MEMBRANE FUSION DURING MAST CELL SECRETION

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INTRODUCTION

Much evidence has been adduced to support exocytosis as the mechanism utilized by a variety of cells to secrete substances stored in intracellular vesicles or granules. Since in all instances the vesicle or granule is bounded or surrounded by a membrane which in turn is separated from the external milieu by the plasma membrane, exocytosis must involve an interaction between two membranes leading to the formation of a channel through which the secretory product leaves the cell.

Secretion of histamine and other secretory granule components by mast cells probably occurs not only at the cell surface but also deep in the cell (24). The extent of membrane interaction required during secretion might be expected to make the mast cell an auspicious object for the investigation of membrane interactions. An ultrastructural search for examples of membrane interaction during mast cell secretion yielded the preliminary results reported here.

MATERIALS AND METHODS

Peritoneal cells including mast cells were collected from the peritoneal cavities of 2–4-month old male rats (CD, Charles River Breeding Laboratories, Inc., Wilmington, Mass.) as previously described (24). The cells were washed once in heparin-free balanced salt solution and samples containing 2–5 $\times$ 10^5 mast cells/ml by hemacytometer count were distributed in 1.5 ml aliquots in test tubes. Cells were kept at either 30° or 37°C, and secretion was induced by the addition of polymyxin B sulfate to a final concentration of 0.5–4.0 μg/ml. Inactive and secreting cells were fixed by the direct addition of an equal volume of 4% buffered glutaraldehyde (0.1 M cacodylate buffer, pH 7.4) to a cell suspension. The cells were fixed for 1 or 2 h at room temperature, washed in 0.1 M cacodylate buffer, postfixed with 1% osmium tetroxide in collidine buffer, pH 7.0, for 1 h at 0°C, washed with pH 5.0 HCl solution, and stained with 0.5% uranyl acetate in collidine buffer, pH 7.0. The cells were washed with water and collected in agar (15). The agar pellet was diced, and the bits dehydrated and embedded in Epon 812. Thin sections were stained with uranyl acetate and alkaline lead reagent (26) and examined in an AEI-6B electron microscope.

RESULTS

In adequately fixed, resting mast cells, each histamine storage granule is surrounded by a unit membrane (Fig. 1). An approach to closer than 100 Å of the outer leaflets of adjacent perigranule membranes or of a perigranule membrane and

the plasma membrane is rare. When mast cells are examined 5 min after their exposure to 0.5 μg/ml polymyxin B sulfate, the cells are seen to be penetrated by channels of extracellular space (Fig. 2). Many altered granules are present in these channels. Under these conditions, 30–40% of the cell histamine is released (24).

When fixative is added to the cell suspension 5 s after polymyxin B sulfate, the peripheral granules are found to be the most frequently involved in the secretory process (Fig. 3). The membranes originally encompassing some of the peripheral granules just beneath the plasma membrane establish continuity with the plasma membrane, effectively externalizing the granules (Fig. 4). Even at this early time some cells exhibit ex-

