

ELECTRON MICROSCOPE OBSERVATIONS
ON COMPOUND 48/80-INDUCED
DEGRANULATION IN RAT MAST CELLS

Evidence for Sequential Exocytosis of Storage Granules

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ABSTRACT

In vitro degranulation of rat mast cells was studied at different intervals ranging from 10 to 60 sec after adding the histamine liberator, compound 48/80 (0.4 $\mu\text{g}/\text{ml}$, 17°C). The ultrastructural changes were followed by electron microscopy, and parallel assays were made to determine the histamine released. In addition, the extracellular tracers lanthanum and hemoglobin (demonstrated by its peroxidative activity) were applied to mast cells to follow communication of the extracellular space with the cavities formed during degranulation. After a lag period of 10 sec, degranulation started in the most peripherally located granules. The perigranular membrane fused with the plasma membrane, resulting in a pore bridged by a thin diaphragm. This was followed by rupture of the diaphragm and extrusion of the granule matrix (exocytosis). The process advanced towards the cell interior by fusion and opening of the deeper situated granules to the formerly opened granule cavities. At the end of the process, the cell was filled by a system of complicated cavities containing a number of altered granules. Extracellular tracers have shown that these intracellular cavities were in unbroken communication with the extracellular space from the very beginning of their formation. Both lanthanum and hemoglobin were found to be adsorbed to the limiting membrane of the cavities and bound to altered mast cell granules. In contrast, no tracer substance was present in nondegranulating mast cells. Degranulation of mast cells by compound 48/80 is regarded as a sequential exocytosis, a process similar to that described for some exocrine gland cells. All the "intracellular" cavities, formed by degranulation, were shown to communicate with the extracellular space; consequently, granules lying in these cavities must be considered as biologically extracellular. The present findings support the view that histamine is released from the granule matrix by the extracellular ionic milieu.

INTRODUCTION

In mast cells the histamine is localized to basophil granules where it is stored in an ionic linkage to the heparin-protein complex (23, 40). There are numerous data which favor the idea that the

release of histamine from this complex is a simple ion exchange between histamine in the granules and cations in the extracellular fluid (22, 35, 36, 38-41). This view requires that the mast cell

granules should be exposed to the extracellular fluid to release their histamine. Since the granules of the mast cell are separated from the extracellular fluid by both the perigranular and the plasma membrane, two theoretical possibilities could fulfill this requirement: either the membranes could be leaking, allowing the free passage of cations and histamine to and from the granules, or the granules could be extruded from the cell.

This first mechanism is probably the one involved in the histamine-releasing action of cytotoxic agents, detergents, hypo-osmosis, freezing and thawing, ultrasound treatment, etc. On the other hand, extrusion of granules from mast cells (degranulation) occurs in histamine release induced by certain drugs, such as the basic polyamine compound 48/80, basic polypeptides (29, 37, 42), as well as antigen (in sensitized cells). During degranulation, which requires an intact cell metabolism, membrane-free granules are expelled into the extracellular space, while the cell remains intact (19) and capable of repeated degranulation (36) and granule synthesis (12, 29).

Electron microscope observations (3-5, 8, 17, 24, 32) of the degranulation process describe changes in the structure of the granules, formation of vacuoles around the granules, fusion of vacuoles to larger ones, and their opening to the cell surface. However, the time sequence of these changes remained unclear, especially since some of these observations were made after histamine release was completed. Some of these papers suggested that the formation of vacuoles preceded their communication with the extracellular space. Since granule alteration is believed to accompany histamine release, it may be concluded that amines are liberated in degranulation partly or entirely intracellularly (5, 6). In fact, Carlsson and Ritzén (7) proposed an intracellular release of 5-hydroxytryptamine (5-HT)¹ since they could show that altered granules situated in the interior of the cell did not exhibit any fluorescence characteristic of the presence of 5-HT.

On the other hand, Uvnäs and collaborators hold the view that histamine release is the second step of a two-stage process (36). The first event is the expulsion of histamine-containing granules to the exterior of the cell, and the second one a cation exchange between granule histamine and extracellular cations (mainly sodium).

¹ Abbreviations: 5-HT, 5-hydroxytryptamine.

The present investigation was undertaken to bridge the gap between conflicting reports as to whether or not histamine is liberated within mast cells or by release of granules from cells. The experimental design made it possible to follow the fine temporal correlation between cytological changes and histamine release. Compound 48/80 was used as a degranulating agent in submaximal doses to allow reproducible results and to avoid damage to the cells. The rapid process of degranulation (10 sec at 37°C) was slowed down by incubating the cells with compound 48/80 at 17°C, and degranulation was stopped at precise time intervals by adding cold fixative.

A careful analysis of electron micrographs from this material suggested that the granules are released by a sequential exocytosis during which fusion of the plasma membrane with the perigranular membranes leads to the formation of passageways connecting the extracellular fluid with granules deep in the cell. To substantiate this, extracellular tracer substances were used. One of the tracers was lanthanum nitrate which has been reported to give reliable results in many cases. Since lanthanum can only be applied at or after fixation, another marker had to be found to trace extracellular spaces in living cells. Instead of using peroxidase, which was reported to induce histamine liberation from mast cells (11), we used hemoglobin having a peroxidative activity (14, 21).

MATERIAL AND METHODS

Preparation of Cell Suspension

For each of the seven degranulation experiments 5-8 male Sprague-Dawley rats (350-450 g) were used. The rats were exsanguinated under light ether anesthesia by cutting the carotids, a small incision was made along the midline of the abdomen, and the peritoneal cavity was washed with 9 ml of a salt solution (NaCl 145 mM, KCl 2.7 mM, CaCl₂ 0.9 mM) buffered with 10% v/v Sørensen buffer (Na₂HPO₄ + KH₂PO₄, 67 mM) pH 7.1, to which 1 mg of human serum albumin/ml was added. The cell suspension was collected in a 15 ml plastic centrifuge tube. After the diaphragm was opened, an additional 4.5 ml of the buffered salt solution was used to collect the cells from the pleural cavity.

Incubation Procedure

The cell suspensions were centrifuged (200 g, 5 min, 22°C), and the cells from all tubes were resus-

pended and pooled, yielding an average of 2×10^6 mast cells/ml.

To follow the initial phase of degranulation, which at 37°C is completed in 5–10 sec, the process was slowed down by performing the incubation with compound 48/80 at 17°C. 7–8 tubes, each containing 0.9 ml of cell suspension, were preincubated for 10 min in a water bath at 17°C. Then 0.1 ml of a buffered salt solution containing 4 µg/ml compound 48/80 was added. In the controls, 0.1 ml of buffered salt solution was added instead of compound 48/80. The degranulation was stopped after 10, 20, 30, 40, 50, or 60 sec by adding 9 ml of cold (+4°C) aldehyde fixative for electron microscopy or 9 ml of ice-cold buffered salt solution for histamine assay.

Electron Microscopy

After 5–10 min of fixation in the cold, the tubes were transferred to room temperature and fixation was continued for an additional 15–120 min. Two fixatives were used: 2% glutaraldehyde (prepared from a 25% solution in water, purified shortly before use with a mixed ion exchange resin) in Millonig phosphate buffer (pH 7.2–7.4), or 1% formaldehyde (prepared by dissolving paraformaldehyde) together with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2–7.4. The fixed cell suspensions were centrifuged and the sediments were resuspended in Millonig buffer containing 6% sucrose. After 5 min, the cell suspensions were again centrifuged and postfixed in 1% osmium tetroxide in Millonig buffer for 60 min at +4°C. After completion of postfixation, the cells were centrifuged, washed in the same sucrose buffer, and centrifuged again. The sediments were resuspended in 5 drops of a warm 10% gelatin solution and transferred to polyallomer tubes (10 mm × 60 mm, Beckman Instruments, Inc., Fullerton, Calif.). These were centrifuged (400 g, 10 min, 22°C) and subsequently cooled to +4°C. The resulting pellets were separated, cut into pieces, dehydrated in an acetone series, and embedded in Vestopal W (Madame Martin Jaeger, Geneva, Switzerland).

The blocks were cut on an LKB Ultratome III (LKB-Produkter AB, Stockholm, Sweden), and the sections were stained with uranyl acetate and diluted lead citrate and observed in a Philips EM 300 electron microscope at 60 kv.

