THE STRUCTURE OF EOSINOPHIL LEUKOCYTE GRANULES IN RODENTS AND IN MAN

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ABSTRACT

The structure of the specific granules of eosinophil leukocytes has been studied by electron microscopy in sections of tissues, buffy coats, and sediments of peritoneal washings of rats, mice, guinea pigs, and men. The core of eosinophil granules is a crystal which has a cubic lattice with a repeat of ~30 A in rodents and ~40 A in man. The chemical composition of the core is discussed in connection with recent cell fractionation studies, and the hypothesis that the core is a crystal of peroxidase is considered.

The characteristic structure of the specific granules of eosinophil leukocytes, hereafter referred to as eosinophil granules, was recognized in several vertebrate species (1–5) and in man (6–9) soon after the development of microtomy techniques suitable for electron microscopy. Early observations were summarized by Low and Freeman (10) and by Bessis and Thiéry (11). The granules were generally described as biconvex discs bounded by a membrane and containing, usually in their equatorial region, one or several inclusions of a dense, "crystalloid" material embedded in a less dense matrix.[1] The fine structure of the internal dense inclusion, or core, has received little attention. Sheldon and Zetterquist (12) mentioned a lamellated structure of alternating dense (50 A) and light lines in the granules of certain mouse leukocytes, later recognized as eosinophils (13). Bargmann and Knoop (13) described the core of eosinophil granules in the cat as a hollow cylinder whose wall consisted of 23 concentric, alternatively dense and light lamellae, each measuring ~45 A across. Osako (14) confirmed these findings, but failed to detect a periodic structure in the eosinophil granule core of a whole spectrum of other vertebrate species examined. Later on, Bargmann and Knoop (15) resumed the work on cat eosinophils, revised the figure given for the thickness of the light lamellae to ~35 A, and claimed to have resolved the dense lamellae into three sublayers of ~15 A each.

In this paper we present some new observations on the structure of the crystalline core of eosinophil granules in man and in a number of laboratory rodents, i.e., the mouse, rat, and guinea pig.

MATERIALS AND METHODS

Bone marrow from the femora of mice, rats, and guinea pigs was removed under ether anesthesia and fixed at ~ 4°C in: (*a*) 1% OsO_4 in 0.1 M Na-phosphate or Na-K-phosphate buffer (pH 7.4) for 2 hr, or (*b*) 2, 3, or 6% glutaraldehyde in 0.05 to 0.1 M Na-phosphate, Na-K-phosphate or Na-cacodylate buffer (final pH 7.2) for 2 to 6 hr. Glutaraldehyde-fixed specimens were washed overnight in 0.1 M buffer containing 0.22 M sucrose, or in 0.15 M buffer and postfixed in 1 or 2% OsO_4 in 0.1 M Na-K-phosphate buffer (pH 7.4) for 2 hr. Sediments of washings from the peritoneal cavity of rats, and pieces of liver and intestinal wall (jejunum) of rats and guinea pigs were also fixed as above. Human eosinophil leukocytes

[1] In the literature (see references 13, 15), the dense inclusion and the matrix of the granule are sometimes referred to as "internum" and "externum," respectively.

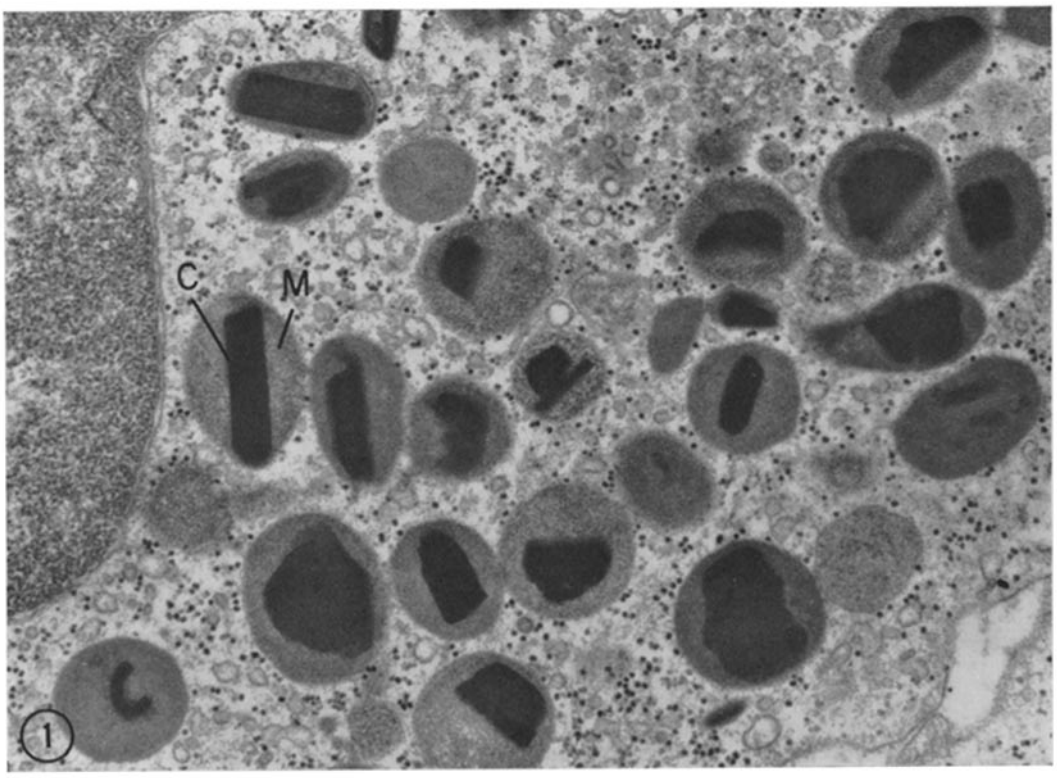


FIGURE 1 Specific granules in a human eosinophil leukocyte showing dense cores (*C*) of various shapes embedded in a less dense matrix (*M*). × 28,000.

were studied in: (*a*) a liver biopsy (41 yr old male icteric patient), and (*b*) peripheral blood (23 yr old normal female, 57 yr old male with allergic eosinophilia, and normal 37 yr old female suffering from Asthma bronchiale). In the latter three cases, 10 to 20 ml of blood were withdrawn by venous puncture, 0.2 ml of Liquemin Roche (heparin) was added, and the blood was spun at 4000 RPM for 5 min. The buffy coats and the liver biopsy were fixed in OsO_4 as above. Some specimens were stained in block, before dehydration, in 0.5% uranyl acetate in acetate-Veronal buffer pH 5.0 (16). All specimens were dehydrated in ethanol and embedded in Epon 812 (17). Thin sections were cut on LKB ultrotomes or Porter-Blum microtomes provided with diamond knives (duPont de Nemours, or R. E. Sugg, Wilmington, Delaware). They were picked up on naked grids and stabilized by a layer of carbon (18), or collected on grids covered with a collodium film reinforced by carbon. The mounted sections were doubly stained with 2% uranyl acetate (19) for 30 min, followed by lead citrate (20) for 10 min, and examined in three different Siemens Elmiskop I electron microscopes operated at 80 kv with double condensors and 50 μ molybdenum apertures in the objective. A few sections were examined by using pointed filament cathodes and an anticontamination device. Micrographs were taken at magnifications ranging from 4000 to 80,000. The instrumental magnification was calibrated with a grating or a cross-linked grating replica provided with 2160 lines per millimeter (E. Fullam, Schenectady, New York). Measurements were made either on prints magnified 3 to 6 times from plates taken at a magnification of 80,000, or on densitometric tracings made directly from negatives taken at a magnification of $\sim$ 40,000 and enlarged 50 times in a Joyce double beam recording microdensitometer (Fig. 12).

