MATURATION OF RAT MAST CELLS

An Electron Microscope Study

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ABSTRACT

Electron microscope study of rat mast cell maturation corroborates certain interpretations of features of mast cell differentiation based on light microscope studies. In addition, the ultrastructural variation observed in the granules of differentiating mast cells suggests that granule formation begins with the elaboration of dense granules about 70 mμ in diameter inside Golgi vacuoles. These progranules appear to aggregate inside a membrane and fuse to form dense cords 70 to 100 mμ in diameter. These dense cords are embedded in a finely granular material possibly added to the developing granule by direct continuity between perigranular membranes and cisternae of rough endoplasmic reticulum. The dense cords and finely granular material then appear to be replaced by a mass of strands about 30 mμ in diameter, thought to be a reorganization product of the two formerly separate components. A process interpreted as compaction of the strands completes the formation of the dense, homogeneous granules observed in mature rat mast cells. The similarity between mast cell granule formation and the elaboration of other granules is considered, with special reference to rabbit polymorphonuclear leukocyte azurophil granules. The relationships between the ultrastructural, histochemical, and radioautographic characteristics of mast cell granule formation are considered, and the significance of the perigranular membrane is discussed.

Light microscope studies in the rat (5) indicate that embryonic mast cells arise from primitive mesenchymal cells by formation of characteristic granules in mitotically active cells. After a certain level of differentiation is reached, mitotic activity ceases; mast cells then mature by continued elaboration of granules. Histochemically and radioautographically definable properties of developing rat mast cells distinguish four stages in the differentiation process. Stage I mast cells contain small numbers of cytoplasmic granules which invariably stain blue with the alcian blue–safranin technique (24) and incorporate radiosulfate at a low level, indicating that the granules contain weakly sulfated polysaccharide. The alcian blue–positive granules also contain histochemically demonstrable specific chymase. In Stage II, safranin-positive granules appear but are less numerous than alcian blue–positive granules. Stage III mast cells contain a majority of safranin-positive granules; a few alcian blue–positive granules are still present. Stage IV mast cells contain large numbers of granules that invariably stain metachromatically with safranin. Radiosulfate uptake increases concomitantly with the shift of granule staining from alcian blue to safranin, reaches a maximum in Stage III, but then falls to a low level in Stage IV cells. These histochemical characteristics and variations in radiosulfate incorporation indicate that the granule synthesis characteristic of immature mast cells increases to a maximum rate in Stage III cells, but virtually ceases in the metabolically sluggish Stage IV mast cells in which large numbers of fully formed granules with the histochemical properties customarily associated with rat mast cells are stored.

Although the histochemical reaction for mast cell chymase is positive in all stages, histamine is first demonstrable in Stage IV cells. Incorporation of tritiated thymidine in mast cell nuclei can be related to the stages in differentiation outlined above, and indicates that Stages I and II comprise a mitotically active proliferating pool, whereas the cells in Stages III and IV have lost their proliferative capability. This report seeks to define the ultrastructural correlates to the process of chemical differentiation, with particular emphasis on granule formation.

METHODS

Mast cells in dermis from the tails of 35-mm crown-rump embryos and newborn rats and in tongue and lymph node of adult female rats were examined. Fixation of rat mast cells for electron microscopy was a problem. Osmium tetroxide and permanganate in a variety of buffers caused extensive granule damage and destruction of the intergranular cytoplasm, but the granules of adult mast cells were sporadically well preserved after fixation in 10% neutral buffered formalin or 6% glutaraldehyde in a variety of buffers and postfixation in 2% osmium tetroxide. These procedures, however, uniformly failed to preserve granule morphology in immature cells. Because of the well documented affinity of alcian blue for acid polysaccharides (19, 21, 25), this copper phthalocyanin dye was added to fixative solutions in an attempt to improve mast cell morphology. Although a thorough study of the effects of alcian blue has not yet been completed, preliminary results (see below) led to the inclusion of the dye in the routinely employed fixative. The tissues on which observations were made were handled as follows:

1. Tissues were placed in a drop of fixative on dental wax and sliced with double-edged razor blades degreased in acetone. Crushed or distorted slices were discarded, and the remainder were transferred to the fixative solution.
2. 4 to 24 hr at 0°–5°C in 6% unpurified glutaraldehyde (Union Carbide Corp., New York) in 0.067 M Sorensen phosphate buffer at pH 7.5 to which 0.1% weight/volume alcian blue 8GX (Allied Chemical Corp., New York) was added immediately prior to use.
3. 4 to 24 hr at 0°–5°C in 0.1 M cacodylate buffer–7.5% sucrose wash, pH 7.4.
4. 1 hr at 0°–5°C in 2% osmium tetroxide in 0.2 M *s*-collidine buffer, pH 7.4.
5. Routine dehydration and embedding in Epon according to Luft (15).
6. Sections were cut on the Porter-Blum microtome, collected on unsupported grids, and stained with uranyl acetate and Millonig's lead stain (16).