For light microscope observations, semi-thin sections were stained with a solution of toluidine blue and azure A containing 60% sucrose.

Histamine Assay

Four histamine assays were carried out, two of them in parallel with the electron microscope series. After the degranulation was stopped by the addition of 9 ml of ice-cold buffered salt solution to the incu-

bation medium, the tubes were kept in an ice bath until centrifugation (300 g, 10 min, 0°C). The supernatants were collected and the histamine was determined. The sediments were each suspended in 2 ml of 0.1 N HCl and heated at 80°C for 5 min to release the remaining histamine from the mast cells. Histamine was measured fluorimetrically according to the method of Shore et al. (33), but because mast cells do not contain any substances which interfere with the histamine assay², the purification steps were omitted and the *o*-phthalaldehyde was added directly to the samples after alkalization.

Tracing with Lanthanum Nitrate

(According to Cohen [9])

Mast cells were degranulated by adding compound 48/80 to a mixed cell suspension at 17°C to give a final concentration of 0.4 µg compound 48/80 per ml. The degranulation was stopped by diluting with cold (+4°C) fixative after 30 or 60 sec. Controls without compound 48/80 were run concomitantly. The fixative used was 2.5% glutaraldehyde in Millonig phosphate buffer, pH 7.3 (25). After initial fixation at +4°C for 10 min, fixation was continued for an additional 60 min at 22°C. The cells were washed (3 × 10 min) in Millonig phosphate buffer. After the last centrifugation, the cells were resuspended in a 1% solution of lanthanum nitrate for 30 min at 22°C. The cells were centrifuged and postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer. After centrifugation and resuspending the cells in several drops of a 10% gelatin solution, the pellet obtained by centrifugation was separated from the bottom of the tube. After thorough dehydration in ethanol and propylene oxide, the pellets were embedded in Vestopal W.

Thin sections made on an LKB Ultratome III were observed in the electron microscope without contrast staining. In order to reduce the disturbingly high osmiophilia of the normal mast cell granules, in the majority of cases osmium was extracted from the sections by floating them on a 1% hydrogen peroxide solution for 2 min.

Tracing with Hemoglobin

The cell suspensions were centrifuged and the pellets resuspended in a salt solution (NaCl 145 mM, KCl 2.7 mM, CaCl₂ 0.9 mM) containing 10% v/v Sørensen phosphate buffer (Na₂HPO₄ + KH₂PO₄, 67 mM) pH 7.1 and 70 mg of bovine hemoglobin/ml. Since Padawer described phagocytosis in mast cells

² Bergendorff, A., and B. Uvnäs. Storage of 5-hydroxytryptamine in mast cells. Evidence for an ionic binding to carboxyl groups in the granule heparin-protein complex. Manuscript in preparation.

(27, 28, 30), we kept the time period between adding hemoglobin to the cells and incubating them with compound 48/80 to a minimum (2–3 min). Because of the high protein content of the incubation medium, an increase in the concentration of compound 48/80 was needed to obtain an effective degranulation. 0.1 ml of compound 48/80 solution (100 $\mu\text{g}/\text{ml}$ in the above-mentioned buffered salt solution) was added to 0.9 ml of cell-hemoglobin suspension. Incubations were carried out for 30 or 60 sec, or for 3 min. Controls without compound 48/80 were run concomitantly. Degranulation was slowed down by immersing the tubes in an ice bath. After centrifugation, the pellets were fixed in Karnovsky's fixative (20) at room temperature for 5 hr. The fixed pellet was separated from the bottom of the tube and washed in 0.1 M cacodylate buffer. 50- μ thick sections were cut with a Smith-Farquhar tissue sectioner and were washed overnight in the same buffer at +4°C. The sections were incubated for 2 hr in the Graham-Karnovsky medium (15) as used for demonstration of hemoglobin by Goldfischer et al. (14). The incubation was followed by a wash in 0.05 M Tris-HCl buffer and the sections were postfixed in 1% osmium tetroxide for 20 min at 22°C. The material was embedded in Vestopal W. Light yellow sections were observed without contrast staining in the electron microscope. Controls for the peroxidase reaction were omitted because our purpose was to compare normal and treated cells, and the hemoglobin was used only as a tracer substance. No endogenous peroxidase activity was found in normal mast cells with the long fixation period used in this study.

RESULTS

Histamine Release and Light Microscopy

The time course of the histamine release induced by 0.4 μg compound 48/80 per ml at 17°C is shown in Fig. 1. The histamine release began after an initial lag period of 10–20 sec and reached about 50% after 50–60 sec.

In the light microscope, a large number of the granules showed a swollen appearance and were stained pink instead of dark blue. Some of these "altered" granules (4, 5) were seen in the intercellular space. The morphological changes (Fig. 2) increased with time and showed a correlation with the magnitude of the histamine release. No alterations were observed in mast cells during the lag phase before the histamine release started.

Electron Microscopy

NORMAL MAST CELLS: Untreated mast cells (Fig. 3) showed the well-known ultrastructural

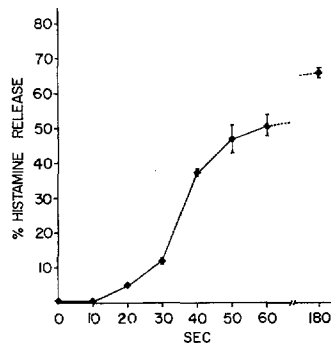


FIGURE 1 Time course of histamine release at 17°C after adding 0.4 $\mu\text{g}/\text{ml}$ compound 48/80. After an initial lag period of about 10 sec, the histamine release increases to about 50% after 60 sec of incubation, and it reaches a maximal 65% after 3 min. The squares represent mean values, the bars range of duplicate samples.

appearance as described in earlier literature (17, 28, 34). Therefore we will stress only some of the ultrastructural features pertinent to the interpretation of the degranulation process. One is the uniform, generally highly osmiophilic and compact appearance of the granules in the normal mature mast cell (Fig. 3). Although there may be some deviations in the form and density of individual granules, altered granules having a loose structure were found only infrequently. There are, however, a few immature mast cells in which the structure of the forming granules differs from the uniformly dense appearance in mature mast cells. In such cells, the progranules usually exhibit a strand-like structure (10) and can be distinguished easily from the evenly swollen granules found in degranulating mast cells.

All granules are surrounded by a membrane (perigranular membrane) which can be discerned only when it is sectioned transversely. Although membranes of neighbouring granules can be very closely apposed, a direct continuity between these membranes could not be observed with certainty. A clear picture of the membranes can be achieved only when the membranes are cut transversely, and therefore a search for continuities between membranes (28) must meet with large difficulties.

Relatively few microtubules, mostly radiating from the centrosomal region, were observed. Some of these could be followed almost up to the plasma membrane. Besides microtubules, numerous cytoplasmic filaments could be found, many of them