OBSERVATIONS

In sections of mouse, rat, guinea pig, and man leukocytes, the eosinophil granules appear as elliptical or, more rarely, circular profiles which measure $\sim$0.3 to 1.2 μ in diameter, are limited by a well defined unit membrane, $\sim$95 A thick, and contain an amorphous or finely granular matrix. Embedded in this matrix is a dense core which appears as a band of varied width usually disposed

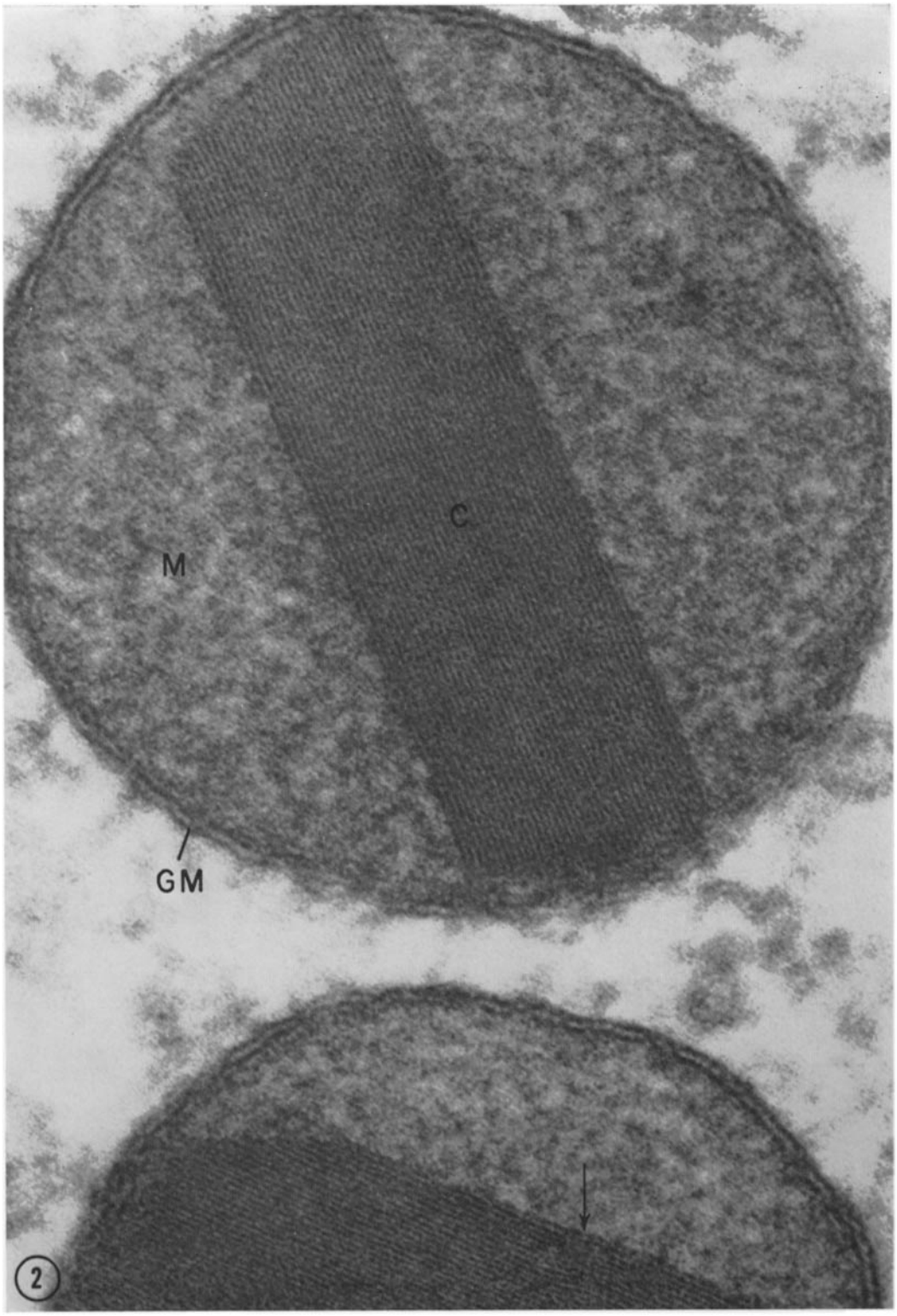


FIGURE 2 Two eosinophil granules in a rat leukocyte infiltrating the intestinal mucosa. The granules are bounded by a single unit-membrane (*GM*). The matrix (*M*) consists of a granular material of medium density. In both granules the cores (*C*) show a regular longitudinal array of dense bands. The arrow points to a slight displacement in ~ 10 consecutive bands. Tissue stained in block with uranyl acetate (16). × 375,000.

