J. Anat. (1977), **123**, 3, pp. 553–562
With 7 figures
Printed in Great Britain

Electron microscopic and enzyme cytochemical studies on granules of mature chicken granular leucocytes

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(*Accepted* 27 *April* 1976)

INTRODUCTION

Application of cytochemical techniques to electron microscopy has made possible the demonstration in *mammalian* granulocytes of several types of granules, each with distinctive enzyme activities (Wetzel, Horn & Spicer, 1967; Ackerman, 1968; Bainton & Farquhar, 1968*b*). It was felt that similar studies should be made of *chicken* granulocytes in view of recent evidence from light microscopy that mammalian and chicken granulocytes differ in their histochemical reactions (Caxton-Martins & Daimon, 1976), possibly reflecting differences in the evolution of blood cells in birds and mammals (Ryerson, 1943).

MATERIALS AND METHODS

Twenty male White Leghorn chickens between one and three months old were used. Buffy coat specimens were obtained from cardiac blood by Anderson's method (1965).

Fixation was carried out with 2 % glutaraldehyde in 0·1 M sodium cacodylate-HCl buffer at pH 7·4 for 30 minutes at 4 °C. Specimens were then washed in the same buffer solution containing 7 % sucrose and stored overnight at 4 °C. After that, fresh tissue sections were cut at 40 μm with a Sorvall TC2 tissue sectioner.

Acid phosphatase method. Sections were incubated at pH 5·0 in modified Gomori's medium (Barka & Anderson, 1962) for 1 hour at 37 °C. Control sections were incubated in media to which 0·1 M NaF was added, or from which substrate was omitted.

Peroxidase method. Incubation of sections was carried out at pH 7·6 in Graham & Karnovsky's (1966) medium for 60 minutes at room temperature. Control sections were incubated in media to which 10^{-2} M 3-amin-1,2,4-triazole was added, or diaminobenzidine or H_2O_2 omitted.

Alkaline phosphatase method. Sections were incubated in medium containing lead citrate (Mayahara, Hirano, Saito & Ogawa, 1967) at pH 9·3. Controls were incubated in substrate-free medium.

After incubation sections were washed three times in 0·1 M cacodylate buffer containing 7 % sucrose and re-fixed in buffered 1 % osmium tetroxide (Caulfield, 1957) for 1 hour at 4 °C. They were then dehydrated through graded ethanols and

