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CORE STRUCTURE, INTERNAL OSMOTIC PRESSURE AND IRREVERSIBLE STRUCTURAL CHANGES OF CHROMAFFIN GRANULES DURING OSMOMETER BEHAVIOUR

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In the adrenal medullary cells, catecholamines are stored in and secreted from specialized secretory vesicles, the chromaffin granules. In order to gain some understanding of both functions of chromaffin granules, it is important to characterize their biophysical organization. Using isolated bovine chromaffin granules we have investigated the osmometer behaviour of chromaffin granules by ^{31}P-NMR and fluorescence spectroscopy, by turbidity measurements and by electron-microscopic determination of chromaffin granule size distributions. On the basis of the osmometer model we have formulated equations predicting the behaviour of the native catecholamine fluorescence quenching and of the size of chromaffin granules as a function of osmolarity and have shown experimentally that the granules' behaviour conforms to these. It was possible to estimate the osmotic activity of the chromaffin granule core solution and the mean absolute water space in chromaffin granules from the determination of the size distributions as a function of osmotic pressure. With NMR spectroscopy a selective line-broadening of the α- and β-resonances was observed with increasing osmolarities, while the γ-phosphorus resonances remained virtually unchanged. Possibly there is an increase in core viscosity with osmolarity which affects only the α- and β-phosphorus groups. While suspending chromaffin granules from lower to higher osmolarities causes no lysis, moving them back to their original osmolarity at which they were previously stable lyses them, thereby releasing a maximum of 70% of their releasable protein. This 'hyperosmolar' lysis is independent of preincubation times in the higher osmolarities and of the absolute dilution applied but depends on dilution beyond the 405 to 322 mosM sucrose range. Under the experimental conditions no uptake of sucrose from the medium into the granules could be measured, thereby suggesting that hyperosmolar lysis is a phenomenon not due to solute penetration. Since with NMR and fluorescence spectroscopy no chemical changes in the core composition can be observed, we conclude that hyperosmolar lysis may be caused by irreversible membrane relaxation upon osmotic shrinking.

Introduction

Chromaffin granules, the secretory vesicles of the adrenal medulla, store catecholamines and other low molecular weight constituents in very high concentrations [1]. They are easily prepared on a large scale and have been extensively characterized biochemically (for recent reviews, cf. Refs. 2 and 3). Within the adrenal medullary cell chromaffin granules appear to have two main functions: they store molecules which are to be secreted in high concentrations in preparation for exocytosis, and they actually effect exocytosis by fusing with the plasma membrane, a process which

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

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is triggered by the influx of calcium [4]. While major advances have been made in elucidating the mechanisms of accumulation of low molecular weight molecules into the granule-core (cf. Winkler and Westhead [2]), the question of how high solute concentrations are stored in the chromaffin granule-store is still controversial. The molecular events associated with exocytosis are poorly understood. The biophysical characteristics of chromaffin granules are of great importance to both questions.

Morris et al. [20] in a careful study have put forth a biophysical model of the chromaffin granule. They describe it as an osmometer, consisting of a flexible, inelastic membrane which bounds an osmotically active core. They demonstrated that the density of the core is proportional to the osmolarity of the suspension medium over a wide range of osmolarities and concluded that within these limits the core consists of a constant number of osmotically active particles in solution. On the other hand there have been two observations reported in the literature in which chromaffin granules were suspended in solutions of high osmolaritis and then transferred back to solutions of lower osmolarities. This treatment was found to cause lysis while the granules not pretreated at higher osmolarities did not lyse [5,6].

Similar observations have been made with cholinergic synaptic vesicles [7] and this may be, like the osmometer behaviour, a widespread biologic phenomenon. This observation, however, is contradictory to the osmometer model which envisages swelling and shrinking to be reversible. Furthermore, this observation is of relevance to isolation techniques of subcellular particles which often involve great jumps in osmolarities and, last but not least, to exocytosis. It has been shown that in vitro fusion of membranes is greatly facilitated by osmotic gradients [8–12] and small osmotic pressure jumps leading to membrane rupture as a 'primer' for exocytosis may very well occur in vivo. These reasons led us to a systematic study of the osmometer behaviour of chromaffin granules with two new, mutually independent methods and to an investigation of the lysis of granules previously exposed to hyperosmotic media.

Materials and Methods

Solutions. All chemicals were of reagent grade and used without further purification. [^{14}C]Sucrose was obtained from New England Nuclear Co., Dreieich, F.R.G. All solutions were buffered with 10 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid, Sigma Co., St. Louis, MO, U.S.A.), pH 7.4, measured at room temperature. Osmolarities of sucrose solutions were calculated from Table 79, p. D-229 [13]. All experiments except for the NMR measurements were repeated several times; this was also meant to exclude artifacts by improper solutions since the osmolarities of the sucrose solutions were not checked separately. Care was taken to avoid settling of the sucrose solutions at low temperatures. The isolation of chromaffin granules was carried out in a cold room at 2–4°C; all experiments were run at 5–6°C. Values given are mean ± S.D. (number of experiments in parentheses).

Preparation of chromaffin granules. Bovine adrenal glands were obtained from a local slaughterhouse and placed on ice within 30 min of the death of the animal. Fractionation was started within 1.5 h of death. The medullae were dissected out of the glands, minced and homogenized in 0.26 M buffered sucrose, centrifuged at $1000 \times g_{max}$ for 10 min and the pellet discarded. The supernatant (S_1) was centrifuged at $27000 \times g_{max}$ for 20 min and the upper layer of the resulting pellet (mostly mitochondria) was washed away with 0.26 M buffered sucrose. The remaining pink pellet (P_2) was resuspended in 0.26 M sucrose and recentrifuged. The resulting pellet was once again washed to remove mitochondria, resuspended, and recentrifuged. The final pellet (P_4) was again washed and resuspended in 0.26 M buffered sucrose and used as starting material for all experiments. It contained 2.11 ± 0.1 ($n = 3$) μmol catecholamine per mg protein.