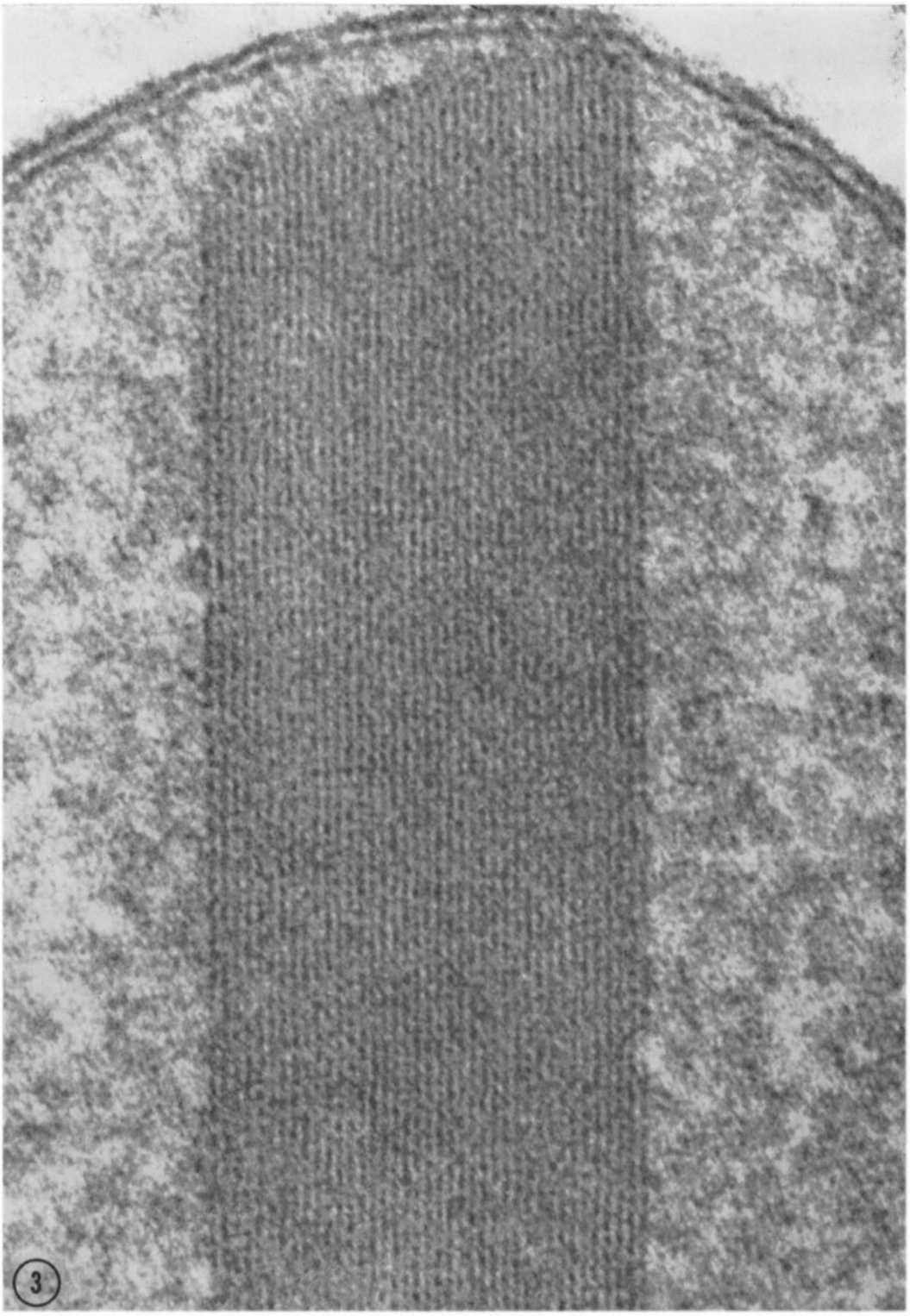


FIGURE 3 Same material as in Fig. 2. At this magnification the bands appear as linear series dots. × 630,000.

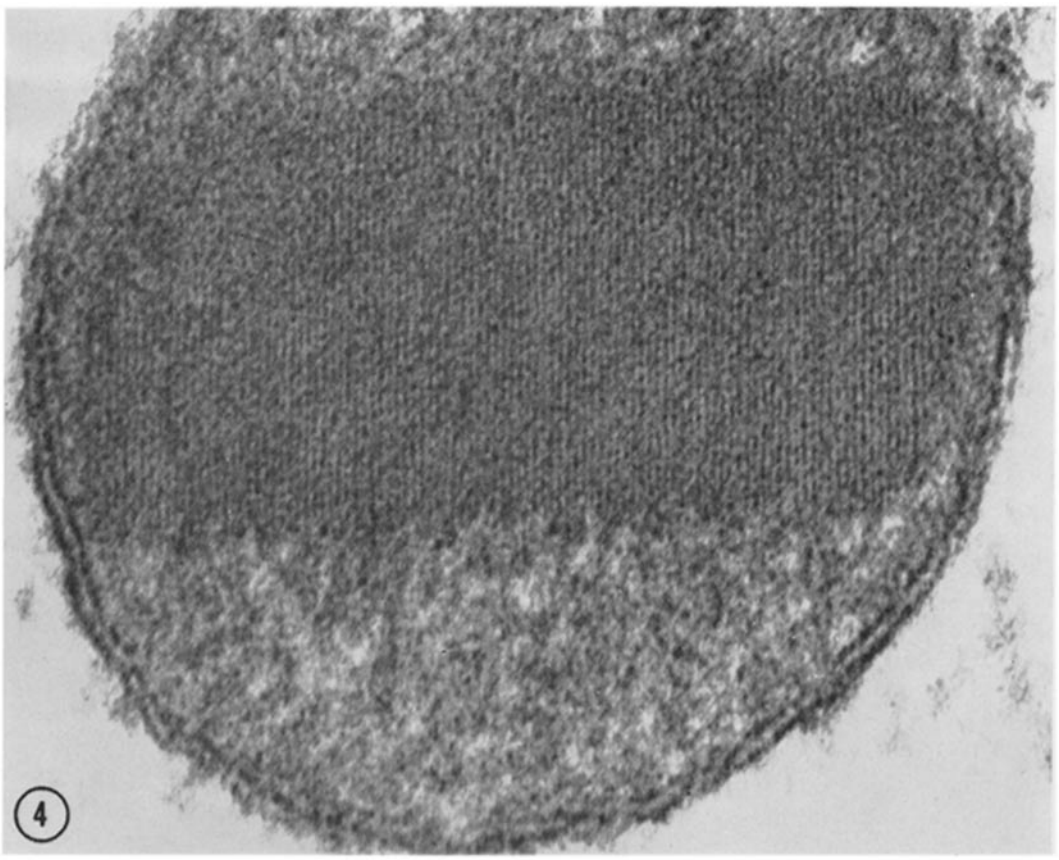


FIGURE 4 Same material as in Fig. 2. In this section the bands appear to run parallel to the short axis of the core. × 440,000.

parallel to the long axis of the granule profile. In human eosinophil granules, the shape of the cores is more varied than in rodents: in addition to rectangular bands, nearly square plates, slender needles, or irregular forms are encountered (Fig. 1). In the eosinophil granules of the guinea pig, the band is occasionally bisected by a narrow (~450 A) strip whose density approaches that of the granule matrix. Sometimes, the central strip can be followed from one end through part of the core only, presumably because it passes out of the plane of the section. In three dimensions, the core could be a plate or a hollow cylinder, as already postulated by Bargmann and Knoop (13) for the "internum" of cat eosinophil granules. Since longitudinal splits were only occasionally observed, and cylindrical cross-sections were never encountered, we assume that, in the species examined, the core of the eosinophil granules is a plate occasionally split parallel to its broad surfaces. Most of the granules contain a single core, but sometimes two to four cores lie parallel to one another or at various angles within the matrix of one granule, especially in human material.

In all species examined, a highly ordered structure, visible on the electron microscope screen at direct electronic magnifications of at least 40,000 times,[2] could be detected in the cores of about one-third of the granules in a given eosinophil leukocyte profile. It consists of a very regular array of alternating and equidistant dense and light bands (Figs. 2 and 7) of which the former frequently appear, at least in part, as linear series of dense dots (Figs. 3 and 10). Usually, the bands run parallel to the long axis of the core from one end to

[2] With a 10× viewing microscope, the image is actually seen magnified ~ 400,000 times.

