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THE MECHANISM OF HISTAMINE RELEASE FROM MAST CELLS*

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Abstract—Mast cell secretion was studied *in vitro* using rat peritoneal cells stimulated by polymyxin B sulfate. The dose–response curve for histamine release was only slightly lowered when mast cells isolated by sedimentation through albumin solution (specific gravity, 1·100) were compared to cells not subjected to the isolation procedure. The release of *N*-acetyl-β-glucosaminidase, a readily soluble component of mast cell granules, closely paralleled the release of histamine. However, little release occurred of two insoluble granule components, mast cell chymase and heparin, and their release was not dose dependent. These results indicated that histamine release from mast cells can occur in the absence of extrusion of their granules. Quantitative studies of the uptake of ruthenium red and morphologic studies of the distribution of ruthenium red and ferritin demonstrated that the granules are effectively extruded into an extracellular space that is confined to the cellular domain by a labyrinth of cytoplasmic processes. The secretory process of mast cells then appears to be effected through a sequence of membrane fusions that produce deep channels of extracellular space penetrating through the cell and enveloping the granules rather than by the propulsion of the granules to the cell surface with extrusion at that site.

INTERPRETATIONS of several experimental approaches to the mechanism of histamine release have converged on the theory that histamine is displaced from its binding site on the granule by extracellular Na^+ after the granule has been extruded from the cell: (1) histamine can readily be removed from isolated granules and replaced by manipulation of the ionic concentration in the suspending media;[1,2] (2) when mast cells maintained in isotonic sucrose are treated with degranulating agents, although granules are extruded, no histamine release is observed, but when the expelled granules are collected and exposed to Na^+ ions, histamine is released;[3] and (3) mast cell ATP,[4,5] LDH and K^+[5] are not released concomitantly with histamine, indicating the absence of a general change in outward permeability of the cell membrane. All these results are consistent with light and electron microscopic observations which have shown granule extrusion associated with histamine release.

However, two observations are at seeming odds with the theory: (1) in electron micrographs, it is evident that many granules which are not extruded from the cell exhibit ultrastructural changes identical with those of obviously extracellular granules;[6] and (2) with histochemical techniques, it has been demonstrated that some granules retained within the cellular domain lose serotonin.[7] Reconciliation of these latter two observations with the extrusion–ion exchange hypothesis is possible if the granules,

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although retained within the cellular domain, are extruded into channels of extracellular medium which penetrate from the cell surface.[8]

In order to test this modified extrusion concept, I have examined the relationship between histamine release and granule discharge using three macromolecular components of the mast cell granule, *N*-acetyl-β-glucosaminidase,[9] mast cell chymotrypsin[10] and heparin,[10] and two markers for extracellular space, ruthenium red and ferritin.

MATERIALS AND METHODS

Mast cells were obtained from the peritoneal cavities of male Sprague–Dawley rats (200–300 g; Charles River Inc., "Specific Pathogen-Free"). Animals were anesthetized with ether and exsanguinated by cardiac puncture. The peritoneal cavities of several animals were lavaged with 10 ml of phosphate-buffered heparinized modified Ringer's salt solution, pH 7·2 (MRS),[11] and the lavages were pooled. The cells so obtained were centrifuged and resuspended in 5 ml of heparin-free MRS. Mast cells were separated from the other peritoneal cells by centrifuging mast cells through 5 ml albumin, specific gravity = 1·100 (Pathocyte-4, Pentex, Inc.), in 20-ml centrifuge tubes at 200 *g* for 20 min at 4°. The nonsedimenting cells at the interface were removed by aspiration, leaving 4 ml albumin solution which was removed and diluted with 4 ml MRS. Mast cells were then collected by centrifugation at 200 *g* for 5 min and washed with 5 ml MRS and resuspended in 10 ml MRS. The mast cells, which routinely comprised better than 80 per cent of the cells by number, were counted and diluted to a final concentration of $2{\cdot}5 \times 10^5$ cells/ml. Routinely 0·5-ml aliquots were used in duplicate. Polymyxin B solutions were added to the aliquots in small volumes, no greater than 50 μl; the cells were incubated for 5 min at 30°, centrifuged at 4° at 200 *g* for 10 min, and the supernatants and residues were assayed for histamine.[12] This centrifugation effectively separated free granules, which remain in the supernatant, from cells and adherent granules which sediment. Assays of *N*-acetyl-β-glucosaminidase,[9] heparin[10] and mast cell chymotrypsin[10] in the supernatants and cell residues were performed as previously reported.

