CHANGES IN THE LIGHT SCATTERING PROPERTIES OF ADRENAL MEDULLARY CHROMAFFIN GRANULES AND CORRELATION WITH MORPHOLOGICAL CHANGES

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Received 3 July 1974

1. Introduction

Information about the in vitro effects on chromaffin granule structure of agents such as Ca^{2+} ions which are implicated in the release of the granule contents in vivo [1] may help to elucidate the processes involved. In this communication the changes observed in the light scattering intensity and dissymmetry ratio of isolated, purified chromaffin granules are examined as a function of time in relation to possible changes in the size and structure of the granules.

The chromaffin granules of the adrenal medulla cells store catecholamines and ATP in high concentrations and release these into the blood stream in response to stimulation of the cells by sympathetic nerves. The release most probably occurs by exocytosis [2], a process in which the granule and plasma membranes become confluent such that the granule contents, but not its membrane constituents, come to lie in the extracellular space (fig. 1).

Size and shape changes in subcellular particles can be studied by electron microscopy, but since the processes used to prepare material for examination in the electron microscope could lead to size and shape changes in the particles, it is desirable to study any changes with additional techniques, especially if these offer a possibility of following changes in size and shape during an extended period of time. Measurement of the light scattered by particles in solution offers such a possibility [3–5].

2. Materials and methods

2.1. Preparation of the purified chromaffin granules and the E/M have been described previously [6].

2.2. Light scattering experiments were performed using either the light scattering photometer described by Hughes and Johnson [7] which was modified to read at angles from 30° to 165° or a FICA 50 thermostated with a LAUDA TUK-30 temperature bath. Scattering blanks with very low dissymmetry and absolute intensity were prepared by filtering the aqueous solvents through successively smaller membrane filters, the final pore size being 50–100 Å. The chromaffin granule gradient fraction was diluted from 20 mM HEPES*-buffered 1.8 M sucrose, pH 7.0 to 0.6 M sucrose and filtered twice through a 4500 Å or 3000 Å carbowax 20 M coated Millipore filter at 0°C to remove the largest granules and contaminating mitochondria.

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* *Abbreviation used:* HEPES, 2-(*N*-2-hydroxyethylpiperazine-*N'*-yl)ethanesulphonic acid.

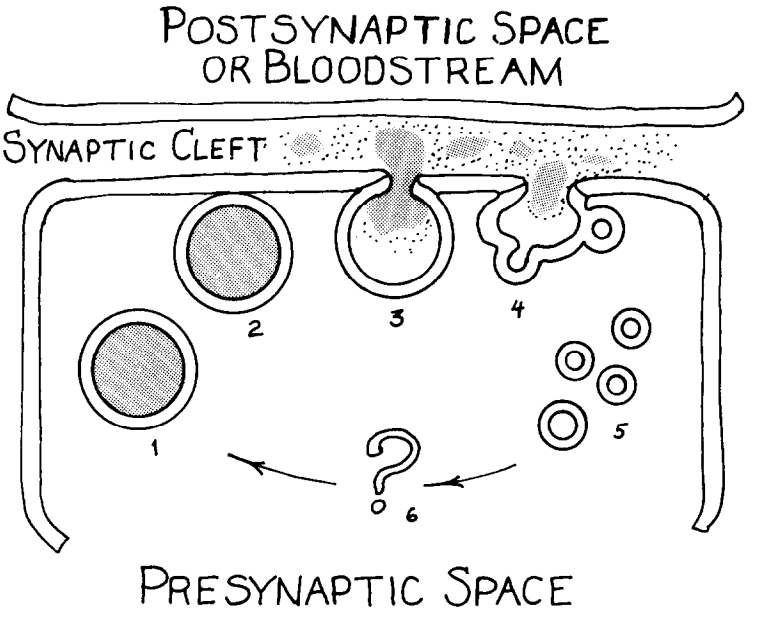

Fig. 1. Model for exocytosis of storage granules. Storage granule (1) fuses with the pre-synaptic membrane (2) establishing a direct connection between the interior of the granule and the exterior of the cell allowing the granule contents to escape into the synaptic cleft (3). The granule membrane is released from the pre-synaptic membrane (4) perhaps at the same time spontaneously vesiculating into smaller hollow pinocytotic spheres (5). The membrane components may be recycled (6).