OBSERVATIONS

In both mature and immature tissues fixed in alcian blue–glutaraldehyde, the morphology of the granules and the intergranular cytoplasm in

Key to Symbols

A, aggregates of progranules	*M*, perigranular membrane
c, dense cords, 70 to 100 mμ in diameter	*mi*, mitochondrion
er, endoplasmic reticulum	*mv*, microvillus
fgm, finely granular material	*N*, nucleus
G, Golgi zone	*p*, progranules
Gr, mast cell granules	*s*, strands, about 30 mμ in diameter

FIGURE 1 Early (Stage I) mast cell from subcutaneous tissue of a 35 mm rat embryo. The cell contains several immature granules. The plasma membrane is not specialized; scattered cisternae of rough endoplasmic reticulum are liberally covered with patterned ribosomes. Aggregates of ribosomes not associated with membranes are numerous. Mitochondria are few in number and globoid in contour. The Golgi apparatus is not unusually prominent. Several progranules are present, both near the Golgi zone and in other regions of the cytoplasm. × 11,500.

FIGURE 2 A typical Stage II–III mast cell from subcutaneous tissue of the newborn rat. Numerous immature granules are present and the plasma membrane is characterized by numerous short microvilli. Mitochondria are more numerous and more complex. The remainder of the cytoplasmic detail seen in this plane of section is similar to that described for the Stage I mast cell. × 11,500.


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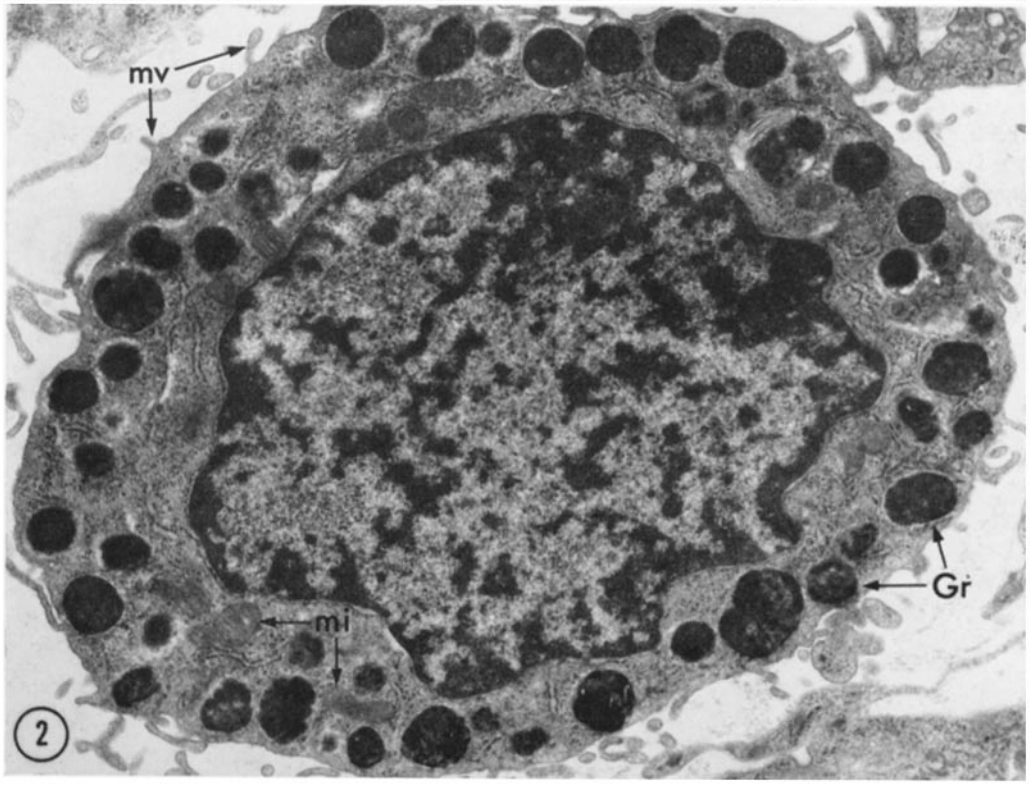
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mast cells was distinctly improved, but even in well fixed material slight granule damage was present in many cells (cf. Fig. 11). Fixation of tissues under identical conditions but without the addition of alcian blue to the glutaraldehyde-buffer solution occasionally yielded approximately comparable preservation of a few mast cells in a tissue specimen, but the majority of mast cells were poorly preserved.

The identification of mast cells in tissue sections was based on the presence of characteristic granules, as described below, and the location of the cells in mesenchyme or loose connective tissue.

While mast cells were generally less differentiated in embryonic tissue than in newborn tissue, cells in all stages of differentiation except Stage IV were present in both 35-mm embryos and newborn rats. Immature cells were rarely observed in normal adult tissues. The presence of granules characteristic of several stages of completion in a single immature cell made it feasible to compare, at the electron microscope level, individual granules in the same cell and in cells at different stages of differentiation.

