ACID PHOSPHATASE LOCALIZATION
IN RABBIT EOSINOPHILS

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ABSTRACT

Eosinophil (and heterophil) leukocytes of glycogen-induced rabbit peritoneal exudates were fixed for $1\frac{1}{2}$ min in 2% glutaraldehyde and examined for acid phosphatase activity both biochemically and cytochemically. Biochemical assays showed that enzymatic activity had been inhibited by only ~10% under these conditions. The cytochemical reaction in the eosinophil was confined to the granules in which the reaction product appeared in the matrix, not in the crystalline core (or in the core region after the latter's extraction). Granules wherein the matrix was disrupted and the crystalline core degraded or extracted showed the most intense deposition of reaction product, whereas well preserved granules with morphologically intact matrix and crystals were unreactive. Yet, not all disrupted granules gave a positive reaction, indicating that disruption was a necessary but not sufficient condition for reactivity. In many eosinophil leukocytes, most if not all granules were acid phosphatase–positive, provided they had become disrupted to a certain degree. Factors possibly involved in converting the granules from an unreactive to a reactive state are discussed.

INTRODUCTION

The enzyme content of granules isolated from horse and rat eosinophil leukocytes has been extensively investigated by Archer and Hirsch (1), who showed that it consists of usual lysosomal hydrolases and a peroxidase. As far as hydrolases are concerned, the eosinophil granules are similar to the heterophil granules of rabbit polymorphonuclear leukocytes previously studied by Cohn and Hirsch (2; see also reference 3). Since leukocytes, as well as granule fractions derived therefrom, have a whole spectrum of acid hydrolases, the question has been frequently asked whether each granule contains the whole set of lysosomal enzymes or only a few, possibly just one, of these hydrolases. The present paper provides a partial answer to this question by showing that, under certain conditions, acid phosphatase activity can be demonstrated cytochemically in all eosinophil granules. These conditions include partial disruption of the granules and possibly extraction of their crystalline core.

MATERIALS AND METHODS

Rabbit leukocytes were obtained from peritoneal exudates as described by Cohn and Morse (4) and by Cohn and Hirsch (2). Differential counts by means of phase contrast microscopy revealed about 2.7% eosinophils but variation was considerable, the corresponding figures being 2.9%, 0.7%, 3.3%, 0.3%, 5.5%, 4.7%, 0%, 4%, and 3.3% in nine different exudates. Since it was of interest to compare the acid phosphatase localization in eosinophil to that in heterophil leukocytes, no attempt was made to separate these two cell types.

The biochemical assay of acid phosphatase activity was carried out as described by Cohn and Hirsch (2).

The histochemical procedure for acid phosphatase localization was carried out as follows. After the peritoneal exudate was filtered through sterile gauze, approximately 3×10^6 leukocytes were centrifuged at 1,500 g for 2 min at room temperature and the supernate discarded. The ensuing pellet was gently vibrated for 2 sec by means of a vortex mixer to disperse the cells in the few drops of remaining

supernate, and 4 ml of 2% glutaraldehyde (in 0.1 M sodium cacodylate-HCl buffer, pH 7.2) (5) were then added to the dispersed leukocytes which were thus fixed in suspension. Clumps of fixed cells which quickly settled to the bottom of the tube were discarded. After a 1½ min period of glutaraldehyde fixation at room temperature (~25°), the preparations were centrifuged at 1,500 g for 30–45 sec, the glutaraldehyde discarded, and the cells resuspended and washed at 4° in either cacodylate-HCl buffer or 0.25 or 0.34 M sucrose. Varying the duration of the wash period between 15 min and 10 hr or the osmolarity of the washes did not change the results. The washed cells were then resuspended in freshly prepared acid phosphatase medium (6) which contained 50 mM Na-acetate buffer, pH 5.0, 10 mM Na β-glycerophosphate (from Eastman Kodak), and 4 mM Pb(NO₃)₂. The ingredients were mixed in the order given, the mixture was shaken and preheated at 56° for 50 min, and finally filtered at 37° just before use. The suspended cells were incubated at 37° for 30–40 min with continuous shaking. After incubation, the cells were recovered by centrifuging the suspension for 1 min, and the ensuing pellet was fixed in buffered 2% osmium tetroxide (7). Although it is common procedure to employ a 1% acetic acid rinse after the incubation period (cf reference 8), this step was omitted since in this material the acid rinse produced irregular losses of reaction product (lead phosphate) deposits. Ten min after the addition of the OsO₄, the pellet (which was less than 1 mm thick) was gently dislodged from the bottom of the test tube, and fixation continued for another 15–20 min. Some of the pellets were then stained in block in 0.5% uranyl acetate for 45 min (see Farquhar and Palade, 9, for details) to enhance the contrast of the membranes; the uranyl acetate treatment did not affect the localization of the acid phosphatase reaction product. The pellets were subsequently dehydrated in ethanol and embedded in Epon. Thin sections cut with a Porter-Blum microtome were picked up on carbon-coated, Formvar-covered grids and examined, either unstained or stained (6% uranyl acetate for 1 hr followed by a 20-min stain on a drop of Karnovsky B lead solution; see reference 10), in an

RCA EMU-3F or a Siemens Elmiskop I electron microscope. A comparison of stained and unstained sections showed that the staining did not affect the extent and localization of the acid phosphatase reaction product.

