THE EFFECTS OF DIVALENT CATIONS ON THE SEDIMENTATION OF CHROMAFFIN GRANULES

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Dedicated

to

My Father

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INTRODUCTION

In the adrenal medulla, the catechloamines, epinephrine and norepinephrine, are stored within membrane bound vesicles named chromaffin granules. Histochemical evidence indicates that there are two types of catecholamine containing cells. One type contains epinephrine, the other norepinephrine (1). There are several different types of proteins found within the vesicles. Dopamine - β - oxidase, which catalyzes the conversion of dopamine to norepinephrine, is present (2). Also present is a protein which has been isolated and characterized by Smith and Kirshner (3). It has been suggested that this protein may be involved in forming a complex with the catecholamines making them unavailable for diffusion through the vesicle membrane. Formation of such a complex might require ATP either as an energy source or as part of the complex. This theory is based upon the fact that total negative charges of the catecholamines is equivalent to the positive charges of the adenosine nucleotides that are also found in the chromaffin granules (4). Since lysis of the granules liberates free catecholamines, this theory has been considered doubtful (5). More recent findings, however, provide direct evidence for a catecholamine-ATP complex in the granules of the adrenal gland (6). The ATP found within the granules may be used in a process which releases the content of the vesicles or it may be used by a specific mechanism that accumulates the catecholamines within the granules (7). One uptake mechanism is stimulated by ATP in the exterior medium of the granules.

The release mechanism seems also to be stimulated by external ATP (8). It has been shown that catecholamines, adenosine nucleotides, and the intergranular protein, chromagranin, appear in perfusion fluids in ratios similar to those found in the granules (9). For this reason it is presently believed that the catecholamines are released directly to the exterior of the cell (10).

In the several different types of tissues such as exocrine pancreas, salivary glands, and the posterior pituitary, material to be secreted is stored in subcellular particles (11, 12, 13). In these tissues Ca^{2+} ions induce a secretory response (14, 15, 16). There is much evidence that indicates that the secretory process is reverse pinocytosis (13, 17, 18). For this phenomenon to occur, the granules must become attached to the inner surface of the cell membrane and then rearrangement of the lipoprotein cell membrane and granular membrane may occur. Calcium ions may serve as a divalent bridge between the negative charges of the granules and cell membrane (19). More recent hypotheses have suggested that the molecular basis of exocytosis (reverse pinocytosis) in the adrenal medulla, and for the release of transmitters and secretory products in general, involves a contractile protein triggered by Ca^{2+} (8).

At present, the details of the synthetic pathway of epinephrine and norepinephrine are still in doubt. It will be necessary to separate the two types of granules so that the uptake mechanisms and enzymatic activities of the segregated granules may be studied. This is a prerequisite to establishing the exact details of the synthesis of the two catecholamines. Separating the granules was the original intention of the present study. It was hoped that the examination of a variety of techniques used in separating organelles would provide a procedure that would separate

the epinephrine and norepinephrine granules.

Various types of chromatographic and electrophoretic techniques were used in attempts to separate the epinephrine and norepinephrine granules. These techniques did not provide any indication that the two types of granules could be separated. While we were experiminting with these techniques, we learned of a report (20) that stated that microsomes, endoplasmic recticulum fragments, will aggregate when exposed to 8mM $CaCl_2$. Low centrifugal forces sedimented these aggregates yielding functionally intact microsomes. Calcium ions may induce the aggregation of chromaffin granules by acting as a divalent bridge between granules having negative charges on their membranes. The main thrust of the present work then turned to a detailed study of the effects of Ca^{2+} and other ions on the sedimentation behavior of epinephrine and norepinephrine containing granules.

MATERIALS AND METHODS

Isolation of chromaffin granules

Bovine adrenal glands were obtained from an abbatoir where they had been placed on ice within 30 minutes of the animals' death. An additional 30 minutes was required in transporting the glands from the abbatoir to the laboratory. The cortex was removed and the medulla was finely chopped. The minced tissue was suspended in ice cold 0.26M sucrose and homogenized in a Potter-Elvehjem homogenizer having a clearance of 0.15mM between a glass mortar and a Teflon pestle. The homogenant was centrifuged at 1,000xg for 10 minutes to remove tissue debris and cell nuclei. A large pellet of chromaffin granules was isolated from the supernatent by centrifugation at 10,000xg for 20 minutes. The weight of the isolated pellet was determined and 1 ml of cold 0.26M sucrose per 0.1g of pellet was used to resuspend the granules.

Aggregation of granules by various ions

To each of two 1 ml samples of the granule suspension was added 1 ml of $CaCl_2$ to yield final concentrations of 4.5mM and 3.5mM. Other $CaCl_2$ granule suspensions were prepared having final concentrations ranging from 0.1mM to 10mM. Calcium was one of several divalent cations examined. Preparations for Zn, Cd, Ba, Be, St, and Mg salt solutions were made in a similar manner. Solutions of Al^{3+}, the only trivalent examined, were

also prepared. All solutions were prepared using chloride salts except Be^{2+} in which case the nitrate salt was used.

Centrifugation of aggregated granules

In early experiments, one ml volumes of the 3.5mM Ca-granule suspension were layered over 10ml of 0.5M, 1.0M, 1.2M, and 1.4M sucrose gradients. Each gradient was then centrifuged 400 xg for 10 minutes. The procedure was repeated using the 4.5mM Ca-granule suspension. This procedure was repeated using 600, 800 and 1,000 xg to determine the minimum force necessary for sedimentation of aggregated granules. In all subsequent experiments in which the effects of widely ranging concentrations of various ions were determined, ion-granule suspensions were layered over 10ml of 1.2M sucrose and centrifuged at 400 xg for 10 minutes.

Analysis of centrifugal fractions

The supernatents of 3.5mM and 4.5mM Ca-granule centrifugations were decanted. Layers that may have remained above the 1.2M sucrose gradients of the other ion centrifugations were removed by pipette and the remaining supernatent was decanted. All of the fractions were suspended in 5% TCA to precipitate endogenous protein and lyse the granules. Each fraction was then centrifuged at 1,000 xg for 10 minutes to remove the debris.