Fixation for electron microscopy was accomplished by addition of an equal volume of 4% glutaraldehyde in MRS directly to the cell suspension. The cells were fixed for 1 hr, washed in 0·1 M cacodylate buffer, pH 7·2, post-fixed for 1 hr in 1% osmium tetroxide in *s*-collidine, washed in 0·001 M HCl, stained with uranyl acetate for 1 hr, washed in 35% ethanol, and the centrifuged pellet was embedded in agar (Bacto-agar, Difco Laboratories).[13] Small cubes of agar were promptly dehydrated and embedded in Epon 812 according to Luft.[14] Thin sections were stained with uranyl acetate and lead hydroxide and examined in an AEI-6B electron microscope.

In the experiments using ferritin, a 5% solution of ferritin in 17% albumin was added to a suspension of washed, glutaraldehyde-fixed cells. The cells were centrifuged to a pellet and the albumin supernatant was gelled by overlaying with 2% glutaraldehyde. The coherent pellets were post-fixed in osmium tetroxide and carried through the procedure as described above.

Ruthenium red was used to supravitally stain mast cells at a concentration of 0·005% in MRS. For quantitative determination of the ruthenium red bound to granules which sedimented with the cells, the absorbance of the solutions was measured at 533 nm after sedimentation of the cells at 200 *g* for 10 min. The difference between

the absorbance of the supernatant from the control cells and that from the cells exposed to polymyxin B sulfate was used to calculate the amount of ruthenium red bound. For electron microscopy, the cells were fixed in 2% glutaraldehyde and then exposed to 0·5% ruthenium red in 2% glutaraldehyde for 1 hr, followed by the same concentration of dye in 1% osmium tetroxide for 1 hr. The cells were subsequently treated in the regular manner described above for electron microscopy.

Polymyxin B sulfate was obtained from Pfizer Laboratories or Sigma Chemical Company and ruthenium red from K & K Inc. or Johnson, Matthey & Company, Inc. Histamine dihydrochloride purchased from CalBiochem was used as standard in the assay. Orthophthalaldehyde obtained from Sigma Chemical Company was recrystallized from ligroin. BioRad AG1X-8, 50-100 mesh, ion-exchange resin was purchased from BioRad Laboratories. The ion-exchange resin was measured out volumetrically from a slurry, and 5-ml plastic syringes fitted with pellon inserts to retain the resin were used as chromatography columns.

RESULTS

It was first necessary to establish the effect on mast cells of sedimentation through concentrated albumin solution (specific gravity = 1·100). The dose-response curve for the release of histamine from isolated cells was very slightly depressed when compared to cells not centrifuged through albumin (Fig. 1). Mast cells isolated with albumin appeared unchanged by electron microscopy (Figs. 2 and 3).

The fraction of histamine released from purified mast cells at different concentrations of polymyxin B was compared to the fraction of *N*-acetyl-β-glucosaminidase released (Fig. 4). The ratio was consistently in the vicinity of 1·2 (Table 1), indicating that only a slightly greater proportion of cell histamine was released at each concentration of the degranulating agent.

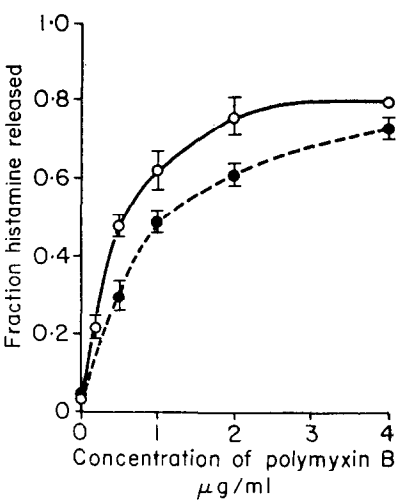

FIG. 1. Effect of isolation procedures on histamine release. Values plotted are the means ±S.E. for at least seven experiments with cells isolated by centrifugation through concentrated albumin (specific-gravity = 1·100), ● – – – – ●; and for at least five experiments with mixed cell population of initial peritoneal wash, ○——○. I Represents ±1 S.E.

