Application of the Electron Microscope to the Cytochemical Peroxidase Reaction in Salamander Leukocytes*

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ABSTRACT

The present study has dealt with the localization by electron microscopy of the products of peroxidase reaction in neutrophil leukocytes in the subcapsular region of the livers of *Triturus viridescens viridescens*.

Small pieces of liver tissue were fixed for 1 hour in buffered osmium tetroxide solution. After fixation they were divided into five groups: (*a*) Not treated with any reagent (control); (*b*) Treated for 4 minutes with the peroxidase reagent containing 0.3 per cent benzidine and 0.014 per cent (0.004 molar) hydrogen peroxide in 50 per cent alcohol; (*c*) Treated for 4 minutes with 0.3 per cent benzidine solution in 50 per cent alcohol alone (control); (*d*) Treated for 4 minutes with 0.014 per cent (0.004 molar) hydrogen peroxide in 50 per cent alcohol alone (control); (*e*) Treated for 5 minutes with pure methanol, washed in water, and treated for 4 minutes with the peroxidase reagent (inhibition test). Each group was then dehydrated and embedded in either methacrylate or epoxy resin.

In electron micrographs, the reaction products of peroxidase activity were evidenced in the form of dense materials localized in the specific granules in the cytoplasm of the neutrophil leukocytes. Neither mitochondria nor any other particles showed increases in density.

The specific granules showed no change of density in the control and inhibition tests. Paraffin-embedded tissues of the above mentioned five groups, when examined with the light microscope, revealed that the brown granules denoting a positive reaction appeared only in leukocytes of the tissue treated with the peroxidase reagent.

Although much further work is necessary before definitive and constant results are to be expected, the possibility that the electron microscope may be applicable to peroxidase cytochemistry in leukocytes has been suggested by the present study.

INTRODUCTION

Sheldon, Zetterqvist, and Brandes (51), Brandes and Elston (10), Barrnett and Palade (3, 4), and Essner, Novikoff, and Masek (14), have shown that the products of certain enzymatic cytochemical reactions can be recognized and localized with the electron microscope with resolutions better than those possible with the light microscope. In this paper, the peroxidase reaction is demonstrated in neutrophil leukocytes of the salamander and provides a basis for characterizing certain granules which display peroxidase activity. It has been found that peroxidase activity survives brief treatment of tissue with osmium tetroxide. This observation may contribute to a solution of the problem of the nature of the peroxidase substance.

The foundations for the peroxidase stain of blood cells were laid by Fischel's investigation (15). He was the first worker who used benzidine, *i.e.* paradiaminodiphenyl, $NH_2 \cdot C_6H_4 \cdot C_6H_4 \cdot NH_2(1,4)(1,4)$, for the purpose of demonstrating peroxidase granules in blood cells.

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It is a general consequence of this benzidine peroxidase reaction that the eosinophil leukocyte, when observed with the light microscope, shows large brown or blue granules which correspond to the eosinophil granules, while the neutrophil leukocyte displays the reaction predominantly in granules much larger in size than the specific granules demonstrated by Wright's stain. Therefore it is quite difficult for cytochemists working with the light microscope to determine the relationship between the peroxidase reacting granules and the specific granules in the neutrophil leukocyte, or to characterize the nature of the particles within the cytoplasm which react with the peroxidase reagent.

The peroxidase reaction can be regarded as having two types of substrates, which we shall call hydrogen donor and hydrogen acceptor respectively. Amongst the hydrogen donors which can be oxidized by the peroxidase–hydrogen peroxide system in biochemical experiments are mono- and diphenols, monoamines, diamines, aromatic acids, amino acids such as tyrosine, cystine, etc. Of these, the hydrogen donors that have been most commonly and effectively used in the localization of the cytochemical reaction of blood cells are benzidine and orthophenylenediamine (32, 33). On the other hand, several kinds of hydrogen acceptors have been studied in cytochemical peroxidase reactions of blood cells (34). Sodium peroxide, barium peroxide, and monoethyl hydrogen peroxide (C_2H_5OOH) can serve as suitable reactants with benzidine (34). However, these experiments did not reveal any hydrogen acceptor more effective than hydrogen peroxide. For these reasons the benzidine-hydrogen peroxide reaction has been chosen for the present study.

In these experiments the developing neutrophil leukocytes of the salamanders, *Triturus viridescens viridescens* and *Ambystoma maculatum* have been used. From the comparative hematological standpoint, urodele amphibians differ from anurans in that in the former the granulocytopoietic aspect of blood formation is more sharply separated from the erythrocytopoietic aspects. Since Danchakoff (12) reported perihepatic hemopoietic tissue in the axolotl (larva of *Ambystoma*) this peculiarity may be regarded as characteristic of urodeles. Erythrocyte formation in the urodele, *Triturus viridescens viridescens* is generally located in the spleen while granulocyte formation, except for very minor intestinal and renal regions, is located in the perihepatic zone (19, 20).

In order to provide suitable reference points, a brief description of the structures of the normal salamander leukocyte is presented, along with a report of the cytochemical localization of the peroxidase activity in the leukocytes as revealed by the electron microscope.

Materials and Methods

The data in this study were obtained from liver tissues of *Triturus viridescens viridescens*. Some of the observations were confirmed on *Ambystoma maculatum*. Both of these animals are generally found in the eastern and midwestern portions of the United States. Dr. Charles Bodemer of the Department of Anatomy, University of Washington, Seattle, generously provided the animals. They were originally procured from commercial animal dealers in North Carolina and Wisconsin.