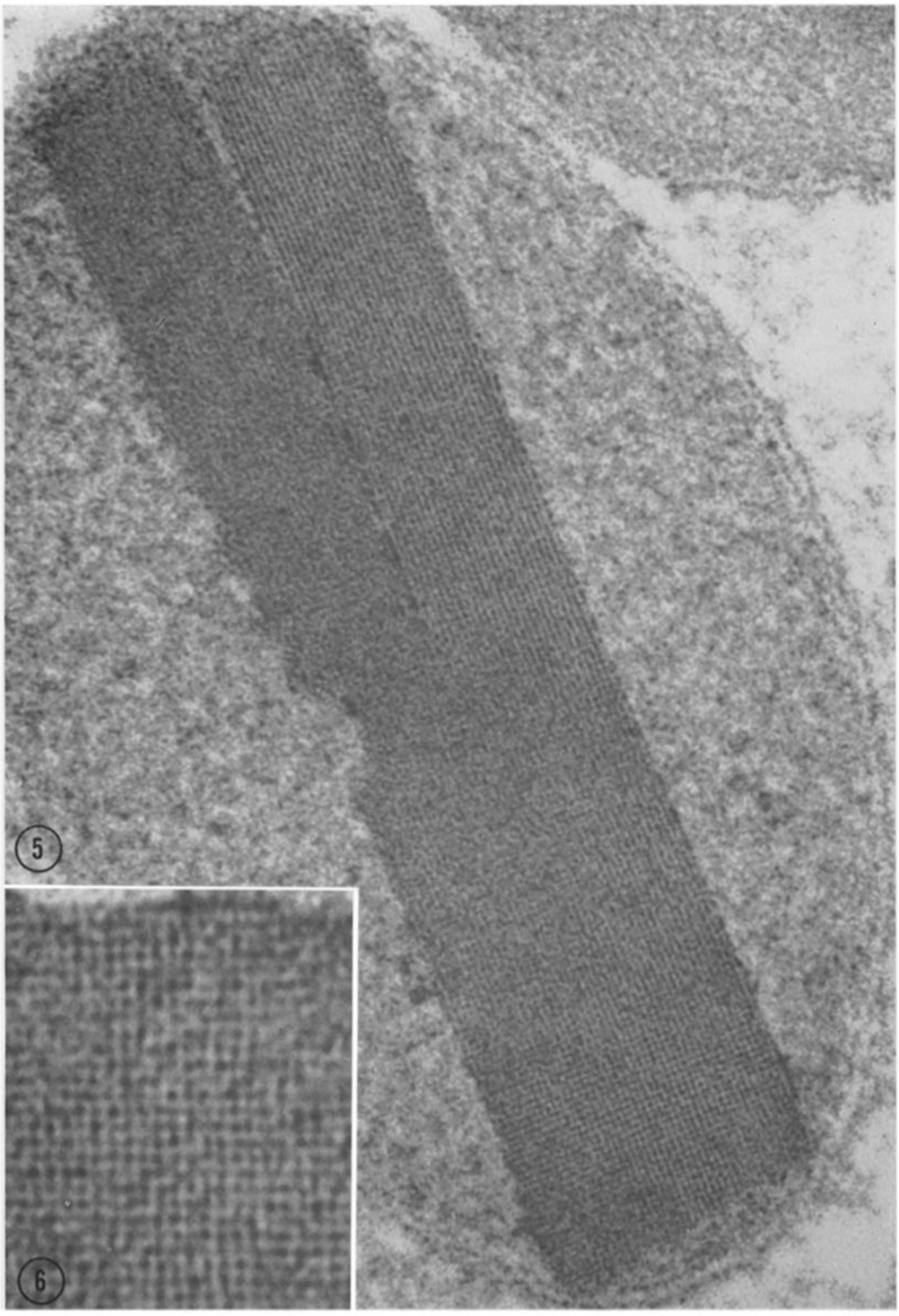
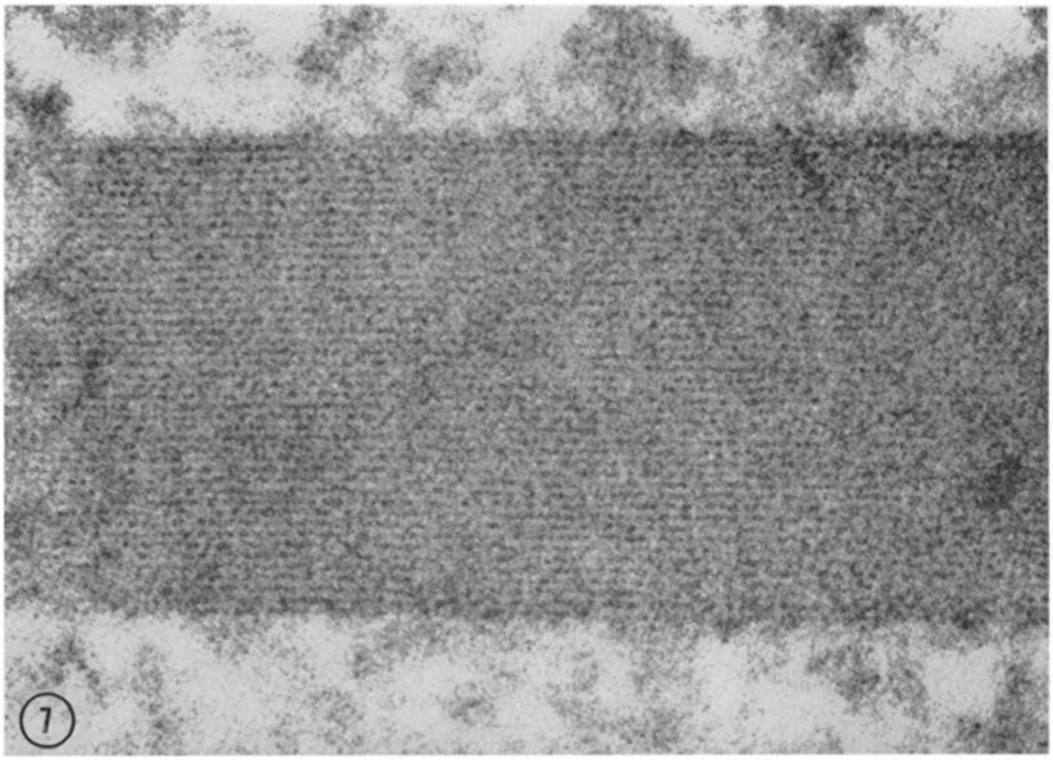


FIGURE 5 This rat eosinophil granule has a partially split core which shows a longitudinal array at one end, and a square lattice at the other, in addition to areas of indistinct structure. × 344,000.

FIGURE 6 Higher magnification showing in greater detail the square lattice seen in Fig. 5. × 800,000.