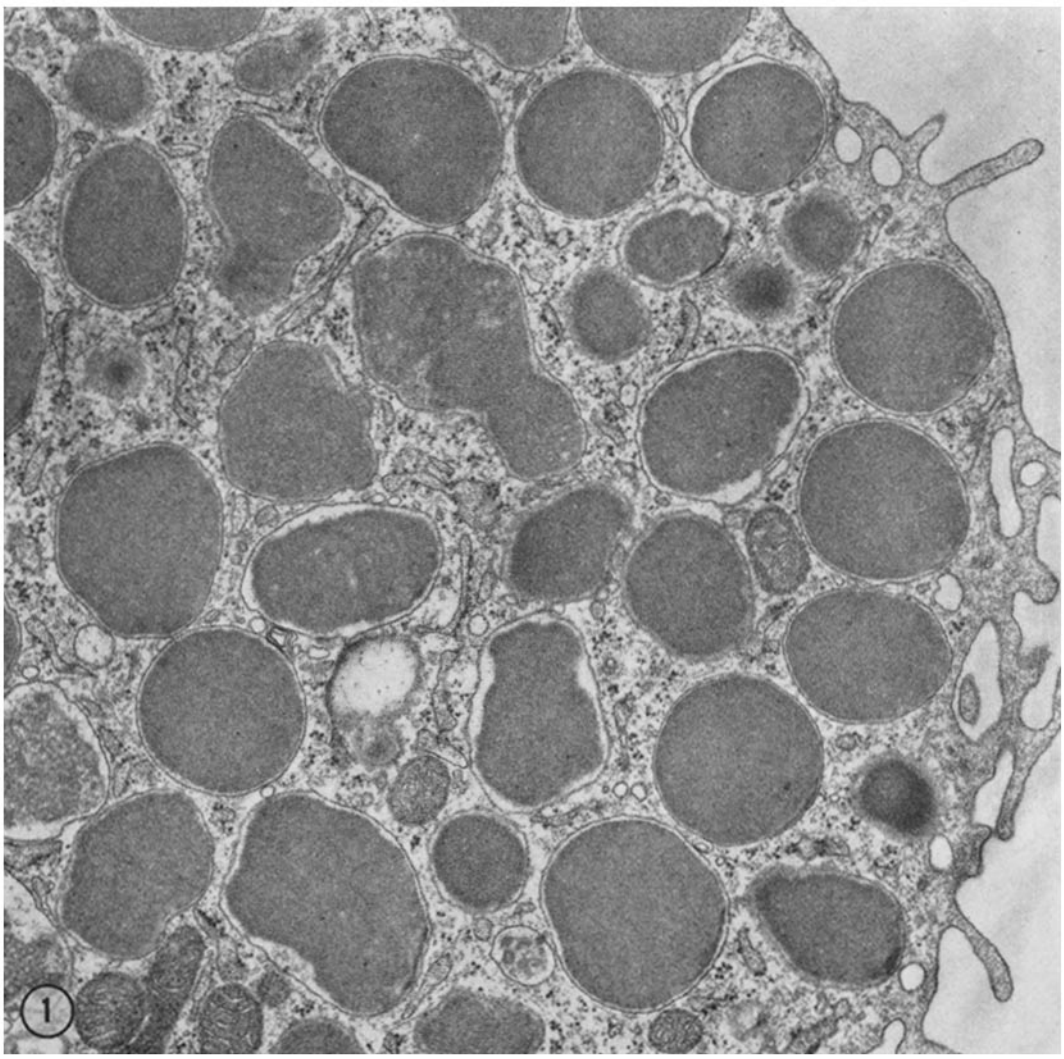


FIGURE 1 A mast cell collected from the peritoneal cavity and incubated in balanced salt solution for 5 min at 30°C. Many of the granules are homogeneous and circular in cross section, others exhibit irregularities in granule structure and contour. All the granules are surrounded by a distinct membrane which is separated from the granule by a narrow electron-lucent region. The perigranule membranes are separated from one another by cytoplasm. Occasionally small vesicles can be seen in the gap between two adjacent perigranule membranes. × 24,000.

tensive involvement of granules including those deep in the cell (Fig. 5). A marked alteration of granule structure is evident in granules that are in contact with extracellular medium. The electron-opaque homogeneous granule (Fig. 1) is progressively transformed to a dispersed fibrous meshwork (Figs. 2–5). Close approach of adjacent perigranule membranes and perigranule and plasma membrane is frequently seen (Fig. 6). However, the membranes of stimulated cells are particularly difficult to preserve and artifacts are frequent (Fig. 7).