The light scattering experiments were started by adding 100–250 μl of the granule suspension (protein content 1–2 mg/ml) to 5–20 ml of the scattering solvent (time 0), rapidly mixing, and replacing the cuvette in the thermostated bath of the machine. The intensity of the light scattered at 30°, 45° and 135° was recorded alternately as a function of time. Expositions of the light scattering theory involved are given elsewhere [8–11].

The light scattering dissymmetry (Z_0) was calculated as the ratio of the intensities at 45° and 135° (I_{45}/I_{135}). Theoretical plots of Z_0 versus the characteristic dimension of several model scattering shapes were generated from the ratio of the Debye particle scattering factors (P(45)/P(135)) as originally described by Doty and Steiner [8] from the models tabulated by Kerker [5] using FORTRAN IV programs.

3. Results

3.1. *Light scattering*

When the granule fractions from the preparative density gradient, diluted to 0.6 M sucrose as described above, are rapidly diluted 50–100-fold at 0°C into 0.3 M sucrose, there is very little change in the light scattering signal as a function of time. If the same sample stored at 0°C, is diluted into a cuvette equilibrated at 20–25°C, the light scattering intensity and dissymmetry decrease rapidly with time (fig. 2). The magnitude of the change varies from preparation to preparation and is greatest when the granules are fresh, and freshly diluted. A diluted and filtered gradient fraction stored over night will show a longer amplitude change than a freshly diluted and filtered sample of the same fraction.

The velocity of the light scattering change at 20–25°C is dependent upon the pH of the suspension medium. Fig. 3 shows the relative change in I_{45} for a given chromaffin granule preparation at pH's ranging from 3.5–5.5. Below pH 4, the scattering intensity and dissymmetry increase with time. Of special interest is the curve for pH 3.75 which shows first a decrease and then an increase suggesting that two competing processes are occurring simultaneously. The effect of low pH can be reversed by titrating the

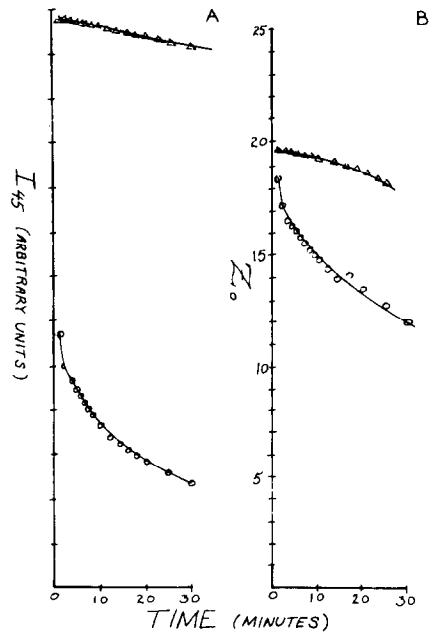

Fig. 2. Time dependent changes in light scattering intensity (a) and dissymmetry (b) for chromaffin granules suspended in 0.30 M sucrose, 20 mM HEPES pH 7.0 at 23°C. ○ – ○ control; △ – △ + 10 mM $CaCl_2$.

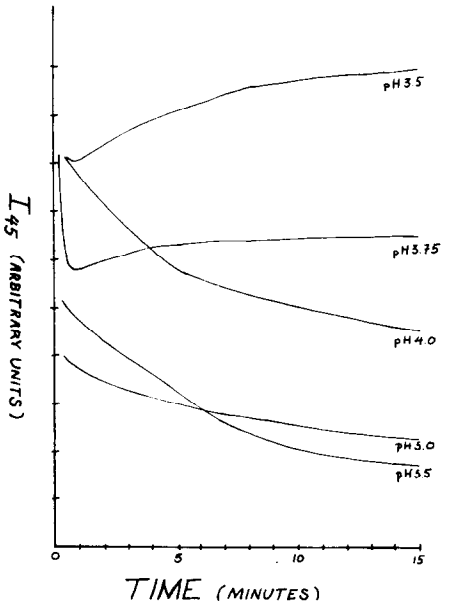

Fig. 3. Time dependent changes in light scattering intensity at 45° for chromaffin granules suspended in 0.3 M sucrose at various pH's (temp 23°C continuous recordings). Buffers were 20 mM citrate.

suspension back to pH 7.0.