Extracts from each fraction were analyzed for their catecholamine content by the fluorometric procedure described by Vol Euler (21). The procedure determined both norepinephrine and epinephrine in each fraction.

Microscopic visualization of granule aggregation

Some of the ion-chromaffin granule suspensions were viewed under a light microscope. The granules were observed under the Zeiss WL microscope using either phase contrast or Nomarski interference optics at a magnification of 200x.

Dialysis of aggregated granules

One ml of 8.0mM Ca^{2+} containing granule suspension was placed by pipette into dialysis tubing (.984" flat width). Both ends of the tubing were sealed and the tube placed into 1 liter of cold 2.6M sucrose. After 4 hours the tubing was removed from the sucrose and the granules were examined under the Zeiss WL microscope with Nomarski interference optics. This dialysis experiment was repeated removing the tubing from the sucrose at the end of 20 hours. In each case the experiment was repeated with 8.0mM versinate (EDTA) added to each 0.26M sucrose mixtures.

RESULTS

Demonstration of cation induced aggregation of chromaffin granules

Control experiments indicate that granules which have not been exposed to divalent cations will not sediment through the sucrose densities used when centrifuged at the low forces used. Divalent cations will cause aggregation of the granules as seen in Fig. 1 and Fig. 2. After being exposed to the cations for 10 minutes, the chromaffin granules will sediment through sucrose densities using low centrifugal force.

Effect of sucrose densities on sedimentation of aggregated granules

As expected, sedimentation was retarded in the more dense sucrose mediums. As shown in Table 1, movement through concentrations less than 1.2M was not hindered, while concentrations greater than 1.2M inhibited sedimentation completely. The 1.2M sucrose was the most dense solution which allowed sedimentation.

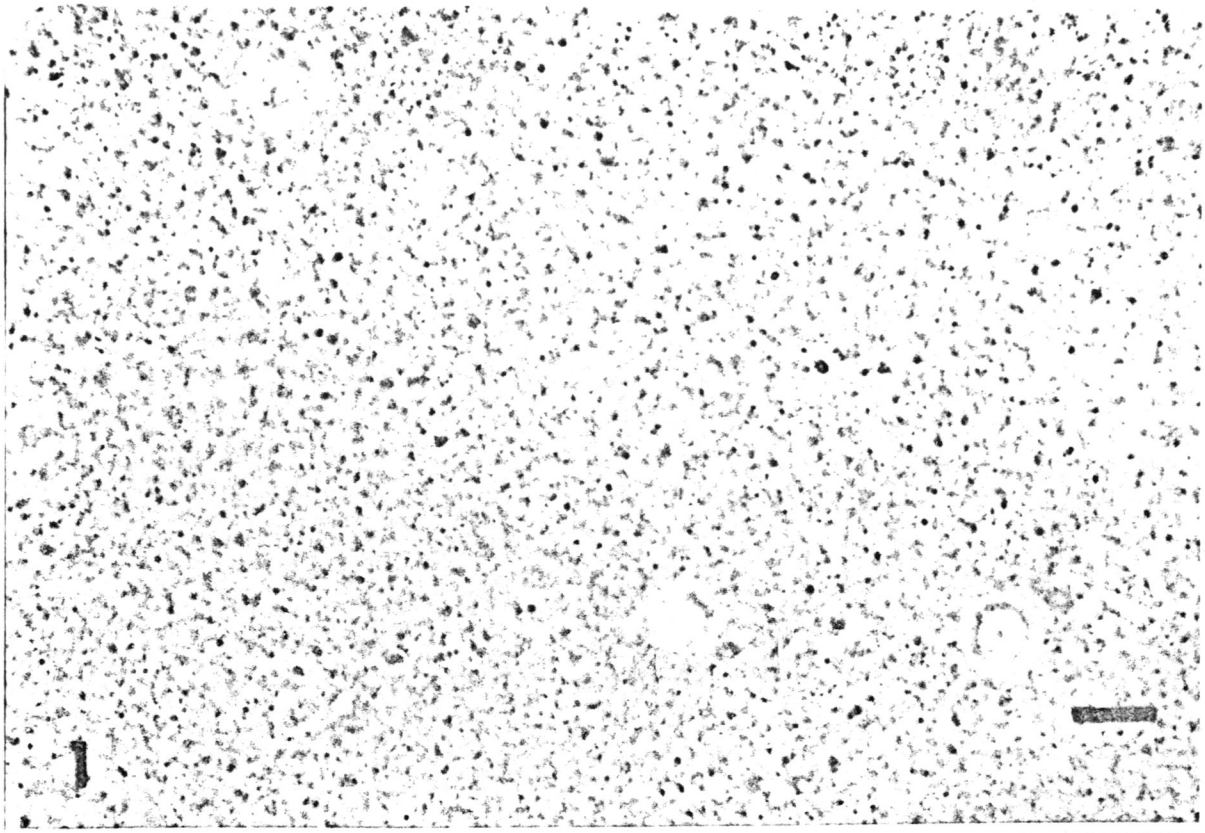
Effect of varying centrifugal force on sedimenting aggregated chromaffin granules

Increasing the speed of centrifugation from 400 to 1,000 did not increase sedimentation in any of the concentration with the exception of the 1.2M sucrose. As seen in Table 1, maximum sedimentation occurred

