared as described [7] by differential centrifugation in 0.26 M sucrose solution, 10 mM Hepes, 1 mM EDTA (pH 7.4) without further purification. Briefly, fresh bovine adrenal glands were cut and the medullary tissue was dissected. After homogenization it was centrifuged at $1000 \times g$ for 10 min. The supernatant was centrifuged at $22\,000 \times g$ for 20 min and the resulting pellet was washed twice in isotonic buffered sucrose at $22\,000 \times g$ for 20 min. A final centrifugation at $2000 \times g$ for 5 min yielded a homogeneous supernatant. The granule stock suspension contained about 5 mg/ml protein as determined by a modified biuret method [14] with HSA as standard. Preparation was carried out at 4 °C.

Lysis determination. Lysis of granules was determined by a fluorimetric catecholamine release assay [15,7]. Catecholamines show maximum fluorescence excitation at 285 nm and emission at 320 nm. Their dilution due to lysis of the granules causes a dequenching of their fluorescence and thus an increase in fluorescence intensity. 0% lysis refers to the intensity of an aliquot of granules in isotonic or hypertonic solutions whereas 100% lysis refers to the intensity in 10 mM Hepes.

Photon correlation spectroscopy (PCS)

Theory. Photon correlation spectroscopy (or dynamic light scattering) allows a rapid determination of translational diffusion coefficients and sizes of submicron particles [16]. For a monodisperse suspension of spheres the first order autocorrelation function of the scattered laser light $g^{(1)}(\tau)$ has the simple form

$$|g^{(1)}(\tau)| = \exp(-\Gamma\tau), \tag{1}$$

where τ is the delay time and Γ is the decay constant. Γ is related to the translational diffusion coefficient D:

$$\Gamma = DK^2, \tag{2}$$

where K is the scattering vector. Assuming the particles to be solid, noninteracting spheres one can calculate their diameter, d, using the Stokes-Einstein relationship

$$D = k_B T/(3\pi\eta d). \tag{3}$$

Here k_B is the Boltzmann constant, T the temperature and η the medium viscosity. Usually the particles are not monodisperse but show a size distribution. In this case the autocorrelation function becomes an integral over weighted exponentials

$$|g^{(1)}(\tau)| = \frac{\int W(r,\theta)\exp[-D(r)K^2(\theta)\tau]\,dr}{\int W(r,\theta)\,dr} \tag{4}$$

where the weighting function $W(r, \theta) = N(r) \cdot I(r, \theta)$ depends on the number N of particles of the radius r and the intensity $I(r, \theta)$ scattered in the direction θ by a particle of the radius r. The real particle size distribution function $N(r)$ can be obtained from the PCS experiment only if $I(r, \theta)$ is known [16].

In most practical cases the methods of cumulants is more suitable for the analysis of PCS data. It gives the first and the second moment of the distribution function of decay times, i.e., its mean value, Γ, and the width, μ. The ratio $Q = \mu/\Gamma$ is called polydispersity. It is essential to note that in this case Eqn. 1 and Eqn. 2 give the diffusion coefficient, D, and the diameter, d, as intensity-weighted parameters but not as mean parameters for the particle size distribution function $N(r)$. The intensity-weighted parameters are called z-average diffusion coefficient, D_z and $1/z$-average diameter, $d_{1/z}$ [17]. If the weighting function $W(r, \theta)$ is unknown, D_z and $d_{1/z}$ are the only parameters available from PCS data. As the intensity of the scattered light is proportional to d^4 to d^6, larger particles give a considerably higher fraction of the signal than smaller ones. Therefore, the $1/z$ average diameter is overestimated as compared with the number average diameter d_n.

Measurements. PCS measurements were performed using a 'Zetasizer-2', Malvern, U.K. the 632.8 nm line of a helium neon laser was focused onto the sample in an glass tube maintained at constant temperature (20 °C). The correlation function of the scattered light was detected over a period of 10 min for each sample. The samples contained chromaffin granules at a concentration of 50 μg protein/ml in solutions of varying sucrose concentrations, containing 10 mM Hepes (pH 7.4) and 0.5 mM EDTA. At such a low granule concentration aggregation or secondary scattering should not occur [18]. Buffer and sucrose solutions were prefiltered through 0.22 μm filters, Millipore. Granules were incubated and diluted with 4 °C solutions and allowed to equilibrate before the measurements. PCS data were analysed using the method of cumulant ex-

pansion which yielded $1/z$ average diameter and polydispersity. Refractive indices, viscosities and osmolarities of sucrose solutions were taken from Ref. 19, p. D-262, Table 88.