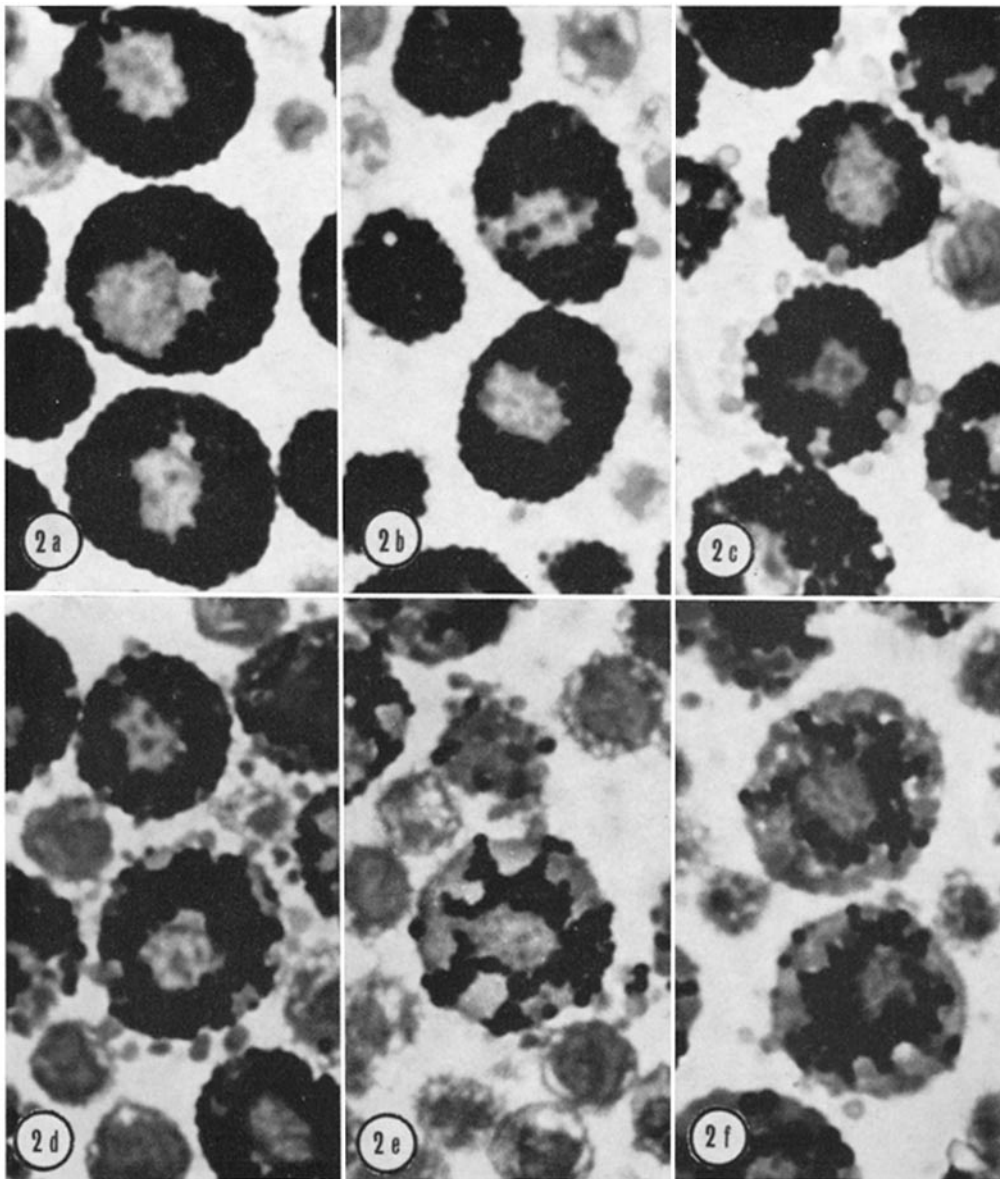


FIGURE 2 Light micrographs of semi-thin sections showing different stages of degranulation. (a) Normal, untreated mast cells; (b) mast cells treated with $0.4 \mu\text{g/ml}$ compound 48/80 at 17°C for 20 sec; (c) for 30 sec; (d) for 40 sec; (e) for 50 sec; and (f) for 60 sec. Note gradual degranulation starting from the periphery of the cell. $\times 1500$.

situated adjacent to the granules and at the periphery of the cell.

COMPOUND 48/80-TREATED MAST CELLS: Mast cells treated with compound 48/80 did not show any detectable morphological changes in the lag period before histamine release. Nor

could alterations of the plasma membrane be observed with our technique.

A small proportion of the mast cells started to respond to compound 48/80 treatment after 20 sec. The initial changes were consistently found in the most peripherally located granules (Fig. 4).



FIGURE 3 Low-power electron micrograph of a normal mast cell. The cytoplasm of the cell is tightly packed with granules having a uniformly dense, osmiophilic structure. No altered granules can be observed. A circumscribed paranuclear region is occupied by the Golgi apparatus. A few mitochondria are scattered in the cytoplasm. $\times 20,000$.

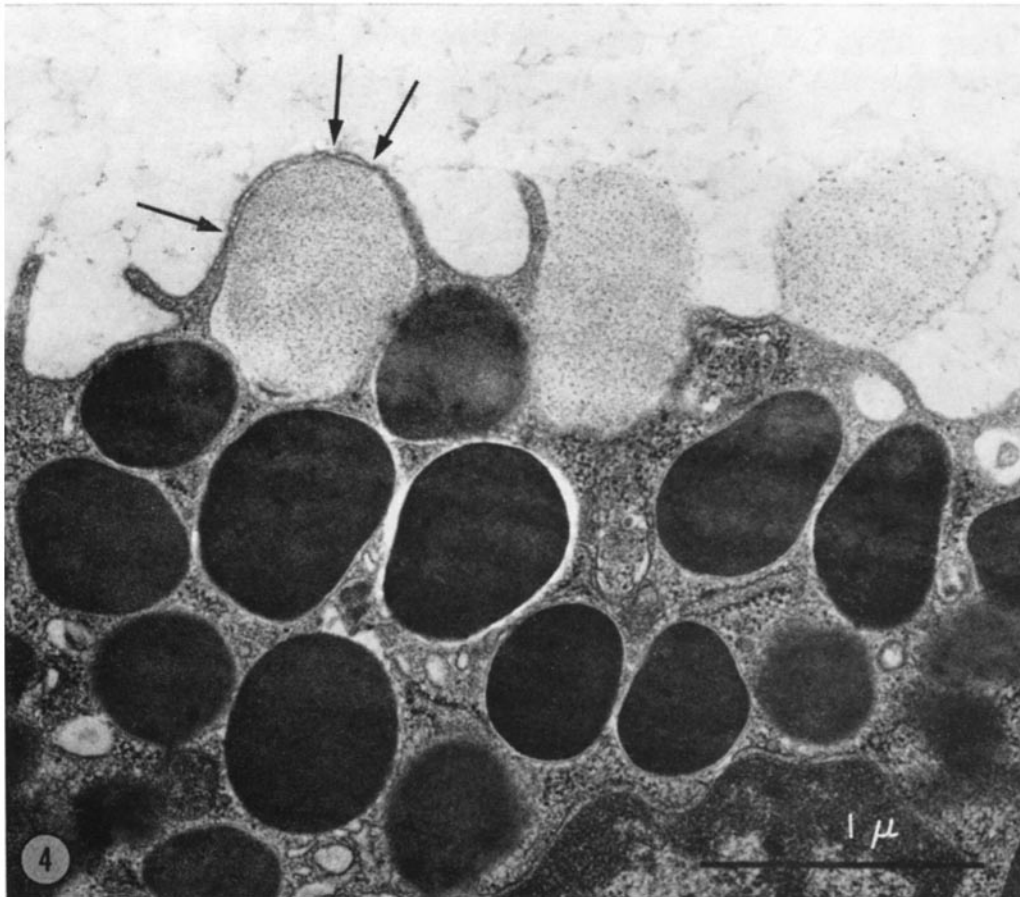
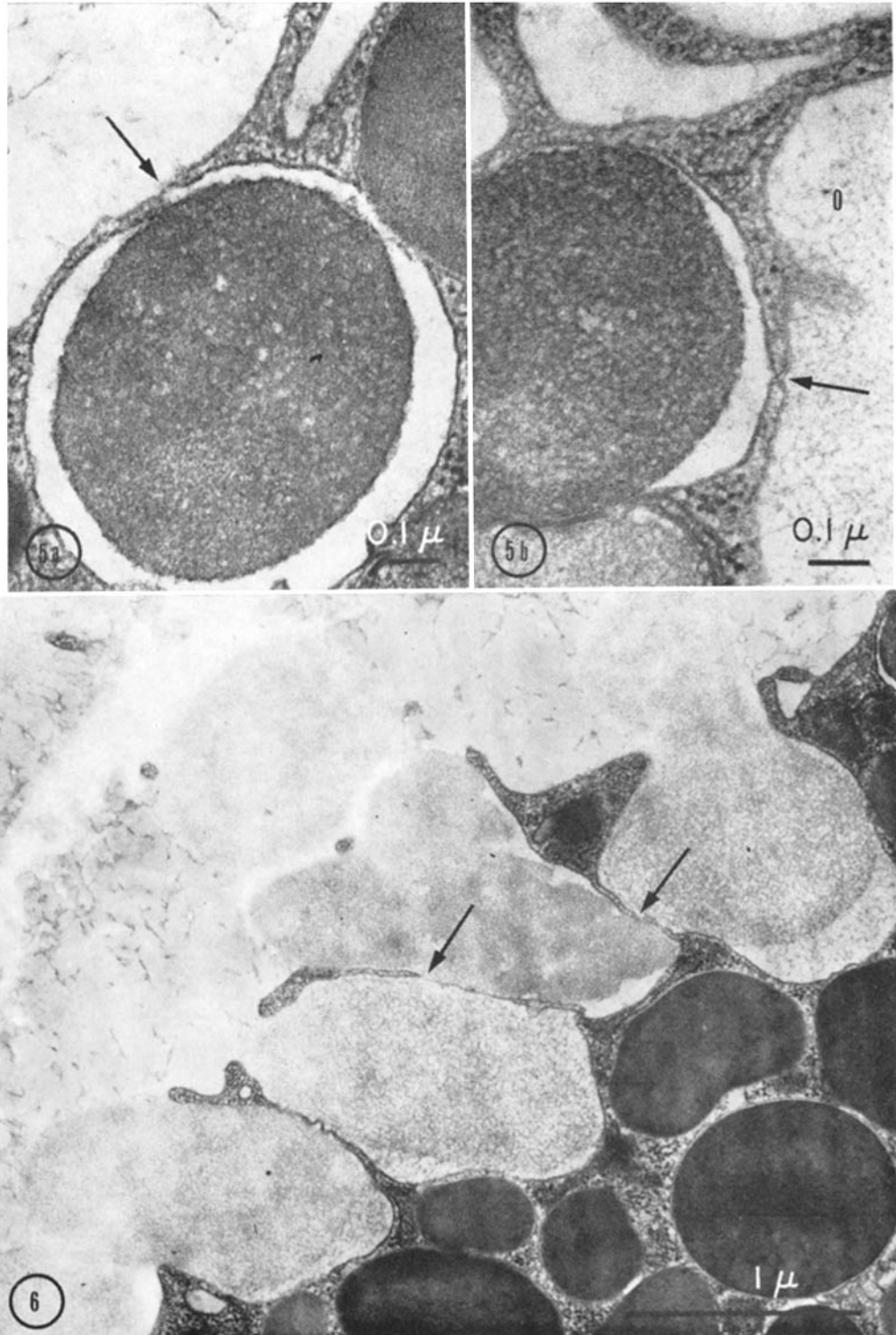