RESULTS

Biochemical

To determine the degree of enzyme inactivation that resulted from glutaraldehyde fixation, we suspended peritoneal exudate luekocytes in 2% glutaraldehyde for ~1 min, centrifuged them for 30–60 sec, separated them from the glutaraldehyde, and froze and thawed them six times in an acetone-dry ice mixture before assaying biochemically their acid phosphatase activity. The average acid phosphatase activity of fresh, unfixed peritoneal leukocytes (activated by the same freezing-thawing procedure) was about 3.2×10^4 μμmoles phosphate released per cell per hr (average of nine peritoneal exudates). Washing the cells in 0.25 M sucrose for 1 hr or overnight did not significantly reduce this value. Exposing the cells to 2% glutaraldehyde in cacodylate-HCl buffer for a total time of ~1½–2 min resulted in a drop of 10–12% of the acid phosphatase activity. After 3–10 min of glutaraldehyde fixation, the level of enzymatic activity preserved was constant at ~80%. Each value represents an average obtained from at least three di erent rabbit peritoneal exudates. Almost identical results were obtained using 1% glutaraldehyde. Exposure to glutaraldehyde for 1½ hr resulted in 80% inactivation of the enzyme.

The results indicated that after a 2-min exposure to glutaraldehyde there was a substantial amount of enzymatic activity preserved, which accounted for ~90% of that found in unfixed cells. It is not known whether fixation affected equally the acid phosphatase of the eosinophils and heterophils of the exudate.

FIGURE 1 Large field in a rabbit eosinophil leukocyte. The nucleus is marked n and the cell membrane cm; the numerous lacunae (l) in the cytoplasm were left behind by extracted glycogen granules. With the exception of a fine, presumably nonspecific precipitate in the nucleoplasm, opaque deposits of reaction product (lead phosphate) are restricted to the matrix (m) of eosinophil granules (eg). The light regions left behind by the extracted crystalline cores (c) are generally free of deposits. Some of these areas still contain core remnants (cr). In this cell, *all* eosinophil granules gave an equally intense reaction. Glutaraldehyde (90 sec); Gomori incubation (30 min); OsO₄ postfixation; Epon-embedded; uranyl and lead stains. × 40,000.
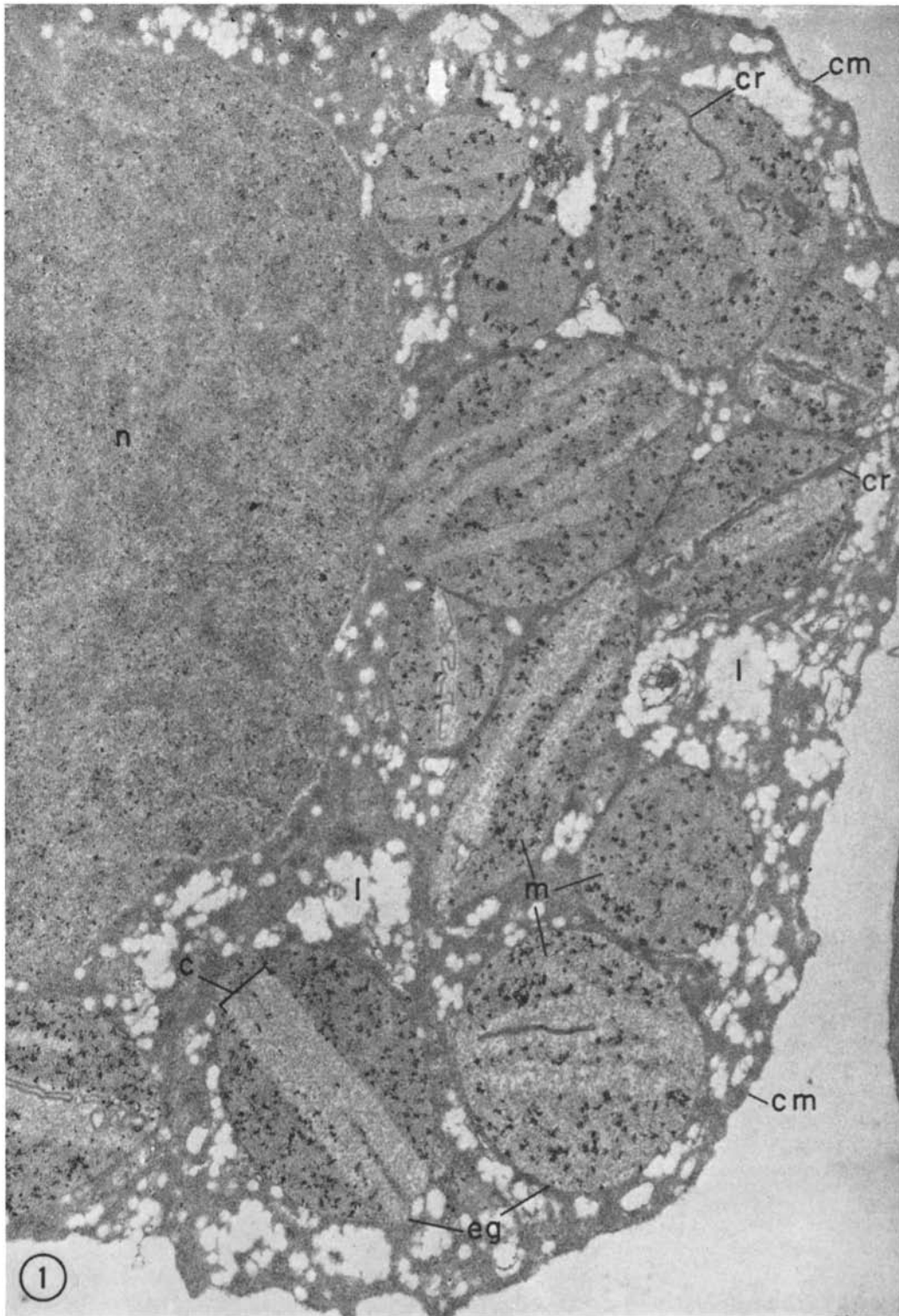
Cytochemical

The localization of the acid phosphatase reaction product in rabbit eosinophil granulocytes is demonstrated in Figs. 1–9.