The presence of Ca^{2+} in the suspension has an effect similar to that of low pH. The decreases in intensity and dissymmetry are much reduced (fig. 2), or, in many cases, are reversed. The effects of Ca^{2+} and low pH are additive, but the Ca^{2+} component is not reversed by raising the pH. Mg^{2+} produces effects which resemble those due to Ca^{2+}, but higher concentrations are required.

3.2. *Electron microscopy*

The purified granules from the gradient were diluted 5-fold to 0.6 M sucrose and immediately fixed iso-osmotically at 0°C (fig. 4a). They are morphologically similar to granules fixed in situ [12] and consist of membrane-bound spheres with densely staining core material [12–14]. However, many of the granules show an electron lucent space between the membranes and the core and often the membranes are broken or contain outfoldings which suggests that core material is being lost and the particles become smaller. The excess membrane may bud off into small hollow vesicles.

The effects of dilution and aging were studied: chromaffin granules were rapidly diluted at room temperature, as for the light scattering studies. After 45 min they were fixed with buffered isotonic glutaraldehyde and prepared for electron microscopy as described previously [6].

Compared to the controls, the relative number of larger granules seems to decrease in the diluted and aged preparation (fig. 4b) and one sees an increase in the relative incidence of membrane outfoldings, granules with broken or incomplete membrane envelopes and granules with coarse grain substructures (fig. 4c). The last has been associated with catecholamine and ATP depletion in sympathetic nerve trunk granules [15].

The effects of diluting and fixing the granules in 0.3 M sucrose at pH 4.2 (fig. 4d) are similar to fig. 4b except for an increase in the relative number of coarse grain centers and the appearance of large numbers of adjacent granules with their membranes tightly apposed. These areas of apposition are often quite extensive and suggest that the granules try to maximize their area of contact, which is opposed by the non-deformability of the still intact spheres.