Fig. 1. Unclumped Granules

Fig. 2. Maximumly Clumped Granules

Legend Bar is 10μ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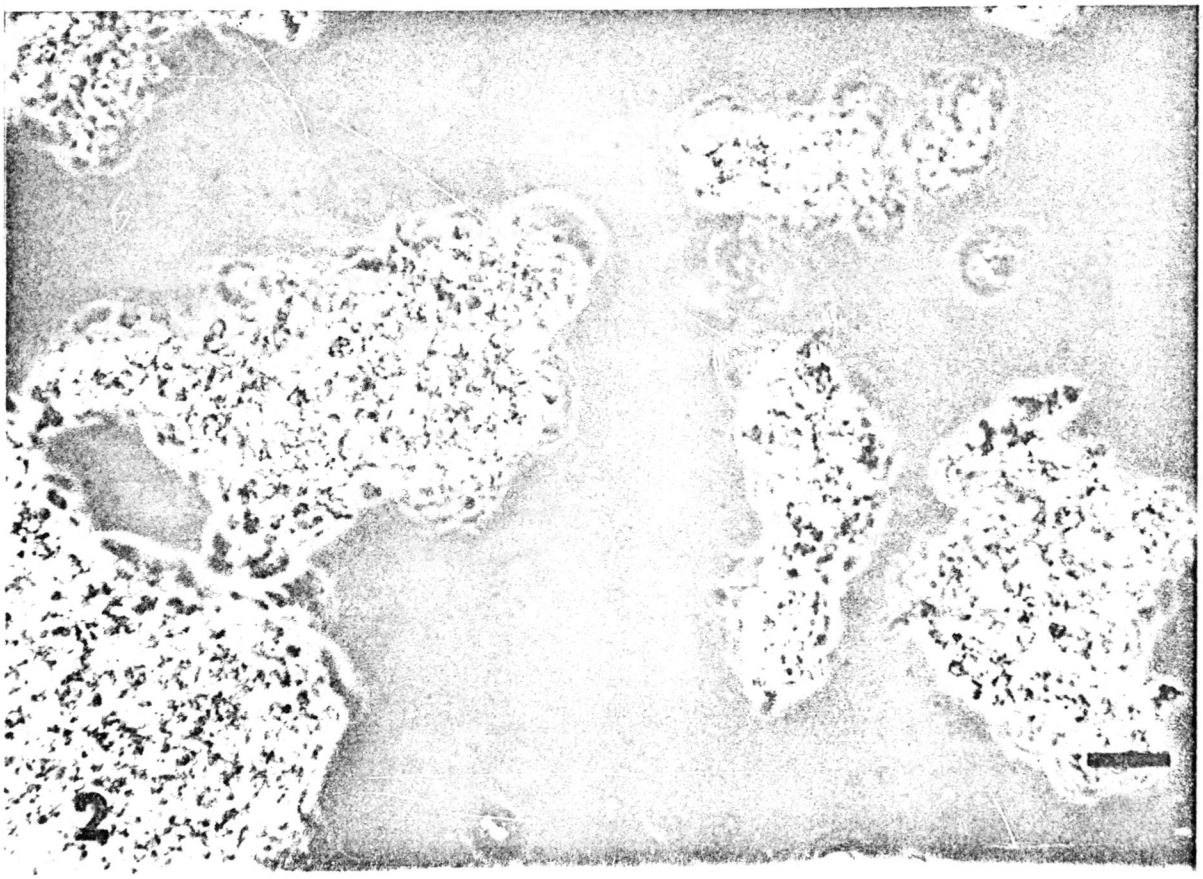
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Table 1. Effect of Sedimentation Force and Sucrose Densities on Sedimentation of Aggregated Granules

Force, xg	Percent Sedimentation			
	Sucrose Concentration, M			
	0.5	1.0	1.2	1.4
400	95	96	30	1
600	96	95	20	1
800	97	96	92	2
1000	94	96	85	1

Fig. 3. Effect of Variou Ca^{2+} Concentrations on the Sedimentation of Chromaffin Granules.