Spectroscopic measurements and lysis determinations. For the spectroscopic determination of osmometer behaviour, a 50 μl sample of fresh ice-cold chromaffin granule suspension in 0.26 M sucrose was added to 2 ml of the appropriate solution preequilibrated at 6°C. After 1 h of incubation the samples were transferred to water jacketed cuvettes kept at 6°C and their turbidity

and fluorescence were read, the former in a PMQ3 Zeiss spectrophotometer at 320 nm, the latter at 285 excitation and 317 emission wavelength with an emission filter opaque to radiation below 310 nm and slits of 2 nm in a MPF-4 Perkin-Elmer fluorescence spectrophotometer. All readings were corrected by subtracting the readings of the corresponding sucrose solutions. The fluorometer setting was chosen to minimize scattering contribution to the readings. With this setting, dow latex beads (diameter 312 nm, Serva Co., Heidelberg, F.R.G.) in concentrations up to 1.0 A_{320} showed only minimal signals. In several experiments the suspensions were centrifuged afterwards and the protein release was determined as described below. This was done in order to control for the influence of lysis on the spectroscopically measured parameters. However, no net protein release was ever measured (cf. Fig. 3).

^{31}P-NMR spectra of chromaffin granules as a function of osmotic pressure were read on highly concentration chromaffin granule-suspensions which had been centrifuged and washed in the appropriate sucrose solutions before measurements. NMR spectra were recorded in a Bruker WH 270 FT spectrometer interfaced to a Nicolet B-NC computer. The temperature was kept at 5°C.

For the hyperosmolar lysis measurements, chromaffin granules were resuspended into the required sucrose solutions in ice and 50-μl samples transferred to 2 ml 0.26 M or 0.52 M sucrose solutions pre-equilibrated at 6°C. After thorough mixing and one hour of incubation the suspensions were centrifuged at $40000 \times g_{max}$ for 10 min. The percent protein in the supernatant of the total protein in the incubation was measured and percent protein release was calculated from the same measurements in totally lysed chromaffin granules (in 10 mM Hepes, defined as 100% release) and in chromaffin granules transferred from 0.26 M to 0.26 M sucrose (defined as 0% release).

Electron microscopy. Whole mounts of chromaffin granules in the appropriate sucrose solutions were prepared on copper grids (coated with colodion and carbon) by rapid blotting with filter paper of a small volume (10 μl) of the suspensions [14]. The samples were electronmicrographed directly after preparation in a JOEL 100 B electron microscope and photographs of the samples (see plate) used for measuring the size frequency of chromaffin granules with a Zeiss TGZ 3 particle size analyser.

Protein assay. Protein concentrations were measured according to Ref. 14 with bovine serum albumin as standard.

Theory

Osmometer behaviour of a membrane bound biologic particle can be defined by the existence of an osmotic pressure range of the suspension medium during which the osmotic pressure inside the particle equals the osmotic pressure of the suspension medium. In the case of the chromaffin granule this definition can be written as

$$\mathrm{osM} = \frac{m}{V_{CG} - V_D} \qquad (1)$$

where osM denotes the osmolarity of the suspension medium, V_{CG} the total particle volume, V_D the particle volume minus the solvent space and m the apparent number of osmotically active molecules in the solution space of the granules in moles. The right hand term of the equation is not equal to the solute concentration because in such highly concentrated ionic solutions as exist in the chromaffin granules [1] no relation of the solution molarity to the osmotic pressure can be predicted. Osmometer behaviour of a biological particle does not imply any specific structural features in the particle except for the existence of a solution space inside and of water permeability of the membrane. However, it leads to changes in several physical parameters of the particle whose measurements allows to calculate some interesting biophysical characteristics. It has to be kept in mind, however, that osmometer behaviour implies that the solute activity coefficients remain constant over its range. This will not be the case especially for high osmolarities, leading to deviations from ideal osmometer behaviour at high concentrations. On the other hand, in media of very low osmolarities there will be a point beyond which the particle can swell no further, leading to the establishment of an osmotic pressure gradient across the membrane which after a small range of osmolarities will result in lysis of

the particle since biologic membranes are not elastic [16,17]. In the following, physical changes of chromaffin granules associated with osmometer behaviour are described mathematically.

(i) The particle density is a complex function of the osmolarity of the suspension medion. The equations have been developed by De Duve et al. [18] and the principle has been applied to chromaffin granules by Morris and Schovanka [19] and Morris et al. [20]. However, the quantitative interpretation of Morris et al. [20] of their excellent experimental work is wrong due to a mistaken definition of osmolarity in their starting equations.

(ii) As can be seen from Eqn. 1, the solvent space $V_{CG} - V_D$ is an inverse function of the osmolarity. This has been confirmed [1,2]. Diminution of the solvent space leads to concentration of the solutes, which in the case of the chromaffin granules can be directly measured by other methods. First, with ^{31}P-NMR spectroscopy linebroadening of the ATP-phosphorus resonances may be demonstrable with increasing osmolarity as a consequence of increasing core solute concentrations. Second, chromaffin granules contain catecholamines in high concentrations whose native fluorescence is greatly quenched by a dynamic mechanism [22] but increases by a factor of approximately four upon particle disruption [21]. If lysis is ruled out by independent measurements of protein release, catecholamine fluorescence measurements can be used to measure the core solute concentration. The Stern-Vollmer equation for dynamic fluorescence quenching states that

$$\frac{F_0}{F} = 1 + K_Q[Q] \tag{2}$$

where F_0 is the fluorescence of the unquenched fluorophore, F the fluorescence of the sample, K_Q a quenching constant and [Q] the concentration of the quencher [23]. Although the quencher in the granules is not identified it follows from Eqn. 1:

$$\text{osM} = kK_Q[Q] = k\left(\frac{F_0}{F} - 1\right) \tag{3}$$

where k is another constant. This equation can be directly tested experimentally.