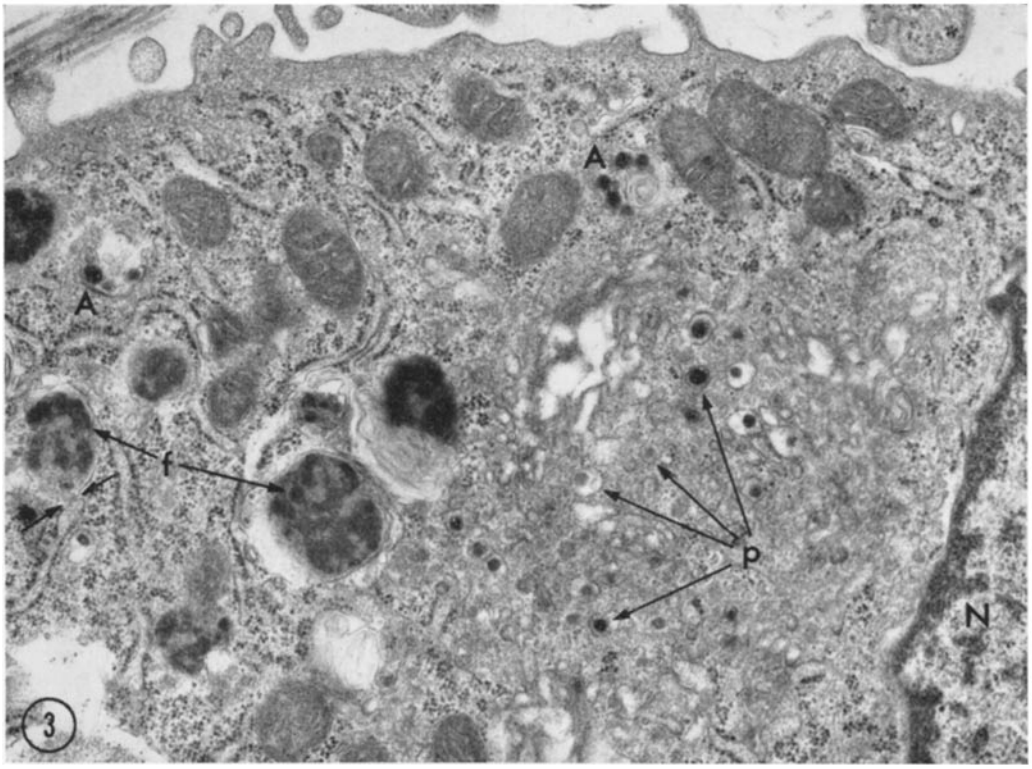
The earliest identifiable mast cells closely resemble the surrounding undifferentiated mesenchymal cells, but are recognizable by the granules they contain. These granules often lie near a small Golgi zone. The cell surface shows no specialization and the cytoplasmic matrix is flocculent. Irregular cisternae of rough endoplasmic reticulum are scattered throughout the cytoplasm. The cisternae contain finely granular material and on tangentially cut surfaces are liberally covered with typical patterned arrays of ribosomes. Ribosomal aggregates unassociated with membranes are generally distributed in the cytoplasm. The few mitochondria present are globoid (Fig. 1).

As differentiation proceeds, the plasma membrane develops numerous short microvilli. Mitochondria become elongated and more numerous; otherwise the nongranular cytoplasm is little altered. Characteristic granules increase in number and become distributed throughout the cytoplasm (Fig. 2). In cells of this stage the Golgi zone is very prominent and has associated with it dense granules 70 to 100 mμ in diameter inside smooth membrane-limited vacuoles. These granules are often but not invariably separated from the vacuolar membrane by a less dense, granular material. For the sake of convenience they are termed "progranules" (Fig. 3). Mast cells with the characteristics so far described probably correspond to Stages I or II and as expected are occasionally found in mitosis (Fig. 4). Maturation is completed by the continued formation of granules, a process that finally reduces the intergranular cytoplasm to a few fragments of rough endoplasmic reticulum, scattered ribosomal aggregates, and an occasional mitochondrion wedged between granules (Fig. 5).

Of particular interest are the morphologic variations seen in the cytoplasmic granules of immature rat mast cells. These variations have been interpreted as morphologic correlates to granule formation. A postulated sequence for the major steps in the process of granule formation is depicted schematically in Table I. The hypothetical steps may be outlined as follows: granule synthesis begins with the formation of single progranules contained inside membrane-limited vacuoles in the central part of the Golgi zone. The progranules appear to migrate to the periphery of the Golgi zone and become more dense (Fig. 3). Several progranules aggregate inside a common membrane, usually at or near the periphery of the Golgi zone; however, both single and aggregated progranules are occasionally seen in peripheral portions of the cell (Figs. 3, 6, and 8). Finely granular material apparently derived from the rough endoplasmic reticulum is added to the

FIGURE 3 Golgi apparatus in a Stage II–III mast cell from subcutaneous tissue of the newborn rat. Numerous progranules are scattered throughout the Golgi zone. The progranules lying near the center of the Golgi apparatus are generally less dense. Several progranules have aggregated at "*A*" and appear to be in various stages of fusion in the larger granules (*f*). Note the intimate association of the rough endoplasmic reticulum with the perigranular membrane (arrows near *f*). × 23,000.