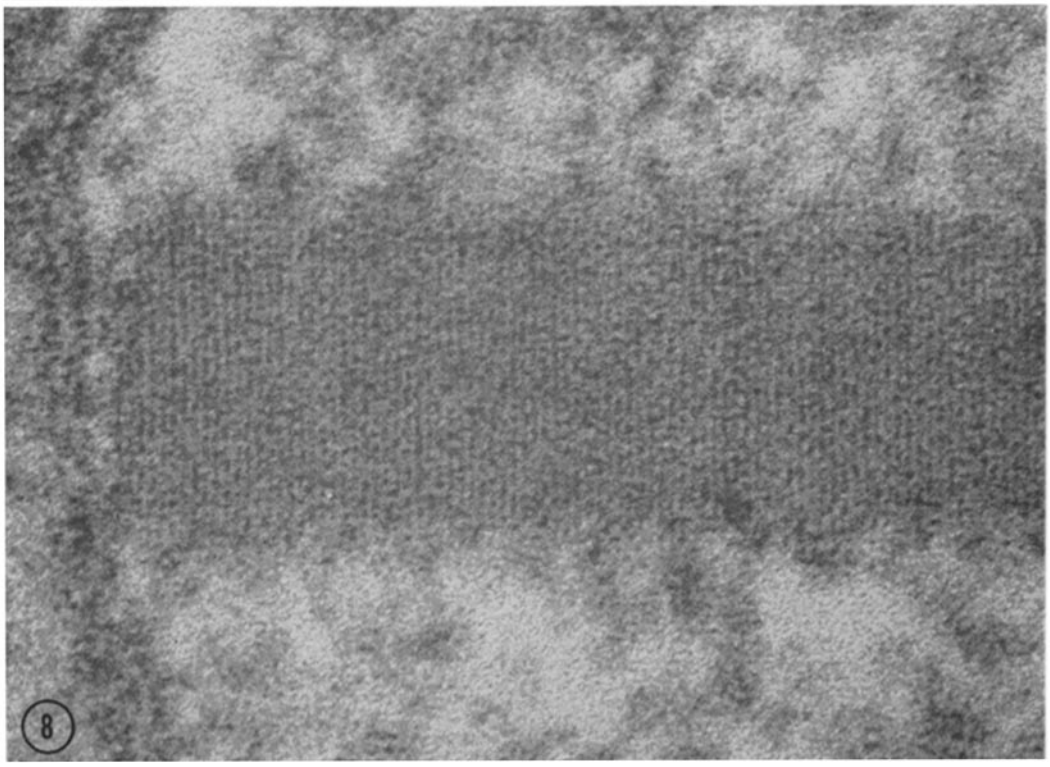


FIGURE 7 Part of a core of an eosinophil granule in a Swiss mouse myelocyte showing the longitudinal repeat. × 470,000.

FIGURE 8 Same material as in Fig. 7, showing the transverse repeat of the core. × 470,000.

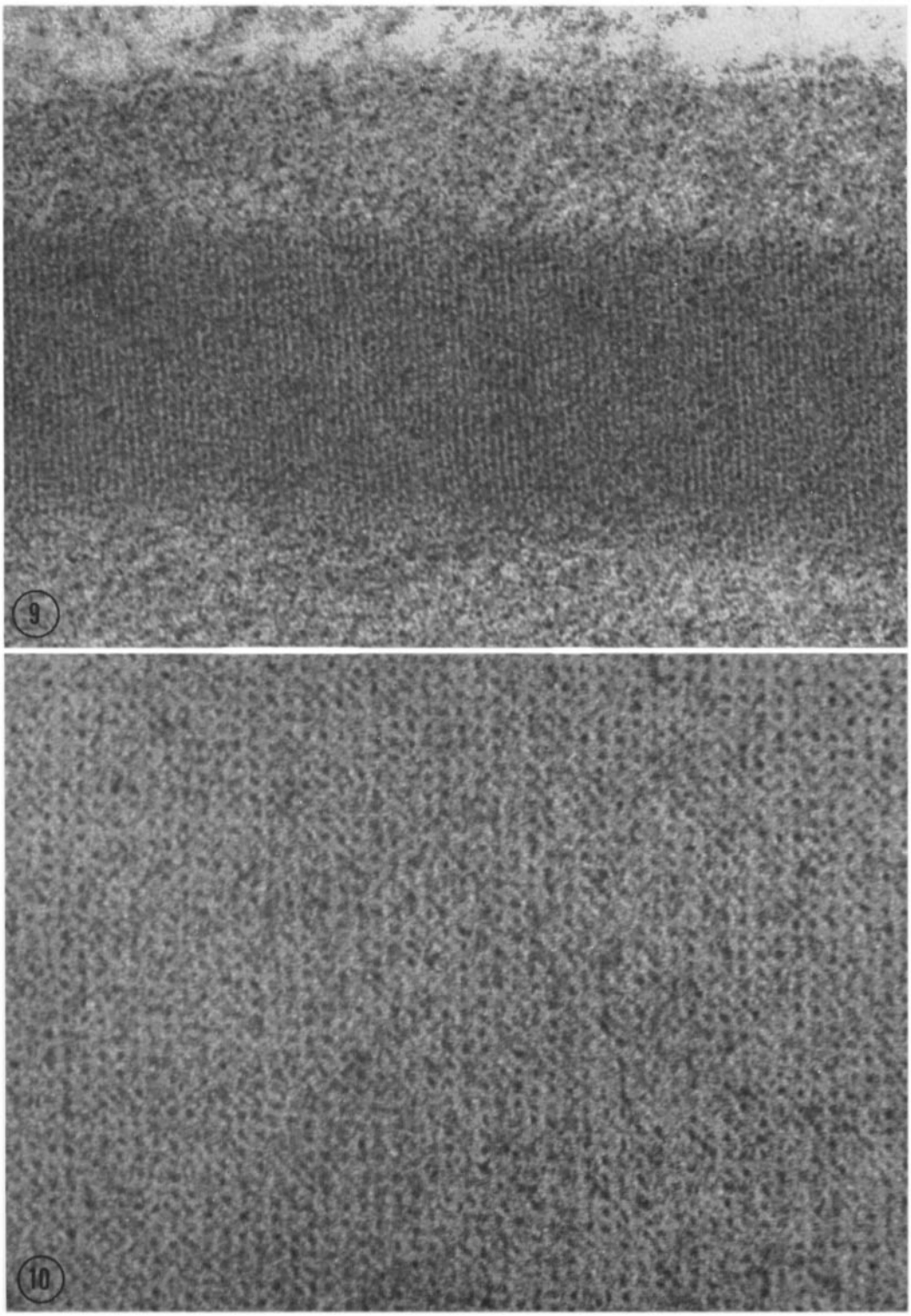


FIGURE 9 Transverse repeat in the core of a human eosinophil granule. The bands appear as linear arrays of dots. × 320,000.

FIGURE 10 This small field in the core of a human eosinophil granule shows a square array of dots, presumably because in this case the plane of the section is nearly parallel to one plane (100) or (010) of the cubic lattice of the core crystal. × 640,000.

TABLE I

Spacings in the Crystalline Lattice of Eosinophil Granule Core

The center-to-center distance between consecutive dense or light bands was measured on either (*a*) prints of negatives taken at a magnification of 80,000 and enlarged 3 times photographically (10 to 46 repeats being included in one measurement), or (*b*) densitometric tracings of negatives taken at a magnification of 40,000 (Fig. 12), and enlarged 50 times in the densitometer. In the latter case, 17 to 49 repeats were included in one tracing.