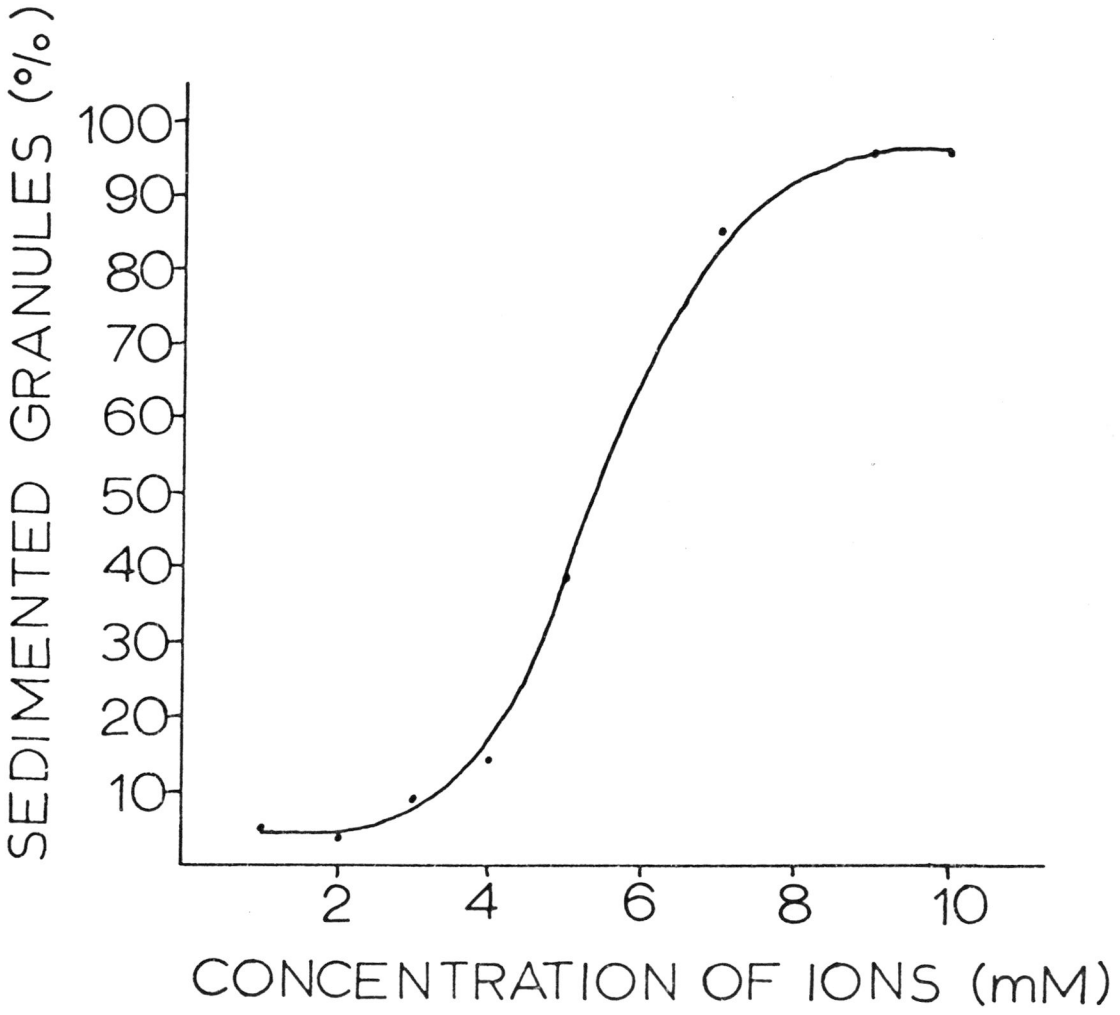
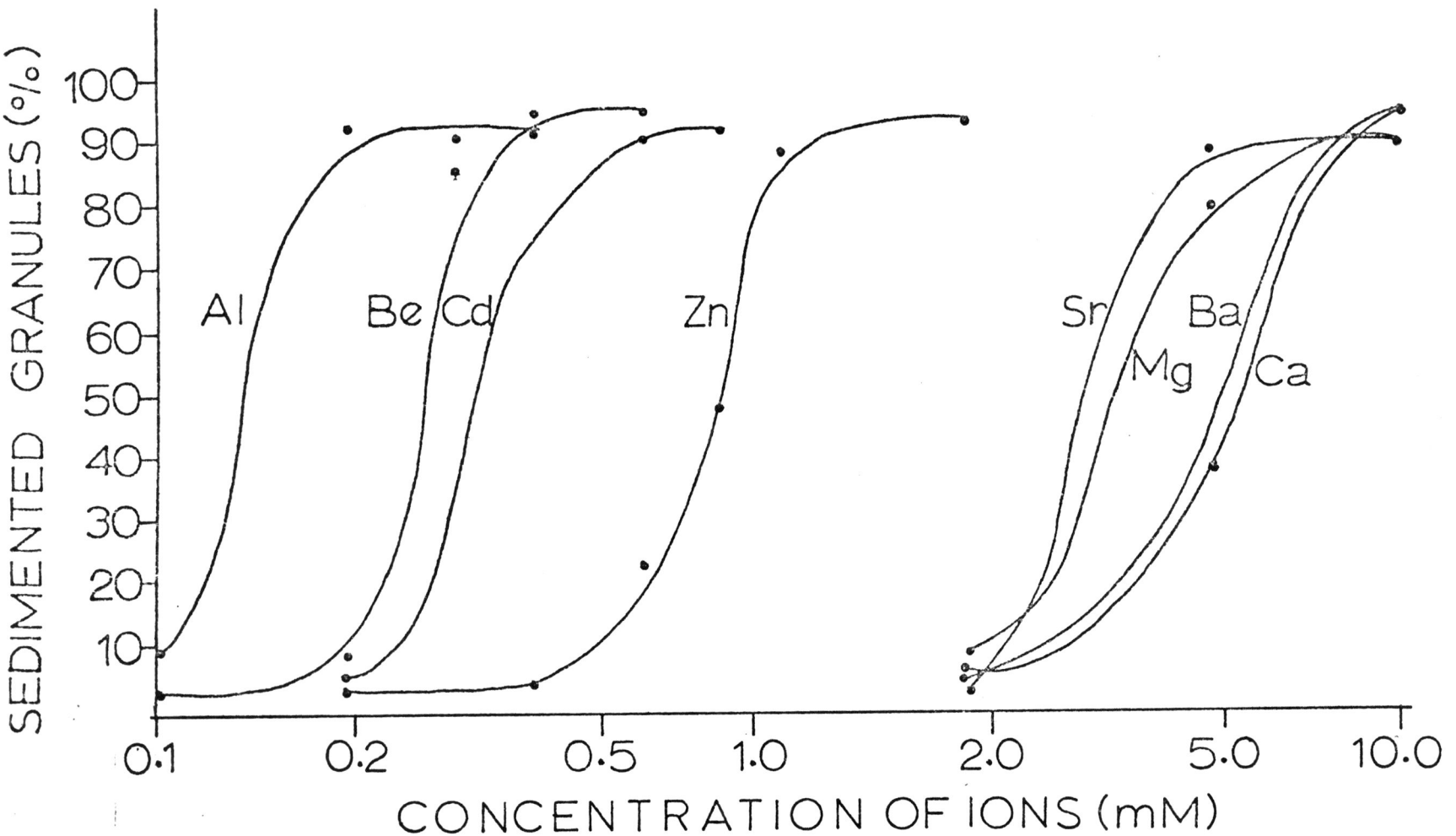

SEDIMENTED GRANULES (%)
100
90
80
70
60
50
40
30
20
10
2
4
6
8
10
CONCENTRATION OF IONS (mM)

Fig. 4. Effect of Various Ions on the Sedimentation of Chromaffin Granules


SEDIMENTED GRANULES (%)
100
90
80
70
60
50
40
30
20
10
Al
Be
Cd
Zn
Sr
Ba
Mg
Ca
0.1
0.2
0.5
1.0
2.0
5.0
10.0
CONCENTRATION OF IONS (mM)

in all concentrations less than 1.2M even at the lowest forces used. In sucrose concentrations greater than 1.2M, no sedimentation occurred even with the maximum centrifugation force used of 1,000 xg. Table 1 also indicates that decreasing the force when sedimenting granules through 1.2M sucrose decreased sedimentation.