FIGURE 4 The first signs of granule extrusion are always found in the most peripheral granules. Arrows: multiple fusions between cell- and perigranular membrane. All other granules deeper in the cytoplasm exhibit the compact structure characteristic of normal mast cells. Incubation with compound 48/80 for 20 sec. $\times 38,000$.

Only mast cells sectioned through their largest diameter were used to determine the exact location of these granules. The substance of such altered granules showed a somewhat swollen appearance and a decreased density when compared with normal granules. At some places a close apposition and fusion between plasma membrane and the perigranular membrane could be observed. The trilaminar structure of both membranes disappeared at such places, leaving behind a thin electron-opaque single layer (Fig. 5 *a*). At the same stage of degranulation we could observe such peripheral granules on their way out of the cell (Fig. 4). The perigranular membrane became part of the plasma membrane by this process. All granules in the cell interior remained unchanged.

At 30, 40, and 50 sec after exposure to the releaser, the above changes affected more and more mast cells and/or penetrated more deeply into individual cells (Fig. 6). With progressing degranulation, alteration of the granular structure was observed first to spread to granules lying in the immediate vicinity of the already affected granules (Fig. 7). A fusion of the perigranular membranes of altered granules was occasionally found (Fig. 5 *b*).

After 50 and 60 sec of incubation, numerous granules showed the loose, swollen appearance characteristic of degranulating mast cells. The changed granules were situated in large membrane-bounded labyrinthic cavities (Fig. 8). A continuity of these cavities with the extracellular



space was evident at favorable planes of sectioning, mostly where the cells were sectioned through their largest diameter. In many places, however, the cavities appeared as closed vacuoles. The fusion process did not always spread towards the center of the cell; it also affected laterally located granules. Even in the latest stages the mast cell retained its original shape due to a remaining peripheral layer of cytoplasm (Fig. 8). This cytoplasmic layer was connected with the perinuclear cytoplasm by cytoplasmic strands. The formed cavities contained many of the altered granules. The present technique, however, did not allow us to calculate the ratio between completely released granules and granules retained in the cavities.

Not all mast cells were found to be in the same stage of degranulation at a given time. Even after 60 sec we could observe normal mast cells, although they were few in number. The cytoplasmic organelles did not show any observable alterations even after the longest incubation with compound 48/80.

Lanthanum Tracing

Semi-thin sections of mast cells exposed to compound 48/80 revealed in the light microscope that the so-called altered granules, characteristic of mast cell degranulation, did not show the typical pink metachromasia, but were deeply blue in color.

On electron microscope observation, the control cells (Fig. 9 *a*) showed a lanthanum precipitate as a thin layer adsorbed to the cell surface. The cell interior was not stained by lanthanum, indicating that the lanthanum could not penetrate the cell. The high density of normal granules was due to their osmiophilia, which could be decreased by treating the sections with hydrogen peroxide.

Compound 48/80-treated cells showed a different picture. In addition to the lanthanum precipitate adsorbed to the cell surface, all the cavities formed in the cell interior were coated with a thin lanthanum precipitate. The granules found inside

these cavities and between cells were deeply stained by lanthanum (Figs. 9 *b* and 10). In the 30 sec material the size and number of the cavities were rather low, and they were located near the cell surface (Fig. 9 *b*). After 60 sec of incubation, in addition to superficial cavities, others could be followed deep into the cytoplasm (Fig. 10).

We would like to mention that a few slightly swollen granules did not show a lanthanum precipitate (Fig. 10). Such granules were usually found close to the extracellular space (i.e., adjacent to the cell surface or the cavities).

Hemoglobin Tracing

The swollen granules of compound 48/80-treated cells, which usually exhibit a pink metachromasia, were not stained by toluidine blue in semi-thin sections. Instead, they showed a yellowish-brown coloration characteristic of the peroxidase reaction. In contrast to this, granules of normal mast cells or intact granules in degranulating cells appeared, as usual, dark blue.

In the electron microscope, the hemoglobin appeared between individual cells as fine, electron-opaque particles of the peroxidase reaction. A thin layer of reactive material was found to be adsorbed to the surface membrane of the cells (Fig. 11 *a*). Although several endocytosis vacuoles containing hemoglobin were found in macrophages, no signs of endocytosis could be observed in mast cells. The swollen granules of degranulating cells, either inside the cavities or in the extracellular space, gave an intense peroxidase reaction, indicating the presence of hemoglobin (Figs. 11 *b* and 12). Such granules had a rather homogeneous structure when compared to similar granules in the absence of hemoglobin. Often they were increased in size to a considerable extent. The cavities with the swollen granules were not so voluminous as observed in the lanthanum experiments. The inner surface of the cavity membrane was coated with a fine layer of reactive substance (Fig. 12).

FIGURE 5 (a) Pore formed by fusion of the cell membrane with the perigranular membrane (arrow). The edges of the pore are bridged by a thin diaphragm. The granule matrix is slightly swollen when compared to normal mast cells. Incubation time: 30 sec. $\times 75,000$. (b) Pore formation (arrow) between two perigranular membranes. O, opened granule. Incubation time: 40 sec. $\times 90,000$.

FIGURE 6 A more advanced stage of peripheral granule extrusion. All swollen granules are opened to the cell surface and, besides this, they also seem to be fused with each other (arrows). Incubation with compound 48/80 for 40 sec. $\times 35,000$.