Species	Tissue	Type of measurement	No. of prints	No. of repeats	Repeat mean + standard error		Standard deviation
					longitudinal	transverse	
Rat	Bone marrow	Micrographs	39	935	29.97 ± 0.28	—	1.77
" *	Jejunum	Densitometer tracings	10	318	26.99	—	
" *	"	" "	4	165	—	25.47	
Guinea pig	Bone marrow	Micrographs	18	351	30.75 ± 0.24	—	1.02
"	Jejunum	Densitometer tracings	6	209	31.42	—	
Mouse	Bone marrow	" "	5		27.63	—	
"	" "	" "	2		—	28.75	
Human	Buffy coat of peripheral blood	Micrographs	25	488	40.7 ± 0.31	—	0.95
"	" "	"	6	82	—	41.1 ± 0.26	0.63
"	" "	"	5	116	no preferential direction 28.0 ± 0.13		0.29

* Specimens treated with 0.5% uranyl acetate before dehydration.

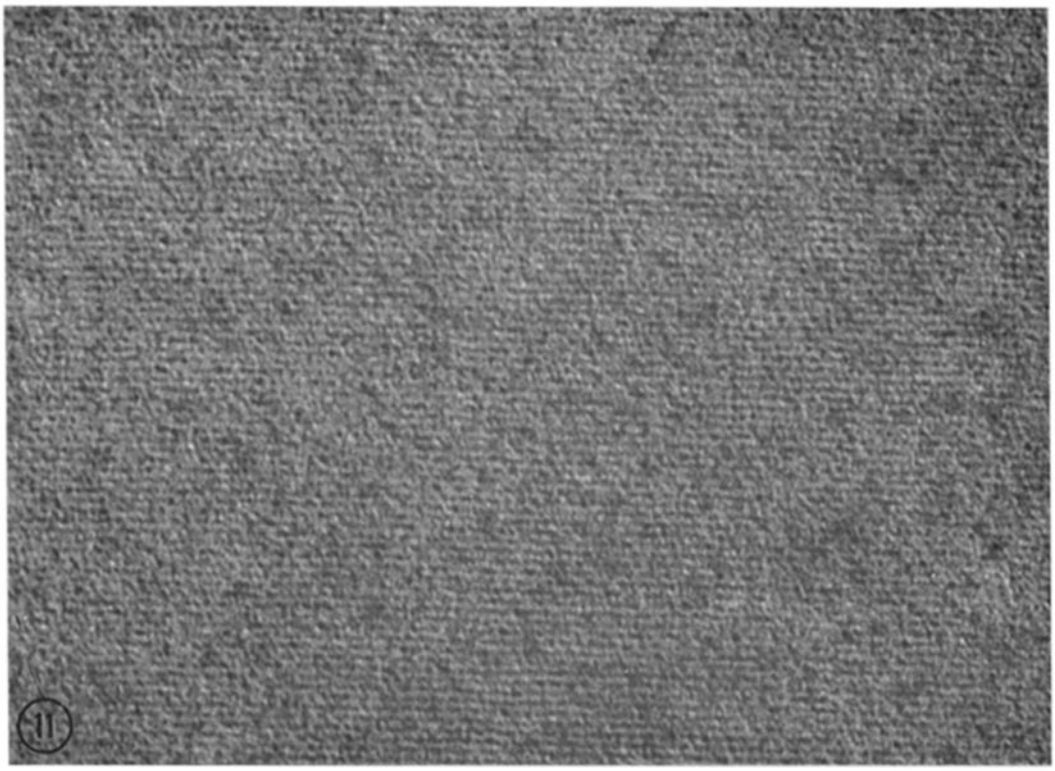


FIGURE 11 Small field in the core of a human eosinophil granule showing the 28 A repeat occasionally seen in sections through the core crystal. Geometric considerations explaining the occurrence of 2 repeats (the usual ~40 A and the less frequent 28 A) are given in the Discussion. × 480,000.

the other (Figs. 2, 3, and 7); less frequently, they run parallel to the short axis (Figs. 4, 8, and 9); and occasionally they form a square lattice by running in both directions (Figs. 5, 6, and 10). The structure of the core is not always faultless along its entire length: the longitudinal arrays may be disturbed by the displacement of one or more bands (Fig. 2), or become locally indistinct, being apparently replaced by more or less homogenous areas beyond which the regular arrays reappear. Finally, a square lattice may be found in one part of the core, and a linear array in the rest (Figs. 5 and 6).

Table I gives the measured spacings in the crystalline lattice of the core and shows that, in conventional preparations, the longitudinal repeat is ~30 A in the rat, ~31 A in the guinea pig, ~28 A in the mouse, and ~40 A in man.[3] In a few cores of human eosinophil granules, a band pattern with a smaller repeat of ~28 A was observed (Fig. 11). The two repeats (40 and 28 A) were found in different granule cores in the same eosinophil leukocyte. In uranyl-treated rat leukocytes, the longitudinal repeat (Figs. 2 and 3) is smaller by ~10% (27 A). The transverse repeat in the rat (Fig. 4), mouse, and man is nearly equal to the longitudinal spacing, the differences being $\lesssim 5\%$. Each dense band or light interspace in the lattice measures accordingly ~14 to 15 A across in rodents and ~20 A in man (see Discussion).

The features described apply to eosinophil granules in all locations mentioned, i.e., bone marrow, peripheral blood, peritoneal fluid, liver, and lamina propria of the intestinal mucosa. In the guinea pig and in man, the granules are

[3] Preliminary observations on the eosinophil granules of the rabbit also showed an array of dense and light bands (mean center-to-center distance 30.1 ± 0.42 A; s = 1.45) in the very slender and needle-shaped cores.