Concentration of the cation affects sedimentation of the chromaffin granules

The concentration of ion used to induce sedimentation will determine what proportion of the available granules will sediment. A Ca^{2+} concentration of 5.0mM induced sedimentation of 38% of the available granules as seen in Fig. 3. Increasing the concentration to 5.6mM yielded sedimentation of 50% of the granules sedimented. It is also observed in Fig. 4 that Al^{3+}, the only trivalent cation examined, induced maximum sedimentation at much lower concentration than the other ions. The remaining ions followed in the order of Be^{2+}, Cd^{2+}, Zn^{2+}, Sr^{2+}, Mg^{2+}, Ba^{2+}, and Ca^{2+} in their aggregation abilities. Microscopic examinations verified that many of the concentrations used did not result in complete aggregation as seen in Fig. 5 and Fig. 6. It was observed that after maximum aggregation of the granules, increased Be^{2+} concentration resulted in a loss of aggregation ability. Excessive concentrations of $CaCl_2$, and $AlCl_3$ yielded similar results. $Ca(NO_3)_2$ was examined, producing sedimentation similar to $CaCl_2$. This diphasic characteristic is illustrated in Fig. 7. The nitrate salt of Ca was examined to determine if the NO_3^- ion affected the sedimentation of the chromaffin granules.

Separation of the types of chromaffins granules

The fluorometric procedure indicated there was no separation of the granule types. The ratio of norepinephrine to epinephrine was similar in layers and pellets.

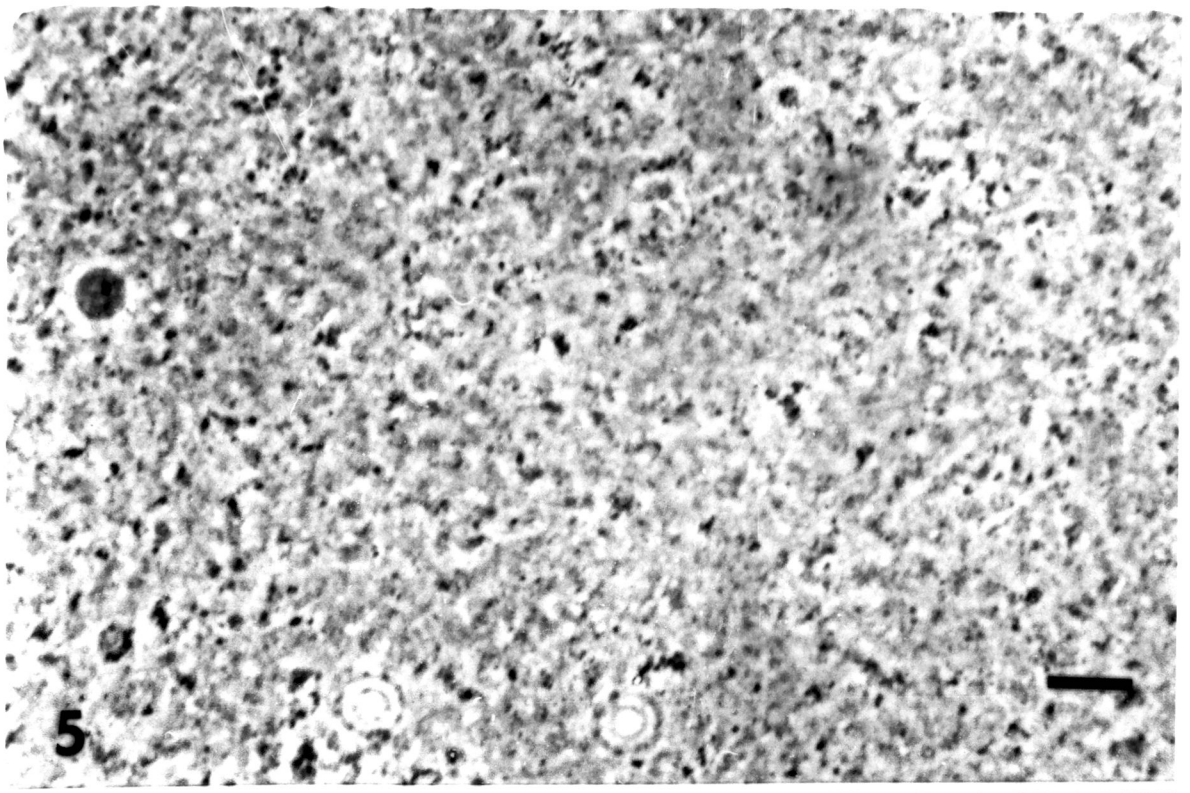
Reversibility of Aggregation

Maximumly aggregated granules remained partially clumped after being dialysed against 0.26M sucrose and the addition of EDTA to the 0.26M sucrose did not cause further disaggregation. As Fig. 8-9 indicates, granules remained partially aggregated after dialysis of 4 hours and extended dialysis of 20 hours.