In general, there were few or no deposits in the nucleus[1] and in the cytoplasm outside the eosinophil granules. The reaction was preferentially localized to these granules which, as already known (cf. 12, 13), have a discoidal form (diameter $\simeq 0.5$ μ), are limited by a unit membrane, and contain a fine granular matrix in which a crystalline core (14) is embedded. In the rabbit, the core is usually represented by one or more thin, needle-like or platelike crystals. Within the granule, the acid phosphatase reaction was preferentially localized in the matrix.

Although the reaction varied considerably from preparation to preparation and from cell to cell, the following findings were consistently made on all the numerous specimens examined:

(a) Intact eosinophil granules characterized by the presence of a well defined crystalline core and a compact, dense matrix were generally nonreactive.

(b) The acid phosphatase reaction was positive in slightly damaged granules in which the crystalline core was either degraded or extracted and the matrix was less dense and compact than in nonreactive specimens. During incubation in the acid phosphatase medium, pH 5.0, the core was extensively altered prior to complete extraction, its usual crystalline lattice being replaced by waving sheets of dense material with a much wider center-to-center spacing ($\sim$80 A instead of $\sim$30 A in the crystal) and by structures which had the appearance of fragments of unit membranes (Figs. 4 and 5). In reactive granules, the deposits of reaction products were usually (Fig. 1) but not always (Fig. 2) restricted to the matrix; they spared the spaces occupied by disrupted cores or left behind by extracted crystals which were often as sharply outlined as by negative staining. A negative image of a partially disrupted crystalline core appears at eg_1 in Fig. 3.

(c) Disruption appeared to be a necessary, but

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[1] The erratic, usually small and diffuse lead phosphate deposits present in the nucleus are considered "nonspecific" and partly nonenzymic in origin. Complete references and a detailed discussion of this problem are given by Deane (11).
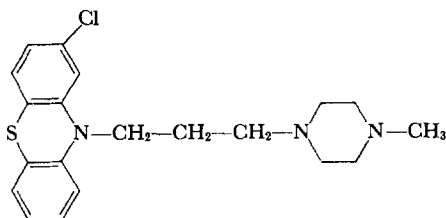
not sufficient condition for reactivity: when a cell contained intact as well as damaged granules, deposits of reaction product were found only in the latter. Although a large percentage of the altered granules gave a positive reaction, unreactive disrupted granules were rather frequently encountered. The finding could not be ascribed to a microtomy artifact since serial sections showed that the reaction was uniformly positive throughout the matrix of reactive granules and uniformly negative throughout adjacent nonreactive granules (Figs. 6–9).

(d) When a cell contained only altered granules, all or practically all these granules showed an acid phosphatase positive reaction in their matrix (Fig. 1).

To ascertain the enzymatic character of the reaction, we carried through the following controls: (a) incubation of active cells in the absence of substrate; (b) inactivation of the enzyme either by heating the fixed cells at 80° for 5 min in 0.1 M cacodylate-HCl buffer, pH 7.0, or by incubating them in the presence of 5 mM NaF. The results were all negative. To check spurious localizations by diffusion and adsorption of either enzyme or reaction product, we incubated heat-inactivated cells in: (a) the usual medium in the presence of excess (10^6-fold) enzyme added in the form of a granule extract, or (b) an incubation medium from which active cells have just been removed. the granules remained free of reaction product in both cases.