Several micrographs of pentalaminar fusion of membranes have been obtained with cells incubated at 30°C (Figs. 8 and 9). All of these involve adjacent perigranule membranes. A few micro-

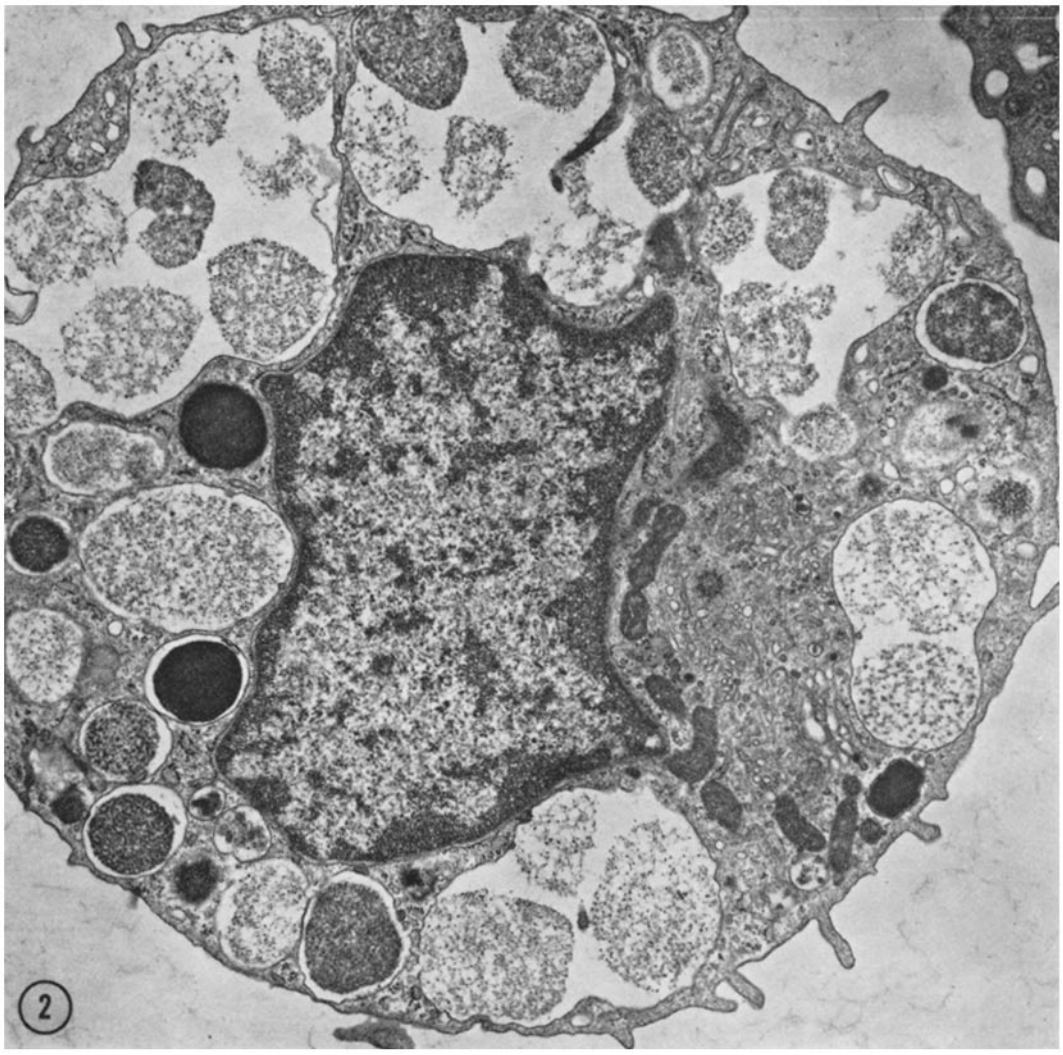
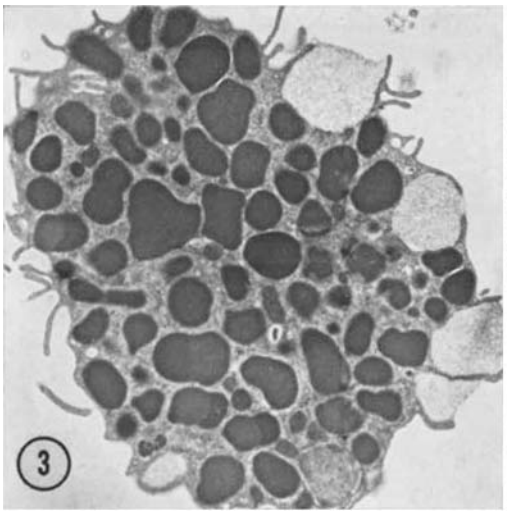