FIGURE 4 Mitosis in a Stage I–II mast cell from subcutaneous tissue of a 35 mm rat embryo. All the cytoplasmic characteristics associated with the differentiating mast cell are present. *Chr*, chromatin. × 11,500.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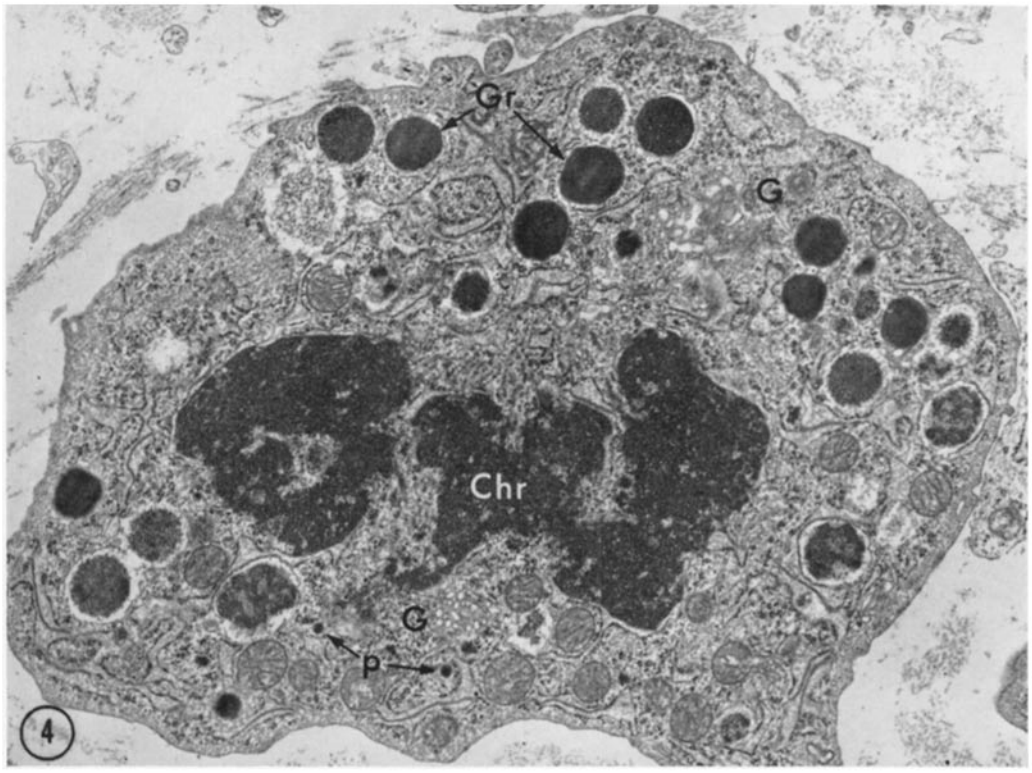
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vacuoles containing aggregates of progranules, possibly by direct membranous continuity with cisternae of rough endoplasmic reticulum (Fig. 8). The progranules then appear to fuse, forming dense ropy cords about 70 to 100 mμ in diameter embedded in the finely granular material (Fig. 7). Progranules and finely granular material continue to accumulate inside the perigranular membrane until the granule reaches its maximum size of approximately 1 μ in diameter (Figs. 6 and 7). The dense and the finely granular components then appear to reorganize, forming a mass of electron-opaque strands 20 to 30 mμ in diameter. The strands have a beaded appearance and are tightly packed inside the perigranular membrane (Fig. 9). The granule is then thought to undergo compaction and perhaps further reorganization to form the dense, homogeneous, chemically complete mast cell granule (Fig. 10).

This postulated sequence accounts for the appearance of most of the granules; however, granules are often observed to consist of a coarse, open, fibrillar network with indistinct margins surrounded by an empty halo. This latter morphologic appearance is interpreted as the equivalent of dissolution of the granule that accompanies degranulation and histamine release in mast cells following a variety of insults (4, 7, 17, 22, 23), including less than optimal fixation (Fig. 11).

DISCUSSION

Light microscope studies have yielded much information concerning the differentiation and proliferation of mast cells, the constituents of mast cell granules, and the response of the mast cell to injury (3, 5, 22). The usefulness of the electron microscope in extending these studies has been limited by difficulties in obtaining adequately fixed mast cells. A combination of careful handling of tissue specimens, glutaraldehyde in a suitable buffer, and the addition of alcian blue as described above, results in fixation adequate for morphologic study of differentiating mast cells at the ultrastructural level. Observations made with the electron microscope add to the evidence that (*a*) mast cells arise in the embryo in primitive mesenchyme from typical mesenchymal cells, and (*b*) the essential process in the maturation of the individual mast cell is the synthesis and accumulation of characteristic mast cell granules. In addition, the electron microscope reveals variations in granule appearance that can reasonably be arranged in a morphologic sequence that may correlate with the accumulation of the major constituents of the granule, i.e. heparin, and basic protein (Table I).