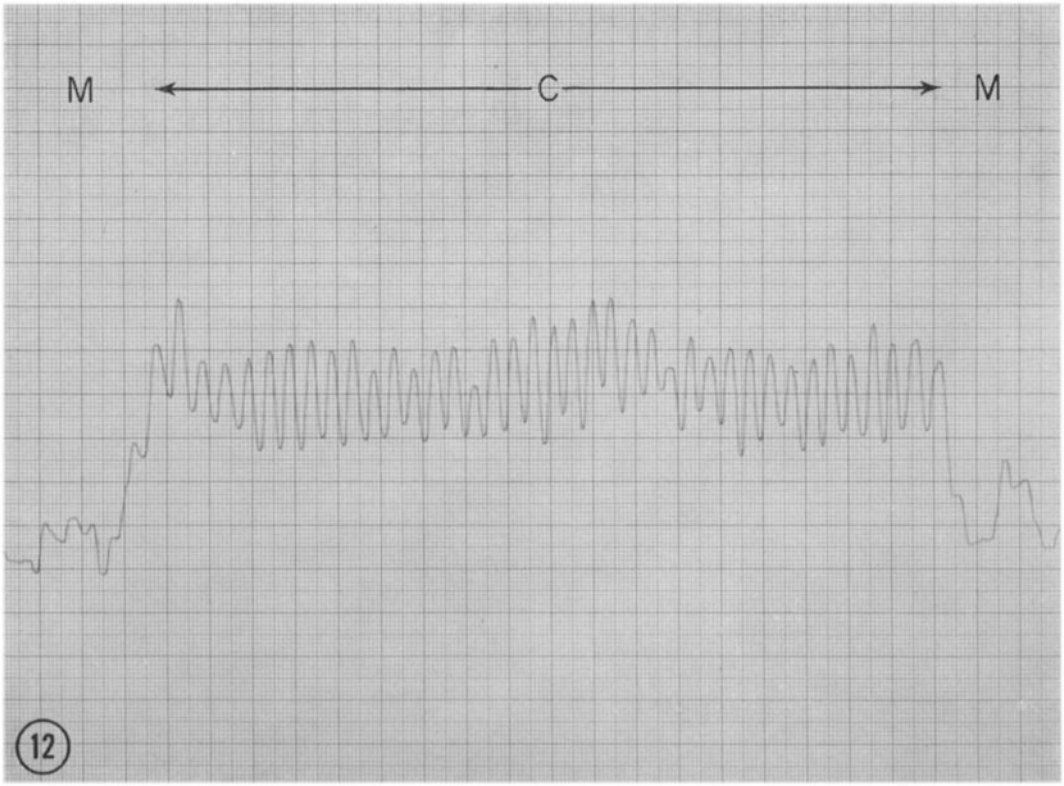


FIGURE 12 Densitometric tracing of the entire width of a core (*C*), showing 39 consecutive bands of equivalent dimensions and comparable density.

more irregular in shape, and many of them have more than one core; in addition, the matrix frequently contains small membrane-bounded vesicles and other polymorphic inclusions.

DISCUSSION

Our observations show that parallel bands or a square lattice can be demonstrated in the case of eosinophil granules of rats, mice, guinea pigs, and men. In all cases, the repeat is ~30 A in rodents, and ~40 A in men; in the latter, an additional 28 A repeat is occasionally encountered.

The regular patterns observed cannot be interpreted as real images of a structure in which contrast is provided by scattering (mass-thickness contrast) because the sections are relatively thick, namely 200 to 400 A (22), and are penetrated by stain to a depth of at least 400 A (22). Assuming a section thickness of 200 A and a molecular diameter of ~15 A, a tilt of ~4° of the (100) or (010) lattice plane on the optical axis would be sufficient to obscure the banded patterns which, accordingly, should be encountered with a frequency $\lesssim 2\%$. The corresponding figure would be $\lesssim 1\%$ for sections 400 A thick. In our observations, banded patterns were recorded at a considerably higher frequency.

The micrographs obtained can be interpreted as interference patterns of crystals (21) with a cubic lattice. In this situation, the spacings in the micrographs correspond to true spacings in the lattice, but the thickness of the dense bands and the diameter of the dense dots in the image do not represent the dimensions of the molecules of the crystal.

The presence of square lattices and of bands parallel to the long as well as to the short axis of the cores, and the fact that the repeats are nearly equal in two directions in the plane of the section, suggest that the cores are crystals with a cubic lattice. This assumption is compatible with the observation of two different repeats in the human

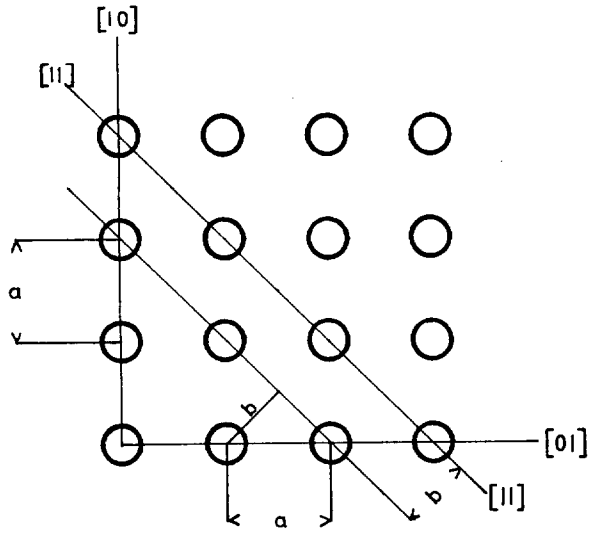


FIGURE 13 Diagram of the crystalline lattice of the human eosinophil core. The repeat b observed in the (*11*) direction is smaller than the repeat a seen in the (*10*) and (*01*) directions. The calculated value of this repeat ($40/\sqrt{2} = 28.2$ A) agrees well with the smaller repeat (28 A) found in some micrographs (see Fig. 11).

material. As shown in the diagram (Fig. 13) the repeat a (40 A) can be obtained in the (*10*) and (*01*) directions, and the repeat $b = a/\sqrt{2} = 28.2$ A in the (*11*) direction. The ratio $a/b = \sqrt{2}$ fits well with the ratio $40/28 = 1.43 \approx \sqrt{2}$ measured in the human material.

From these observations it appears that in the species examined the structure of the core is quite different from that described by Bargmann and Knoop (15) in cat eosinophil granules; the core of the latter is a lamellar structure with a periodicity 2 to 3 times larger than those found in rodents and man.

The chemical composition of these crystals remains unknown. Histochemical observations show that the "core" of the eosinophil granules contains a protein rich in arginine, positively charged at physiological pH's (23) and responsible for the staining with acidic dyes (24, 25), whereas the "cortex" is PAS-positive (24) and rich in phospholipids (25–27). Yet there is no clear correspondence between the "core" (or "body") and "cortex," distinguished in these tests by light microscopy, and the structural elements, i.e. crystalline core, matrix and membrane, resolved by electron microscopy. Half the total cell protein of rat eosinophils is localized in the granules, and only about one-fourth of it is soluble after freezing and thawing (28). These data imply a high concentration of protein in the eosinophil granule and suggest that the crystalline core consists of protein. The dimensions of the lattice are compatible with this assumption (29).

Some of the proteins of isolated rat and horse eosinophil granules have been identified as lysosomal enzymes by Archer and Hirsch (28) who showed, in addition, that cathepsin, ribonuclease, arylsulfatase, and β-glucuronidase are almost fully released in soluble form upon disruption of the granules, while acid and alkaline phosphatases are only partly solubilized. Under these conditions, the entire peroxidase activity remains with the insoluble granule debris from which it can be extracted with weak acid (28).[4] A hemoprotein with the enzymic and spectral properties of a peroxidase has been extracted and partially purified from rat eosinophil granules, and found to be different from the myeloperoxidase or neutrophil peroxidase present in neutrophil granulocytes (30). Of all the enzymes so far identified in eosinophil granules, the specific peroxidase seems to be the most likely candidate for the protein of the crystalline core. In the future, this hypothesis could be put to test by subfractionating eosinophil granules and by using the parameters of the lattice here described to correlate the presence of the crystals with the activities of the subfractions.