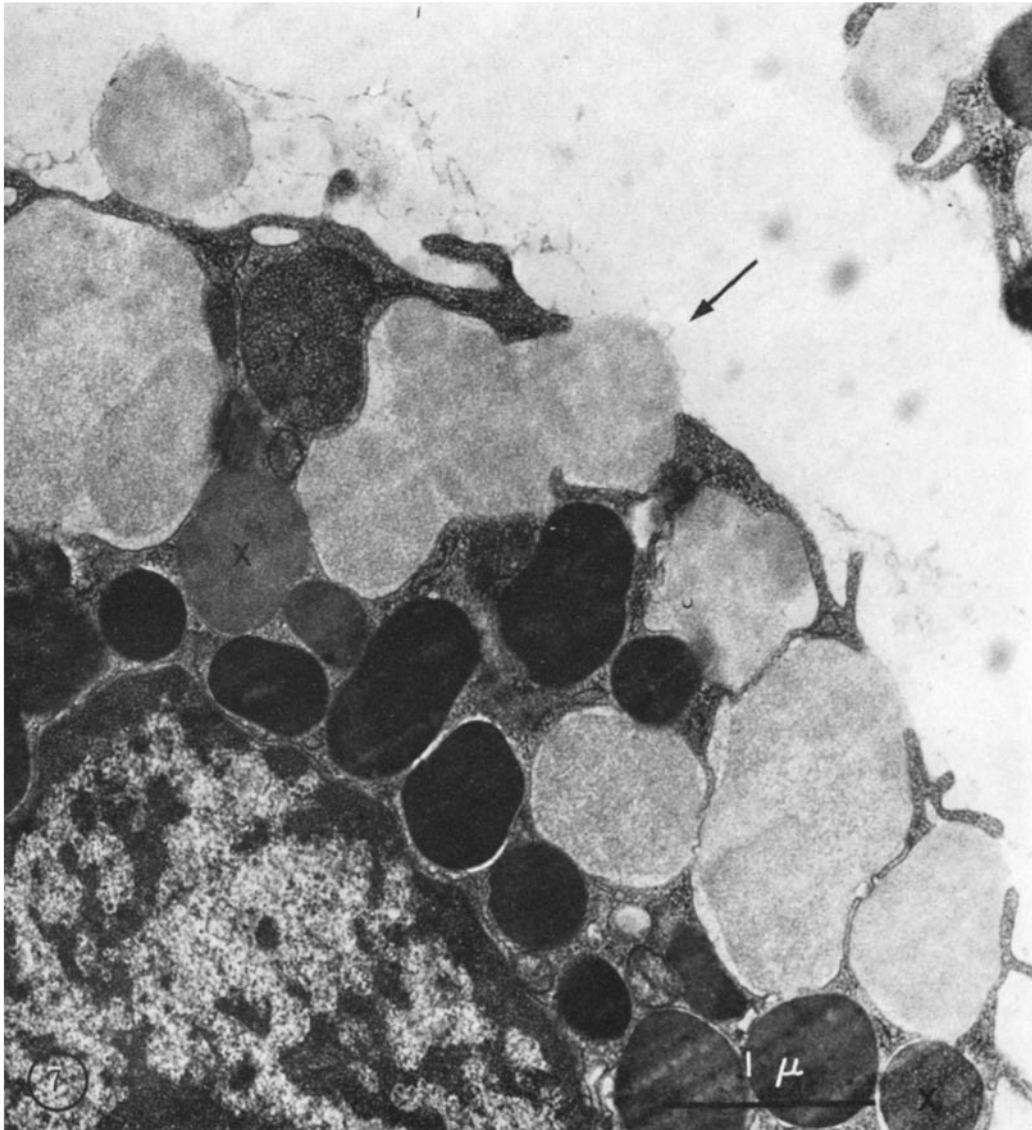


FIGURE 7 An advanced stage of degranulation. Fusion of adjacent granules and their contact with the extracellular space is evident at arrow. Granules at the initial stage of swelling (X) are always seen in the immediate vicinity of opened granules. Incubation time: 40 sec. $\times 28,000$.

As in the lanthanum experiments, some slightly swollen granules were observed which did not bind hemoglobin. These granules were usually situated along the surface membrane or along the membrane of the cavities (Fig. 12).

DISCUSSION

After the initial lag period the first structural changes in the granules of degranulating mast cells

are seen at the periphery of the cell, immediately under the cell membrane, indicating that the effect of compound 48/80 starts from the cell membrane. The granule substance undergoes gradual loosening, revealing a finely filamentous inner structure. Its density is between that of the compact normal granule and that of the very loose, released granule. At this stage, careful searching can reveal close apposition and fusion of the cell membrane with

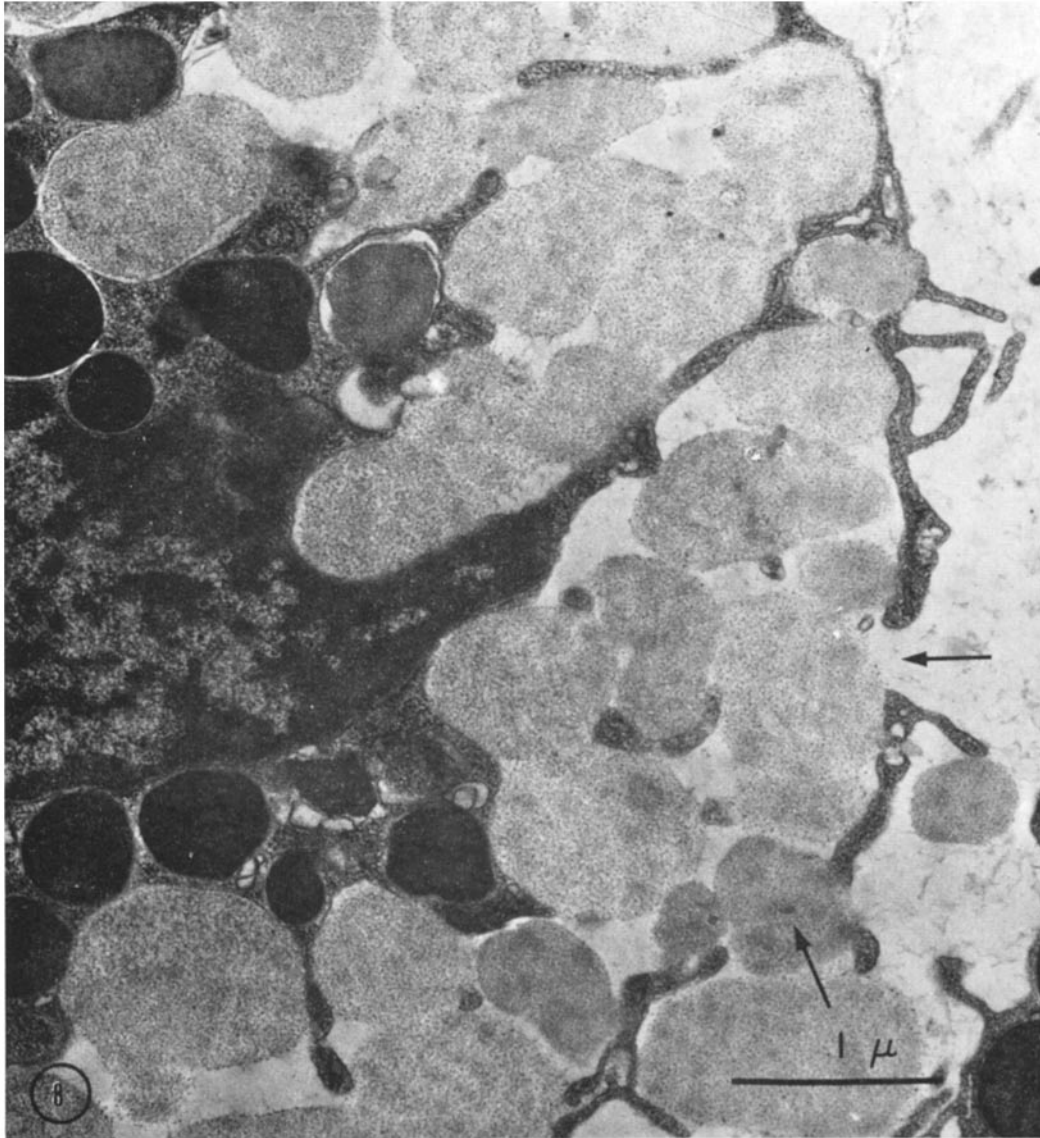


FIGURE 8 At the final stage of degranulation the cell is filled with large anastomosing cavities containing many of the changed granules. Arrows: exits of the cavities to the cell surface. A thin marginal cytoplasmic layer, which at several places is connected to the perigranular cytoplasm, delineates the original shape of the cell. 60 sec after incubation with compound 48/80. $\times 23,000$.

the perigranular membrane. What is left behind is a thin, structureless diaphragm bridging the edges of the fused region. The resulting "pore" resembles very much the endothelial pores of fenestrated capillaries, where a similar mode of pore formation has been proposed (43). Since the diaphragm of endothelial pores is supposed to have permeability characteristics differing from those of the endo-

thelial layer, we suggest, by analogy, that in the mast cell the permeability of the diaphragm is also different from that of the membranes from which it was formed by fusion. A diaphragm acting as a dialyzing membrane and allowing free passage of small molecules and ions would then explain the initiation of structural changes in the granule matrix. The increasing internal pressure in the