FIGURE 2 This mast cell was fixed after 5 min of exposure to polymyxin B sulfate 0.5 μg/ml at 30°C. Altered granules are present in spaces formed by fusion of the membranes around the individual granules. No continuities between the extracellular region and the spaces containing the altered granules are evident. A few granules are present that retain their homogeneously dense appearance. The cytoplasm shows no degenerative changes; mitochondria, microtubules, small vesicles, and the centriole are unaffected by the secretory process. × 15,000.



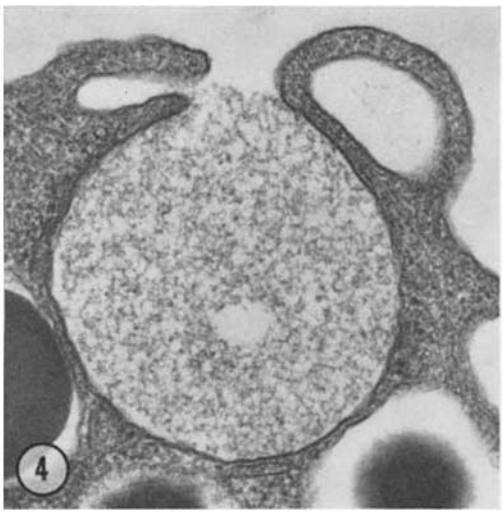


FIGURE 3 A mast cell fixed by the addition of glutaraldehyde to a suspension of cells 5 s after the addition of 0.5 μg/ml polymyxin B sulfate. At this early time period only a few of the most peripherally situated granules have been extruded. × 4,500.

FIGURE 4 A higher magnification of a granule being discharged at the surface of a mast cell treated as described in Fig. 3. An intact unit membrane is traceable around the altered granule and is continuous with the plasma membrane. × 60,000.

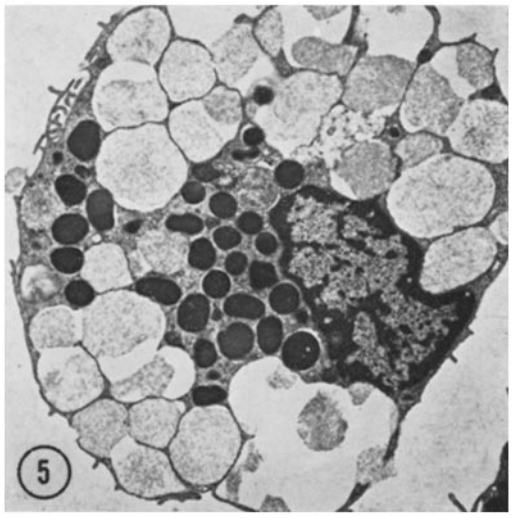


FIGURE 5 A mast cell treated as described in Fig. 3. Involvement of most of the granules is evident at this early time. × 4,500.

graphs are suggestive of an early stage of pore formation involving a perigranule membrane and plasma membrane (Fig. 10), but in none of these are the membrane leaflets distinct enough to be entirely convincing. I have observed neither trilaminar membranes nor diaphragms lacking unit membrane structure separating two granules or a granule and the extracellular medium.

DISCUSSION

Secretion by an extrusive process involving fusion of the membranes and channel formation was clearly described for synaptic vesicles by De-Robertis and Bennett in an abstract published in 1954 (6). "Some of the vesicles seem to perforate the presynaptic membrane so that portions of the vesicle lie in the intermembranal space and come into direct contact with the post-synaptic membrane." DeRobertis and Vaz Ferreira (7) found a similar process occurring in the adrenal medulla, and Palade (29) provided ultrastructural evidence for the extrusive secretion of pancreatic zymogen granules. Since 1961, extrusion has been established as a common cellular mechanism for secretion (1, 9, 11–13, 17, 18, 20–22, 27, 31, 34, 37). Several terms, reversed pinocytosis, emiocytosis (39), and exocytosis (5), have been suggested for the process; it is the latter that seems to have been sanctioned by widespread use.