(iii) The chromaffin granule-volume will be an inverse function of the osmolarity as can be seen from a different formulation of Eqn. 1

$$V_{CG} = \frac{m}{\text{osM}} + V_D \tag{4}$$

Measuring the volume changes as a function of osmolarity will yield by far the most information about the structure of chromaffin granules because there are only two well-defined unknowns in the equation which can be determined that way. It will allow direct measurements of the apparent osmotic pressure in chromaffin granules as well as of the lower limit of osmometer behaviour and of the solvent space.

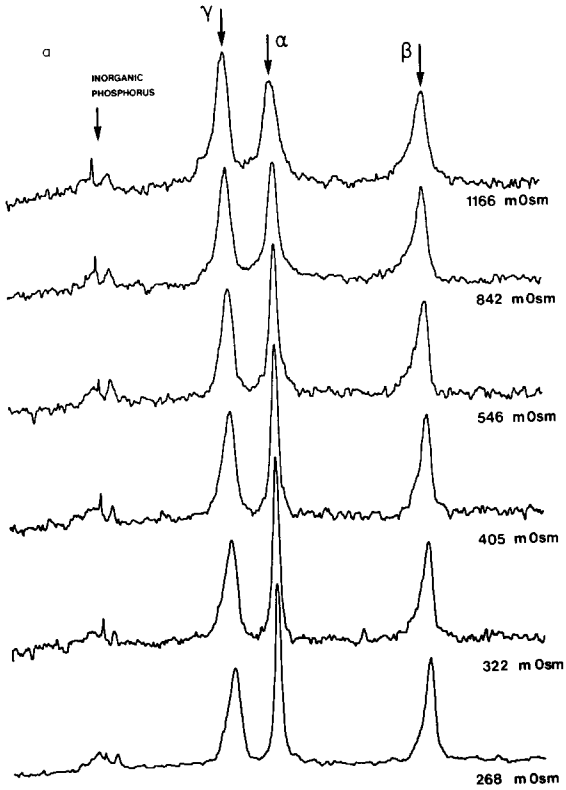
Results

Spectroscopic studies

The ^{31}P-NMR spectra depicted in Fig. 1a compare well with spectra of intact chromaffin granules published by other groups [23,24]. No chemical shifts of the phosphorus resonances can be observed with increasing osmolarity, indicating that no lysis or changes in the intragranular pH occur during osmometer behaviour. Fig. 1b depicts the half-height linewidths of the phosphorus resonances as a function of osmolarity. It demonstrates that linebroadening of the α- and β-phosphorus resonances occurs with increasing osmolarity while the γ resonances remain virtually unchanged. The selective linebroadening of the α- and β-phosphorus resonances could indicate that the γ-phosphorus groups are spared from the effect of core solute concentration. Alternatively, linebroadening of the γ-resonances may be obscured by the intergranular heterogeneity in chemical shift suggested by Njus et al. [26].

In Fig. 2 uncorrected fluorescence spectra of lysed and intact chromaffin granules at different osmolarities are shown. No differences between spectra besides a questionable small shift to higher wavelengths with increasing osmolarities can be appreciated, indicating that no sizable chemical changes take place.

Fig. 3 shows the changes in turbidity and fluorescence of a chromaffin granule-suspension as a function of osmolarity. It can be predicted from



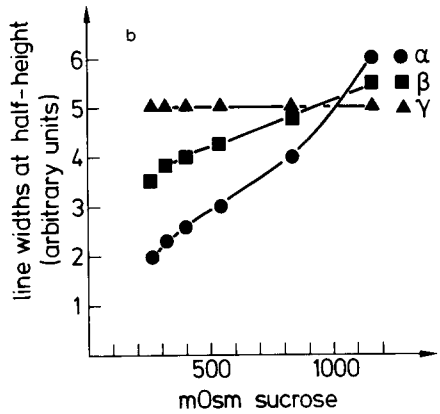


Fig. 1. Fig. 1a depicts ^{31}P-NMR spectra of chromaffin granules at different osmolarities of the sucrose suspension medium. A selective broadening of the α- and β-phosphorus resonances can be observed with little change in γ-phosphorus resonances. In Fig. 1b this is illustrated by a plot of the lnewidths at half height vs. the sucrose osmolarities. (Spectra were taken at 5°C with 2000 pulses per spectrum and an interpulse time of 1 s.)

Eqn. 3 that in the case of osmometer behaviour

$$1/\mathrm{osM} = 1/\left(\frac{F_0}{F} - 1\right) \qquad (5)$$

In Fig. 3 the fluorescence of lysed granules of the same concentration was taken as F_0. Fig. 3 demonstrates excellent agreement with the predicted behaviour which is limited in the lower osmolarities by the beginning lysis as demonstrated with the protein release data in the same figure. As soon as there is measurable protein release from the chromaffin granules the fluorescence greatly increases. The fact that the line does not cross the zero-point as predicted in Eqn. 3 can be accounted for by the presence of a small amount of background lysis which is unavoidable when resuspending chromaffin granules. Fig. 3 also shows that there is no systematic variation of turbidity with osmotic pressure. This is contrary to the results of Johnson and Scarpa [21] but not surprising in view of the strong dependence of turbidity on refractive indices as well as particle volumes [27] which will all change as a function of particle hydration. However, it is interesting to note that there are parameters which do not vary linearly with osmotic pressure or its inverse.