Since it was clear that morphologically intact granules remained unreactive although there was little enzyme inactivation during fixation, we carried out a number of experiments to define the conditions under which these granules become "activated." Variations in fixation time from 3–30 min, and in incubation time from 15–100 min did not change the results. Reduction of glutaraldehyde concentration (0.1%, 0.75% and 1%) in the fixative solution resulted in unsatisfactory preservation (e.g., extracted cytoplasmic matrix and disrupted membranes), but did not increase the frequency of positive reactions and did not change their localization. Similar results–i.e., increase in over-all specimen damage without a noticeable increase in the frequency of positive reactions—were obtained by: (a) fixing the cells in glutaraldehyde at acid pH (pH 5, 4, 3, or 2); (b) exposing them to pH 4.0 before fixation; (c) treating them before or after fixation with
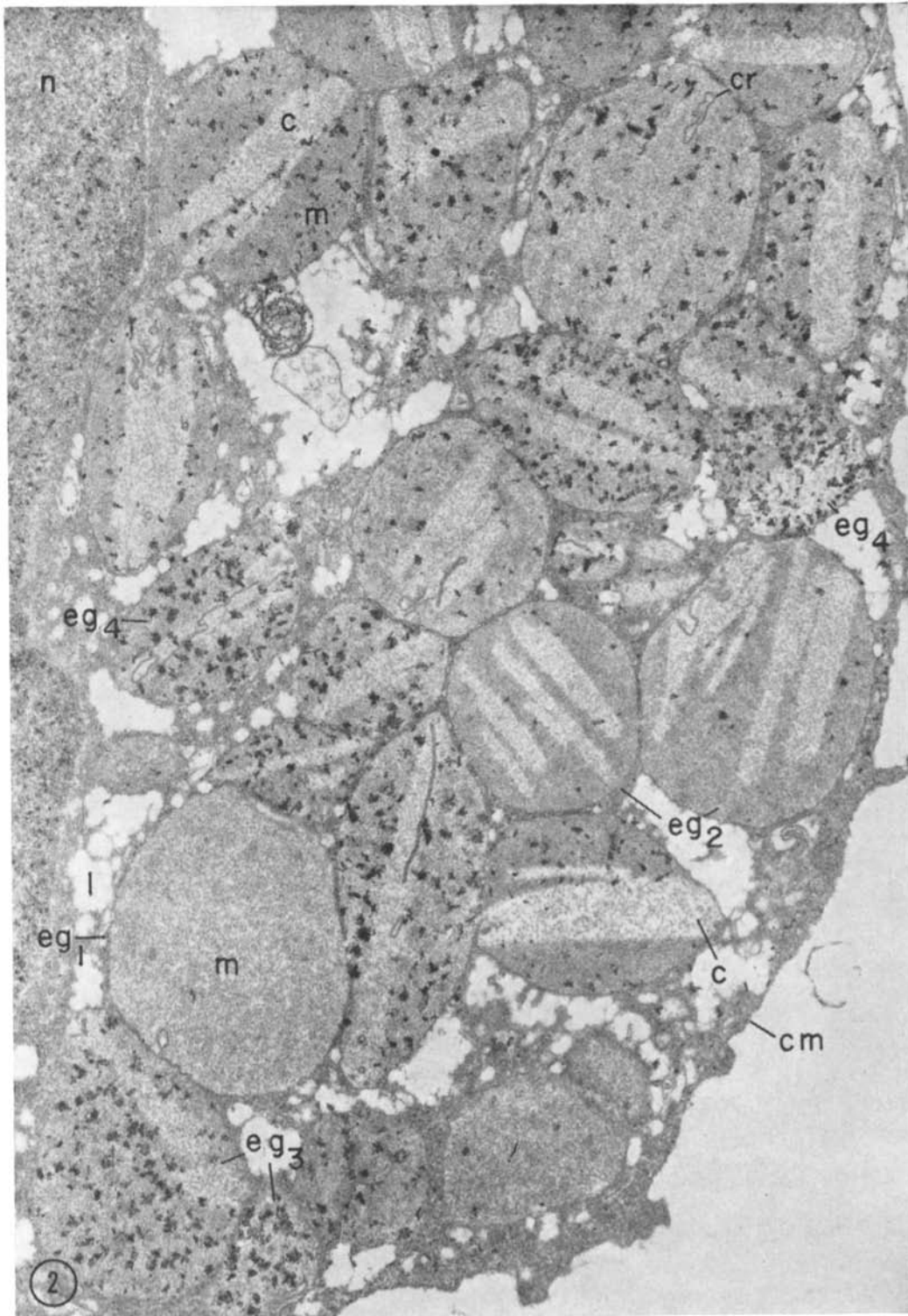
FIGURE 2 Large field in an eosinophil leukocyte in which acid phosphatase reaction varies from none (eg_1) to intense (eg_3) from granule to granule. Note that, in general, the reaction is less intense in granules with relatively dense matrix (eg_2). Note further that deposits of reaction product occur also in the core region in certain granules (eg_4). In this cell, some lead phosphate deposits appear scattered in the cytoplasm. For preparationprocedures and other indications, see Figure 1. $\times$ 40,000.

detergents (Triton X-100, prochlorperazine[2]); (d) subjecting them to hypertonic or hypotonic shock. The only procedure that appeared to be effective in increasing the frequency of an acid phosphatase–positive reaction among eosinophil granules with the treatment of fixed cells with 0.1 M citrate buffer, pH 2 or 3, for 30 min before incubation. After such a treatment, practically all eosinophil granules in all cells became reactive, although the granules were extensively extracted (Fig. 10), as shown by the complete absence of of the core or core remnants and by the highly decreased density of the matrix.

DISCUSSION

The salient points of our observations on the localization of acid phosphatase in rabbit eosinophils are: (1) the reaction product is never found in well preserved or intact granules, but predominantly in those somewhat damaged or extracted; (2) the deposits of reaction product are localized primarily in the granule matrix and not within the crystals or the spaces left by the extracted crystals; and (3) under certain conditions, all the granules in the same eosinophil are acid phosphatase–positive. The same observations were made on human blood eosinophils in several preliminary experiments. Recent preliminary reports have shown that acid phosphatase reaction product can be localized by electron microscopy to the matrix of eosinophil granules in rodents (17) and man (17,

18). Both reports pointed out that the crystalline core is unreactive.