The association of mast cell granule dispersal

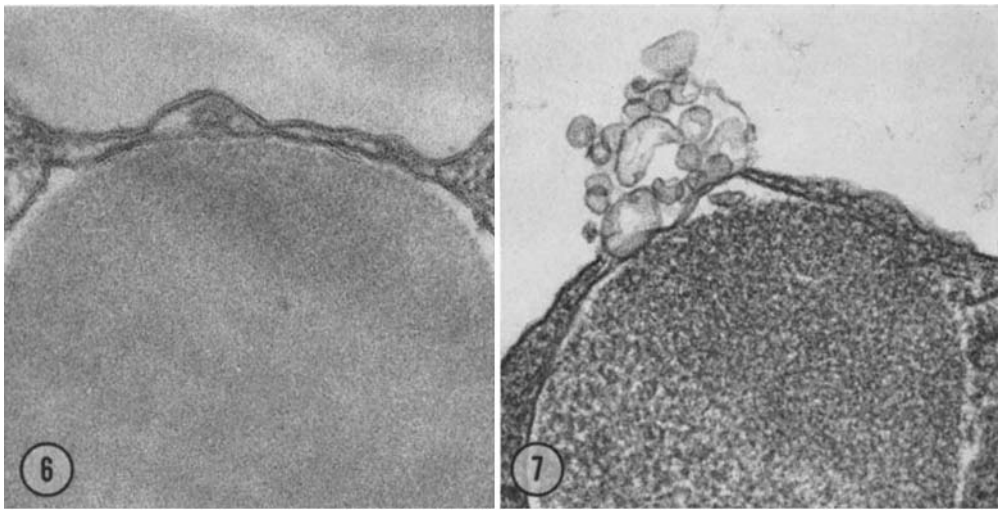


FIGURE 6 Close approach of a perigranule membrane to the plasma membrane is shown in this micrograph. It is difficult to determine if fusion has occurred. Cell treated as described in Fig. 3. × 100,000.

FIGURE 7 An example of an artifact in which the membrane is thrown in redundant coils in the vicinity of a close approach of perigranule and plasma membranes. Cell treated as in the description for Fig. 3. × 48,000.

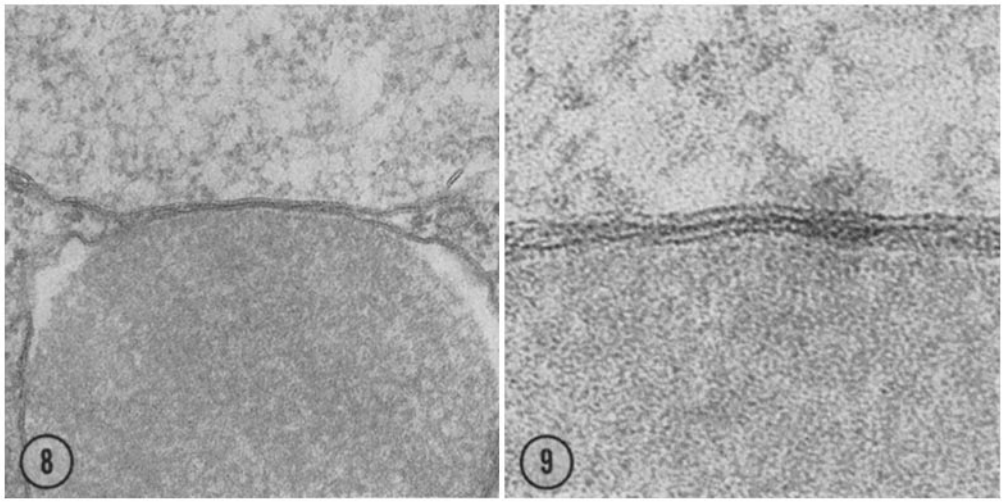


FIGURE 8 Pentalaminar fusion of adjacent perigranule membranes. The granule at the top has already undergone alteration while the granule below shows early rarefaction. × 80,000.