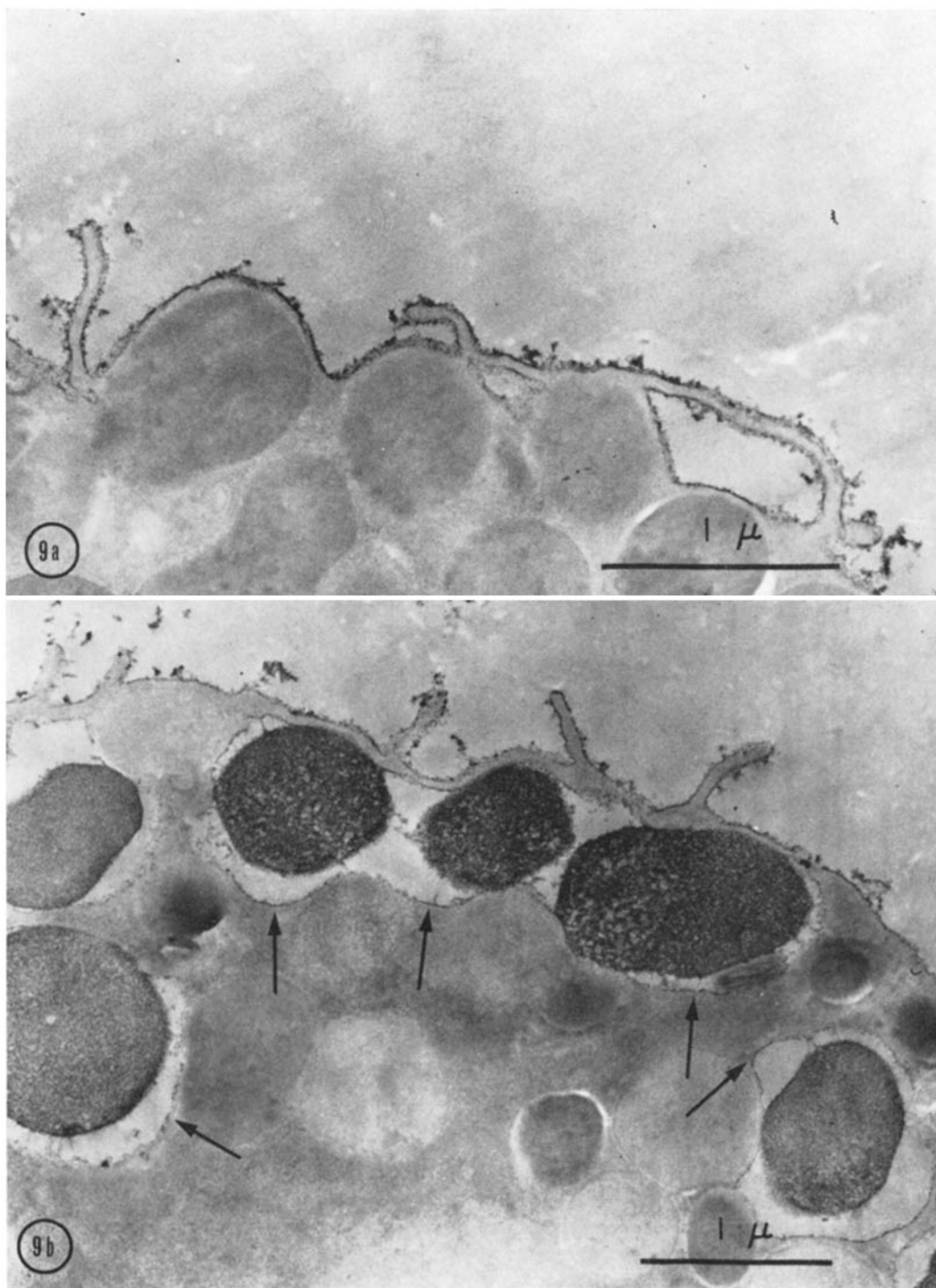


FIGURE 9 (a) Normal, untreated mast cell showing a fine lanthanum precipitate at the cell surface. The granules are not stained by lanthanum. $\times 35,000$. (b) Mast cell treated with compound 48/80. The swollen granules in the cavities of the degranulating mast cell are stained by the lanthanum method. The cavities are delineated by a thin layer of lanthanum precipitate (arrows) adsorbed to the limiting membrane. In both sections (Figs. 9 a and 9 b) osmium was extracted by hydrogen peroxide. $\times 28,000$.

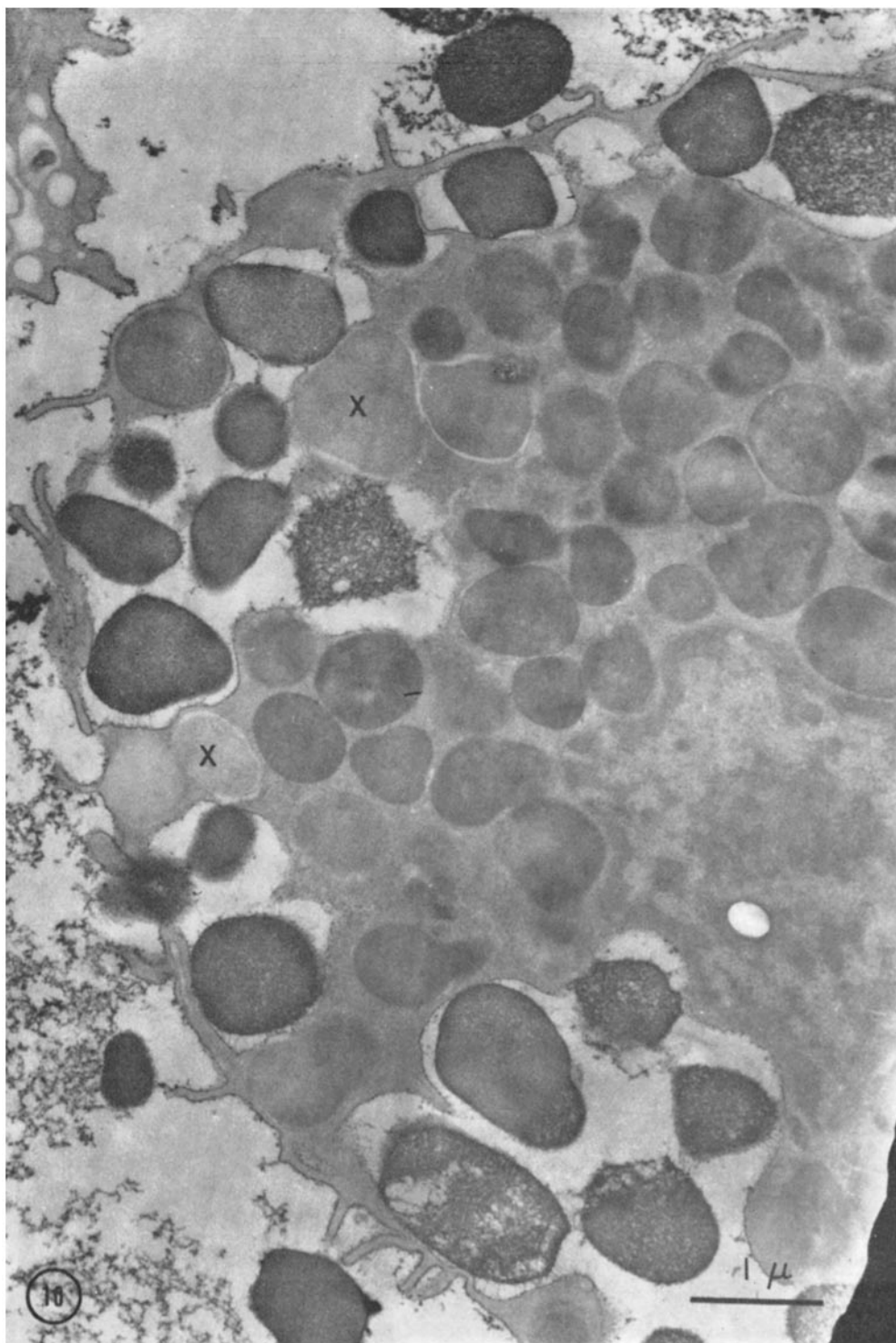


FIGURE 10 Advanced stage of degranulation. Swollen granules in the cavities or outside the cell bind lanthanum. The cell surface and inner surface of the lobated cavities adsorb a fine layer of lanthanum precipitate. Some of the looser granules which are not stained by lanthanum (*X*) are situated close to the extracellular space. $\times 19,000$.