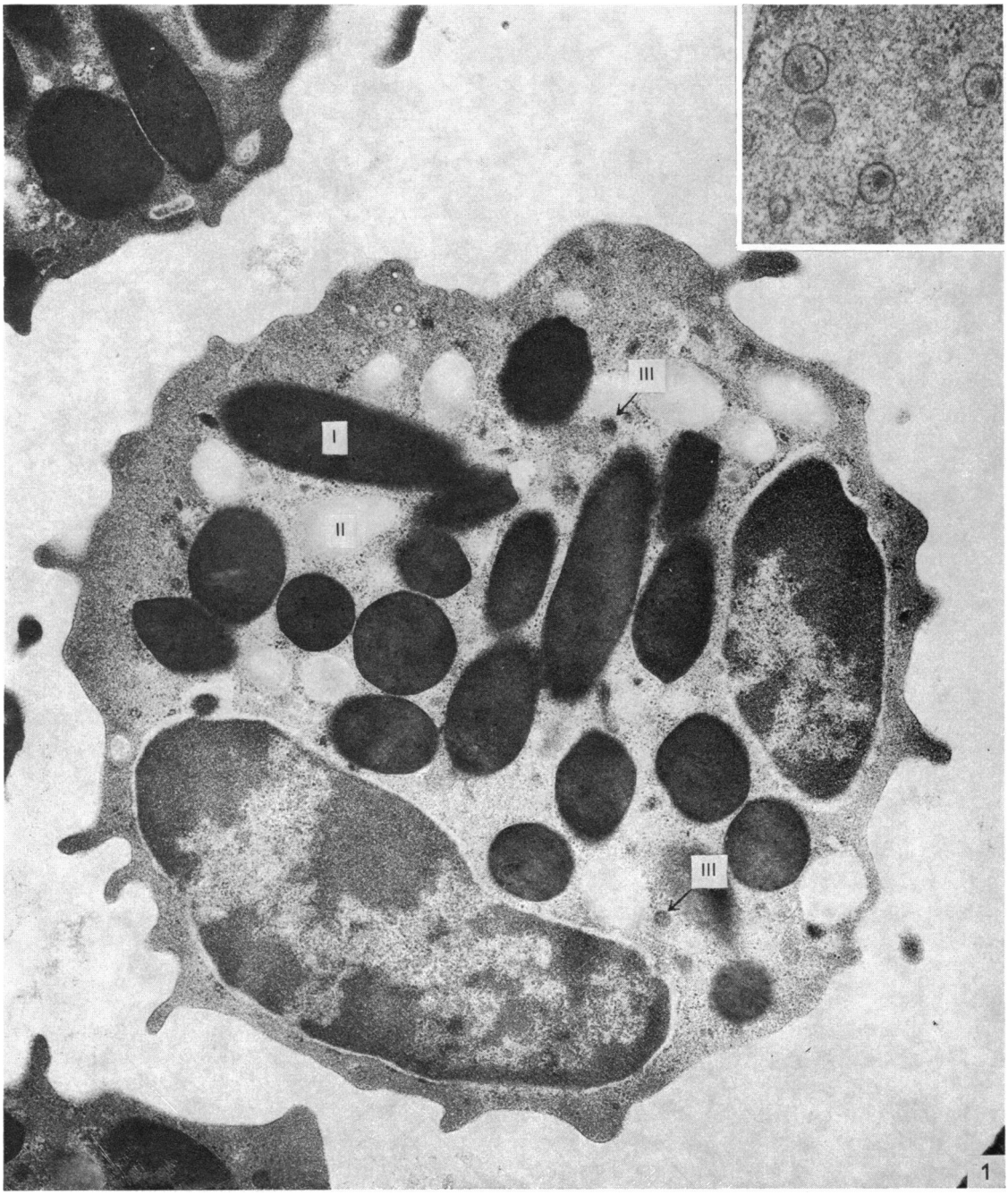


Fig. 1. Mature heterophil fixed with glutaraldehyde followed by osmium tetroxide. Type I granules (I) are large, rod-shaped and have an electron-dense content. Type II granules (II) are smaller and less dense than Type I granules. In addition to these granules, much smaller Type III granules are to be seen (arrows). The inset shows a core surrounded by an electron-lucent area in Type III granules. Uranyl acetate–lead citrate stained. × 24000. Inset. × 30000.

propylene oxide, and embedded in Epon 812 (Burke & Geiselman, 1971). Ultrathin sections cut on a Porter–Blum I Ultramicrotome with a glass knife were examined either unstained or after staining with 2 % uranyl acetate, followed by lead citrate (Venable & Coggeshall, 1965) and examined in a JEM 100-C electron microscope. Granule counts were made on equatorially-sectioned heterophils in 50 micrographs. In all, 2488 granules were counted and classified.

RESULTS

Electron microscopy

Heterophils possessed three types of granules. Type I, which accounted for 48·3 % of the granules, was large and rod-shaped, approximately 1·5 μm long and 0·6 μm wide, and had a homogeneous electron-dense content (Fig. 1). Type II, which accounted for 24·7 % of the granules, was smaller, approximately 0·5 μm in diameter, and less dense than Type I granules. Fine fibrous material was often observed in Type II granules in acid phosphatase preparations (Fig. 5). Type III, which accounted for 27·0 % of the granules, was 0·1 μm in diameter, and had a core surrounded by an electron-lucent area (Fig. 1): the density of the contents varied. All three types of granule were bounded by a unit membrane.

Only one type of granule was observed in chicken *eosinophils* (Fig. 2). It was circular, from 0·25 to 0·5 μm in diameter, and had an electron-dense, homogeneous content. Central angular dense bars, such as have been reported in the eosinophils of some mammals, were not observed in the chicken.