Fig. 5. Minimumly Aggregated Chromaffin Granules

Fig. 6. Moderately Aggregated Chromaffin Granules

Legend Bar is 10μ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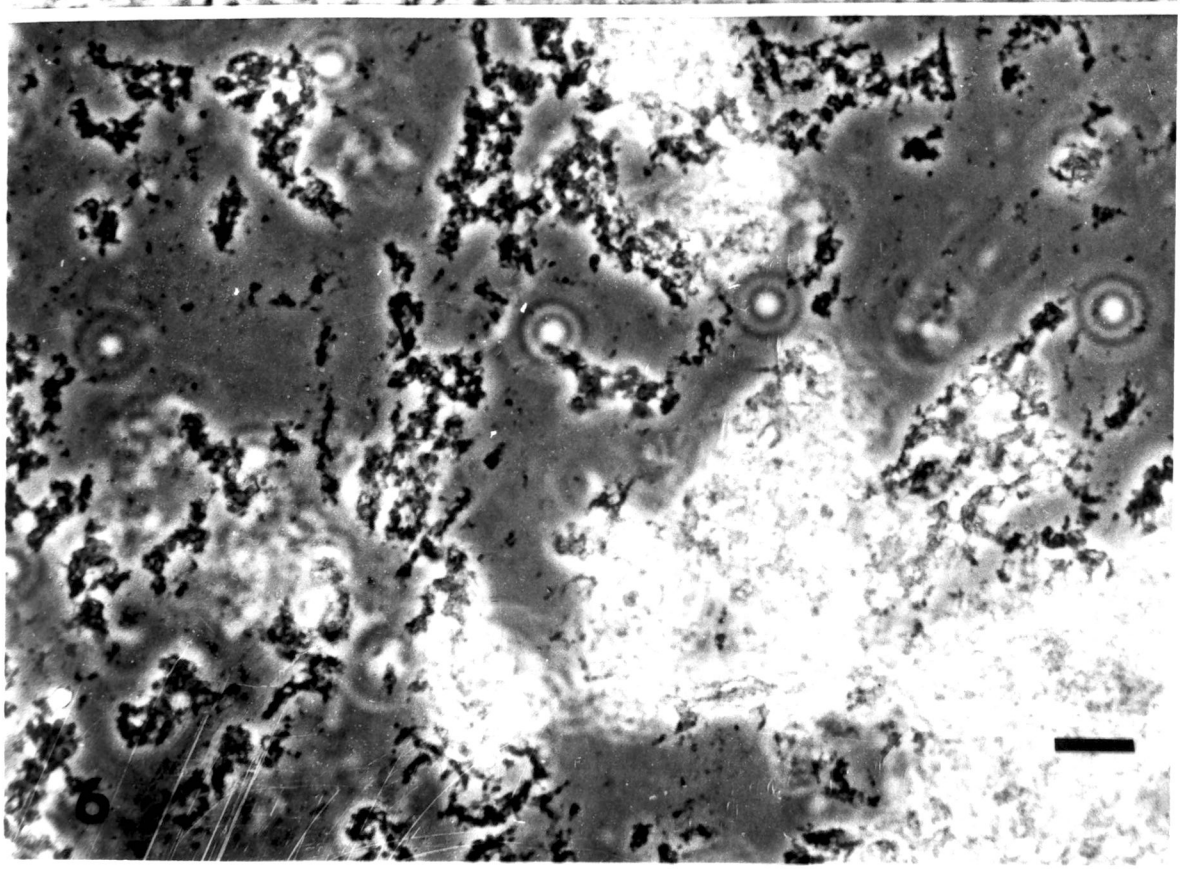
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Fig. 7. Effect of Excessive Concentration of Cations on Sedimentation of Chromaffin Granules