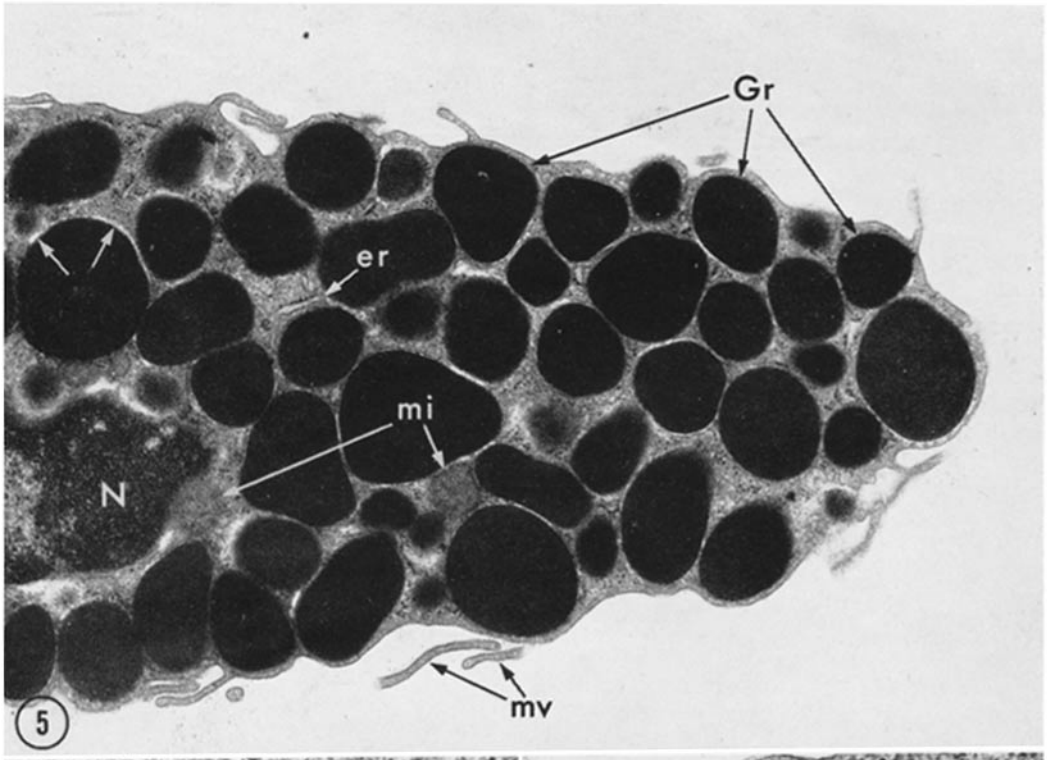
The first detectable material that contributes to the formation of mast cell granules is intimately

FIGURE 5 A mature mast cell (Stage IV) from adult rat tongue. Dense, homogeneous, fully formed granules pack the cytoplasm, reducing the intergranular cytoplasm to fragmentary rough endoplasmic reticulum, scattered ribosomes, and an occasional mitochondrion. The microvilli are less numerous in this cell than usually observed in a mature mast cell. Lifting of perigranular membranes (arrows) is interpreted as fixation damage. × 18,000.

FIGURES 6 to 10 Examples of mast cell granules, depicting steps in the postulated sequence of events in granule formation (cf. Table I).

FIGURE 6 At "*A*" two vacuoles containing aggregates of progranules and a vacuole containing a single progranule are in intimate contact, with apparent fusion of the vacuolar membranes (indicated by diamonds). In the lower granule, a process interpreted as reorganization of the fused material from progranules (cords, *c*) into smaller subunits (*s*) in the size range of 30 mμ is evident. This reorganization occurs after the steps shown in Fig. 7. × 34,000.