Freeze-fracture electron microscopy. Isolated chromaffin granules were incubated in sucrose solutions of different osmolarities. The granules were centrifuged for 60 min at $55000 \times g$ or for 20 min at $22000 \times g$ depending on whether or not they were lysed before, and kept at 0 °C. A sandwich technique was used in which the unfixed material was placed between specimen holder plates for the double-replica stage of Balzers. After freezing in liquid propane the samples were fractured at −150 °C using a BAF 400 D apparatus (Balzers, Lichtenstein). The replicas were examined in a JEM 100 B electron microscope (Jeol, Japan) at 80 kV.

Results

Lysis determination

Fig. 1 shows the amount of catecholamine release upon hypoosmotic lysis and hyperosmotic relaxation lysis. For the latter type of lysis, granules were incubated for 15 min at 0.5 M or 1.0 M sucrose, respectively. No catecholamine release was observed when the granule suspension was diluted with the respective incubation solution (see 0% values for the incubation concentration). When the sucrose concentration was lowered, however, hyperosmotic relaxation lysis occurred.

PCS measurements

The sizes of granules as a function of the sucrose concentration were measured for different ways of sample preparation, see Fig. 2. In most cases, the data are

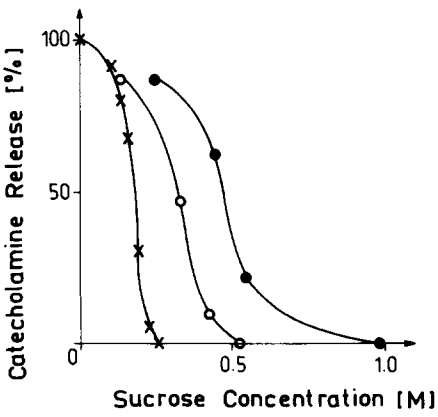

Fig. 1. Osmotic lysis of chromaffin granules in terms of catecholamine release as a function of sucrose concentration. The crosses refer to hypotonic lysis, when the osmolarity of the granule suspension was lowered starting from isotonicity. The other curves reflect hyperosmotic relaxation lysis. After incubation at 0.52 (○) or 0.99 (●) M sucrose the granules were transferred to solutions of lower sucrose concentration as indicated.

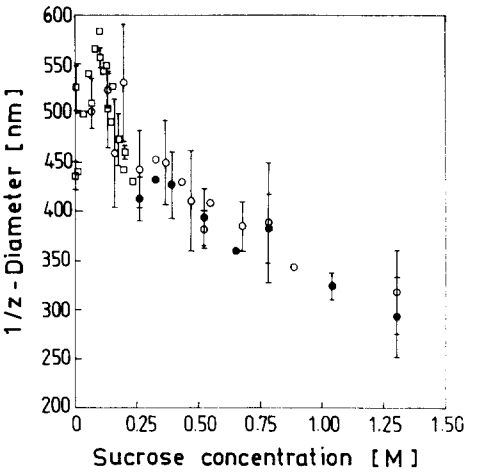

Fig. 2. $1/z$ average diameters of chromaffin granules determined by PCS. ●, mean of control granules ± S.D. suspended at the sucrose concentration indicated. ○, granules which were incubated at 0.78 M or 1.3 M sucrose and transferred towards lower osmolarities. □, granules which were transferred from isotonic sucrose concentration to hypotonic osmolarities (see also Fig. 3a).

mean values ± S.D. from three different granule preparations. Filled circles in Fig. 2 represent mean sizes of granules that were transferred from isotonic to hypertonic solutions. On increasing the osmotic pressure their size decreased indicating osmometer behaviour. Open circles show mean diameters of granules that were preincubated at 0.78 or 1.3 M sucrose and transferred back towards lower osmolarities. This procedure causes hyperosmotic relaxation lysis which amounts to 70% or 90%, respectively, at isotonic sucrose concentration. Within the error of our measurements there was no significant difference regarding the sizes of intact and lysing granules.

Open squares in Fig. 2 refer to chromaffin granules that were transferred from isotonic to hypotonic sucrose solutions. A striking nonmonotonic behaviour of PCS diameters can be observed. The $1/z$ average diameter reached a maximum of as much as 585 nm at approximately 0.1 M sucrose. On further decreasing the osmotic pressure, the diameter dropped again.

This behaviour is shown more clearly in Fig. 3a. The size maximum was observed for each of three different granule preparations. Usually the osmotic pressure was lowered directly in one step from isotonic concentration to the sucrose concentration indicated. A stepwise protocol did not change the nonmonotonic behaviour.