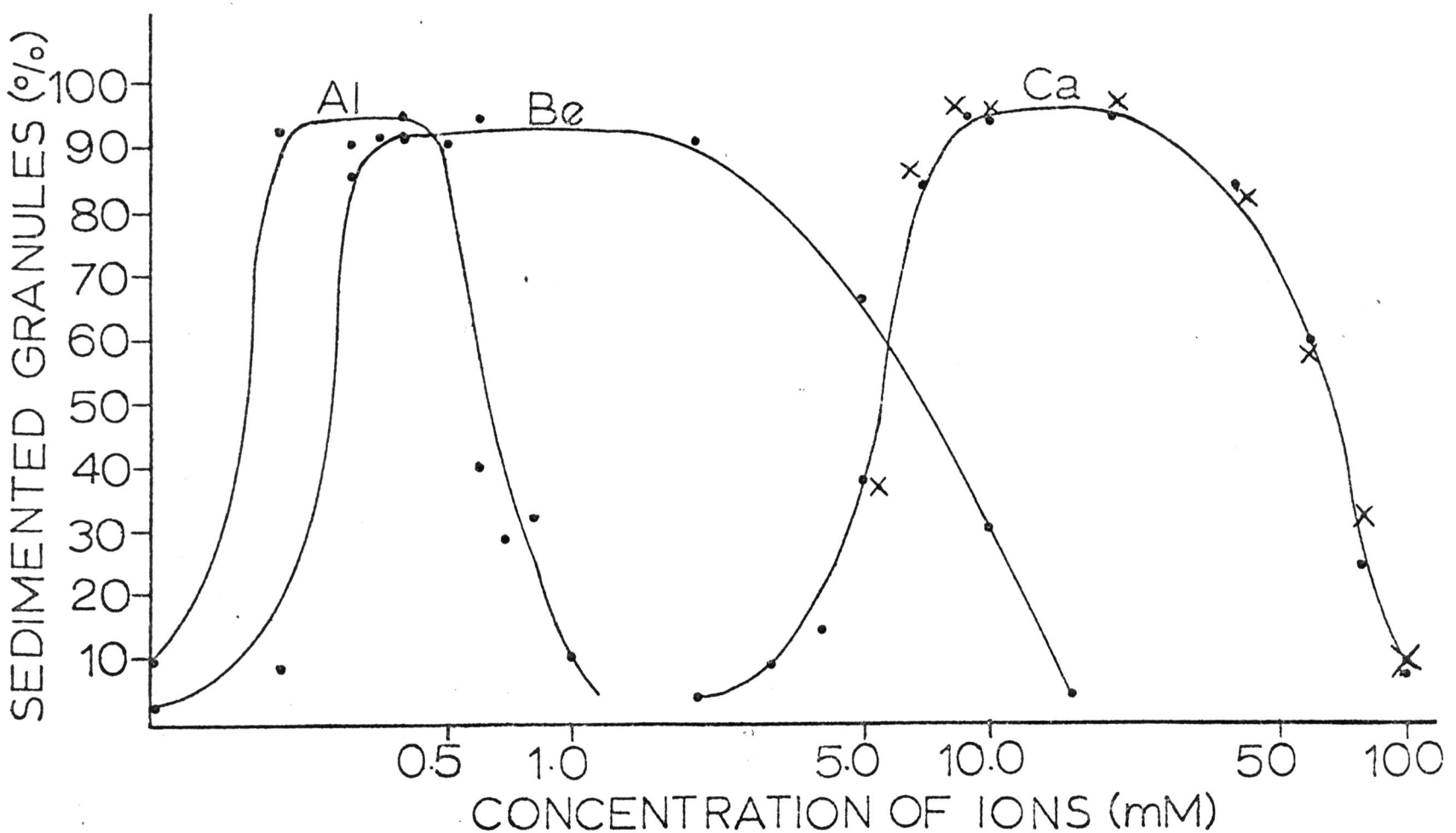
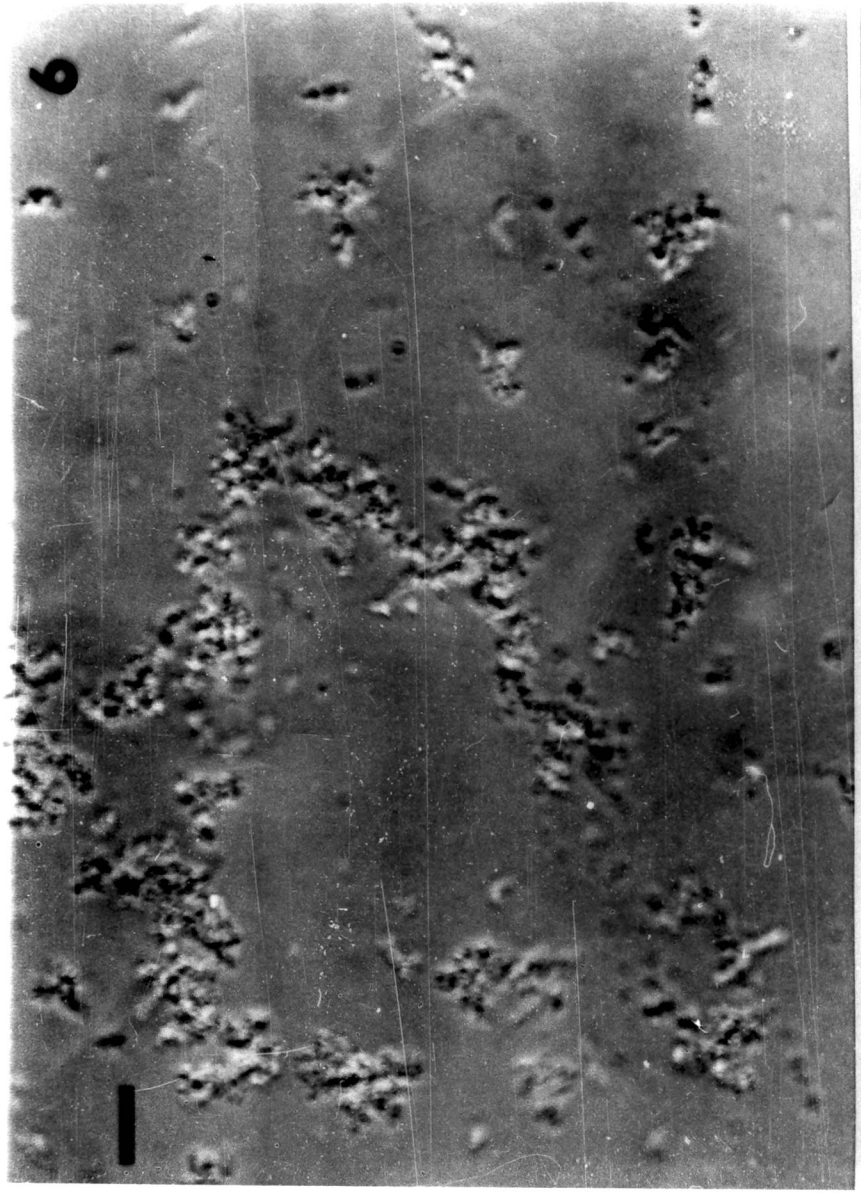

SEDIMENTED GRANULES (%)
100
90
80
70
60
50
40
30
20
10
Al
Be
Ca
0.5
1.0
5.0
10.0
50
100
CONCENTRATION OF IONS (mM)

Fig. 8. Aggregated Granules Dialysed for 4 Hours

Fig. 9. Aggregated Granules Dialysed for 20 Hours

Legend Bar is 10μ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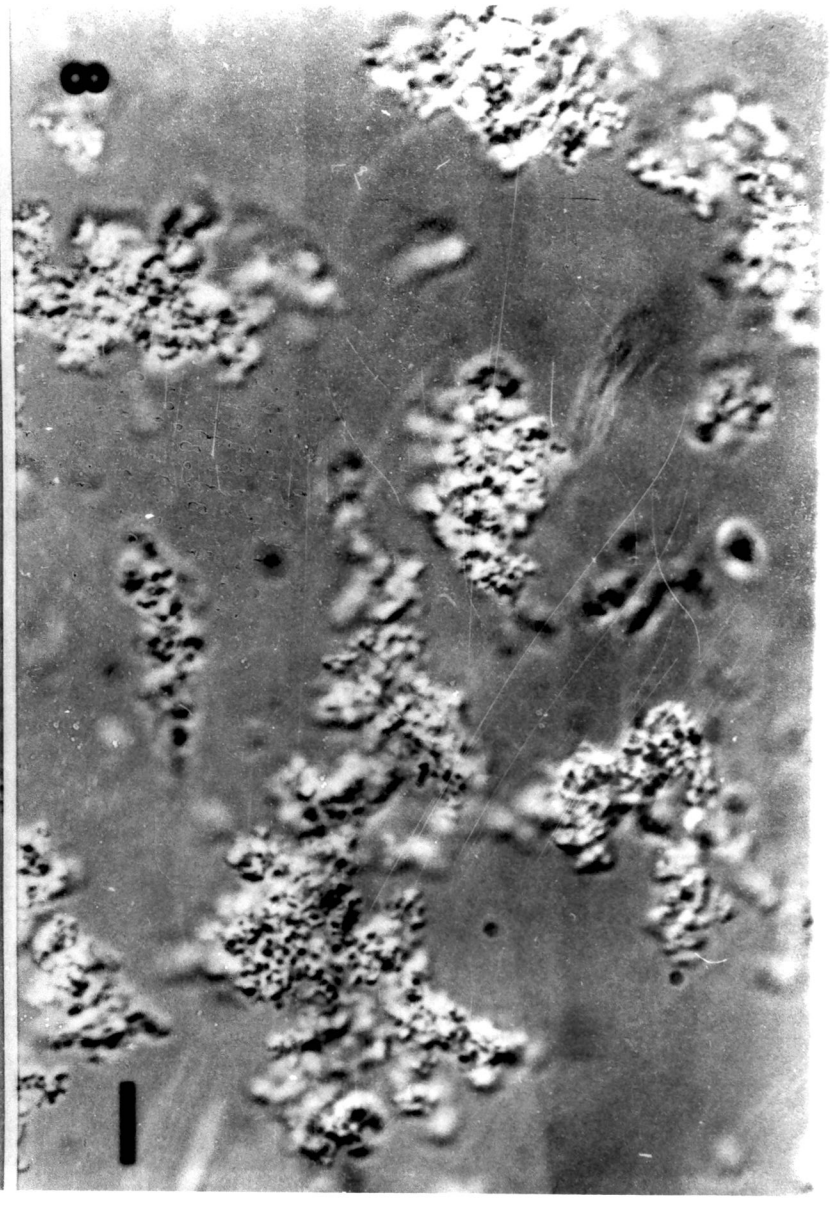
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DISCUSSION