Basophil granules were of two types (Fig. 3). The majority, Type I, were round, approximately 0·6 μm in diameter, and the density of their contents varied with the degree of their maturation. Type II granules were also round, but were less frequent and much smaller, being only 0·1 μm in diameter and their contents were homogeneous and very electron-dense. These small granules were frequently found in contact with the larger Type I granules (Fig. 3).

Cytochemistry

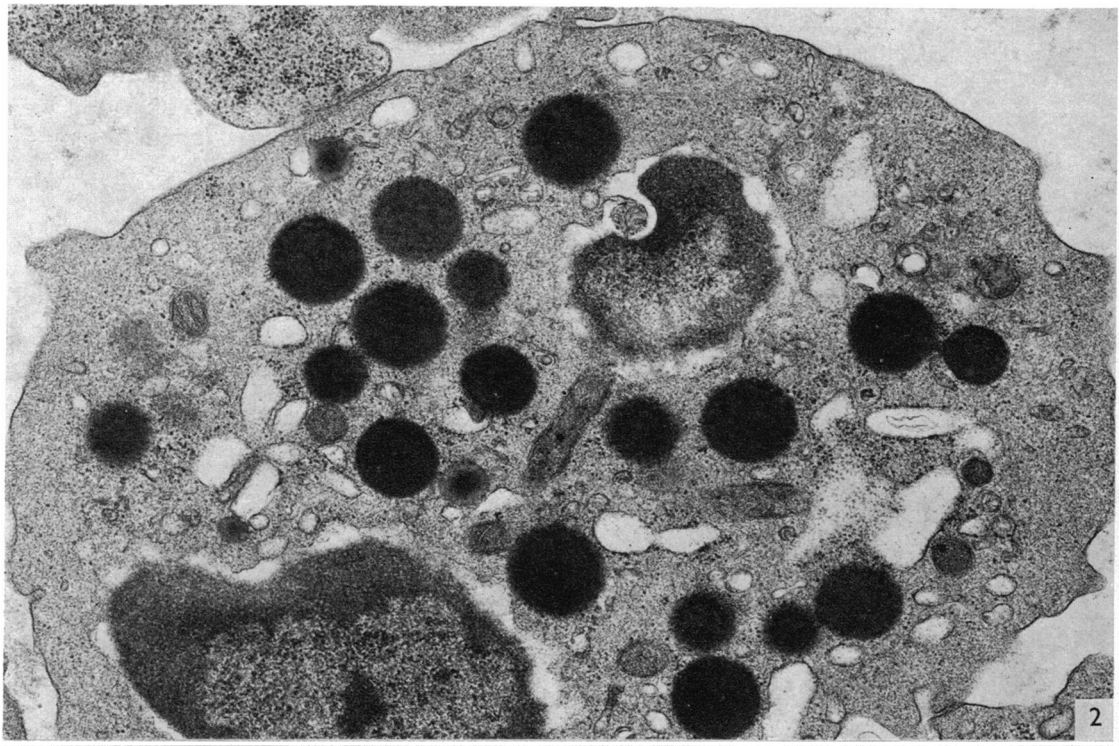
Peroxidase. Profiles of chicken granular leucocytes showed strong peroxidase activity in eosinophils (Fig. 4), but heterophils and basophils did not stain for this enzyme. Diaminobenzidine-reaction product was limited to granules of eosinophils, and appeared as a heavy electron-opaque deposit throughout the granules.

Controls. No reaction was observed when diaminobenzidine was omitted. However, weak reaction products were often seen at the periphery of granules when only H_2O_2 was omitted.

Acid phosphatase. Enzyme activity was seen in all of the large, dense, Type I granules of heterophils (Fig. 5). The reaction product appeared as needle-like crystals. No reaction products were observed, however, in medium-sized, light, Type II granules or in small core, Type III granules in these cells.

Most granules in eosinophils were highly reactive and showed the heaviest deposits (Fig. 6).