Size changes as a function of osmolarity

In our experiments, chromaffin granules in whole mount electron microscopic preparations appeared as homogeneously highly electron dense circles with sharp borders. Occasionally indented, elongated or fragmented forms were seen and sometimes the center of the circle was slightly more bright than the periphery. However, no systematic variation of these atypical forms with osmotic pressure was observed; even at the highest osmolarities used the granules still seemed to be round with an increased electron density (Fig. 4). Ghosts, i.e. lysed chromaffin granules, on the other hand presented as electronlucent round structures. Thus the sizes measured in the electron micrographs are measurements of only the core diam-

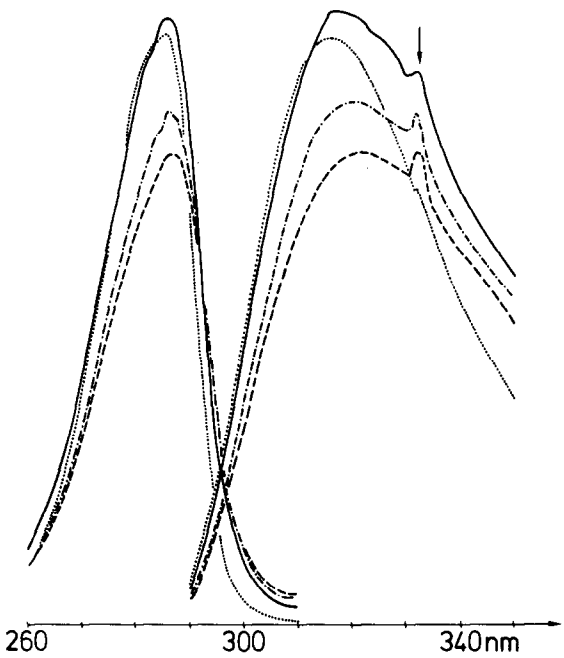


Fig. 2. Uncorrected fluorescence excitation and emission spectra of the native catecholamine fluorescence of chromaffin granules at different osmolarities. ·····, chromaffin granules in 10 mM Hepes (lysed granules, sensitivity one third of the sensitivity used in the other samples); ————, granules in 268 mosM sucrose/10 mM Hepes; ·–·–·, granules in 405 mosM sucrose/10 mM Hepes; — — —, granules in 844 mosM sucrose/10 mM Hepes. The excitation scans were done at 317 nm emission wavelength with a 310 nm filter, the emission scans at 285 nm excitation wavelength with a 290 nm filter. Excitation and emission slit widths were 2 nm; protein concentration in all samples 55.5 μg/ml. The ordinate represents relative fluorescence intensity. The emission peak marked with an arrow is due to Raman scattering.

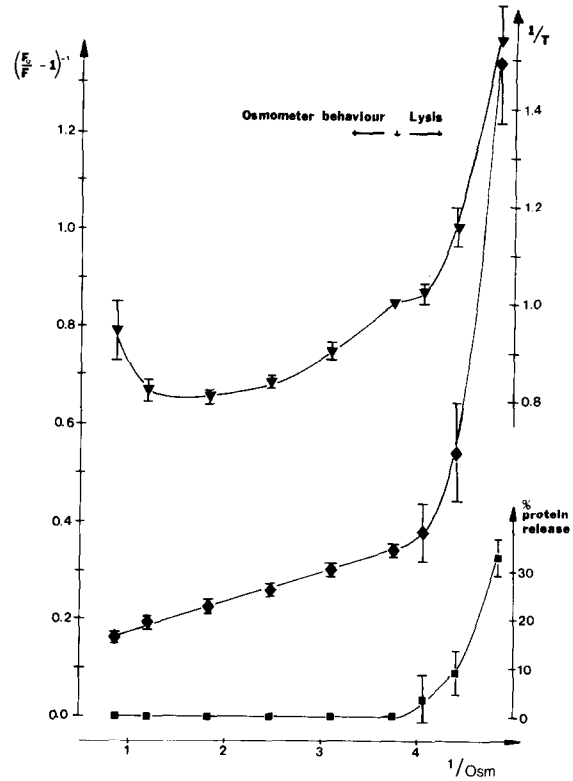


Fig. 3. Fluorescence quenching (◆, $((F_0/F)-1)^{-1}$ as developed in Eqn. 3), turbidity (▼, $1/T$) and protein release (■) in a chromaffin granule-suspension as a function of osmotic pressure. At osmolarities lower than the isolation medium (arrow 'lysis') chromaffin granules lyse, leading to great fluorescence increases, turbidity decreases and protein release. At higher osmolarities (arrow 'osmometer behaviour') the fluorescence behaves as predicted for an osmometer while the turbidity shows a complex, nonlinear variation with osmotic pressure and there is no protein release. The results depicted represent means ± S.D. from five experiments with triplicate determinations at 6°C; protein concentrations were 61.1 ± 17.0 μg/ml. For fluorescence quenching calculations the fluorescence of lysed granules was taken as F_0; the turbidity values were normalized to one for the value at 268 mosM to make results comparable.

eters in a two-dimensional view. No systematic attempt to identify contaminating mitochondria was made, although their typical fairly electronlucent structure with small dense granules [28] was sometimes encountered.

Fig. 5 shows the size distribution of chromaffin granules at three different osmolarities in a representative experiment. The radii appear to be fairly well normally distributed. Table I gives the experimental results of the four experimens conducted. The inverse relationship between volume and osmolarity as predicted in Eqn. 4 can be appreciated from Fig. 6 in which the results are graphically represented. While the experiments are in themselves consistent, there is some variation between experiments in the absolute volumes determined. Although there are only a few points for each experiment, Fig. 6 clearly demonstrates that above an osmolarity of 336 mosM sucrose a linear relationship between the inverse of the osmolarity and the mean granule-volume exists. It is striking that between 268 mosM and 336 mosM sucrose the mean volume does not decrease but in fact

TABLE I

CHARACTERISTIC PARAMETERS OF THE SIZE DISTRIBUTIONS OF CHROMAFFIN GRANULES AS A FUNCTION OF OSMOTIC PRESSURE

The table gives the means ± S.D. of the radii of chromaffin granules in nm with the number of granules counted in parentheses. n.d., denotes not determined.