FIGURE 7 In the lower granule, the dense materials from the progranules have fused to form dense ropy cords (*c*) about 70 mμ in diameter embedded in a finely granular material. The upper granule is probably in a similar stage but contains more of the dense material. Note the close apposition of a cisterna of rough endoplasmic reticulum to the perigranular membrane (arrows). × 34,000.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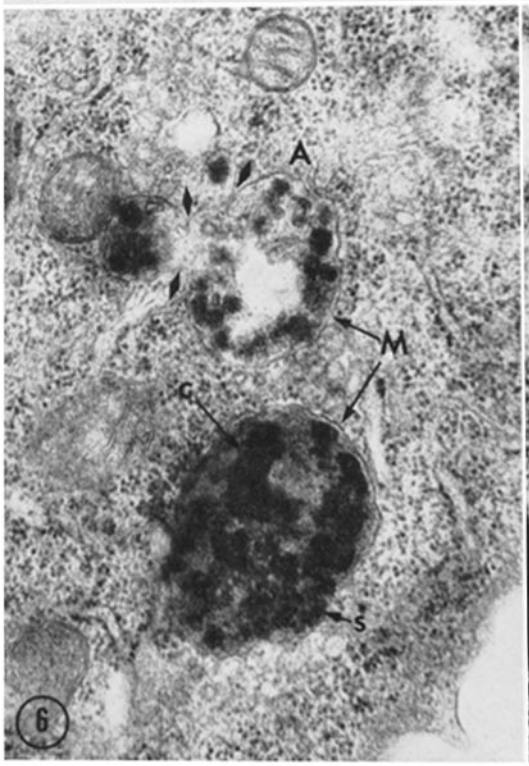
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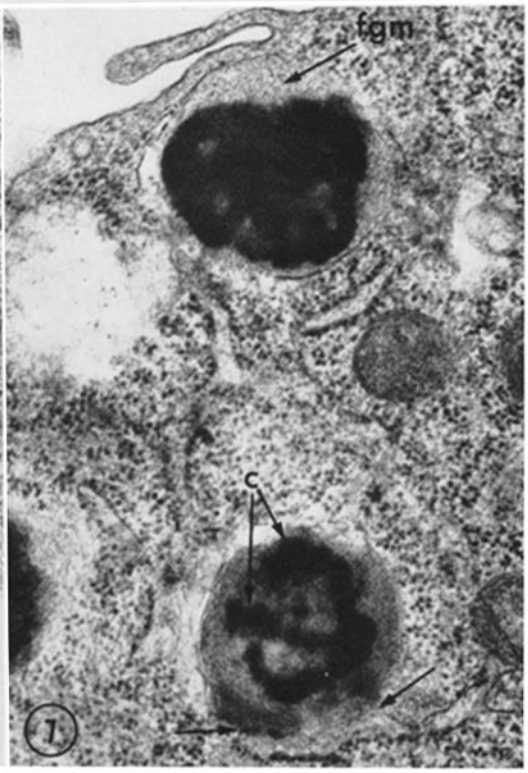
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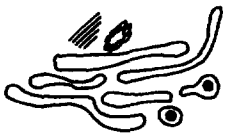
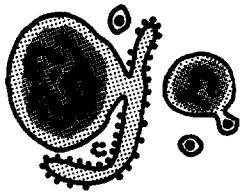
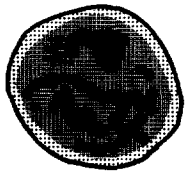
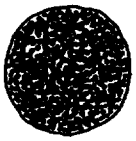
Table I

A Postulated Sequence of Events in Rat Mast Cell Granule Formation

Morphologic steps		Hypothetical chemical correlates
Step 1 Progranule formation		Formation of heparin precursor; O-sulfation
Step 2 Aggregation of progranules		Accumulation of heparin precursors, forming a nidus for the future mast cell granule
Step 3 Addition of finely granular material and continued aggregation of progranules		Accretion of basic proteins including mast cell chymase
Step 4 Fusion of progranules and continued addition of finely granular material		N-sulfation
Step 5 Reorganization to form strands with disappearance of the finely granular and dense components		
Step 6 Compaction		Maximum ionic binding between heparin and basic proteins

associated with the Golgi apparatus and probably includes partially sulfated heparin precursors. Elaboration of sulfated polysaccharides has previously been attributed to the Golgi complex in chondrocytes, on the basis of morphologic, cytochemical, and radioautographic data (10, 11, 18, 20).

More recently, Bainton and Farquhar (1, 2) described a process of granule formation in rabbit polymorphonuclear leukocytes (PMN) involving elaborate participation of the Golgi apparatus. The initial postulated steps in the formation of rat mast cell granules closely resemble the scheme proposed by Bainton and Farquhar for the elaboration of the azurophil granule of the rabbit PMN. The progranules seen in immature mast cells are morphologically similar to the "dense cored vacuoles" described in the rabbit PMN. In both types of cells the aggregation of material apparently originating in the Golgi apparatus is a prominent part of granule formation. A general chemical similarity may underly the nearly identical appearance of the formative process in PMN azurophil granules and immature rat mast cell granules. Isolated mast cell granules contain approximately ⅓ heparin, ⅓ basic protein of which a substantial amount is mast cell chymase, and about 10% histamine (14). Basic protein and sulfated polysaccharide have been detected in rabbit azurophil granules by histochemical and radioautographic techniques (12) and by analysis of isolated rabbit PMN granules (9). The small amount of sulfated polysaccharide found in isolated rabbit PMN granules is explicable on the basis of the small number of azurophil granules present in the mature (peripheral) PMN. As few as 1% azurophil granules in the isolates would account for the amount of sulfated polysaccharide observed (9) if the azurophil granule contains about the same amount of sulfated polysaccharide as the mast cell granule.

The aggregation of several progranules and fusion of their vacuolar membranes probably represents the accumulation of partially sulfated heparin precursor, providing a nidus for the formation of a larger and more complex granule by the accumulation of additional progranules and material, presumably protein originating in rough endoplasmic reticulum. The combination of the progranules and the finely granular material from the rough endoplasmic reticulum may bring together the necessary elements for the formation of a granule, consisting of an ionically bound complex of heparin and basic protein as proposed by Lagunoff (13, 14).

The observation of direct continuity between rough ER (endoplasmic reticulum) and perigranular membranes in immature mast cells raises the possibility that constituents may be donated directly to the developing granule from the cisternae of the endoplasmic reticulum. Good examples of these membrane continuities are only occasionally seen on electron micrographs of immature mast cells; however, membranes of rough ER and perigranular membranes are frequently seen in intimate contact (Figs. 3 and 7). The rarity of actual connections may indicate that the amount of material traversing this pathway is small. Alternatively, their low frequency may be a result of the small size of the connections, the number of connections with a given granule being small, or the existence of the actual continuity being ephemeral. The site of origin and the routes in the cell followed by the major constituents of the granule are presently under study.