TABLE 1. COMPARISON OF THE RELEASE OF MAST CELL GRANULE COMPONENTS

	Ratios of fraction released*			
	Polymyxin B concentration (μg/ml)			
Granule components	0·5	1·0	2·0	4·0
Histamine: *N*-acetyl-β glucosaminidase	1·2 ± 0·1	1·2 ± 0·1	1·2 ± 0·1	1·2 ± 0·1
Histamine: heparin	7·9 ± 4·7	7·8		4·4 ± 0·9
Histamine: chymase	5·5 ± 1·6	8·5		5·0 ± 1·9
Heparin: chymase	1·1 ± 0·5	1·0		1·1 ± 0·2

* Ratios of fractions were calculated from the results of the experiments graphed in Figs. 4 and 5 (±S.E.).

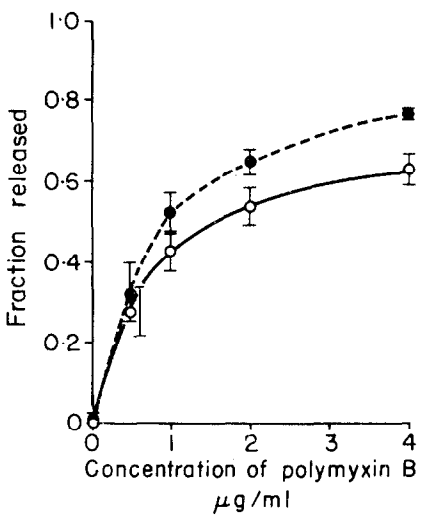

FIG. 4. Comparison of histamine and *N*-acetyl-β-glucosaminidase release by polymyxin B for mast cells isolated in albumin. Fraction of histamine released, ●– – –●; fraction of *N*-acetyl-β-glucosaminidase released, ○——○. Values are the mean ±S.E. for three experiments.

Of the three macromolecular granule components presently known, *N*-acetyl-β-glucosaminidase is most readily dissociated from the granule so that, if all granules were retained within the cellular domain, contact of granules with the extracellular ionic concentrations should be sufficient to solubilize this enzyme.[9] Contrariwise. neither mast cell chymotrypsin nor heparin is solubilized by the ionic constituents of the extracellular medium.[1] When release of these two granule components was investigated, only a small proportion of either could be found free in the medium at the highest concentrations of polymyxin B used (Fig. 5). Two washes with 0·1% albumin in MRS, pH 7·2, did not significantly increase the release of chymotrypsin or heparin.

Ruthenium red is a complex inorganic cation used as a polysaccharide stain by plant histologists and introduced by Luft[15,16] as a reagent for identifying extracellular