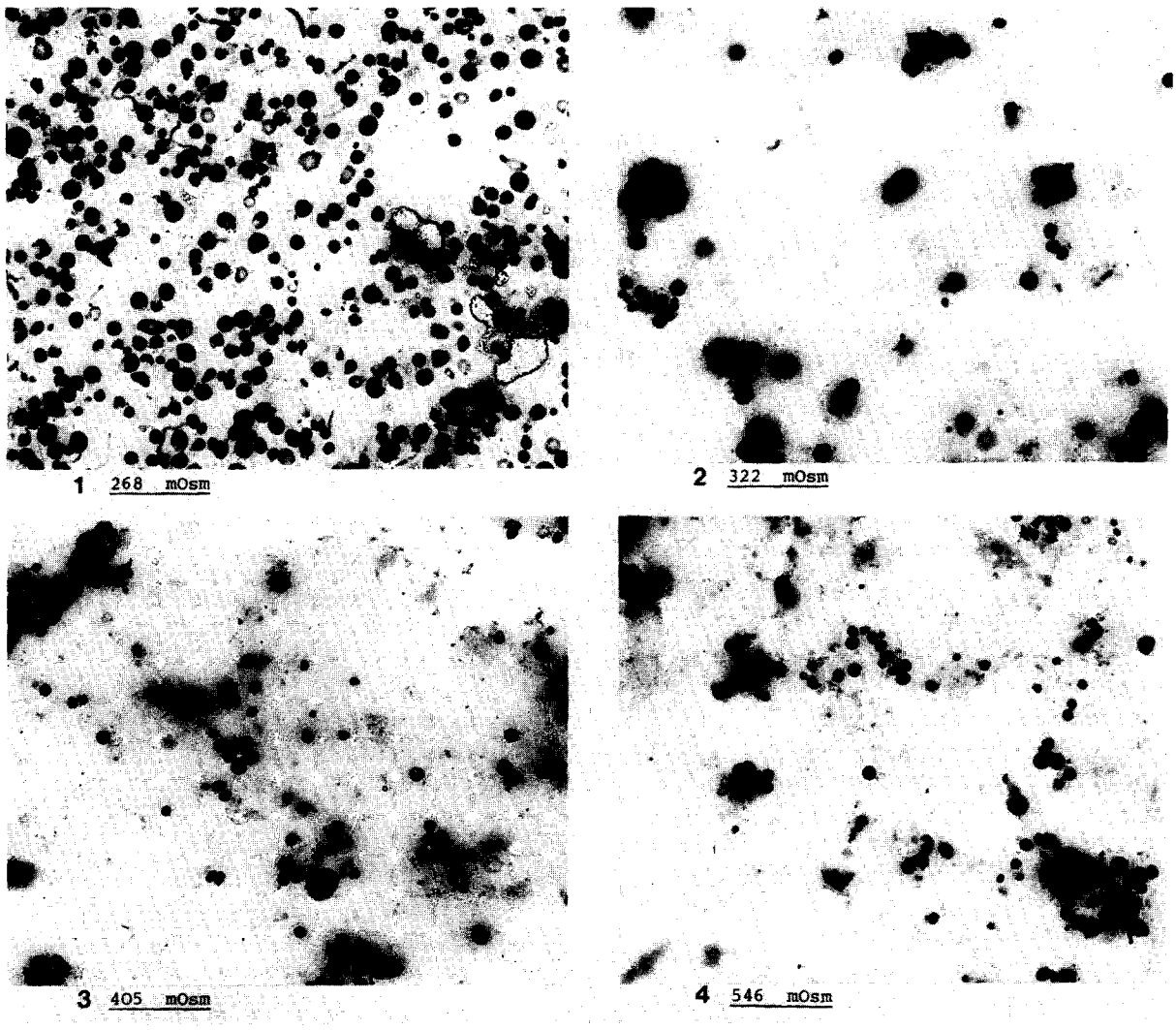
Osmolarity of sucrose solution (mosM)	268	322	405	542	844	1166
Expt. 1	189 ± 64 (n = 193)	189 ± 66 (n = 439)	n.d.	158 ± 57 (n = 567)	138 ± 50 (n = 200)	n.d.
Expt. 2	190 ± 60 (n = 639)	194 ± 65 (n = 475)	195 ± 65 (n = 932)	171 ± 63 (n = 452)	157 ± 57 (n = 362)	n.d.
Expt. 3	185 ± 64 (n = 631)	184 ± 67 (n = 1020)	178 ± 57 (n = 1306)	160 ± 57 (n = 175)	n.d.	134 ± 54 (n = 936)
Expt. 4	164 ± 56 (n = 109)	n.d	166 ± 54 (n = 926)	140 ± 48 (n = 507)	116 ± 40 (n = 59)	97 ± 24 (n = 42)

slightly increases, which has also been observed for the red blood cell [29]. In our four experiments, the lower limit of osmometer behaviour was 336 ± 25 mosM sucrose. From fits of individual experiments to straight lines according to Eqn. 4 the following biophysical parameters of chromaffin granules could be calculated: a solvent space ($V_{CG} - V_D$) of $(34.7 \pm 1.6) \cdot 10^{-21}$ m^3, a dehydrated core volume (V_D) of $(2.6 \pm 4.7) \cdot 10^{-21}$ m^3, and the average number of osmotically active molecules per granule (m) as $(11.72 \pm 0.78) \cdot 10^{-18}$ moles. (All values in means ± S.D.).

Volumes in Fig. 6 and the calculations above are based on the assumption of spherical shape which may not be true because our electron microscopy technique allows only vertical views onto the preparation. Chromaffin granules might have lenticular shapes induced by gravitation. This would somewhat flatten the line in Fig. 6 without changing the linear relationship and would change the calculated parameters accordingly. As an internal control the internal osmotic pressure can be determined from the calculated parameters by dividing m by V_{CG} which gives 317 ± 35 mosM. This is in good agreement with the observed lower limit of osmometer behaviour of 336 ± 25 mosM which is not affected by any systematic errors as the one described above. If one supposed that lenticular shapes of chromaffin granules formed only at higher osmolarities because of a decrease in core volume by osmotic dehydration the volumes at higher osmolarities would be smaller than actually measured, leading to a negative dehydrated volume (V_D). This is implausible.