In the case of isotonically and hypertonically suspended granules the parameter of polydispersity varied from 0.05–0.2 in an irregular manner. In hypotonic lysis experiments, however, the polydispersity showed the same nonmonotonic behaviour as a function of the sucrose concentration (Fig. 3b) as the $1/z$ average diameter did (Fig. 3a). The polydispersity seemed to be correlated with the $1/z$ average diameter in the hypo-

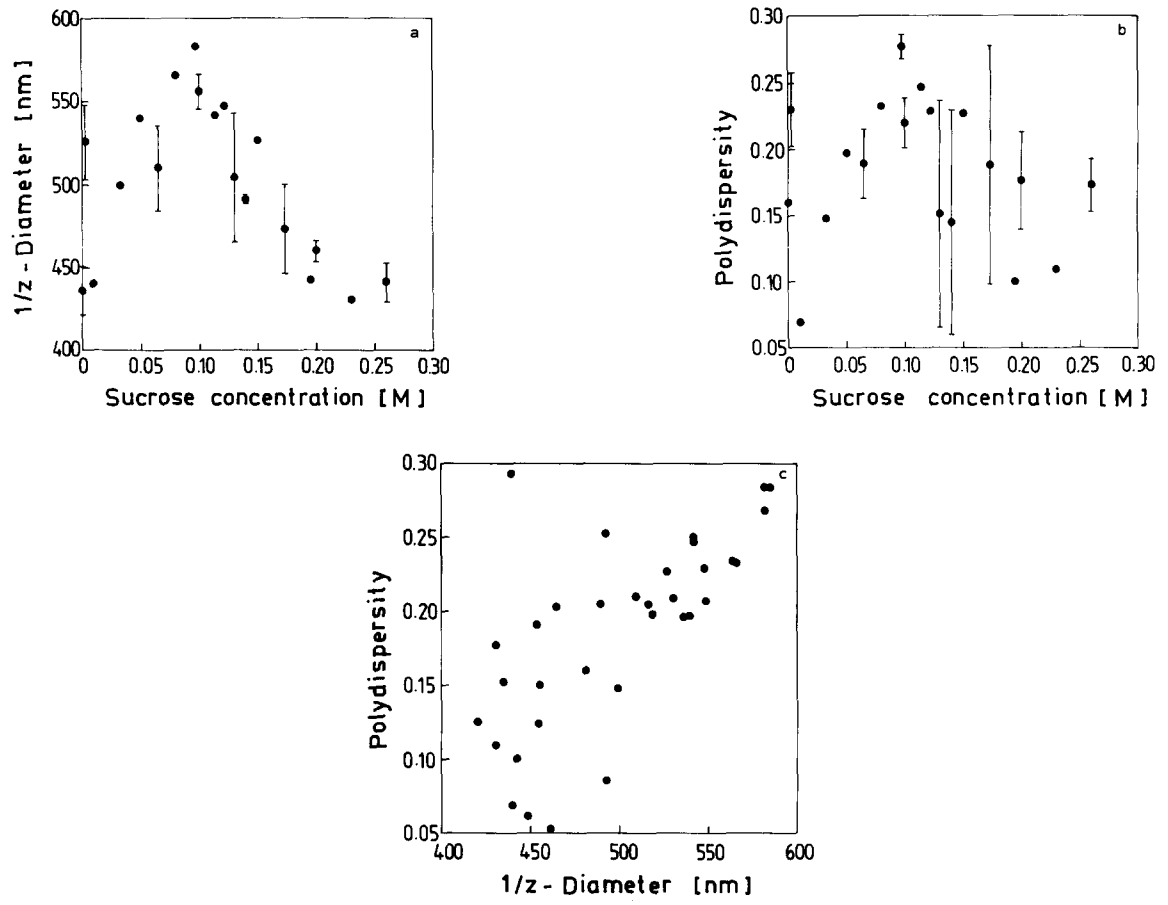

Fig. 3. PCS measurements of granules in the hypotonic range. (a) shows the $1/z$ average diameter as a function of the sucrose concentration. In (b) the corresponding polydispersities are shown. (c) suggests a correlation between polydispersity and the $1/z$ average diameter.

tonic range, see Fig. 3c. A correlation coefficient of 0.68 was determined using all data. When two nontypical points with low diameter, but high polydispersity (probably due to contamination) were omitted the correlation coefficient was equal to 0.86.

The correlation between both parameters led us to the assumption that the striking size maximum at 0.1 M sucrose need not exist in reality. Instead, it may be due to a change in the size distribution which is induced by granule lysis, see Discussion.

Electron micrographs

Isotonically suspended granules are shown in Fig. 4a. They were mostly of a spherical shape with a mean diameter of 334 nm $\pm$ 111 nm. Intramembraneous particles (IMPs) were distributed randomly.

For the examination of hyperosmotic relaxation lysis, the granules were first incubated at 0.5 or 1.0 M sucrose. Upon osmotic shrinkage they underwent changes in their shape which resembled a continuous 'budding' process (Fig. 4b,c). Usually one 'bud' was formed by each granule which gave rise to a 'twin'-shaped granule. Sometimes, however, there were two 'buds' leading to 'triplet' granules. Interestingly, the 'buds' were free of IMPs (Fig. 4c).