In both the chondrocyte and the rabbit PMN the sulfated polysaccharides are of the chondrotin type containing O-sulfate esters (9, 12). Heparin and heparan sulfate are unique among sulfated polysaccharides in containing sulfamido groups as well as the more common ester sulfates. The pattern of radiosulfate uptake in the immature mast cell granules and the shift from alcian blue to safranin binding and finally to safranin metachromasia seen in granules as they mature indicate that active sulfation occurs in the incompletely synthesized granules in the peripheral cytoplasm during their maturation process (5). The conversion of mature granules from safranin- to alcian blue–staining by dilute nitrous and dilute hydrochloric acids, both of which selectively hydrolyze sulfamido bonds, suggests that a major part of the sulfate bound by peripheral immature granules is incorporated into heparin as *N*-sulfate (5, 6). The reorganization of the coarse cords formed by the fusion of progranules and of the finely granular material to form smaller strands observed in the immature granules may result from progressive *N*-sulfation of heparin precursor. As the polysaccharide becomes fully sulfated, maximum ionic binding between the heparin polymer and basic proteins may lead to compaction of the granule matrix to form the dense,

relatively homogeneous granule observed in the mature mast cell.

The structural specializations seen in guinea pig basophils (paracrystals, reference 8), human mast cells (scrolls and paracrystals, references 8, 17) and human basophils (26) might be explained in terms of variations in (*a*) the patterns and rates of sulfation, (*b*) the nature of the basic proteins in the granules, (*c*) the relative amount of polysaccharides and proteins present, and (*d*) the rates at which polysaccharides and proteins arrive inside the granules. Interplay of these factors might produce structure at a level detectable in the electron microscope, ranging from amorphous as in the rabbit azurophil granule and mature rat mast cell granule to the highly organized structure of the human mast cell granule, without presuming any major differences in the chemistry of the main structural elements. It is interesting in this regard to note a close morphologic similarity between the granules of the normal human basophil and the granules of the rat mast cell just prior to compaction.

Mature mast cells also contain histamine and/or serotonin, depending on the animal species (3). Lagunoff and coworkers consider these amines to be electrostatically and reversibly associated with sulfate groups on the heparin polymer (13, 14). Under appropriate conditions histamine can be released from and reassociated with isolated rat mast cell granules without discernible ultrastructural alteration (14), but the isolated granule has no perigranular membrane. In the developing mast cell, histamine is histochemically detectable only in mature (Stage IV) mast cells and hence in mature granules surrounded by perigranular membranes (5); thus, the association of amines with the mast cell granule may not have a discernible ultrastructural expression. In the studies (4, 7, 17, 23) which correlate histamine release with morphologic alteration in the mast cell granules, the granule disintegration observed may invariably release histamine, but histamine release per se may not require any detectable morphologic change.

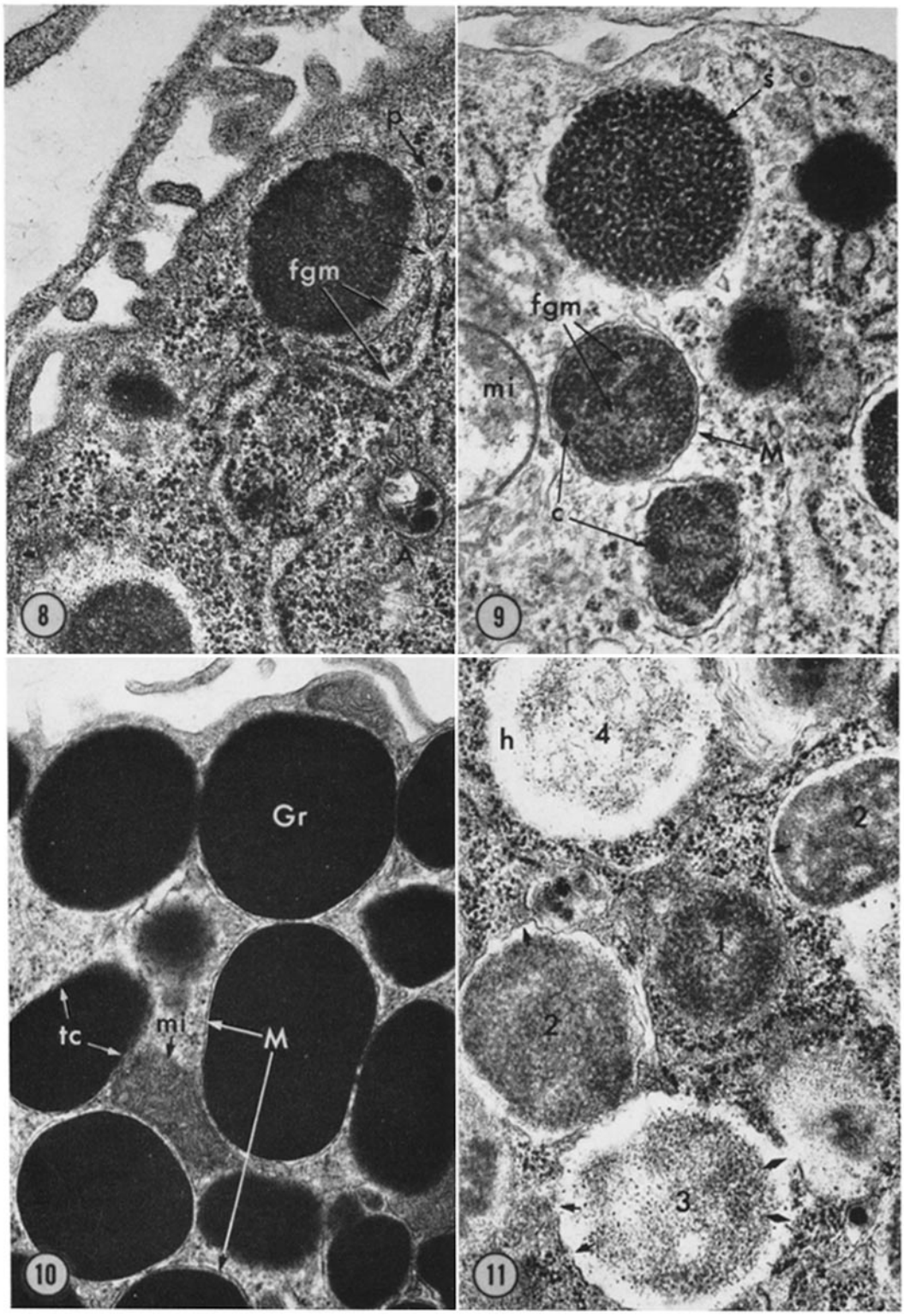
The mast cell granule, with its proteolytic capability and high content of polyanionic heparin polymer, must remain isolated from surrounding cytoplasm while the granule is "stored" in the intact mature mast cell (3, 13). Thus, the status