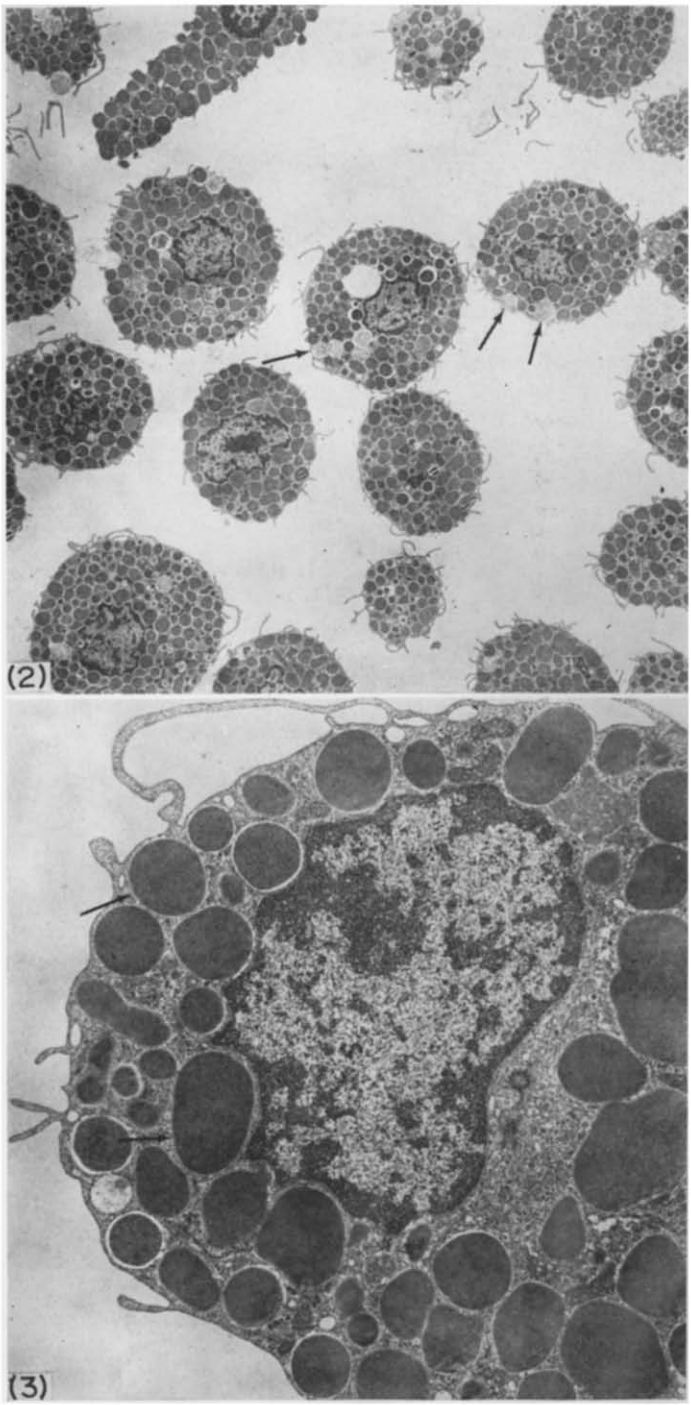

FIG. 2. Appearance of a typical field in a pellet of mast cells centrifuged into concentrated albumin. Most of the granules in the cells, and the cells themselves, appear normal. A few granules (arrows) exhibit a decrease in density, typical of those releasing histamine. Glutaraldehyde fixation (×2160).

FIG. 3. Higher magnification of a mast cell isolated with albumin. The surface projections typical of mast cells are unaffected by the procedure, and the granules do not show any evidence of secretory activity. In particular, the perigranular membranes (arrows) are usually separated from the granule matrix by only a small gap (×11,300).

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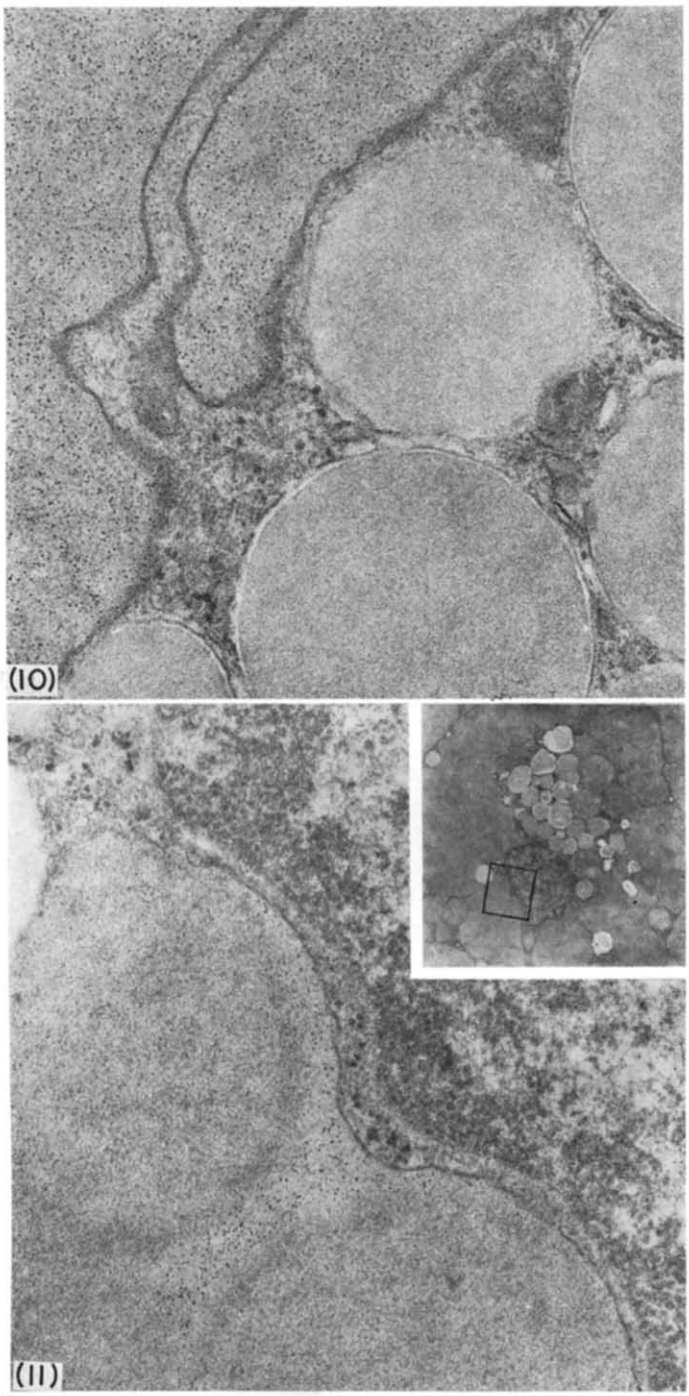