FIGURE 9 At high magnification the pentalaminar character of the fusion shown in Fig. 8 is evident, with increased density and probably increased thickness of the middle dark line. × 236,000.

and histamine release was suggested by Riley (32) and definitively demonstrated by Fawcett (10) who made the important distinction between cytotoxic effects of distilled water and nonlethal effects of the histamine-releasing agent, compound 48/80. Ultrastructural evidence for the maintenance of mast cell integrity during secretion was provided by Thiéry (38) and amply corroborated by subsequent electron microscope (3, 4, 16, 23, 28, 33, 36, 41) and biochemical studies

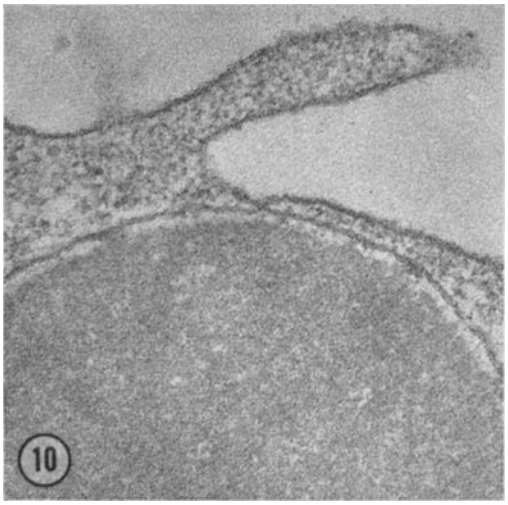


FIGURE 10 A possible example of early pore formation at the cell surface. The precise relationship of the membranes at the apparent point of juncture is not clear. × 75,000.

(8, 19) of the degranulation process with a range of agents. The possibility has been considered that alterations in granule structure and the expansion of the perigranular space precede the establishment of continuities between the external milieu and the perigranular space (2). The sum of present evidence suggests on the contrary (16, 23, 24, 33) that the alterations depend on the exposure of the granules to extracellular medium which is carried deep into the cell in channels formed by a series of membrane interactions, first between perigranule membranes and the plasma membrane and then between adjacent perigranule membranes. Padawer (28) has raised the possibility that preformed channels, capable of opening and closing, control access of extracellular medium to the granules.

The ultrastructural studies of mast cell secretion to date have not clearly indicated the mechanism of membrane interaction. Röhlich et al. (33) have interpreted their electron micrographs as providing evidence for fusion of perigranule and cell membranes with the formation of "a thin, structureless diaphragm bridging the edge of the fused region." They suggest that the formation of the diaphragm is analogous to the formation of the pore diaphragm of fenestrated capillaries as proposed by Wolff and Merker (40). A similar mechanism was previously suggested by Palade and Bruns (30) on the basis of their detailed study of the fusion of pinocytotic vesicles and endothelial cell plasma membrane.

A five-layered membrane with a thickened dense layer where the two unit membranes abut (fu_1 of Palade and Bruns) is clearly evident between perigranule membranes during mast cell secretion (Figs. 8 and 9), but I have observed no convincing examples of three-layer membrane fusion (fu_2) or of diaphragms lacking unit membrane structure (fu_4). Failure to observe images of attenuated intermediates between pentalaminar fusion of two membranes and a pore might be attributable to (*a*) an exceedingly short lifetime of the intermediate structures, (*b*) their instability under the conditions of fixation, or (*c*) their nonexistence.[1] The rare observation of what appears to be an incipient pore (Fig. 10) offers an alternative to the attenuation model for exocytosis, namely direct pore formation from pentalaminar fused membranes.