With increasing osmotic pressure the number of 'budding' granules or 'twins' increased. Also, the 'twins' became more pronounced (Fig. 4b,c). Table I shows the percentage of 'twinned' granules as a function of sucrose concentration for controls, shrunken granules, and partially osmotically reexpanded granules. Even in controls a few granules were observed which had a lenticular shape. However, they did not exhibit the typical segregation of IMPs. At 1 M sucrose, more than one third of all granules showed these characteristic shape changes. The actual percentage should be even larger. As the twin-shaped granules are fractured randomly, a considerable number of fracture planes will yield circular profiles rather than 'twin'-profiles. Thus, the real number of 'twins' is underestimated in our analysis of two-dimensional micrographs.

When the granules were transferred from 1 M sucrose towards lower osmolarities the number of 'twins' quickly decreased. At 0.7 M there were 8.4% 'twins' and at 0.5 M as little as 0.6% 'twins' left (Table I). The latter value is close to the percentage of twins in controls. As shown

TABLE I

Percentage of 'twin-shaped' granules ± S.D. per micrograph and lysis of granules in terms of catecholamine release as a function of sucrose concentration

(a) – granules were incubated without further treatment; (b) – granules were preincubated at 1 M sucrose for 15 min and subsequently transferred to the concentration indicated.

Sucrose concn. (M)	Twins ± S.D. (%)	Number of all granules counted	Catecholamine release (%)
(a)			
0.26	1.3 ± 0.7	852	0
0.5	10.6 ± 2.9	165	0
1.0	36.1 ± 4.2	1404	0
(b)			
0.7	8.4 ± 0.6	614	10
0.5	0.6 ± 0.8	817	45

in Fig. 4d,e the granules seemed to round up again. Apart from the few twins that were still visible at 0.7 M there was no IMP segregation in granules any longer. IMPs were distributed over the whole granule again, partly forming small clusters.

Hyperosmotic relaxation lysis was not only accompanied by the reduction of 'twins' but also by the formation of small vesicles. All these vesicles had a similar size of approx. 60 nm. They already occurred at 0.7 nm sucrose but much more frequently at 0.5 M after incubation at 1 M sucrose.

For the investigation of hypotonic lysis, micrographs of granules were taken at 0.27, 0.18, 0.10, and 0 M sucrose concentration, 10 mM Hepes, 5 mM EDTA. At these concentrations the catecholamine release amounted to 0, 50, 90 and 100%, respectively (crosses in Fig. 1). On decreasing the osmotic pressure, the granule membranes became flaccid and crinkled. Some disrupted granules and pieces of membrane occurred. IMPs formed clusters within the granule membranes. Especially at 0.1 M and 0 M sucrose many small vesicles were visible. Fig. 4f shows granules lysed in sucrose-free buffer where these effects were the most pronounced.

The decrease in osmotic pressure was accompanied by an increasing number of cross-fractured granules. At 0 M sucrose, there were 30-times more cross-fractured granules as compared with isotonicity. This effect is caused by the decreased stability of the membranes due to lysis. At 0.18 M sucrose, 40% of the cross-fractured granules revealed intragranular vesicles. At 0 M sucrose, however, this percentage amounted to 16% only. Thus we conclude that 60% or more of the IGVs were re-