FIG. 10. Electron micrograph of the surface of a normal mast cell fixed in glutaraldehyde and suspended in a solution of albumin and ferritin which was then gelled with glutaraldehyde. The ferritin molecules (small black dots) do not penetrate the intact plasma membrane (70,000).

FIG. 11. Electron micrograph of the perinuclear region of a mast cell stimulated by polymyxin B and then treated in the same manner as the cell in Fig. 10. Under these circumstances, the ferritin is seen to have penetrated very close to the nucleus in one of the apparent vacuoles containing altered granules. The location of the field shown is indicated in the insert. The indistinctness of the altered mast cell granules is the result of the method of preparation (70,000).

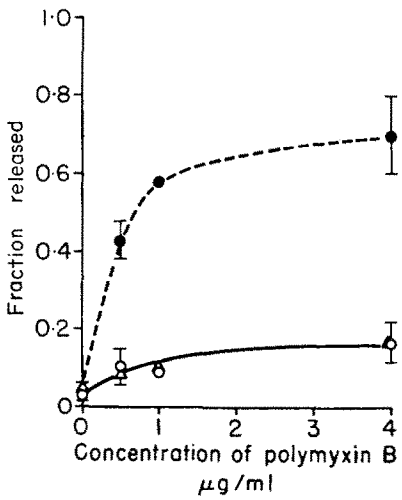

FIG. 5. Comparison of histamine, heparin and chymase release by polymyxin B from mast cells isolated in albumin. Fraction of histamine released, ●---●; fraction of chymase released, ○——○; fraction of heparin released, △——△. Each value is the mean ±S.E. for three experiments.

polysaccharides in electron microscopy. Ruthenium red has a high extinction coefficient and a high binding affinity for heparin.[16] When ruthenium red was incorporated into MRS, at a concentration of 0·005 per cent, mast cell granules did not stain, indicating that the cells were not permeable to the dye. When polymyxin B was added, obviously extruded free granules stained intensely with ruthenium red. In addition, many granules retained within the cell also stained (Fig. 6). Microscopic inspection suggested that the preponderance of granules was associated with cells rather than free. Since cells other than mast cells did not stain, the amount of dye bound to cells collected in a low speed sediment (175 $g \times 5$ min) yielded a measure of ruthenium red bound to mast cell granule heparin still associated with cells. Few free granules were collected under these conditions. The fraction of histamine released with increasing concentrations of polymyxin B roughly paralleled the amount of ruthenium red bound (Fig. 7).

Since sites of binding of ruthenium red can be demonstrated in electron microscopic images by virtue of the reaction of the dye with osmium tetroxide,[16] it was possible to determine the location and ultrastructural appearance of those cell-associated granules that stained with ruthenium red. Peritoneal mast cells treated with polymyxin B were fixed in glutaraldehyde containing 0·5% ruthenium red and post-fixed in osmium tetroxide containing the same concentration of ruthenium red. Increased density indicative of ruthenium red binding was not evident in mast cell granules in intact cells (Fig. 8), while in cells treated with polymyxin B, many granules within the mast cell were more compact and electron dense than their altered counterparts in cells not exposed to ruthenium red (cf. Figs. 8 and 9). Many ruthenium red-stained granules appeared to be deep within the cell.