The effects of the addition of Ca^{2+} or Mg^{2+} have

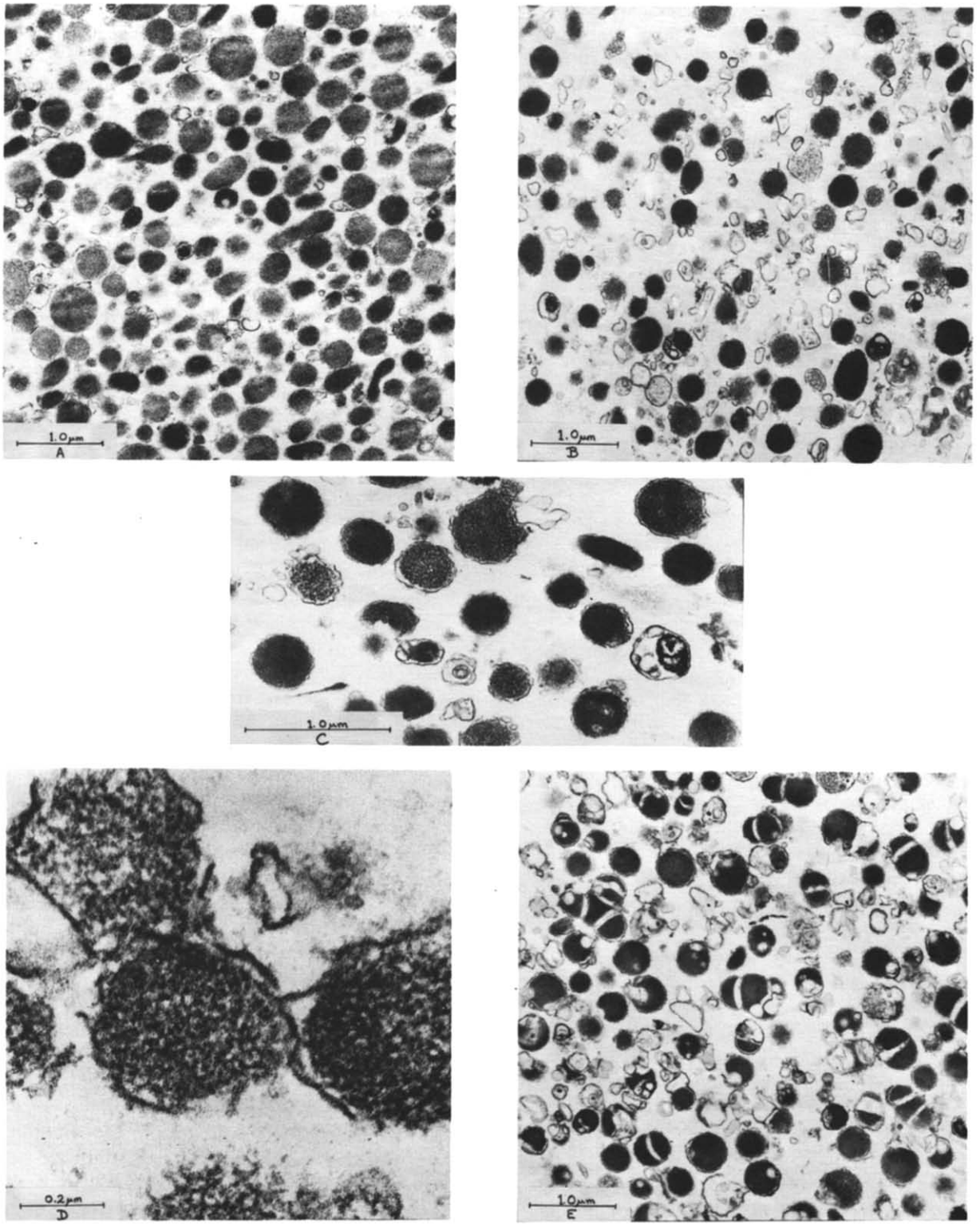

Fig. 4. Electronmicrographs of the granule preparations. See text for explanation.

been described extensively elsewhere [6]. Briefly, in addition to the changes seen upon dilution at neutral pH, the granules not only aggregate but appear to 'fuse' along their contact borders (fig. 4e). The core material appears to dissolve or contract away from the area of contact, producing an electron lucent stripe. The membrane at the area of apposition often contains breaks or is almost entirely absent.

4. Discussion

In order to determine both the size and shape of a particle by light scattering it is necessary to have observations of the scattered light intensity (I_θ) at a series of angles for a series of particle concentrations [3,5]. However, if one can make a reasonable assumption about the shape of the particles, one can estimate their average size by methods which depend upon the ratio of the light scattered at only two angles. This approach has been used successfully to determine the size of polystyrene beads [8,16], soap micelles [9], phospholipid bilayer vesicles [10], tobacco mosaic virus [17] and microsomal vesicles [11].

Our granule preparations show no scattering intensity changes when diluted at 0°C. Zimm plots [4,11] may therefore be constructed from measurements made at this temperature. Our results suggest that the granules are polydisperse spheres of average diameter 3500–4500 Å. We therefore assume that the material at the beginning of the light scattering experiments at 25° consists of solid spheres, and attempt to correlate their time-dependent changes with biochemical and morphological observations, in order to assess possible changes in their size and shape.

Trifaro and Poisner [18] observed a decrease in turbidity (increase in transmission) of purified chromaffin granules suspended in 0.3 M sucrose at 25°C. This would be consistent with our observations of a decrease in scattering intensity. We found that a large change occurred within the first ten minutes of the dilution (the curves in fig. 1a may be assumed to have the same origin, although it was not possible to record from the suspensions at times less than 1 min. after mixing, for technical reasons).

We have found that changes in the catecholamine and ATP content of the granules occur even more rapidly [21]. When measured under the exact conditions of the light scattering experiments, 80% of granule-bound catecholamines (but less than 50% of their protein) were lost within one minute of dilution; the remaining catecholamine was stably bound for at least an hour, however, in contrast to the situation of hypotonic lysis, when 99.8% is lost within the first minute. Similar results were reported by Hillarp [19,20] who noted that catecholamines, although stable at 0°C, were more readily released than protein on warming, and that granules were less stable if purified in high concentrations of sucrose. In general, the light scattering changes had a slowly decreasing component in addition to their initial rapid decrease (fig. 1a), suggesting that the structure of the granules was altering secondarily to the loss of their stored components. Measurements showed that their light refractive index increment (dn/dc) was constant over time under the conditions of the scattering experiments, implying that the observed changes in intensity and dissymmetry reflect changes in their size and/or shape.