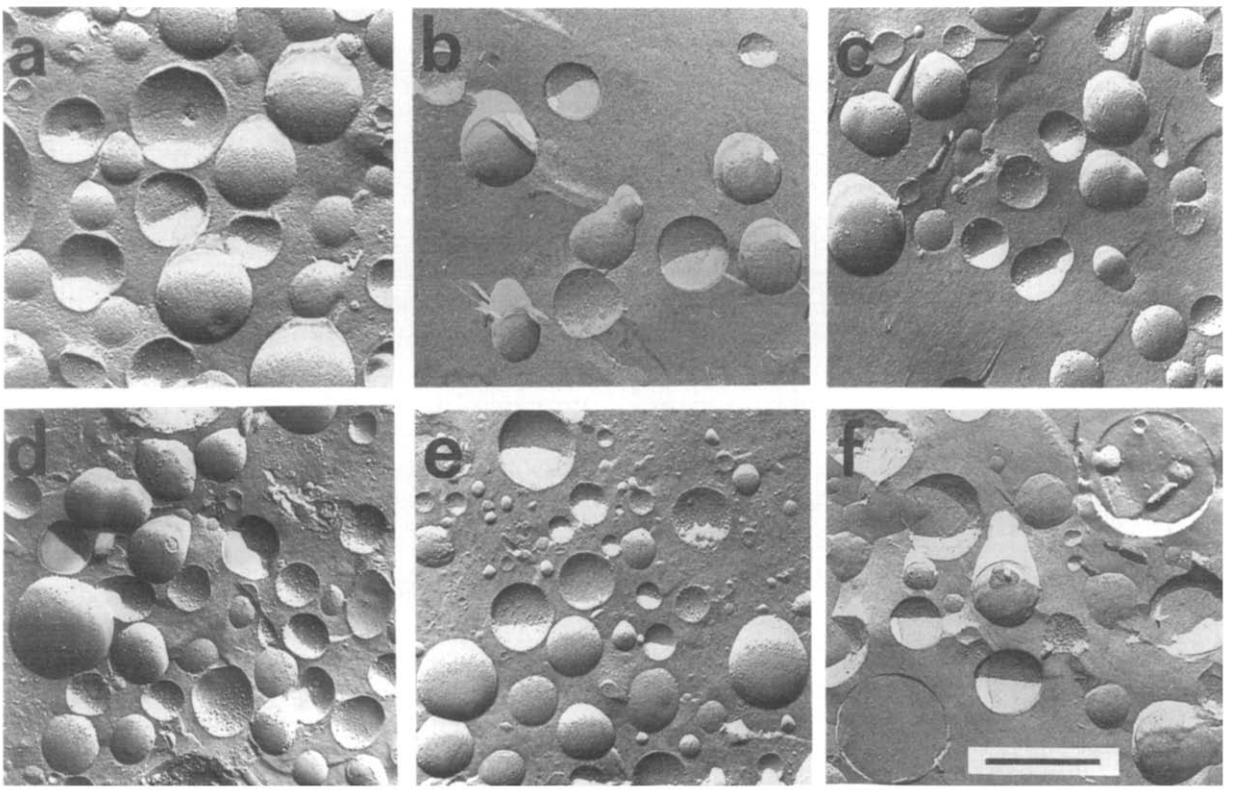

Fig. 4. Freeze-fracture micrographs of chromaffin granules. (a) Control granules at 0.27 M sucrose; (b) granules at 0.5 M sucrose, only one single 'twin' is visible; (c) granules at 1.0 M sucrose, a number of 'twins' are formed, one part of each twin is free of IMPs; (d) granules at 0.7 M sucrose after incubation at 1.0 M sucrose, twins begin to disappear; (e) granules at 0.5 M sucrose after incubation at 1.0 M sucrose, spherical shape is regained, IMPs are no longer segregated, small vesicles appear; (f) granules lysed in sucrose-free Hepes buffer showing deformed membranes and IMP cluster formation, cross-fractured granules reveal no IGVs; inset: a cross-fractured granule at 180 mM sucrose which still contains 3 IGVs. The shadow direction is from bottom to top, bar: 500 nm.

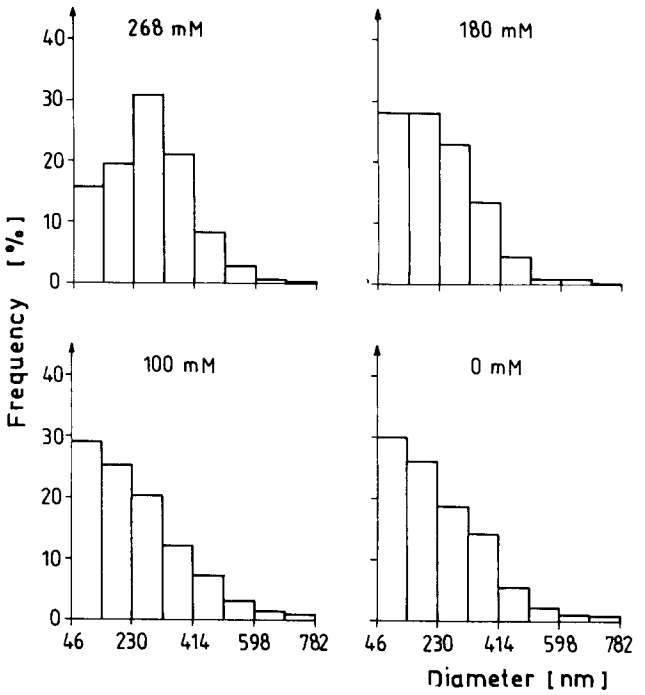

Fig. 5. Histograms of the diameters of the fracture profiles of chromaffin granules for four different sucrose concentrations.

leased together with the soluble contents of the granules during lysis.

With respect to the size maximum of granules, which was observed by PCS, we analysed the size distributions of granules at the four sucrose concentrations. Fig. 5 shows the distributions of the fracture profiles. Isotonically suspended granules possess a unimodal size distribution with a symmetrical frequency maximum. At lower osmotic pressure, the size distribution becomes highly asymmetric. Eventually, the frequency maximum is located at the lowest sizes (50–150 nm).