Ferritin is a large molecular weight protein (450,000 Daltons) with an iron-hydroxide core; it is readily evident in electron micrographs and has found use as a marker for extracellular space. Ferritin was added to a suspension of fixed normal mast cells and

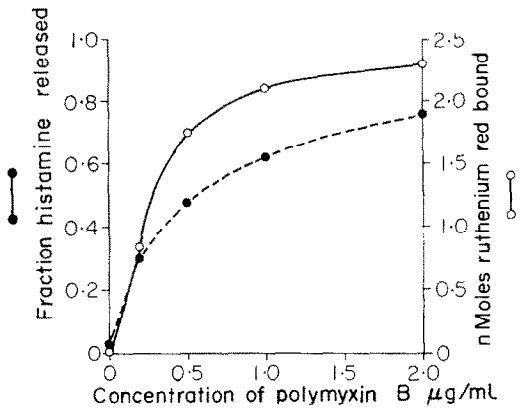

FIG. 7. Comparison of histamine release and ruthenium red binding by mast cells after exposure to varying concentrations of ruthenium red and polymyxin B for 5 min *in vitro*. Dye bound to the cells was calculated as the difference between the initial absorbance at 533 nm and that of the supernatant after sedimentation of the cells.

its distribution compared to that in mast cells exposed to polymyxin B and then fixed. Whereas normal mast cells exhibited no penetration of the plasma membrane by ferritin (Fig. 10), cells treated with polymxin B contained ferritin in many deep channels (Fig. 11).

DISCUSSION

While granule discharge is undeniably an important mechanism for mast cell secretion, several observations suggest that histamine or serotonin, or both, can be released from the cell prior to or even without complete exocytosis of granule matrices. Ritzén[7] observed that after stimulation of mast cells with 48/80, some granules retained within the cell had lost serotonin. Bloom and Haegermark[6] have described alterations in the ultrastructural appearance of granules that precede apparent extrusion but closely resemble changes seen in extracellular granules. Padawer[17] suggested that the granules may reside in extracellular domains in complex invaginations of plasma-lemma from where they may be extruded in channels of extracellular fluid formed by membrane fusion. Röhlich *et al.*[18] and I favor the view that the granules are extruded into the extracellular milieu by virtue of a series of membrane fusions forming deep channels of extracellular medium. The granules in these channels, whatever their origin, are extracellular but retained within the cell domain, and they may conceivably be reincorporated by the cell through the re-establishment of membrane barriers.

Evidence contradictory to the hypothesis that histamine release and overt granule discharge are dissociable has come from Uvnäs's laboratory.[19,20] He and his co-workers have presented evidence that the ratios of per cent histamine released to per cent protein or heparin released are between 1·1 and 1·4 when examined kinetically or in terms of the dose–response curve for degranulating agents. Slorach,[21] working in Uvnäs's laboratory, finds somewhat higher ratios for percentage histamine release/percentage heparin release. To achieve the low ratios, it was necessary to wash the cells three times. I have found little release of insoluble granule components associated

with histamine release, and two washes did not significantly affect the release of heparin or chymase. It must be pointed out that Uvnäs *et al.* have used 48/80 to induce histamine release and the cells were isolated with Ficoll.

Substantiation of the mechanism of contained extrusion was provided by study of the distribution of two markers for extracellular space, ruthenium red and ferritin. There are two possible explanations for the staining of granules retained within the confines of the cell; granules may be extruded into a cellular compartment continuous with the extracellular environment or, alternatively, there may be a substantial increase in inward permeability of the cell membrane to ruthenium red. The latter explanation taken in conjunction with the observations on granule matrix markers would require an unlikely change in outward permeability that would permit both histamine and *N*-acetyl-β-glucosaminidase, the latter with a molecular weight in the vicinity of 100,000,[9] to move out while retaining cell ATP and K^+.[4,5] The access of ferritin to the deep channels supports the former interpretation of the results with ruthenium red.

It seems most reasonable then to consider the results with ruthenium red and ferritin as supportive of the hypothesis that mast cell secretion of histamine can occur by extrusion of granules into channels of extracellular fluid, where the granules are exposed to the extracellular ionic milieu but are retained within the domain of the cell. The importance of this mechanism *in vivo* remains to be established, and the possibility of re-utilization of insoluble granule components requires further investigation.