Acid phosphatase activity was localized in a small proportion of Type I granules



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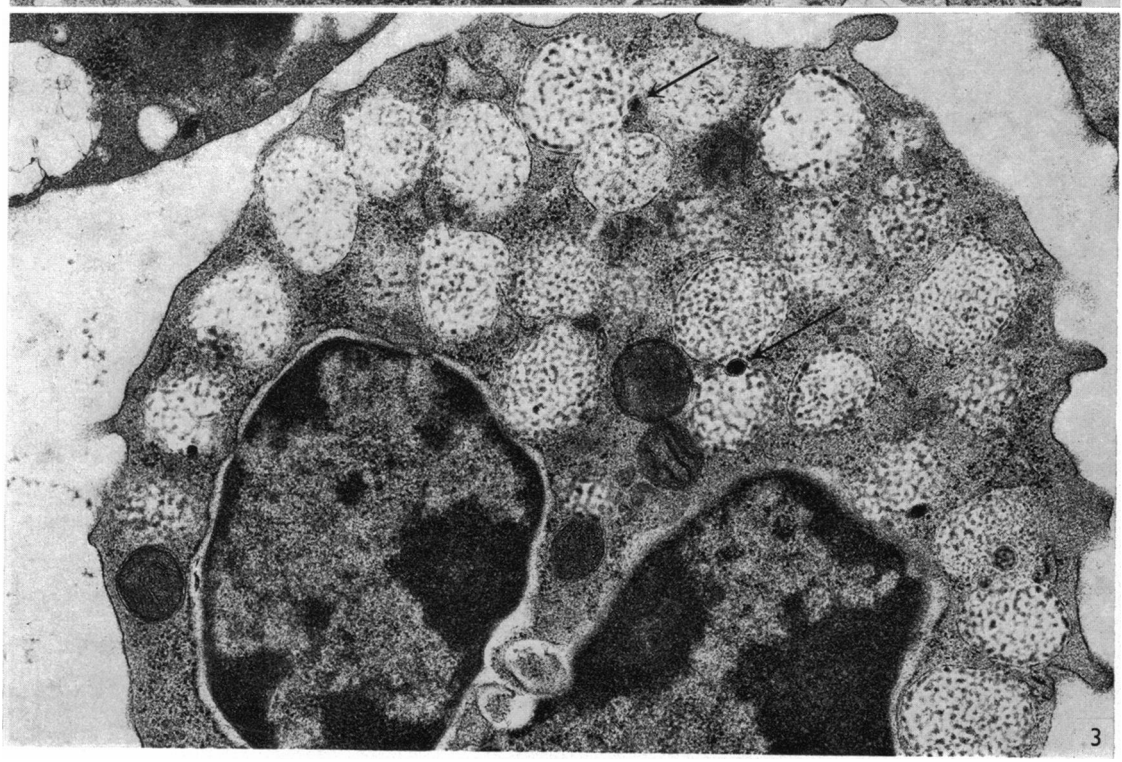

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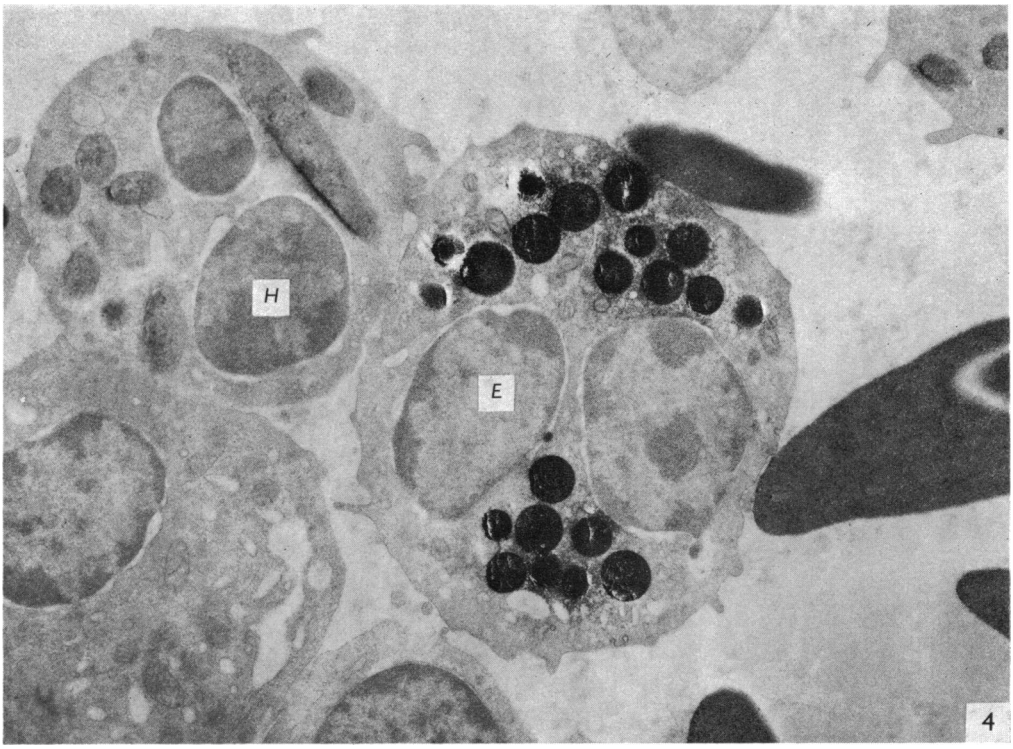