Note that we show the size distributions of the profiles after freeze-fracturing. As the fracture plane cuts the spherical granules at random levels the profile size distribution will differ from the actual size distribution of the spheres. Unfortunately, the profile size distributions cannot easily be transformed into the underlying granule size distribution [20,21]. However, at least the first (mean diameter) and the second moment (standard deviation) of the distribution function can be transformed as follows:

$$d_g = 4d_p/\pi \tag{5}$$

$$s_g = 3s^2{}_p/2 - (16/\pi^2 - 3/2)d_p^2 \tag{6}$$

Here d_g is the mean diameter of the granules and d_p the mean diameter of the fracture profiles and s_g and s_p are the respective standard deviations. Eqn. 5 was taken from Ref. 21 whereas Eqn. 6 was deduced by ourselves.

Corrected mean diameters, d_g, as a function of sucrose concentration and corrected corresponding standard deviations s_g are shown in Fig. 6. At 0 M sucrose concentration, the mean diameter (weighted mean of all micrographs) dropped to 85% of that of control granules. On the other hand, the width of the size distribution of the lysed granules increased. The standard deviation was 30% higher than that of the control.

Discussion

Both techniques, PCS and freeze-fracture electron microscopy, revealed considerable differences between hypoosmotic lysis and hyperosmotic relaxation lysis of chromaffin granules. When the granules were transferred from isotonic into hypotonic solutions, we observed a striking nonmonotonic behaviour of their mean size by means of PCS (Figs 2,3a). The maximum size observed at 0.1 M sucrose amounted to 585 nm. Given a spherical shape of the granule at 0.26 M as well as 0.1 M sucrose, this increase in diameter corresponds to an area increase by more than 75% and an increase in volume by 135%. As biological membranes can be stretched up to 2–3% of surface area increase only [22], the values must be reconsidered critically.

Not only did we observe a nonmonotonic behaviour of the mean diameter but also a correlation between the mean diameter and the corresponding polydispersity (Fig. 3a–c). A polydispersity different from zero indicates that the decay rates, Γ, and hence the diameters, are not monodisperse but show a distribution. The method of cumulant analysis, however, yields only average, i.e., intensity-weighted parameters. Therefore larger particles contribute to this average value much more than smaller ones do.

For some particle size distributions such as log normal there are analytical methods that allow to transform the intensity-weighted parameters into number-average or weight-average values [17]. Electron microscopic size analyses of chromaffin granules using the whole mount technique [7] suggested that the granules are distributed as log normal.

We transformed the intensity-weighted diameter $d_{1/z}$ obtained by PCS into the weight-average diameter d_w as follows [17]:

$$d_w = d_{1/z}/(1+Q)^2 \tag{7}$$

This was done in order to calculate the mean of the granules. Fig. 7 shows the volumes before (circles) and after this correction (squares) as a function of inverted osmotic pressure. Before the correction, the volume shows a nonmonotonic behaviour with a steep maximum similar to the size curve in Fig. 3a. The correction by polydispersity, however, caused the maximum to disappear. There is a slight increase of the corrected

mean volume in the leftmost part of the curve, corresponding to the range from 0.26–0.13 M sucrose. Towards lower osmolarities, the volume remains essentially constant.

Is such a correction by the parameter polydispersity verified? What is actually measured by the PCS method is the correlation time of fluctuations of the scattered light. As a result of calculations, we obtain intensity-weighted parameters. In order to determine the number-average diameter or the weight-average diameter a correction should be done generally. Since chromaffin granules possess a log normal size distribution a correction of $d_{1/z}$ according to Eqn. 7 was accomplished which yielded reasonable results.

Comparing the data of the PCS method and FFEM we obtain a contradiction: Both parameters of cumulant analysis show a nonmonotonic behaviour but the mean diameter and its standard deviation taken from the micrographs show small but monotonic changes in opposite directions (Fig. 6). This contradiction can be explained by the appearance of particles ranging from 50–200 nm caused by lysis. These vesicles could be generated from the granule membranes. When pores are formed, membrane edges can curl up and spontaneously form vesicles [23]. The lack of intragranular vesicles in cross-fractured granules at 0 mM sucrose suggests, however, that just these internal vesicles were released, at least in part. Additionally, their size [11,13] is comparable with that of vesicles appearing during hypotonic lysis. As this size is in the range of the sizes of the granules the new vesicle population could be detected by PCS. If there were a considerable distance between