The original intent of this work was to achieve separation of epinephrine and norepinephrine chromaffin granules. In all the experiments using various ions to aggregate the chromaffin granules, little significant separation was achieved. The complete separation of chromaffin granules from mitochondria and lysosomes was not achieved. However, the most effective cations, Be^{2+} and Al^{3+}, for aggregation of chromaffin granules were not examined for their ability to aggregate and hence sediment mitochondria and lysosomes. Work with the more effective cations may provide a means of preparing chromaffin granules free of other organelles.

The mechanism of aggregation presumably is the formation of cation bridges between negative groups in the membranes of the organelles. Membranes of all known organelles are composed of phospholipids and proteins. The phospholipids are arranged with their negatively-charged phosphate groups more or less exposed to the exterior of the membrane. Membrane proteins are also known to be exposed to the exterior and proteins usually have negatively-charged groups on their surfaces. Thus there is ample opportunity for cations to bind to exposed negative groups of the membranes. Since Ca cations will aggregate microsomes (20), chromaffin granules, mitochondria and lysosomes, it seems probable that it is the phospholipid that is the agent participating in the binding of cations since phospholipids are relatively more constant in makeup than proteins are from one organelle membrane to another. But perhaps both phospholipids and

proteins are involved in the cations bridges.

Dialysis experiments with aggregated granules were designed to show reversibility of cation binding. Reversibility was only partially demonstrated. Under the conditions employed, the granules should have shown complete disaggregation if the binding was reversible, since the cation level was reduced by dialysis below levels that produce aggregation. The large clumps never disaggregated to the original condition of free granules. This may mean that there are two mechanisms involved in the binding of the cations--one which is reversible and one which is not readily reversible. This may be an argument for the participation of both phospholipids and proteins in the cation binding process.

Another observation that may be relevant to a discussion of the mechanism of aggregation is that of the three cations, Be^{++}, Ca^{++}, Al^{+++}, that were used in excess of the concentration required for complete aggregation of granules. These three cations exhibited a reversal of their ability to aggregate at high cation concentrations. This phenomena may be analogous to other similar situations, for example: the Biuret reagent for protein determinations consists basically of copper ions which complex with two peptide bonds to produce a color. An excess of copper ions, however, will allow two copper ions to bind with each pair of peptide bonds so that no color is produced. Thus, in the present work, excess cations may cause each phospholipid binding site to bind its own cation. This would inhibit binding to an adjacent granule. Each membrane would have its own bound cations and thus no bridge between organelles can form.

Further work may clarify the mechanism of aggregation; but from a practical point of view, the present work indicates that various cations may be used to separate organelles by aggregation centrifugation when

other procedures fail. Since many organelles are surrounded by phospholipid membranes, the above suggested procedure may be of rather general applicability.

SUMMARY

The catecholamines, epinephrine and norepinephrine, are stored within the membrane-bound vesicles found in the adrenal medulla. It has been reported that Ca^{2+} ions will aggregate endoplasmic reticulum fragments. It is also known that Ca^{2+} will not only stimulate the release of catecholamines from the granules, but will also induce the aggregation of the chromaffin granules by acting as a divalent bridge between independent granules. Chromaffin granules were isolated from bovine adrenal medulla and exposed to various divalent cations. Several concentrations of each cation were examined.

The cation granule suspensions were layered over monogradients of sucrose ranging from 0.5M to 1.4M. Each gradient was then centrifuged at 400xg. Centrifugation was repeated using speeds of 600xg, 800xg and 1000xg. The maximum concentration that would allow sedimentation of the granules was 1.2M. Granules would sediment through 1.2M sucrose using low forces. Some of the cations demonstrated biphasic sedimentation patterns for the granules. A fluorometric analysis indicated there was no distinction in sedimented and non-sedimented granules as far as their catecholamine content was concerned.

Of the cations examined, the chromaffin granules were least sensitive to Ca^{2+}. This suggests that it could be possible to induce sedimentation of microsomes and other organelles by using divalent cations other than Ca^{2+}. The ion-sedimented granules were examined for their content

of mitochondria and lysosome contaminants. The presence of these organelles indicate that the cations may aggregate any organelle with a phospholipid membrane.

At higher concentration of cations, all the negative charges on the surface of the granules may be bound to cations. This would prevent the divalent bridge formation and yield the biphasic characteristic.

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