Hyperosmolar relaxation lysis

In this laboratory chromaffin granules are routinely isolated in sucrose solutions of 268 mosM buffered with 10 mM Hepes, pH 7.4. When they are resuspended into solutions of that or higher osmolarities at low temperatures as was done for the determination of the osmometer behaviour, chromaffin granules are stable and do not lyse for many hours and even days apart from a constant amount of background lysis inherent in the resuspension procedure. Lysis of granules isolated in 268 mosM sucrose occurs only when they are resuspended into sucrose solutions of lower osmolarities with 10% lysis at 227 mosM and 50% lysis at 196 mosM (publication in preparation). Surprisingly, chromaffin granules resuspended in sucrose solutions of higher osmolarities lyse when referred back to their original osmolarity at which they were previously stable. The dependence of this hyperosmolar lysis on osmolarity ranges is shown in Fig. 7. In this experiment granules were resuspended into various osmolarities shown in the abscissa and then transferred to sucrose solutions of 268 and 542 mosM. After 1 h incubation time the protein release in the samples was determined as percent soluble protein present and the percent lysis calculated from the percent soluble protein



present in samples completely lysed. Soluble protein measured in the samples resuspended in 268 and 542 mosM sucrose and transferred to solutions of their own osmolarity was considered to represent background lysis and subtracted from all data. Fig. 7 clearly demonstrates that not the size of the osmolarity range jumped determines the amount of lysis. Chromaffin granules resuspended into 844 mosM sucrose and transferred to 542 mosM sucrose lyse very little compared to granules transferred from 542 to 268 mosM. Fig. 7 demonstrates unequivocally that a certain osmolarity range exists which has to be passed in order to cause a great amount of lysis, namely osmolarities between 320 and 400 mosM. These characteristics were confirmed with independent spectroscopic methods (data not shown). The first and most obvious interpretations of hyperosmolar lysis would be either slow sucrose permeation into the granules or a chemical effect of sucrose. In view of the specific distribution of hyperosmolar lysis they appear improbable but control experiments were done in order to rule them out. Hyperosmolar lysis in our experiments is not specific for sucrose because it can also be demonstrated when chromaffin granules are resuspended into hyperosmolar NaCl solutions. When the time of resuspension into the hyperosmolar sucrose solution is varied before they are transferred to 268 mosM sucrose, there is no change in the amount of lysis, indicat-

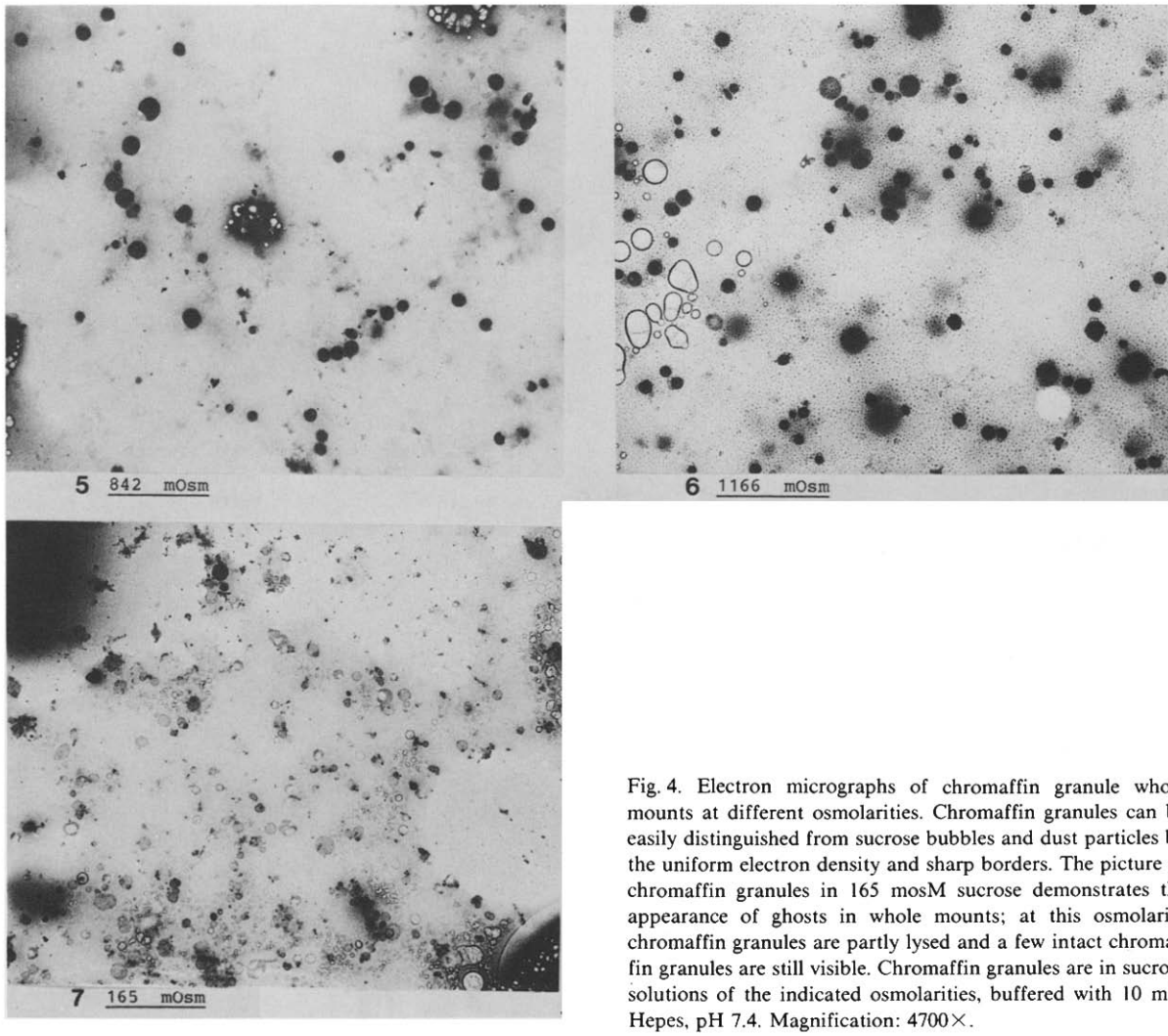


Fig. 4. Electron micrographs of chromaffin granule whole mounts at different osmolarities. Chromaffin granules can be easily distinguished from sucrose bubbles and dust particles by the uniform electron density and sharp borders. The picture of chromaffin granules in 165 mosM sucrose demonstrates the appearance of ghosts in whole mounts; at this osmolarity chromaffin granules are partly lysed and a few intact chromaffin granules are still visible. Chromaffin granules are in sucrose solutions of the indicated osmolarities, buffered with 10 mM Hepes, pH 7.4. Magnification: 4700×.