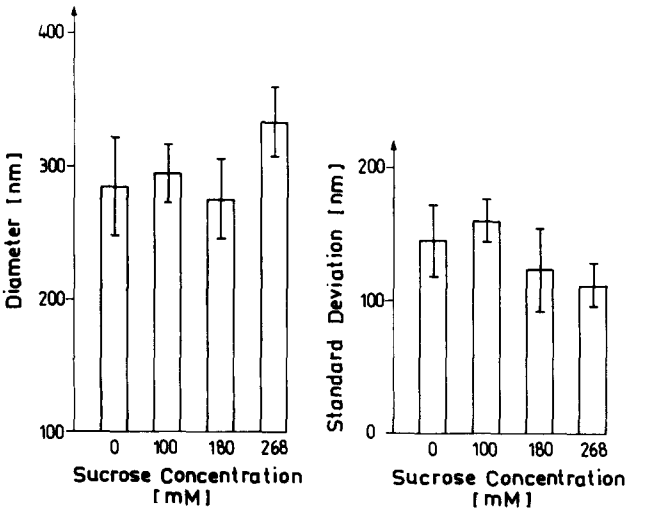

Fig. 6. Mean corrected diameter and mean corrected standard deviation of chromaffin granules at four different hypotonic sucrose concentrations. The correction for the random fracturing process was accomplished according to Eqns. 5 and 6. Values are given ± S.D. that refer to the standard deviation of different micrographs in each group. The total numbers granules counted are 1216, 482, 318, 728 for the concentrations 0, 100, 180, 268 M sucrose, respectively.

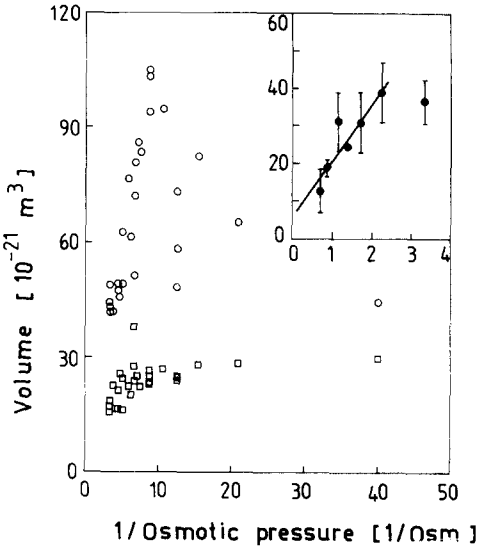

Fig. 7. Volume of chromaffin granules as a function of inverted osmotic pressure. The volumes are calculated from PCS data. The inset shows the osmometer behaviour of granules above isoosmotic pressure. The main diagram refers to the hypoosmotic range. ○, volumes of granules calculated from PCS data (see Fig. 3); □, volumes corrected by the polydispersity (Fig. 3b).

the mean sizes of both populations the signal intensity of the population of the smaller particles would be negligible as compared with that of the larger particles unless the smaller particles were much more abundant than the larger ones [16,24].

The appearance of this new vesicle population leads to an increase of the width of the distribution and a decrease of the mean diameter which have opposite effects on the behaviour of $d_{1/z}$ as shown in Eqn. 7. Upon decreasing the osmotic pressure, the polydispersity may grow faster than the weight-average diameter decreases due to the formation or release of small vesicles. According to Eqn. (7) the $1/z$-average diameter should increase in this case. At still lower external osmolarities this behaviour may reverse. Then, $d_{1/z}$ must obviously decrease.

Hence, we can explain the maximum in $d_{1/z}$ as a function of external sucrose concentration by the specific properties of our system. Probably, the granules spontaneously released internal vesicles during the process of hypotonic lysis. In order to estimate the number of IGVs released the histograms (Fig. 5) prior to and after complete lysis were compared. The scale of the histogram at 0 M sucrose was changed such that all bars except the first and the second one did coincide with those from the unlysed granule population as shown in Fig. 8. The deviation in the first two size classes between lysed and unlysed population then expresses the amount of vesicles that appeared during lysis. From this estimation it follows that less than one internal vesicle (on average 0.6–0.7) was released by each granule. This value is in agreement with the result obtained from the

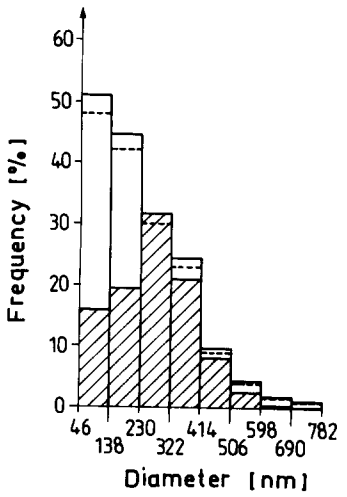

Fig. 8. Comparison between the size distribution of control granules (hatched bars) and that of granules lysed at 0 M sucrose. During hypotonic lysis many small vesicles appear the sizes of which fall into the first and second size class. In order to estimate the number of vesicles released, the histogram at 0 M sucrose (Fig. 5) was fitted to that of control granules such that the bars 3–8 show minimal deviation. Solid bars, 0.7 small vesicles were released by each granule; dashed bars, 0.6 vesicles were released by each granule.

analysis of IGVs in cross-fractured granules. Thus, hypoosmotic lysis of chromaffin granules offers a way to isolate and analyse the intragranular vesicles.