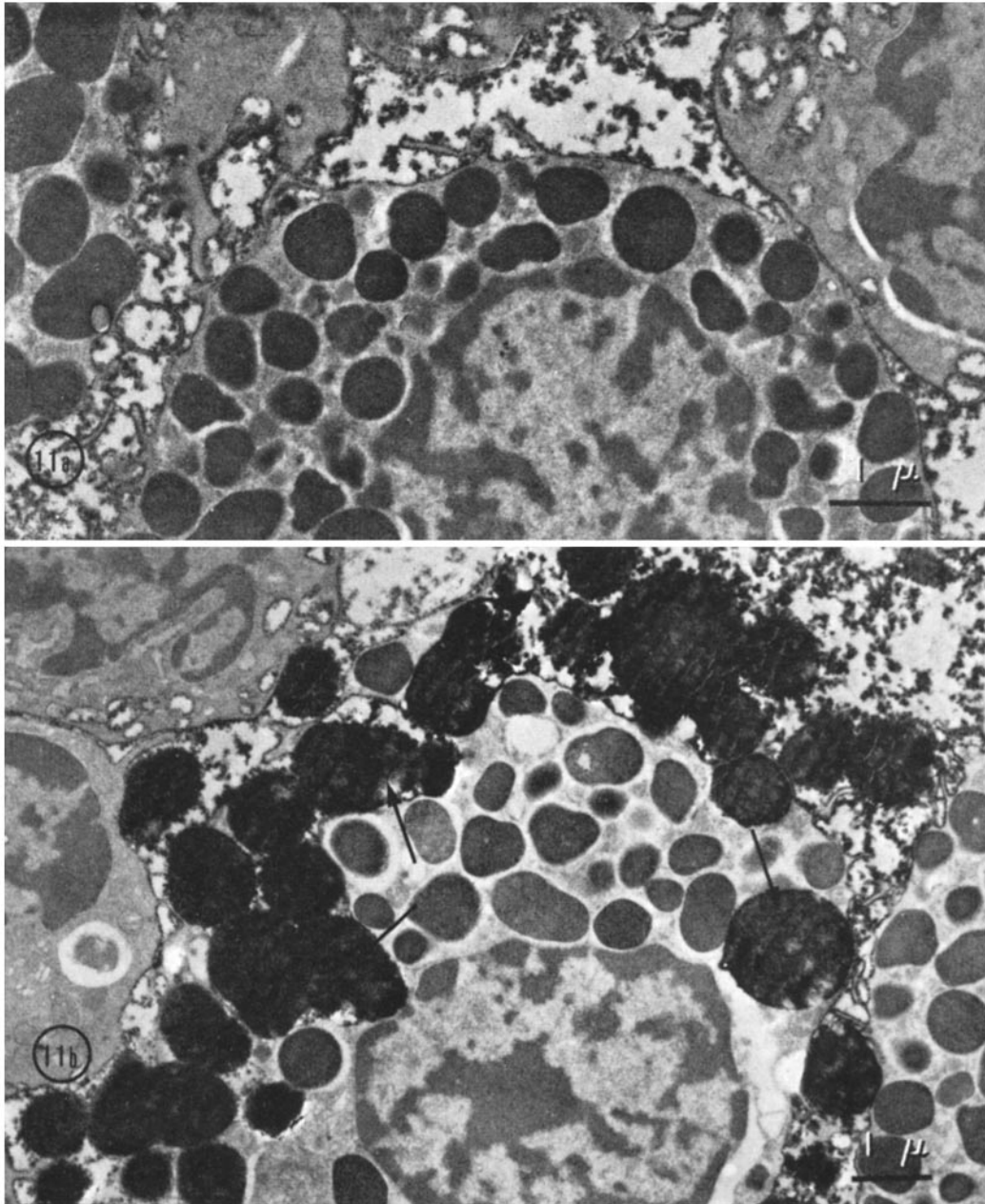


FIGURE 11 *a*) Hemoglobin tracing on normal, untreated mast cell. The hemoglobin is adsorbed to the mast cell surface in a thin layer. $\times 14,000$. *b*) Swollen granules of degranulating mast cells to which hemoglobin is bound. Some of these granules are extruded from the cell (upper right corner) while many of them are still in the cavities of the mast cell (arrows). $\times 11,000$.

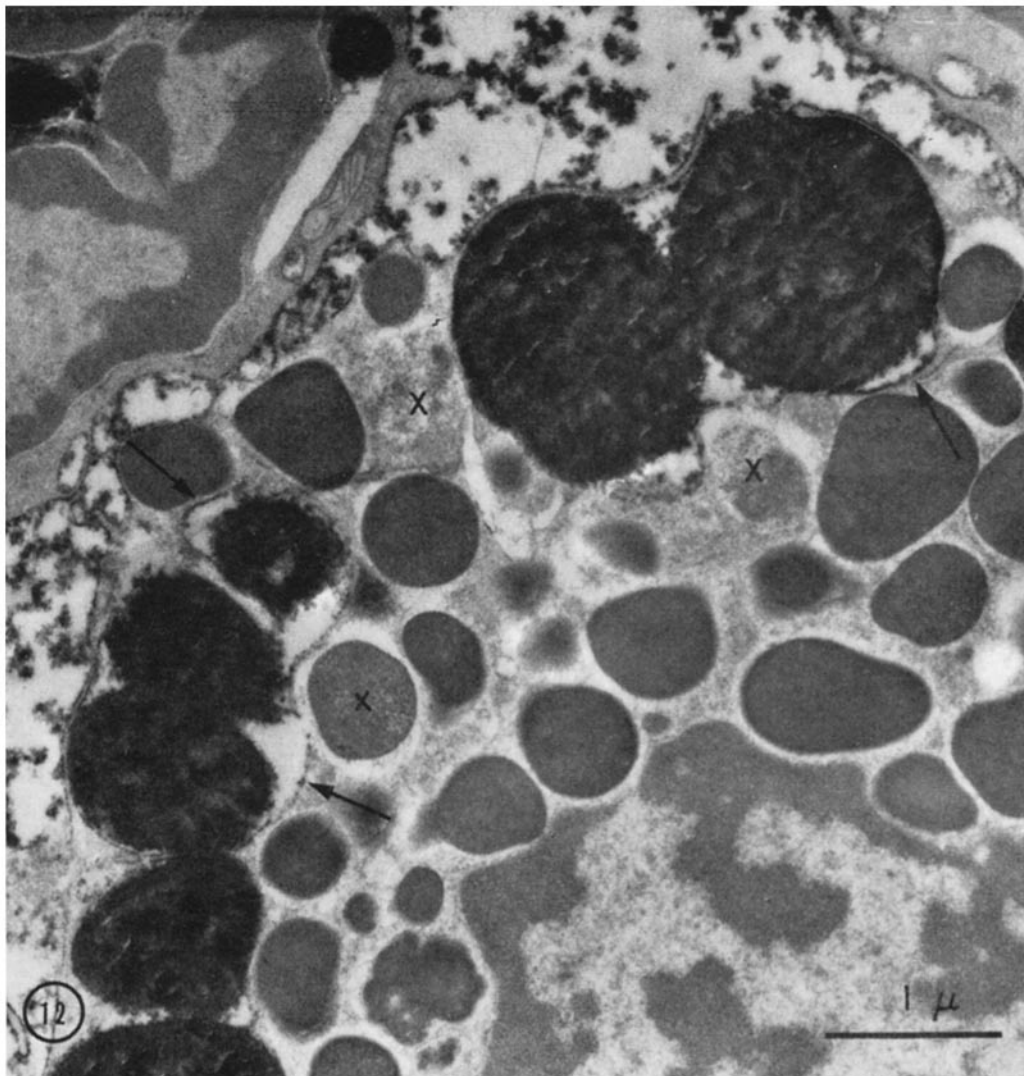


FIGURE 12 Altered granules to which hemoglobin is bound are lying in the cavities of the mast cell. The membrane of the cavities—like the cell membrane—shows a fine layer of hemoglobin adsorbed (arrows). Three (X) granules in the initial stage of swelling and adjacent to the cavities are not stained by hemoglobin. $\times 23,000$.

vacuole of the swelling granule may break the fragile diaphragm, resulting in extrusion of the granule matrix into the extracellular space, as was observed in the present material. It must be said, however, that pores were observed only in the minority of cases and, furthermore, it was not possible to determine how both processes (i.e., formation of pores and loosening of the granule matrix) are related to each other in time. Therefore further electron microscope observations,

mainly on serial sections, are needed to clarify this problem.