FIGURE 8 An example of direct continuity between a cisterna of rough endoplasmic reticulum and the perigranular membrane is seen at the arrow. Material of similar appearance (*fgm*) fills both the cisterna and the region around the dense portion of the granule. A solitary progranule is closely applied to the perigranular membrane, and a single membrane contour at "*A*" contains several progranules. × 34,000.

FIGURE 9 The upper granule consists of a packed mass of strands about 30 mμ in diameter, replacing the dense and finely granular components seen in less mature granules. In the two lower granules the presence of several dense areas (*c*) and patches of finely granular material indicates that reorganization is incomplete. × 34,000.

FIGURE 10 The granules observed in mature mast cells stain intensely and usually appear homogeneous. Membranes invariably surround granules cut near the midplane, but are obscured by the dense matrix of tangentially cut granules (*tc*). × 34,000.

FIGURE 11 Granules in a Stage II or III mast cell from the same tissue specimen as Fig. 2. The granules are numbered to correspond to increasing granule alteration. In adequately fixed granules, the matrix is compact and in close contact with the perigranular membrane (granule No. 1 is tangentially cut, obscuring the membrane). Minimal alteration is seen as a slight loss of granule density and the appearance of a space between the granule and its perigranular membrane (No. 2). The membrane is irregular but largely intact (No. 2, arrows). More obvious alteration is characterized by progressive loss of granule density and widening of the space around the granule as in No. 3. The membrane is fragmentary (No. 3, arrows) or absent (diamonds). In severely altered granules (No. 4) the matrix is reduced to a loose meshwork surrounded by an empty halo (*h*) with no discernible perigranular membrane. × 34,000.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of the membranes surrounding the mature mast cell granule is probably crucial in maintaining cellular integrity. Our observations concur with those of other workers who have described a perigranular membrane surrounding the unaltered mature mast cell granules (3, 4, 13). Further, a membrane invariably surrounds developing granules and progranules in well preserved specimens, except when the plane of section is sufficiently tangential as to smudge any contrast between the perigranular membrane and the granule contents. Granule dissolution appears to involve alteration of the perigranular membrane in its earliest phases, and membrane fragments are commonly observed around granules which have undergone minimal morphologic alteration (Fig. 11). The appearance of the membrane surrounding granules is thus a useful criterion for evaluating the structural integrity of granules, as well as a criterion for adequate fixation.

The association of the synthesis of proteins and polysaccharides with ultrastructural features in the differentiating mast cell provides a context for evaluation of the status of any particular mast cell population. That is, the presence of an elaborate Golgi apparatus, progranules, large numbers of ribosomal aggregates, irregular cisternae of rough endoplasmic reticulum, and multiphasic granules in a mast cell are indicative of active granule synthesis. The virtual absence of rough endoplasmic reticulum, a diminutive Golgi zone, and the presence of large numbers of dense, homogeneous granules in the mast cell are indicative of cellular maturity and synthetic inactivity. Although the observations and interpretations presented here provide no explanation of the role of the mast cell in either normal physiology or the inflammatory response, the ultrastructural detail of the differentiating and mature mast cells should prove useful in assessing the status of mast cells in experiments designed to test their function.

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