Apart from the increase in the width of the size distribution and the decrease in the mean weight- or number average diameter there may be additional factors which caused the nonmonotonic behaviour of $d_{1/z}$ and polydispersity. One reason could be the changing geometry of the granules due to lysis. The evaluation of $d_{1/z}$ by the method of cumulants is based on the assumption that the particles have a spherical shape. If the granules become irregularly shaped there will be 3 different diffusion coefficients (one for each direction). Such a particle is thus counted as three particles with different diffusion coefficients and, therefore, diameters $d_{1/z}$. In electron micrographs we observed nonspherical granules with crinkled membranes as well as pieces of membranes. These changes could have affected the polydispersity as discussed.

Under hypertonic conditions, the mean size of granules decreased with increasing sucrose concentration (see Fig. 2; the same was observed in electron micrographs). This decrease is due to the osmometer behaviour of the granules [7]. For an ideal osmometer the volume is proportional to the inverted osmotic pressure. In more detail, it is

$$V_t = m/\pi + V_i \tag{8}$$

with V_t being the total volume of the osmometer, m the number of osmotically active molecules inside the osmometer, π the external osmolarity and V_i the osmotically inactive volume. Fig. 7 shows the dependence of the volume on the inverted osmotic pressure. The inset demonstrates that for osmolarities above 0.35 osM the granules behaved in fact as osmometers. With regard to their sizes this behaviour was reversible as shown in Fig. 2 (open symbols).

Within the hypertonic range, we did not observe a systematic change in polydispersity for both intact granules and granules lysing, because of hyperosmotic relaxation. Therefore, no correction was accomplished. Within the accuracy of these measurements, there was no significant difference in the size, $d_{1/z}$ between these two types of granule. Electron micrographs contradicted this result. They revealed many small vesicles of the same size (60 nm) that appeared upon relaxation and thus decreased the mean diameter. It is unlikely that these vesicles are IGVs because IGVs are larger and do not remarkably shrink in hypertonic media [11,13]. Probably they were formed from disrupted granule membranes. Although these vesicles were frequent, they probably had no effect on PCS data since their light scattering intensity was too small as compared with the granules. Taking into account that these vesicles were 2–3-times smaller than those which appeared in hypotonic lysis their light scattering intensity was 2 to 3 orders of magnitude smaller than than that of the latter. This could explain the lacking influence of the small vesicles on PCS data. In a previous paper [11] we have shown that hyperosmotic incubation causes the intragranular vesicles to adhere to the granule membrane. It was our hypothesis that this adhesion was irreversible in the time scale of re-swelling. The changed geometry of the twin being equivalent to an effective reduction of surface area that is available for reexpansion was assumed to cause relaxation lysis.

In this paper, we present data on twin formation not only for shrunken granules but also for granules after osmotic relaxation. Table I shows that for a given hypertonic sucrose concentration there were more twins before than after incubation at 1 M sucrose and subsequent transfer back to this concentration. If the adhesion process of intragranular vesicles to the granule membrane were reversible in the time scale of water entry into the granules one would expect a similar percentage of twins prior to and after the incubation-relaxation procedure and one would not expect lysis to occur.

It has been established that the process of contact formation between membranes indeed need not be a reversible process [25]. The work of separation per unit area ('adhesion energy') may be significantly greater than the chemical affinity that causes adhesion. Dissipative processes as well as chemical reactions can increase the work of separation considerably [25]. The segregation of IMPs from those membrane areas which we assume to adhere to intragranular vesicles alters the

composition of these membrane patches and could induce such 'irreversible' processes. Since IMP redistribution requires some time the separation of IGVs from the inner granule membrane might be too slow as compared with granule swelling.

Given a lateral diffusion coefficient for membrane proteins of 10^{-13}–10^{-14} m^2/s [26] and a mean distance of 100 nm, the characteristic time for the redistribution of IMPs is equal to 0.1–1 s, respectively. The time for the influx of water, t_{in}, can be estimated as

$$t_{in} \approx V/AP_w \quad (9)$$

with V and A being the volume and surface area of the granule and P_w the water permeability. Assuming $P_w = 4 \cdot 10^{-6}$ m/s [27] and a radius of the granule of 150 nm the characteristic time for water influx is $1.2 \cdot 10^{-2}$ s and thus one to two orders of magnitude smaller than that for IMP redistribution.

The granules in Fig. 4d,e indicate that IMP redistribution occurs and that IGVs become separated from granule membranes after hyperosmotic relaxation lysis. For technical reasons we were not able to perform the freeze-fracturing immediately after hyperosmotic relaxation. An interval of several hours obviously was sufficient for separation and IMP redistribution to occur.

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