A careful analysis of the stages of advancing degranulation led us to the conclusion that the process described above for the individual granule is repeated with adjacent granules. The only difference is that most of the granules do not open to the cell surface but to already opened granule cavities lying adjacent to them. In a biological sense, however, the membranes of the latter granules are

identical with the cell membrane. The cavities of the newly opened granules are in free communication with the extracellular space as was shown in the present study by extracellular tracers. Thus the same ionic environment will exist in the cavities as exists at the outer surface of the cell membrane. At the end of the process (Fig. 13), large and labyrinthine cavities are formed delimited by a membrane, which originates from the earlier perigranular membranes and which is continuous with the surface membrane of the cell. Such cavities were observed to retain swollen granules.

The observed process corresponds to what is called exocytosis in cell biology. Palade (31) was the first to describe this mechanism for the secretion process in the pancreatic acinar cell. Further observations on the pancreas (18) and on other glandular cells (2, 16) confirmed this hypothesis and implied a sequential fusion of perigranular

membranes of secretory granules by which the extracellular space penetrates deep into the cell interior. Our present observations are in good accordance with this mechanism of granule release.

Recently, Padawer (28) proposed for mast cells a cytoarchitecture consisting of several sets of granules enclosed by single membranous sacs. This concept was derived from thorotrast uptake studies in which the particles became associated with younger granules and then rapidly appeared in more mature ones. It was suggested that the sets are independent physiological units and that they remain readily accessible to the extracellular environment. In a more recent paper (29) he studied the degranulating effect of polylysine on the fine structure of mast cells and found rows of fused granules in the cell interior. He regarded them as additional support for his concept and raised the

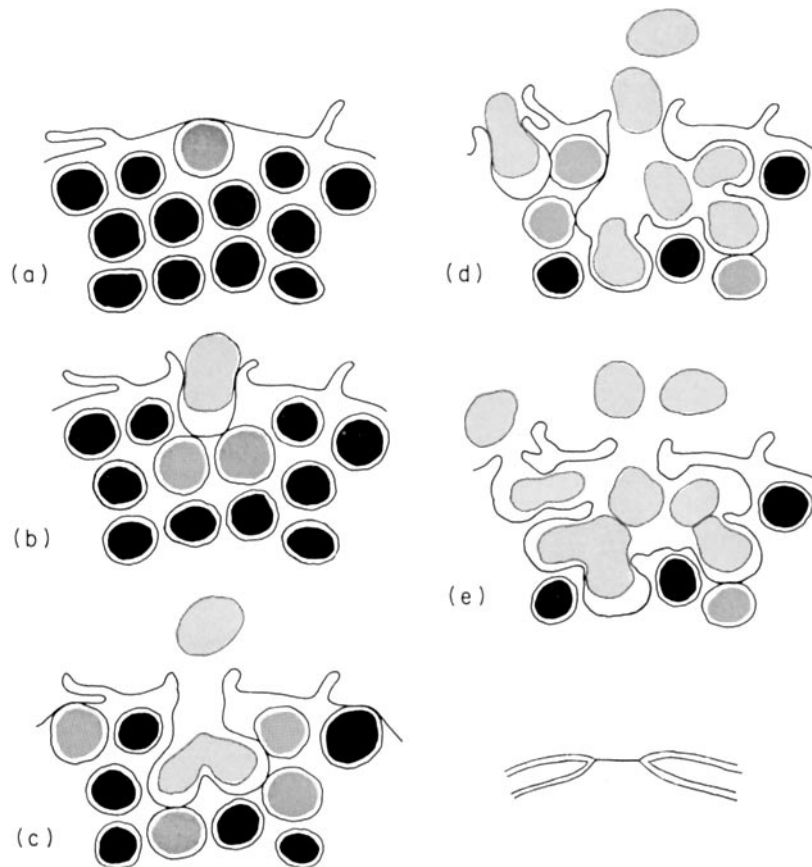


FIGURE 13 Schematic drawing to show the sequential exocytosis of the granules. See explanation in text. At the right lower corner the initial fusion of cell membrane and perigranular membrane is represented.

possibility that the channels of normal, nondegranulating mast cells are infoldings of the plasma membrane, i.e., the granules lie topologically outside the cell.

An alternative interpretation of our present findings in the sense of Padawer's concept can not be excluded theoretically. In this case, the labyrinthic channels in our degranulating cells would be preexisting in nondegranulating mast cells, and all that happens during degranulation would be a dilatation of the entrance and individual parts of the channel system. We think, however, that several considerations make the concept of sequential exocytosis more likely. (a) We did not succeed in finding the entrance of the channels even after a careful search on a large material of nondegranulating mast cells, and extracellular tracers were similarly unsuccessful in detecting the opening of the channels in normal mast cells. Our preliminary freeze-etch studies were also without success in this respect. (b) Continuity of perigranular membranes with each other was an exceptional finding in our material of normal mast cells. However, as we mentioned, the reconstruction of membrane continuities in mast cells meets large difficulties. (c) If a set of granules functioned as a physiological unit, it is difficult to explain why at the beginning of degranulation only a few granules immediately beneath the plasma membrane react and not a whole set of granules. The gradual progression of degranulation towards the cell interior and also sideways seems to be more compatible with sequential fusions. (d) The appearance of thorotrast particles associated with mast cell granules may have an alternative interpretation: they may fuse with the granules like phagosomes fuse with lysosomes.

The present paper may clarify some of the contradictory opinions concerning the relationship between degranulation and histamine (and 5-HT) release. As is known, the matrix of the granules consists of a complex of heparin and basic protein; histamine and 5-HT² are probably bound to carboxyls of the protein by ionic linkages (1, 40). According to experimental data (22, 35, 36, 38-41), the biogenic amines are instantly released from this complex by a simple ion exchange when the granules are exposed to a cation-containing extracellular fluid. Histamine release from degranulating mast cells was therefore proposed by Thon and Uvnäs (36) to be a two-stage process:

the first step is the expulsion of the granules from the cell, while the second one represents the release of histamine in the extracellular ionic milieu.

On the other hand, some electron microscope observations (5, 6) suggested that histamine is released partly or entirely in the cell proper. The main argument for this idea was that altered granules, assumed to be devoid of histamine, were usually found enclosed in intracellular vacuoles. A similar conclusion was reached by Carlsson and Ritzén (7) when studying the release of 5-HT induced by compound 48/80 and anaphylaxis. Their view was based on the finding that altered granules found in the cell interior did not contain any 5-HT detectable with fluorescence, and furthermore that the percentage loss of dry mass due to granule extrusion was less than the percentage 5-HT released.

The present findings might give a satisfactory explanation of this contradiction. As was seen, compound 48/80 induced a sequential exocytosis of mast cell granules. At the end of this process, a complex system of cavities containing a certain number of altered granules remained in the cell body, while another part of such granules had left the cell. The granule-containing cavities have been in unbroken communication with the extracellular space from the beginning of their formation, as was shown using extracellular markers. The appearance of granule-containing closed "vacuoles" in the papers cited can be explained easily by the section profiles of this labyrinthic system of cavities. Therefore altered granules situated in the vacuoles are in contact with the extracellular fluid and must have lost their amines by cation exchange. In other words, there is no need to assume any alternative amine-releasing mechanism for these apparently intracellularly located altered granules.

The retention of altered (possibly amine-free) granules in cavities of the cell body gives a simple explanation to the finding that granule release does not parallel the histamine release (13, 26). Using the release of ³⁵S-labeled heparin from mast cells as an index of granule release, the ratio of the percentage histamine release per granule release was found to be higher than unity (1.1-1.4). To what extent retention of amine-free granules occurs when mast cells are exposed to degranulating agents *in vivo* is unknown. In our experiments, the expulsion of granules may have been depressed by the relatively low temperature (17°C) used. However, recent work by Padawer (29) shows a considerable retention of fused granules in many de-

granulating mast cells when the degranulating agent (polylysine) was applied in vivo.

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