ing that the process leading to the change in the chromaffin granules associated with hyperosmolar lysis is very fast. And finally we have incubated chromaffin granules in [^{14}C]sucrose solutions of 268 and 542 mosM for 5 h and then rapidly centrifuged the granules five times and measured the radioactivity of the supernatants and the final pellet in order to determine if any sucrose permeation occurred. The pellet of each centrifugation was resuspended into nonradioactive sucrose of the same osmolarity. In the experiments an exponential decrease of the radioactivity in the supernatants could be observed with much less than one percent of the radioactivity left after the third centrifugation. The final pellets containing the chromaffin granules exhibited only 0.1% of the starting radioactivity, no more than the final supernatant contained. There was no difference between the samples incubated in 0.52 and 0.26 M sucrose. These results demonstrate that no sucrose is taken up into chromaffin granules under experimental conditions and that there is no difference in the samples incubated at different osmolarities. Thus there is no basis on which to suppose that hyperosmolar lysis is a consequence of sucrose uptake.

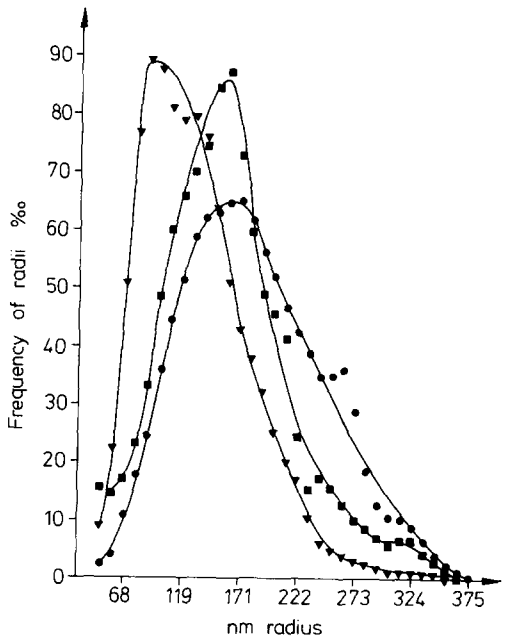


Fig. 5. The size distribution of chromaffin granules at three osmolarities (●, 268; ■, 542 and ▼, 1166 mosM of experiment 3 in Table I). Experimentally determined curves were smoothed by four point averaging. The sizes appear in a normal distribution with a slight tilt to lower sizes. As can also be appreciated from Table I, the width of the size distribution decreases with increasing osmotic pressure.

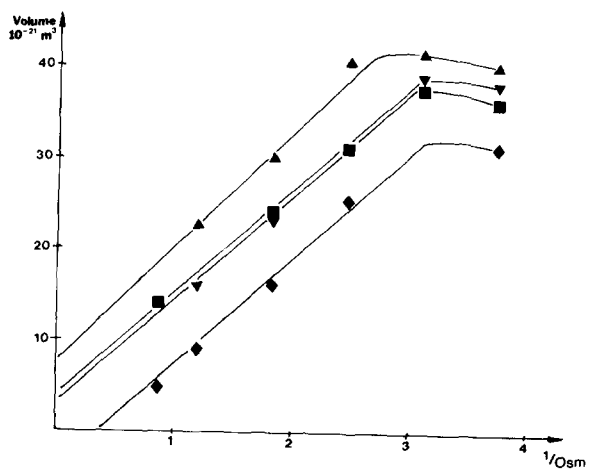


Fig. 6. The mean volume of chromaffin granules as a function of osmotic pressure. The four lines represent results from four different experiments. Mean volumes were calculated from the size distributions on the assumption of spherical shape. Chromaffin granules behave like osmometers above an osmotic pressure of 336 mosM sucrose as shown by the linear relationship between the inverse of the osmotic pressure and the mean volume.

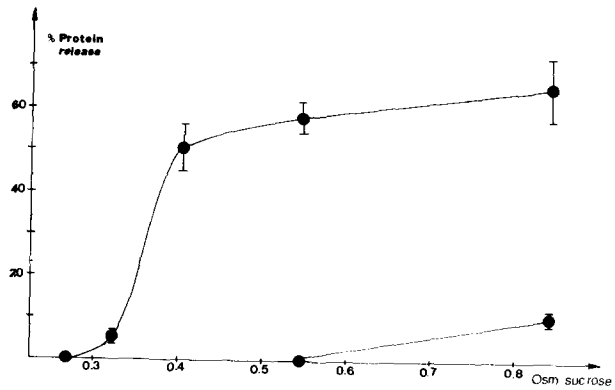


Fig. 7. Hyperosmolar lysis of chromaffin granules as determined by protein release. Chromaffin granules were resuspended into sucrose solutions of the osmolarities stated in the abscisse and then transferred into sucrose solutions of 268 mosM (upper curve) of 542 mosM (lower two points). The protein release was determined after one hour of incubation. The graph gives the means and standard deviations from four experiments. It demonstrates that hyperosmolar lysis is independent of the osmolarity range over which chromaffin granules are jumped. Greater degrees of lysis only occur when chromaffin granules are diluted over the 400–325 mosM sucrose osmolarity range. Hyperosmolar lysis maximally amounts to 70%.