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REFERENCES

1. AMSTERDAM, A., I. OHAD, and M. SCHRAMM. 1969. Dynamic changes in the ultrastructure of the acinar cell of the rat parotid gland during the secretory cycle. *J. Cell Biol.* **41**:753.
2. BENDITT, E. P., and D. LAGUNOFF. 1964. The mast cell: its structure and function. *Prog. Allergy.* **8**:195.
3. BLOOM, G. D., B. FREDHOLM, and Ö. HAEGERMARK. 1967. Studies on the time course of histamine release and morphological changes induced by histamine liberators in rat peritoneal mast cells. *Acta Physiol. Scand.* **71**:270.
4. BLOOM, G. D., and Ö HAEGERMARK. 1965. A study on morphological changes and histamine release induced by compound 48/80 in rat peritoneal mast cells. *Exp. Cell Res.* **40**:637.
5. DE DUVE, C. 1963. The lysosome concept. *In* Ciba Foundation Symposium, Lysosomes. A.

[1] Lucy has previously proposed a micellar model for direct pore formation that would not be expected to yield electron microscope images of intermediates beyond the pentalaminar state (25). However, the increasing evidence for the bilayer structure of membranes (14, 35) diminishes the likelihood that Lucy's hypothesis is valid.

V. S. de Reuck and M. P. Cameron, editors. Little, Brown and Company, Boston. 1.

6. DeRobertis, E. D. P., and H. S. Bennett. 1954. Submicroscopic vesicular components in the synapse. *Fed. Proc.* **13**:35. (Abstr.)
7. DeRobertis, E., and A. Vaz Ferreira. 1957. Electron microscope study of the excretion of catechol-containing droplets in the adrenal medulla. *Exp. Cell Res.* **12**:568.
8. Diamant, B. 1967. The effects of compound 48/80 and distilled water on the adenosine triphosphate content of isolated rat mast cells. *Acta Physiol. Scand.* **71**:283.
9. Diner, O. 1967. L'expulsion des granules de la médullo-surrénale chez le hamster. *C. R. Hebd. Seances Acad. Sci. Ser D Sci. Nat. (Paris).* **265**: 616.
10. Fawcett, D. W. 1954. Cytological and pharmacological observations on the release of histamine by mast cells. *J. Exp. Med.* **100**:217.
11. Fillenz, M. 1971. Fine structure of noradrenaline storage vesicles in nerve terminals of the rat vas deferens. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* **261**:319.
12. Fujita, H., and Z. Matsuro. 1967. Some observations on the fine structure of the pancreatic islet of rabbits, with special reference to β cell alterations in the hypoglycemic state induced by allo treatment. *Arch. Histol. Jap.* **28**:383.
13. Hand, A. R. 1970. The fine structure of von Ebner's gland of the rat. *J. Cell Biol.* **44**:340.
14. Hendler, R. W. 1971. Biological membrane ultrastructure. *Physiol. Rev.* **51**:66.
15. Hirsch, J. G., and M. E. Fedorko. 1968. Ultrastructure of human leucocytes after simultaneous fixation with glutaraldehyde and osmium tetroxide and "post-fixation" in uranyl acetate. *J. Cell Biol.* **38**:615.
16. Horsfield, G. I. 1965. The effect of compound 48/80 on the rat mast cell. *J. Pathol. Bacteriol.* **90**:599.
17. Ichikawa, A. 1965. Fine structural changes in response to hormonal stimulation of the perfused canine pancreas. *J. Cell Biol.* **24**:369.
18. Jamieson, J. D., and G. E. Palade. 1967. Intracellular transport of secretory proteins in the pancreatic exocrine cell. I. Role of the peripheral elements of the Golgi complex. *J. Cell Biol.* **34**:577.