The fact that acid phosphatase in the intact eosinophil granule is cytochemically unreactive, although not irreversibly inhibited, may partly explain the negative results reported in earlier investigations. Gomori (19) found that "all (peripheral) blood cells of all species studied" were histochemically acid phosphatase–negative. Rabinovich et al. (20) and Rabinovich and Andreucci (21) were the first to demonstrate acid phosphatase in the eosinophils of bone marrow smears, but obtained negative results in peripheral blood smears. Wislocki et al. (22) and also Rheingold and Wislocki (23) found that the eosinophils of bone marrow and of peripheral blood were negative for acid phosphatase.[3]

Granule Homogeneity

Our results partly answer the abiding question of whether the eosinophil granules are homogeneous or heterogeneous with respect to enzyme content (see Introduction). The cytochemical findings suggest that these granules are homogeneous insofar as they all contain acid phosphatase. The manifestation of this granule homogeneity, however, is invariably associated with a certain amount of structural damage.

The crystalline cores present in each eosinophil granule may represent another enzyme, possibly peroxidase (cf. 14). If so, the eosinophil granule population would be homogeneous for at least two enzymes. It remains to be shown, however, whether these granules are homogeneous or heterogeneous with respect to the other acid hydrolases of the lysosome group.

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[2] Prochlorperazine is a potent surface-active tranquilizer which has been used to lyse red blood cells (15, 16) and neutrophil granules. It has the following structure:

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[3] Another possible explanation for these negative results is total enzyme inactivation caused by the prolonged fixation periods used in these earlier investigations. By comparison, the brief fixation used in the present series of experiments produced only minimal enzyme inhibition.