It is noteworthy that, although the eosinophil granule contains a large number of different enzymes, only one of them seems to crystallize. A comparable situation has been found and studied in hepatic microbodies (31) in which the crystal seems to consist of urate oxidase (32). A potentially similar situation exists in the specific granules of basophil leukocytes in which a crystalline content has been recently detected (33, 34).

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[4] In cytochemical tests for acid phosphatase, which are carried out at pH 5.0, the crystalline core of rat and man eosinophil granules is partly extracted during the incubation. The reaction product is preferentially localized in the matrix of the granules (35–37).

REFERENCES

1. PEASE, D. C., Marrow cells seen with the electron microscope after ultrathin sectioning, *Rev. Hematol.*, 1955, **10,** 300.
2. PEASE, D. C., An electron microscopic study of red bone marrow, *Blood*, 1956, **11,** 501.
3. BERNHARD, W., HAGUENAU, F., and LEPLUS, R., Coupes ultrafines d'éléments sanguins et de ganglions lymphatiques étudiées au microscope électronique, *Rev. Hematol.*, 1955, **10,** 267.
4. BESSIS, M., Structures cellulaires découvertes par le microscope électronique dans les leucocytes, *Rev. Hematol.*, 1956, **11,** 295.
5. BARGMANN, W., and KNOOP, A., Über das elektronenmikroskopische Bild des eosinophilen Granulozyten, *Z. Zellforsch. u. Mikr. Anat.*, 1956, **44,** 282.
6. BERNHARD, W., and LEPLUS, R., La méthode des coupes ultrafines et son application à l'étude de l'ultrastructure des cellules sanguines, *Schweiz. med. Woch.*, 1955, **85,** 897.
7. MILLER, F., Elektronenmikroskopische Untersuchungen an weissen Blutzellen, *Verhandl. deutsch. path. Ges.*, 1956, **44,** 208.
8. BRAUNSTEINER, H., and PAKESCH, F., Elektronenmikroskopische Untersuchungen der Granula menschlicher Leukozyten, *Acta Haematol.*, 1957, **17,** 136.
9. GOODMAN, J. R., REILLY, E. B., and MOORE, R. E., Electron microscopy of formed elements of normal human blood, *Blood*, 1957, **12,** 428.
10. LOW, F. N., and FREEMAN, J. A., Electron Microscopic Atlas of Normal and Leukemic Human Blood, New York, McGraw-Hill Book Company, 1958.
11. BESSIS, M., and THIÉRY, J. P., Electron microscopy of human white blood cells and their stem cells, *Internat. Rev. Cytol.*, 1961, **12,** 199.
12. SHELDON, H., and ZETTERQUIST, H., Internal ultrastructure in granules of white blood cells of the mouse, *Bull. Johns Hopkins Hosp.*, 1955, **96,** 135.
13. BARGMANN, W., and KNOOP, A., Über das elektronenmikroskopische Bild des eosinophilen Granulozyten II, *Z. Zellforsch. u. Mikr. Anat.*, 1956, **44,** 692.
14. OSAKO, R., An electron microscopic observation on the specific granules of eosinophil leukocytes of vertebrates, *Acta Haematol. Japon.*, 1959, **22,** 134.
15. BARGMANN, W., and KNOOP, A., Über das Granulum des Eosinophilen, *Z. Zellforsch. u. Mikr. Anat.*, 1958, **48,** 130.
16. FARQUHAR, M. G., and PALADE, G. E., Cell junctions in amphibian skin, *J. Cell Biol.*, 1965, **26,** 263.
17. LUFT, J. L., Improvements in epoxy resin embedding methods, *J. Biophysic. and Biochem. Cytol.*, 1961, **9,** 409.
18. DE HARVEN, E., A new technique for carbon films, *J. Biophysic. and Biochem. Cytol.*, 1958, **4,** 133.
19. WATSON, M. L., Staining of tissue sections for electron microscopy with heavy metals, *J. Biophysic. and Biochem. Cytol.*, 1958, **4,** 475.
20. REYNOLDS, E. S., The use of lead citrate at high pH as an electron-opaque stain in electron microscopy, *J. Cell Biol.*, 1963, **17,** 208.
21. HEIDENREICH, R. D., Fundamentals of Transmission Electron Microscopy, New York, Interscience Publishers, Inc., 1964.
22. PALADE, G. E., and ADINOLFI, L., unpublished observations.
23. ARCHER, R. K., The Eosinophil Leucocytes, Oxford, Blackwell Scientific Publications, 1963.
24. GROSS, R., and GEDIGK, P., Die eosinophilen Leukozyten, *in* Physiologie und Physiopathologie der weissen Blutzellen, (H. Braunsteiner, editor), Stuttgart, Georg Thieme Verlag, 1959.
25. BLOOM, M. L., and WISLOCKI, G. B., The localization of lipids in human blood and bone marrow cells, *Blood*, 1950, **5,** 79.
26. VERCAUTEREN, R., A cytochemical approach to the problem of the significance of blood and tissue eosinophilia, *Enzymologia*, 1950–51, **14,** 340.
27. VERCAUTEREN, R., The properties of the isolated granules from blood eosinophiles, *Enzymologia*, 1953, **16,** 1.
28. ARCHER, G. T., and HIRSCH, J. G., Isolation of granules from eosinophil leukocytes and study of their enzyme content, *J. Exp. Med.*, 1963, **118,** 277.
29. HALL, C., Introduction to Electron Microscopy, New York, McGraw-Hill Book Company, 1953, 421.
30. ARCHER, G. T., AIR, G., JAKAS, M., and MORELL, D. B., Studies on rat eosinophil peroxidase, *Biochim. et Biophysica Acta*, 1965, **99,** 96.
31. BAUDHUIN, P., BEAUFAY, H., and DE DUVE, C., Combined biochemical and morphological study of particulate fractions from rat liver. Analysis of preparations enriched in lysosomes or in particles containing urate oxidase, D-amino oxidase, and catalase, *J. Cell Biol.*, 1965, **26,** 219.
32. HRUBAN, A., and SWIFT, H., Uricase: localization in microbodies, *Science*, 1964, **146,** 1316.
33. WINQVIST, G., The ultrastructure of the gran-

ules of the basophil granulocyte, *Z. Zellforsch. u. Mikr. Anat.*, 1960, **52,** 475.

34. FEDORKO, M. E., and HIRSCH, J. G., Crystalloid structure in granules of guinea pig basophils and human mast cells, *J. Cell Biol.*, 1965, **26,** 973.
35. SEEMAN, P., and PALADE, G. E., Acid phosphatase localization in rabbit eosinophils, submitted for publication.
36. MILLER, F., Electron microscopic cytochemistry of leucocyte granules, Proc. Intern. Conf. Electron Microscopy, 6th Kyoto, 1966, (R. Uyeda, editor), Tokyo, Maruzen Co. Ltd., 1966, **2,** 71.
37. GHIDONI, J. J., and GOLDBERG, A. F., Light and electron microscopic localization of acid phosphatase activity in human eosinophils, *Am. J. Clin. Path.*, 1966, **45,** 402.