Discussion

In this study we have shown with two independent methods that chromaffin granules behave like osmometers in a mathematically predictable manner above an osmolarity of approx. 322 mosM sucrose and 10 mM Hepes. From the measurement of the volumes of chromaffin granules as a function of osmotic pressure we were able to estimate several biophysical constants of chromaffin granules of great interest. Before discussing these, however, we would like to point out methodological limitations of this study which may partly also represent advantages. As in many other studies on chromaffin granules we did not use highly purified granules but those obtained from differential centrifugation in sucrose. Despite the availability of several sophisticated techniques for further purification [19,30] very little is known about the effects of changes in the physical or chemical suspension conditions on chromaffin granules. Indeed we have shown that an irreversible change in chromaffin granules is induced by treatment with higher osmolarities (Fig. 6) which may have im-

portant implications for tissue fractionation procedures. With our preparatory and experimental procedures the granules have never been brought into contact with anything but sucrose and Hepes, and a constant temperature range was used for preparatory (in the coldroom in ice) and experimental (all at 6°C) manipulations. Thus the only variable changed in our experiments is the sucrose concentration, and all observations must be related to it, greatly reducing the possibility of artifacts. The second methodological feature that could be considered a limitation or an advantage is our electron microscopy technique. Although it has been suggested that the natural electron density of unstained subcellular organelles is specific for secretory vesicles containing biogenic amines [31], this has not been systematically investigated and we do not know how effectively we are singling out contaminating mitochondria in our size distribution determinations. Another disadvantage of the whole mount technique is that it only gives a two-dimensional view of the particle. However, its great avantage over conventional embedding techniques is that with the whole mount technique chromaffin granules are left in their experimental sucrose environment when their sizes are measured, thus evading any uncontrollable influences by the many conventional fixations.

The biophysical structure of chromaffin granules

From this study and from others before [1,20] it can be accepted as an established fact that chromaffin granules behave like osmometers over a certain range of osmolarities. Although this in itself does not imply any specific structural features, we have been able to determine the average number of osmotically active molecules per granule and the average solvent space. Osmometer behaviour can be observed in osmolarities above approx. 336 mosM. Thus chromaffin granules reach their maximal volume at that osmotic pressure and expand no further. This osmolarity can also be calculated for the internal osmotic pressure by dividing the number of osmotically active molecules per granule by the averal maximal volume. Since chromaffin granules are lysed to 50% at approx. 200 mosM sucrose, the osmolarity range over which an osmotic pressure gradient builds up which is high enough to lead to a lysing membrane tension can be considered to amount to approx. 100 mosM. Assuming spherical shape of chromaffin granules, the corresponding membrane tension (S) leading to lysis can be calculated by an equation derived from the van 't Hoff-law and the LaPlace relation:

$$S = 2r\,\Delta\text{osM}RT \qquad (5)$$

where S is the lysing membrane tension, r the radius of the vesicle, ΔosM the osmotic activity gradient leading to lysis, R the universal gas constant and T the temperature in K. The value thus obtained, $S = 0.0882\ N/\text{m}$, is in the same order of magnitude as the value measured by Rand [17] with more exact methods for the erythrocyte membrane, the only other measurement known to us. In view of many approximations involved this lends further support to our conclusions. The solvent space fraction of the granule-core can be determined by dividing the solvent volume, for which excellent agreement exists among our experimens, by the total core volume. In our experiments this yields a value of 0.94, which is even after correction with a term for the membrane volume much greater than that which has been measured by other methods [19,21]. However, our high estimate of the solvent space fraction suggests that virtually the whole core as we see it in the electron microscope is in solution. This in good agreement with the model of core structure put forth by NMR studies on chromaffin granules which demonstrates that all core constituents are in solution and no separate crystalline phase exists [33–36].

Several groups have reported estimations of the core concentration of low molecular weight constituents in chromaffin granules ranging from 0.7 to 1 M [1,32]. This is not in contradiction to our determination of the internal osmotic pressure of 322 mosM and implies that virtually the whole core is osmotically active because such a polyionic solution as the chromaffin granule-core solution, additionally containing a protein lattice structure, will exert unpredictable osmotic pressure far below their actual concentration. Thus in our experiments the granule-core appears to be made up of a concentrated solution whose osmotic pressure is lowered by nonspecific ionic solute interactions.

The nature of hyperosmolar lysis

Hyperosmolar lysis, i.e. osmotic lysis of chromaffin granules at an osmolarity at which they are normally stable, occurs when they are diluted over the specific osmolarity range of 320 to 400 mosM sucrose. It is not dependent on the amount of dilution since Fig. 7 shows that chromaffin granules transferred from 844 to 546 mosM sucrose show no significant lysis as compared to granules transferred from 542 to 268 mosM sucrose. Hyperosmolar lysis is not dependent on sucrose as suspension medium since it also occurs in NaCl. It is not dependent on preincubation times in hyperosmolar media and no sucrose uptake into chromaffin granules could be measured in 268 and 542 mosM sucrose under experimental conditions. These characteristics rule out the most obvious interpretation of hyperosmolar lysis, namely sucrose permeation into the granules, and show hyperosmolar lysis to be a highly specific phenomenon. The fact that it has been qualitatively observed in cholinergic vesicles suggests that it may be a widespread biologic phenomenon [7].

The NMR and fluorescence spectra show that no dramatic changes in the chemical state of the ATP and catecholamines in the core occur with osmometer behaviour, especially none localized to the interval from 322 to 405 mosM sucrose. This suggests that no alteration in the core composition causes hyperosmolar lysis. On the other hand, a comparison of Figs. 6 and 7 demonstrates that hyperosmolar lysis occurs over the osmolarity range where the chromaffin granule-membrane can be thought to relax. We suggest that this relaxation of the chromaffin granule membrane from a condition of natural tension may be irreversible and be the basis for hyperosmolar lysis. Although we cannot at the moment imagine any in vivo relevance of hyperosmolar lysis per se, it seems to demonstrate an important structural feature of secretory vesicles.

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