FIGURE 3   Small field in an eosinophil leukocyte to illustrate extreme variation in the acid phosphatase reaction among adjacent granules. Some granules with dense matrix and partially extracted crystalline core (eg_1) are unreactive, while in others (eg_2) the granular matrix is so heavily loaded with deposits of reaction product that the core region appears in negative image when in the plane of the section (lower half of the field). Other notations as for Fig. 1. $\times$ 72,000.
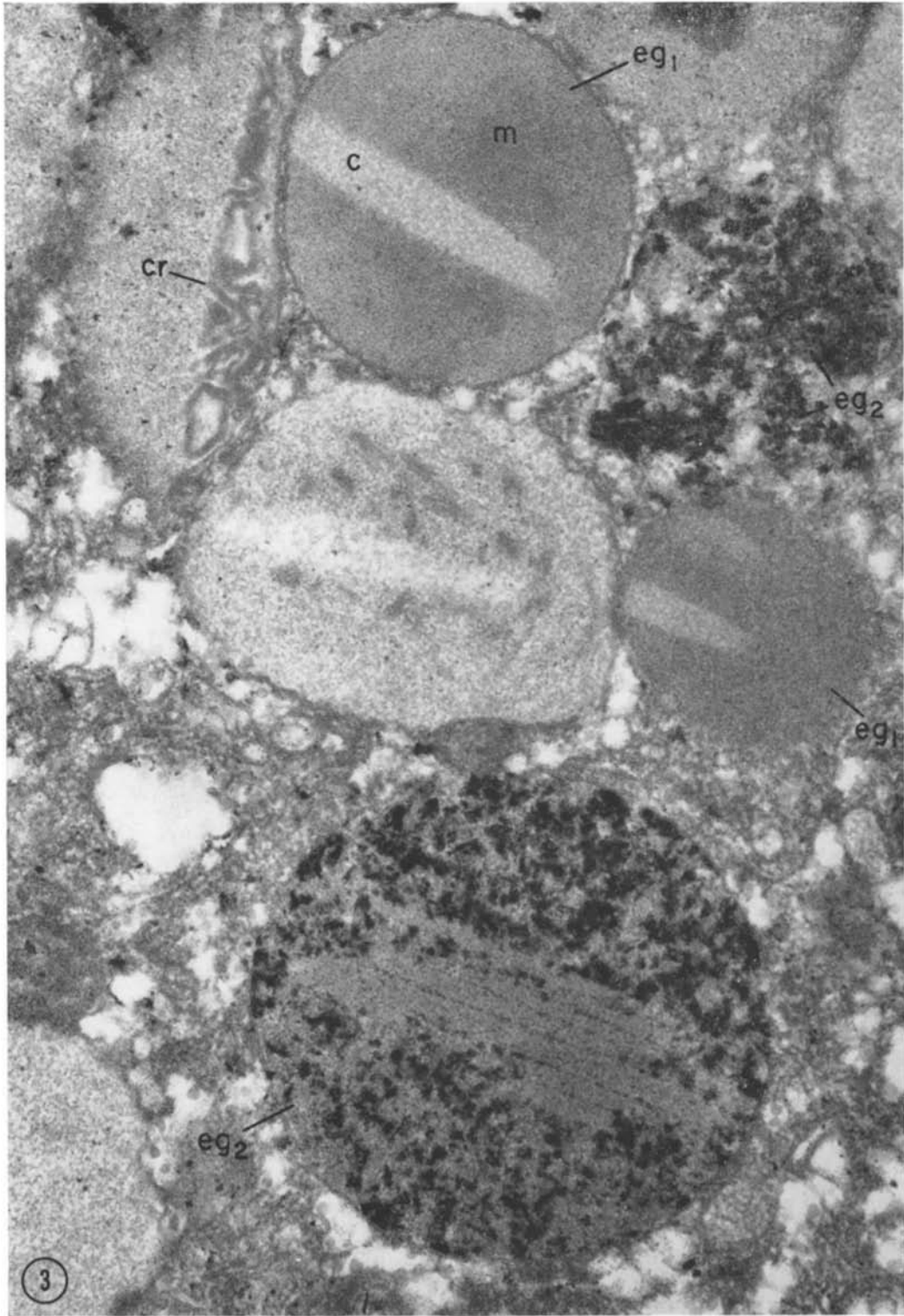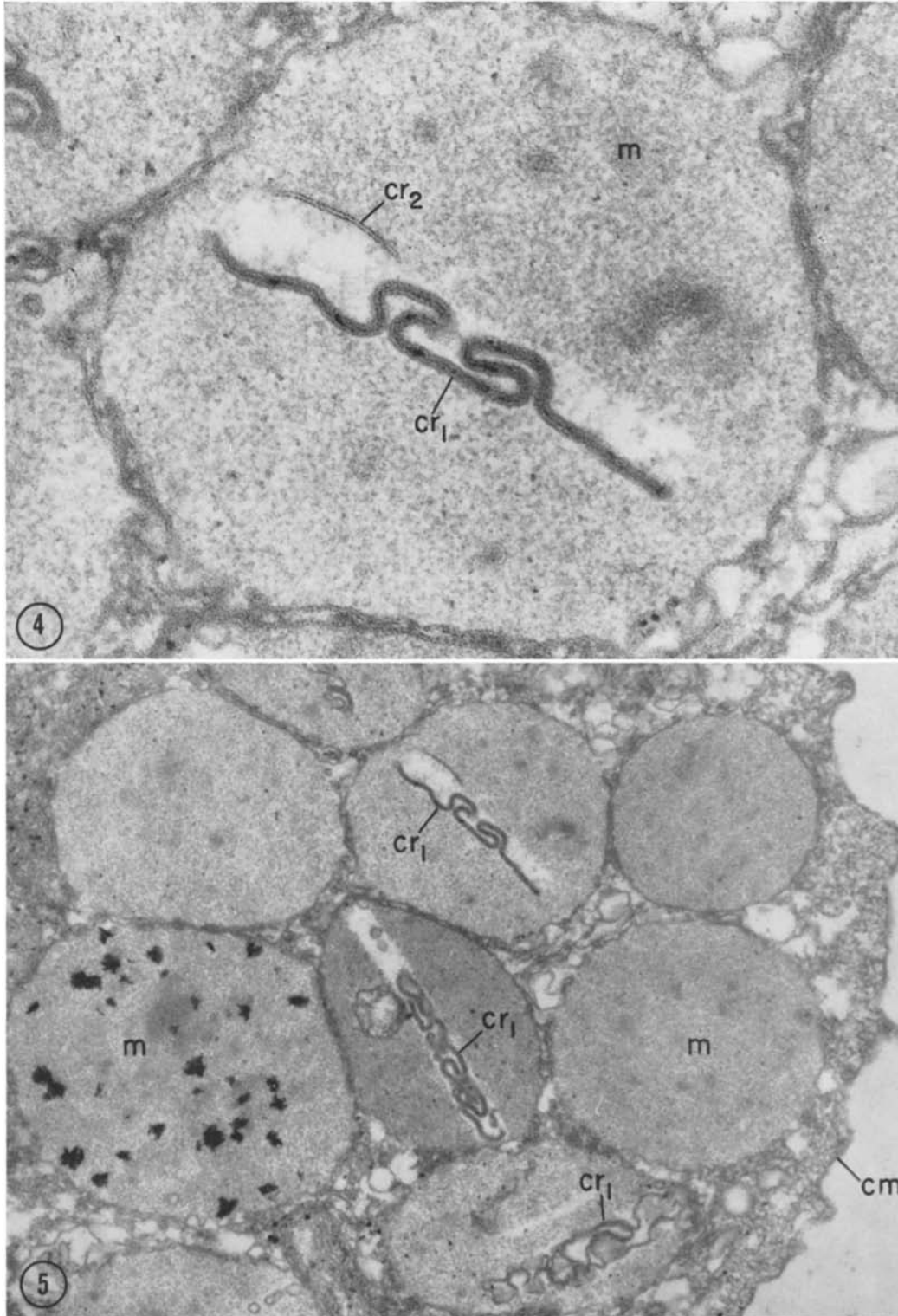
FIGURES 4 and 5 illustrate the wavy sheets of layered material (cr_1) and the structures reminiscent of fragments of unit membrane (cr_2) which appear in the core region during the extraction of the crystalline core. Other notations as in Fig. 1. Fig. 4, $\times$ 120,000; Fig. 5, $\times$ 44,000.