Plausible models for changes in the structure of the granules which are consistent with the light scattering and biochemical observations are summarized in fig. 5 and table 1.

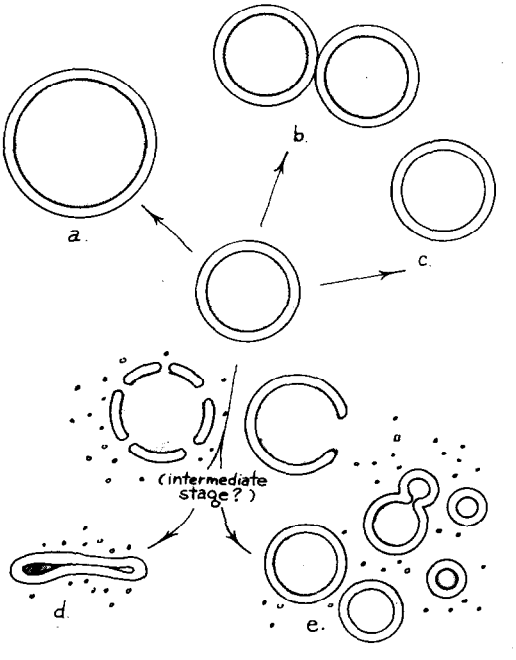

Fig. 5. Models for changes in the size and structure of chromaffin granules. See table 1 for explanation.

Table 1
Expected changes in light scattering parameters predicted by various models for changes in chromaffin granule size and shape

Model No.	Expected direction of change in light scattering	
	Intensity	Dissymetry
a	+	+
b	+	+
c	+	0 or +
d	−	−
e	−	−
f	0 or −	0 or −

a. The granule becomes larger.
b. Aggregation of granules.
c. Release of granule cytoplasm which remains intact. Membrane ghosts remain intact.
d. Release of granule core material; membrane ghost collapses into a disc; cytoplasm core material breaks up into small (< 20 nm) particles.
e. Release of granule core material; membrane breaks up into small hollow vesicles; core material breaks up into small pieces.
f. (a or b) + (d or e).

The electron micrographic results support model e in which the granule membranes spontaneously vesiculate into smaller hollow-cored particles; discs are not seen. These small vesicles seem to bud off from the parent vesicle membrane from polyp-like structures (fig. 4c) and appear similar to the electron micrographs of exocytosing chromaffin granules in the golden hamster adrenal medulla [12]. The vesiculation of the granule membrane secondary to exocytotic release of the storage granule contents has been proposed both by Smith and Winkler [2] for chromaffin granules and by Douglas et al. [22] for the neurosecretory granules of the posterior pituitary. Our results suggest that the vesiculation occurs spontaneously when the granule contents are lost, and that this phenomenon is not dependent upon previous fusion of the granule membrane to the release sites of the cell external membrane in order to proceed.

Both the presence of Ca^{2+} [23] and the lowering of the pH [13] have been observed previously to cause granule precipitation. The light scattering results of both the low pH and Ca^{2+} experiments suggest that either the particles are growing larger or aggregating (fig. 5a,b). The second alternative is consistent with the E/M findings. The biphasic changes in the light scattering seen at pH 3.75 and the reversibility of the low pH scattering intensity increase by readjustment to pH 7 suggest that the aggregation and vesiculation phenomena proceed simultaneously.

It would not have been possible to predict the apparent fusion of the granules by Ca^{2+} from the light scattering data alone, which emphasizes the tremendous potential of the E/M despite its limitations as compared to physical methods which rely upon sensing changes averaged out over the entire sample. The possible relevance of these observations to the phenomenon of exocytosis has been discussed elsewhere [6].

The chromaffin granule membranes thus appear to have two intrinsic properties of great interest. The first is their ability to aggregate and fuse in the presence of divalent cations; the second is their ability to form small vesicles when the granules are depleted of their contents at 23°C.

Acknowledgements

We wish to thank Dr V. P. Whittaker and Dr P. Johnson for their help and encouragement, Miss J. Henderson for technical assistance and Mr J. Kay for the computer programmes. S. J. M. is grateful to the Wellcome Trust for fellowship support.

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