Fig. 4. Peripheral blood cells, showing a reaction for peroxidase in eosinophil (*E*) but not in heterophil (*H*). Unstained. × 9600.

of basophils (Fig. 7). The enzyme activity of small Type II granules was not investigated in this study.

Controls. No reaction was observed in any type of granulocyte when NaF was added or substrate omitted.

Alkaline phosphatase. This enzyme was not found in any of the profiles of mature chicken granulocytes.

DISCUSSION

The electron microscopic findings in this study confirm those of previous light microscopic studies (Caxton-Martins & Daimon, 1976). The present investigation indicates that chicken heterophils contain acid phosphatase, but not peroxidase nor alkaline phosphatase. Previous work on acid phosphatase in the granules of chicken heterophils using electron microscopic techniques and cell fractionation procedure

Fig. 2. Mature eosinophil fixed with glutaraldehyde followed by osmium tetroxide. The granules are circular and electron-opaque. Uranyl acetate–lead citrate stained. × 24000.

Fig. 3. Mature basophil fixed with glutaraldehyde followed by osmium tetroxide. The characteristic basophilic granules are circular in shape, electron-lucent and have particulate contents. Smaller, electron-opaque secondary granules are also present (arrows). Uranyl acetate–lead citrate stained. × 24000.

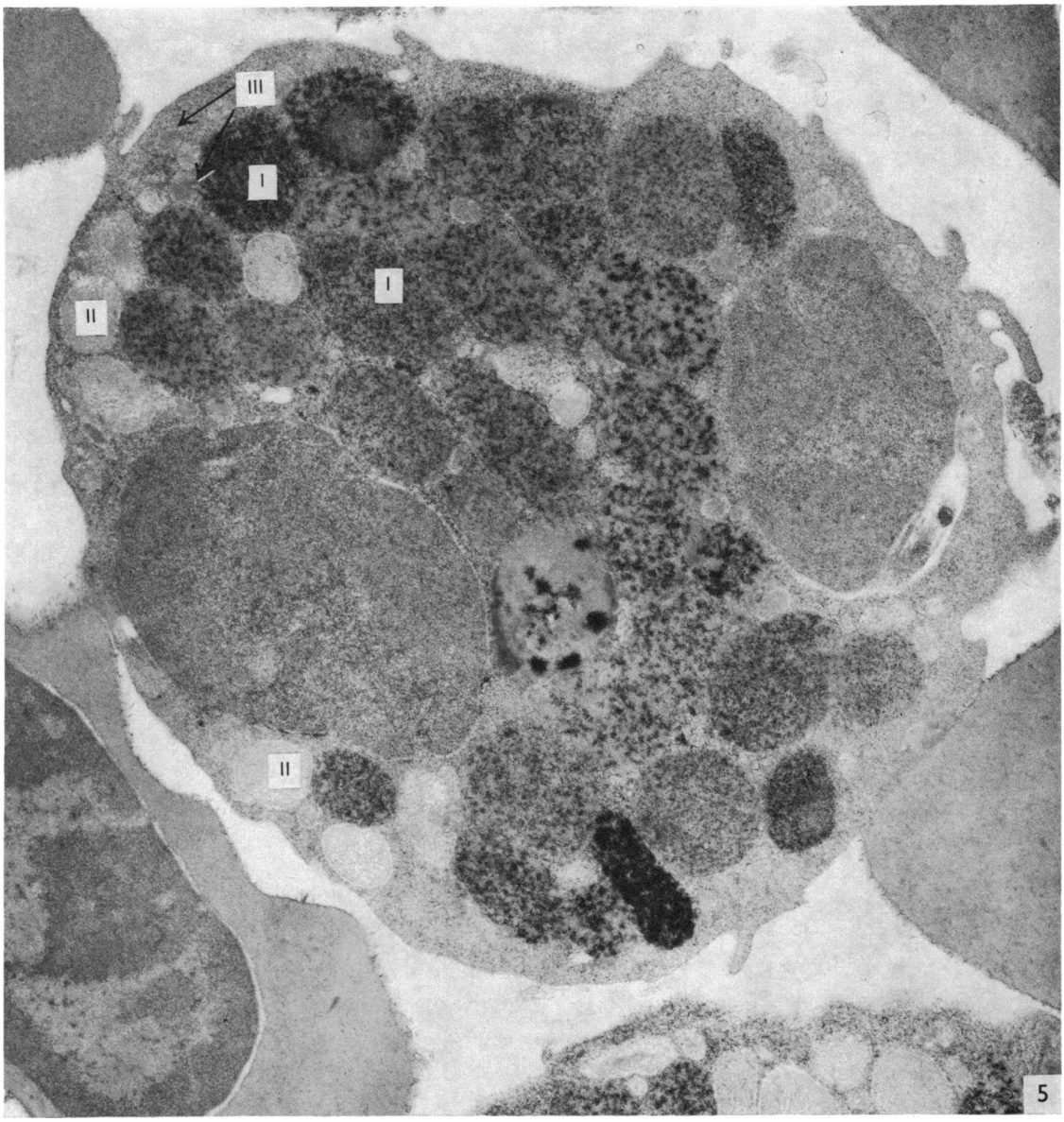

Fig. 5. Acid phosphatase activity in mature heterophil. The large, rod-shaped, dense granules (Type I) contain heavy reaction deposits, while the other types of granules (II and III) show no reactivity. Unstained. × 24000.

has given contradictory results. Ericsson & Nair (1973) suggested that acid phosphatase was limited to some of the large dense granules. Brune & Spitznagel (1973) showed that only the small granules in the light sedimentable fraction (band I) contained acid phosphatase, and concluded that only these were true lysosomes. In the present study, all the large dense Type I granules showed strong acid phosphatase