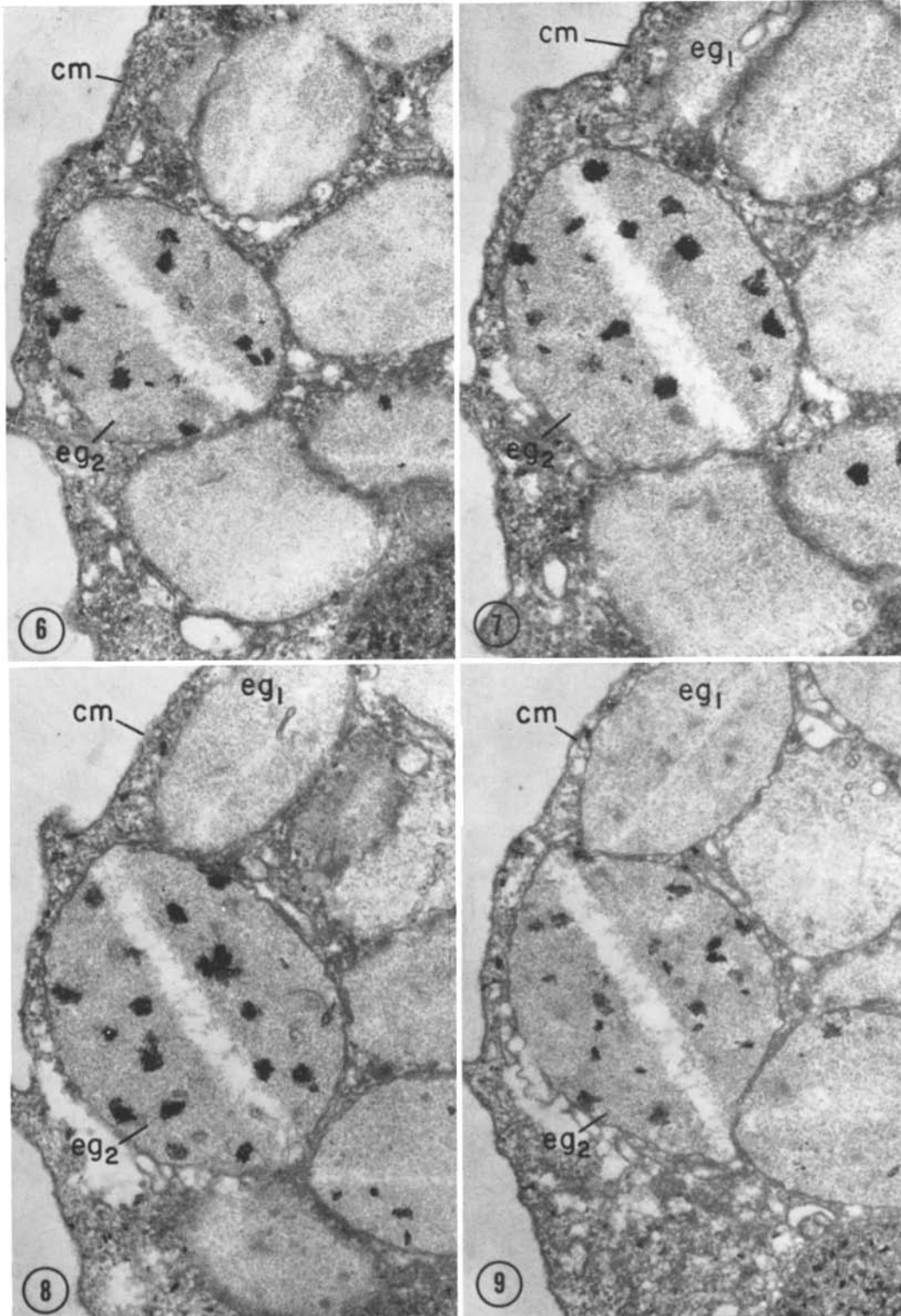
FIGURES 6—9 Serial but nonconsecutive sections through a group of eosinophil granules to show that reactivity (eg_2) or nonreactivity (eg_1) of the granular matrix applies throughout any given granule in the group. Other notations as in Fig. 1. $\times$ 40,000.
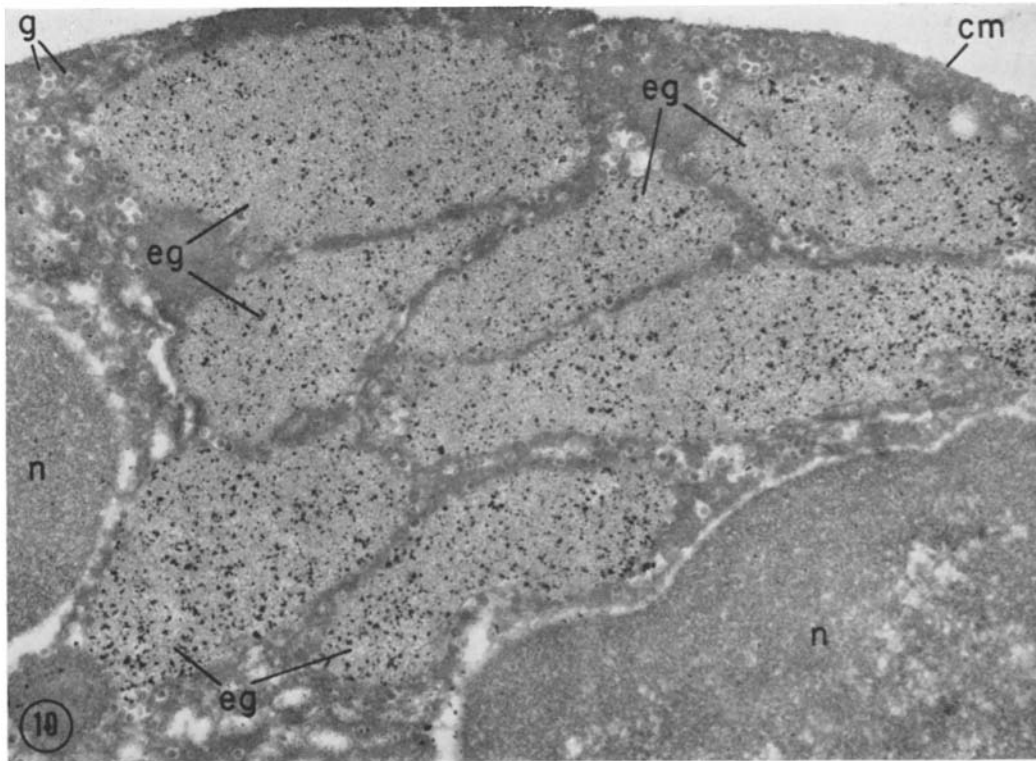
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FIGURE 10 Relatively large field in an eosinophil granulocyte fixed in 2% glutaraldehyde, pH 7, and kept at pH 2.0 in citrate buffer for 30 min before incubation for acid phosphatase reaction. The eosinophil granules (eg) are all acid phosphatase–positive, but appear distorted and extensively extracted. No difference in density or acid phosphatase reaction persists between the matrix and the core region of the granules. Other notations as in Fig. 1. Unextracted glycogen particles are marked g. × 38,000.