19. Johnson, A. R., and N. C. Moran. 1969. Selective release of histamine from rat mast cells by compound 48/80 and antigen. *Am. J. Physiol.* **216**:453.
20. Krisch, B., K. Becker, and W. Bargmann. 1972. Exocytose im Hinterlappen der Hypophyse. *Z. Zellforsch. Mikrosk. Anat.* **123**:47.
21. Kurosumi, K. 1961. Electron microscopic analysis of the secretion mechanism. *Int. Rev. Cytol.* **11**:1.
22. Lacy, P. E. 1970. Beta cell secretion—from the standpoint of a pathobiologist. *Diabetes.* **19**: 895.
23. Lagunoff, D. 1972. Contributions of electron microscopy to the study of mast cells. *J. Invest. Dermatol.* **58**:296.
24. Lagunoff, D. 1972. The mechanism of histamine release from mast cells. *Biochem. Pharmacol.* **21**:1889.
25. Lucy, J. A. 1969. Lysosomal membranes. *In* Lysosomes in Biology and Pathology. J. T. Dingle and H. Fell, editors. North-Holland Publishing Co., Amsterdam. 313.
26. Millonig, G. 1961. A modified procedure for lead staining of thin sections. *J. Biophys. Biochem. Cytol.* **11**:736.
27. Nagasawa, J., W. W. Douglas, and R. A. Schulz. 1970. Ultrastructural evidence of secretion by exocytosis and of "synaptic vesicle" formation in posterior pituitary glands. *Nature (Lond.).* **227**:407.
28. Padawer, J. 1970. The reaction of rat mast cells to polylysine. *J. Cell Biol.* **47**:352.
29. Palade, G. E. 1959. Functional changes in the structure of cell components. *In* Subcellular Particles. T. Hayashi, editor. The Ronald Press Company, New York.
30. Palade, G. E., and R. R. Bruns. 1968. Structural modulations of plasmalemmal vesicles. *J. Cell Biol.* **37**:633.
31. Pelletier, G., F. Peillon, and E. Vila-Porcile. 1971. An ultrastructural study of sites of granule extrusion in the anterior pituitary of the rat. *Z. Zellforsch. Mikrosk. Anat.* **115**:501.
32. Riley, J. F. 1953. The effects of histamine liberators on the mast cells of the rat. *J. Pathol. Bacteriol.* **65**:471.
33. Röhlich, P., P. Anderson, and B. Uvnäs. 1971. Electron microscope observations on compound 48/80 induced degranulation in rat mast cells. *J. Cell Biol.* **51**:465.
34. Santolaya, R. C., T. E. Bridges, and K. Lederis. 1972. Elementary granules, small vesicles and exocytosis in the rat neurohypophysis after acute hemorrhage. *Z. Zellforsch. Mikrosk. Anat.* **125**:277.
35. Singer, S. J., and G. L. Nicolson. 1972. The fluid mosaic model of the structure of cell membranes. *Science (Wash. D. C.).* **175**:720.
36. Singleton, E. M., and S. L. Clark, Jr. 1965. The response of mast cells to compound 48/80 studied with the electron microscope. *Lab. Invest.* **14**:1744.

37. STOECKART, R., H. G. JANSEN, and A. J. KREIKE. 1972. Ultrastructural evidence for exocytosis in the median eminence of the rat. *Z. Zellforsch. Mikrosk. Anat.* **131**:99.
38. THIÉRY, J. P. 1963. Étude au microscope electronique de la maturation et de l'excretion des granules des mastocytes. *J. Microsc. (Paris).* **2**:549.
39. WILLIAMSON, J. R., P. E. LACY, and J. W. GRISHAM. 1961. Ultrastructural changes in islets of the rat produced by tolbutamide. *Diabetes.* **10**:460.
40. WOLFF, J., and H.-J. MERKER. 1966. Ultrastruktur und Bildung von Poren im endothel von porösen und geschlossenen Kapillaren. *Z. Zellforsch. Mikrosk. Anat.* **73**:174.
41. YAMASAKI, H., T. FUJITA, Y. OHARA, and S. KOMOTO. 1970. Electron microscope studies on the release of histamine from rat peritoneal mast cells. *Arch. Histol. Jap.* **31**:393.