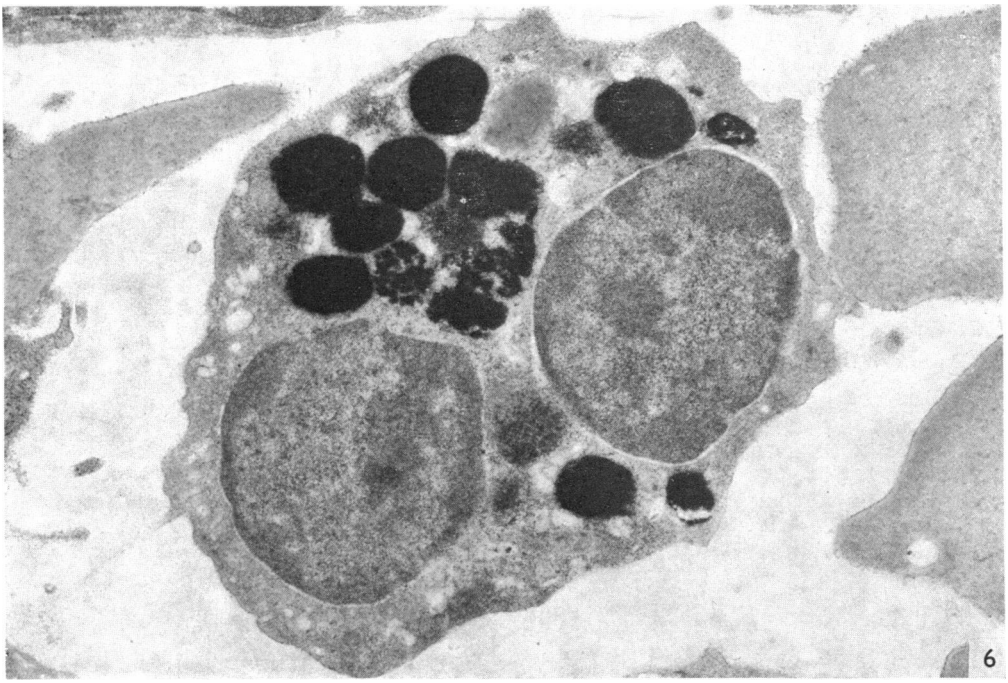

Fig. 6. Acid phosphatase activity in mature eosinophil. The cytoplasmic granules are partly or completely filled with dense reaction product. Uranyl acetate stained. × 15000.

activity and could be regarded as lysosomes, whereas none of the Type II and III granules gave any reaction. These results are similar to those of Ericsson & Nair (1973). The cytochemical nature and the physiological significance of Types II and III granules are unknown.

Unlike mammalian neutrophils (Bainton & Farquhar, 1968*a*, *b*) mature heterophils in chicken peripheral blood do not appear to show alkaline phosphate activity. However, Bainton & Farquhar (1968*b*) suggested that this enzyme was restricted to the granules of immature neutrophils. Immature heterophils in chicken bone marrow evidently need to be investigated.

In rabbit neutrophils Wetzel *et al.* (1967) reported a third type of granule, which was lysosomal in character and smaller than the azurophil, but its existence has been denied by Farquhar, Bainton, Baggiolini & De Duve (1972). In the present study lysosomal granules corresponding to those described by Wetzel *et al.* (1967) were not detected in chicken heterophils.

Because of the difficulty of distinguishing between heterophils and eosinophils in smears of chicken peripheral blood, the histochemical properties of chicken eosinophils cannot be determined with the light microscope (Merkal & Mora, 1962; Caxton-Martins & Daimon, 1975). The present ultrastructural study clearly distinguished eosinophils from heterophils, since the former showed only a single homogeneous population of granules. The central crystalline core which has been reported in the granules of duck and goose eosinophils (Maxwell & Siller, 1972) is