Latent Activity of Acid Phosphatase in Eosinophil Granules

The question arises as to why the granules are not cytochemically reactive when they are morphologically intact. The results indicate that there is some "block" which must be removed or altered before the latent cytochemical reactivity of the enzyme becomes manifest. There are several factors that should be considered in this apparent conversion of an intact phosphatase-negative granule to a slightly disrupted but cytochemically positive granule:

(1) It is possible that the enzyme within the morphologically well preserved granule has undergone total irreversible inhibition, while that in the disrupted granule may be fully preserved. Such differential inactivation seems unlikely, since fixation is expected to occur under uniform conditions

in cell suspensions, and since the fixed suspensions retain so much, i.e., ~90%, of the acid phosphatase activity of the fresh cells.

(2) It may be assumed that the enzyme within the eosinophil granule is fully active, but does not yield a reaction product because the membrane of the granule is impermeable to the substrate. If the heterophil granule is in any way comparable to the eosinophil granule, as it appears to be (1), then it is unlikely that membrane permeability is the complete explanation for the enzyme "block." This is because many heterophil granules were observed (both in intact cells and isolated granule preparations) with a disrupted or missing membrane and yet the reaction product was localized only to the edge of the granule matrix.

(3) Another possible barrier may be represented

by the tightly packed, relatively dehydrated matrix of the granules. Consonant with this assumption is the observation that positive cytochemical reactions were usually found associated with granules having a looser matrix texture than unreactive granules.

(4) A fourth possibility is that an inhibitor or complexing agent is present within the granule and serves to block enzymatic activity until some process (perhaps a drop in pH) dissociates the enzyme-inhibitor complex. The only factor that increased the yield of acid phosphatase–positive granules (both eosinophil and heterophil) was prolonged exposure to acid pH after glutaraldehyde fixation. Since it is known that heterophil granules release their enzyme in active form at low pH (2), it is possible that low pH may normally be responsible for enzyme "activation" in vivo.

Acid Phosphatase Localization in Heterophils

Since our preparations were mixed populations of eosinophil and heterophil leukocytes, a number of observations were made on the acid phosphatase activity of heterophil granules. These observations are far from conclusive; they are briefly discussed only because the reactions of the two types of granules appear to be generally similar. For instance:

(1) All intact heterophil granules were unreactive while disrupted granules were usually cytochemically positive.

(2) While under standard conditions, only about one in ten or one in twenty granules were reactive, after certain treatments (e.g., exposure to low pH or multiple freezing and thawing) many cells were found which contained up to 60% acid phosphatase–positive granules. It appeared that the difference between eosinophil and heterophil activation depended primarily on the extent of granule damage. A positive reaction was seen when the eosinophil granule was mildly extracted, but it could be detected only when the heterophil granule was extensively disrupted. A few heterophil granules were histochemically positive with a matrix of almost normal texture; usually, most phosphatase-positive granules were so extracted and disrupted as to make the cytochemical local-

ization questionable, primarily because relocation of extracted enzyme could not be ruled out.[4]

Since the percentage of reactive granules seems to increase continuously with the degree of disruption of their matrix, it may be tentatively assumed that heterophil granules are also homogeneous in respect to acid phosphatase content. This assumption is in agreement with the findings of Bainton and Farquhar (24, 25) who noted, in addition, that only the immature or less condensed heterophil granules give an acid phosphatase reaction. One may surmise that in the process of granule maturation some enzyme inhibitor or complexing agent is added to the granule matrix. If the situation is comparable in eosinophils, immaturity may explain some apparent discrepancies in the literature (19–21), as well as the occasional finding of an intense reaction in granules that show little structural damage (see Fig. 3).

Depending on the extent and quality of fixation, the cytochemical reaction obtained for acid phosphatase in eosinophils or heterophils will be irregular; well fixed and well preserved granules, even if their enzyme is not irreversibly inhibited, will not be marked by any reaction product deposits. This may explain the irregularity of the histochemical reaction for acid phosphatase in leukocytes, an irregularity first noted by Rabinovich and Andreucci (21). Irregularity of reaction may also be seen in the results of Wetzel et al. (26) in the rabbit heterophil and in the results of Weber (27) in the Xenopus larva macrophage; some of the granules of these cells reacted strongly for acid phosphatase while other granules in the same cell did not react at all. A similar nonuniform cytochemical reactivity from granule to granule has been observed by Seligman et al. (28) for the lipase reaction of pancreatic zymogen granules.

[4] Relocation may occur in cells despite the fact that no adsorptive relocation was observed in control experiments in which a millionfold excess of soluble enzyme was added to isolated granules inactivated by heating.

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