A formally similar process of propagated fusion of perigranule membrane with the cell membrane and then with other perigranule membranes has been proposed for parotid zymogen cells,[22] pancreatic acinar cells[23] and zymogen secretory cells of von Ebner's gland.[24] This mechanism in its extreme form in the non-polarized mast cell would seem to obviate a need to move granules to the surface of the cell as proposed for pancreatic β-cells[25] and α-cells,[26] and might be expected to obviate any role for a microtubular-linked intracellular transport system. However, colchicine and vinblastine, which interfere with the microtubular system, have been reported to inhibit histamine release.[11] No ultrastructural observations as yet support the involvement of microtubules in the secretory process of mast cells, and it is conceivable that colchicine and vinblastine at the relatively high concentrations required to inhibit histamine release are acting by some other mechanism.

The recent electron microscopic study by Röhlich *et al.*[18] concurs with the ultrastructural observations I have made. Their observation effectively documents the formation of extracellular cavities as a mechanism for granule extrusion.

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REFERENCES

1. D. Lagunoff, in *Mechanisms of Release of Biogenic Amines* (Eds. U. S. Von Euler, S. Rosell and B. Uvnäs), p. 79. Pergamon, Oxford (1966).
2. B. Uvnäs and I. L. Thon, in *Mechanisms of Release of Biogenic Amines* (Eds. U. S. Von Euler, S. Rosell and B. Uvnäs), p. 361. Pergamon, Oxford (1966).
3. I. L. Thon and B. Uvnäs, *Acta physiol. scand.* **71,** 303 (1967).
4. B. Diamant, *Acta physiol. scand.* **71,** 283 (1967).
5. A. R. Johnson and N. C. Moran, *Am. J. Physiol.* **216,** 453 (1969).
6. G. D. Bloom and Ö. Haegermark, *Expl Cell Res.* **40,** 637 (1965).
7. M. Ritzén, *Expl Cell Res.* **45,** 178 (1966).

8. D. LAGUNOFF, *J. invest. Derm.*, in press (1972).
9. D. LAGUNOFF, P. PRITZL and L. MUELLER, *Expl Cell Res.* **61,** 129 (1970).
10. D. LAGUNOFF, M. T. PHILLIPS, O. A. ISERI and E. P. BENDITT, *Lab. Invest.* **13,** 1331 (1964).
11. E. GILLESPIE, R. J. LEVINE and S. E. MALAWISTA, *J. Pharmac. exp. Ther.* **164,** 158 (1968).
12. L. T. KREMZNER and I. B. WILSON, *Biochim. biophys. Acta* **50,** 364 (1961).
13. J. G. HIRSCH and M. E. FEDORKO, *J. Cell Biol.* **38,** 615 (1968).
14. J. H. LUFT, *J. biophys. biochem. Cytol.* **9,** 409 (1961).
15. J. H. LUFT, *J. Cell Biol.* **23,** 54A (1964).
16. J. H. LUFT, *Anat. Rec.* **171,** 369 (1971).
17. J. PADAWER, *J. Cell Biol.* **47,** 352 (1970).
18. P. RÖHLICH, P. ANDERSON and B. UVNÄS, *J. Cell Biol.* **51,** 465 (1971).
19. G. M. B. FILLION, S. SLORACH and B. UVNÄS, *Acta physiol. scand.* **78,** 547 (1970).
20. R. NOSAL, S. A. SLORACH and B. UVNÄS, *Acta physiol. scand.* **80,** 215 (1970).
21. S. A. SLORACH, *Acta physiol. scand.* **82,** 91 (1971).
22. A. AMSTERDAM, I. OHAD and M. SCHRAMM, *J. Cell Biol.* **41,** 753 (1969).
23. A. ICHIKAWA, *J. Cell Biol.* **24,** 369 (1965).
24. A. R. HAND, *J. Cell Biol.* **44,** 340 (1970).
25. P. E. LACY, *Diabetes* **19,** 895 (1970).
26. A. C. ESTERHUIZEN and S. L. HOWELL, *J. Cell Biol.* **46,** 593 (1970).