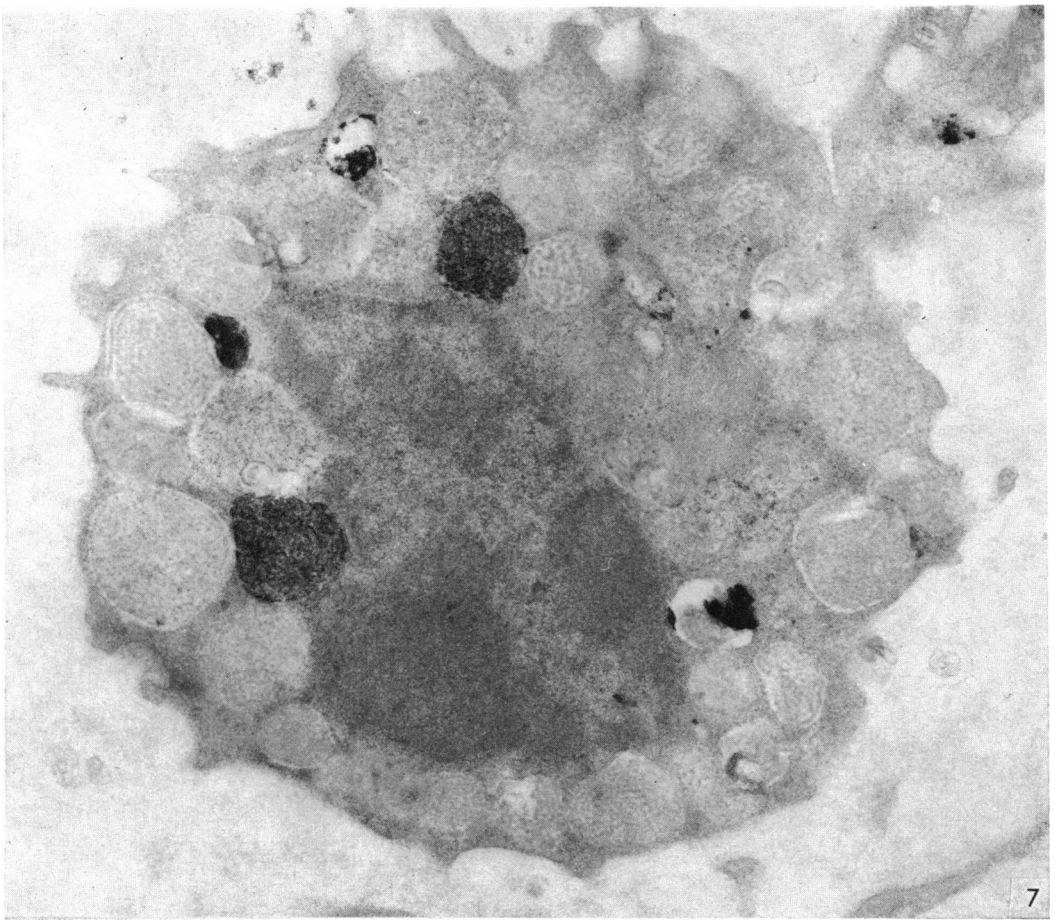

Fig. 7. Acid phosphatase activity in mature basophil. Activity is demonstrable only in a small proportion of the characteristic large basophilic granules. Unstained. × 24000.

not seen in the chicken, in agreement with Maxwell & Trejo (1970). The present cytochemical study has clearly shown peroxidase and acid phosphatase, but not alkaline phosphatase, in the eosinophil granules of the chicken. Chicken eosinophil granules therefore appear to be lysosomes, like those of mammalian eosinophils (Bainton & Farquhar, 1970). The large basophilic granules in the chicken are similar to those of human basophils (Hastie & Chir, 1974) and their characteristics are as described by other investigators (Dhingra, Parrish & Venzke, 1969; Maxwell & Trejo, 1970). However, in addition to the large granules, the present study has shown that another type of granule is present which is very small and electron-opaque. Similar small granules have been reported in mink basophils (Davis, Spicer, Greene & Padgett, 1971) and guinea-pig mast cells (Murata & Spicer, 1974).

The localization of enzymes in chicken basophils was not elucidated in previous light microscopic studies (Merkal & Mora, 1962; Caxton-Martins & Daimon, 1976). The present electron microscopic study clearly reveals that a minority of large Type I

granules contain acid phosphatase, but none of them show peroxidase or alkaline phosphatase activity. In rabbit basophils, Komiyama & Spicer (1974) detected acid phosphatase activity in approximately one-third of the granules, and suggested that enzyme activity was confined to certain stages in the maturation of the granules. The cytochemical nature and physiological characteristics of the small Type II granules in chicken basophils are not known.

SUMMARY

Detailed morphologic and enzyme cytochemical analysis was carried out by electron microscopy on granules of mature granulocytes obtained from the circulating chicken blood.

Heterophils possessed three types of granules: large, rod-shaped, dense (Type I); medium sized, oval, light (Type II); and small-core (Type III). Acid phosphatase activity was present in Type I granules, but peroxidase and alkaline phosphatase were not demonstrable. The cytochemical nature of Types II and III granules remains unknown. *Eosinophils* contained only one type of granule, which was circular and had electron-opaque contents. Both peroxidase and acid phosphatase, but not alkaline phosphatase, were present, indicating that the granules are lysosomes like the granules of mammalian eosinophils. *Basophils* possessed two types of granules, the characteristic large basophilic granules (Type I) and small dense granules (Type II). Acid phosphatase activity was found in only a small proportion of Type I granules: peroxidase and alkaline phosphatase were not demonstrable.

We wish to express our gratitude to Professor T. Ito for his continual encouragement throughout this study and also to Professor V. Mizuhira and Dr S. Yamashina of the Department of Cell Biology, Medical Research Institute, Tokyo Medical and Dental University, for valuable guidance. We are grateful to Dr D. Baxter-Grillo for reading and commenting on the manuscript.

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