Liver tissue was removed from the unanesthetized living animal, cut into small pieces, and quickly immersed in ice cold 1.33 per cent osmium tetroxide solution buffered at pH 7.50 with collidine buffer (Bennett and Luft (5)). Fixation lasted 1 hour, and after washing in distilled water, the tissues were divided into five groups:

Group (*a*) Tissue not treated with any reagent (control).

Group (*b*) Tissue treated with the peroxidase reagent for 4 minutes. The peroxidase reagent is 50 per cent alcohol solution containing 0.3 per cent benzidine and 0.014 per cent (0.004 molar) hydrogen peroxide (36).

Group (*c*) Tissue treated with 0.3 per cent benzidine (paradiaminodiphenyl) solution in 50 per cent alcohol for 4 minutes (control).

Group (*d*) Tissue treated with 0.014 per cent (0.004 molar) hydrogen peroxide in 50 per cent alcohol for 4 minutes (control).

Group (*e*) Tissue treated with pure methanol for 5 minutes, washed in water, and treated with the peroxidase reagent for 4 minutes (inhibition test).

Each group was dehydrated and embedded in either methacrylate or epoxy resin. In the methacrylate method, a mixture of *n*-butyl methacrylate (85 per cent) and methyl methacrylate (15 per cent) was used, and polymerized at 60°C. For epoxy resin embedding, a modification of the method of Glauert and Glauert (16) revised by Dr. Luft of our laboratory was used. (The data of Dr. Luft are not yet published). Observations were made using an RCA model EMU 2-C electron microscope fitted with objective aperture, compensated pole piece, and a specially stabilized power supply.

In general, the liver tissue should be treated with the peroxidase reagent before embedding in resin, because heating or ultraviolet ray used for embedding exerts an inhibiting influence upon the peroxidase (60).

When the osmium-fixed liver tissue is incubated in the peroxidase reagent for a longer period than 4 minutes, for instance 10 or 20 minutes, the reaction occurs more intensely in neutrophil leukocytes in the perihepatic zone. This longer incubation, however, is not recommended, because it causes a slight damage of fine structural details in the cells.

Fine grain-positive sheet films made by the Eastman Kodak Co. were used for electron microscopy in which case D19 as a developer and F5 as a fixer were used as already described by Wood and Howard (64). The corresponding micrographs in controls and positively

reacting specimens went through the same photographic process.

Densitometric tracings in each case were carried out through nucleus, mitochondria, cytoplasmic matrix, and specific granules in order to compare densities in electron micrographs as objectively as possible. The apparatus used was the recording microphotometer, Type MP-3, manufactured by the Rigaku-Denki Co. in Japan. (Wave length of the light source = 3,000 to 5,200 A, slit = 10 μ, width of the measuring line = 0.3 mm., plate travel speed = 8 mm. per minute, chart travel speed = 20 mm. per minute). Densitometric tracings were made on positive transparent prints along pathways shown by the black line in each figure. The densities are plotted on photometer curves with the relative value of the light absorption in per cent on the ordinate and the distance in μ on the electron micrograph on the abscissa. Higher densities are recorded as upward deflections of the curve.

OBSERVATIONS

I. General Features

Sections of *Triturus* and *Ambystoma* liver examined with the light microscope display many granulocytes in the perihepatic zone. The appearance of these cells in *Triturus* liver is shown in Figs. 1 to 4. They occupy a zone 0.02 to 0.2 mm. thick, intervening between the serosa and the liver parenchymal cells. This zone varies in thickness from place to place, but is represented over virtually the entire surface of the liver.

The population of leukocytes in this perihepatic zone consists predominantly of granular neutrophil leukocytes resembling the corresponding mature cells of the circulating blood. Mixed with these neutrophils are smaller numbers of lymphocytes, monocytes, and eosinophils. Mitotic figures are rare in the animals used in this study, and young immature leukocytes were not abundant in this material.

II. Electron Microscope Observations

A. Leukocytes of Group (a). Untreated Control (The Normal Structure of Leukocyte):

The normal neutrophil leukocytes of *Triturus* and *Ambystoma* encountered in the perihepatic zone showed, when examined with the electron microscope, the same general features which have been discovered in mammalian neutrophils by other workers (6, 21, 22, 28, 62). For this reason, a detailed description of the cell is omitted from this paper, and emphasis is confined to those structures which might be implicated in the peroxidase reaction.

The general structural features of the leukocyte of *Triturus* are shown in Fig. 5.

The cytoplasm contains specific granules, mitochondria, endoplasmic reticulum, Golgi bodies, centriole, etc. The most prominent inclusions in the cytoplasm are the specific granules.

1. Specific Granules.—These can be seen to advantage in Figs. 5, 6, 10, and 12. They appear as ellipsoidal profiles of moderate density, limited by a dense line resembling a membrane. The most typical form seems to be that of rounded rod-like structures about 1 μ long and 0.1 to 0.2 μ in diameter. In some cases internal inhomogeneities in density can be discerned. Sometimes these appear as dense inclusions (Fig. 6).

In neutrophil leukocytes of *Ambystoma*, the profiles of the specific granules in sections are generally round or elliptical and rod-like profiles are far less numerous than in *Triturus*. This is the main difference between neutrophil leukocytes in *Triturus* and *Ambystoma*.

In the electron micrographs, the density of these specific granules is much the same or slightly less than that of the matrix of mitochondria as shown by the densitometric tracings (Figs. 18, 20, and 22).

2. Mitochondria.—Mitochondria have been encountered frequently and their basic structure is as described by Palade (41, 42). The mitochondria appear to be elongated, bent filamentous structures. Sometimes horseshoe-shaped profiles are included within a single section (Fig. 16). The length of mitochondria can not be deduced, but the diameter in cross-sections is approximately 0.2 to 0.3 μ. This is generally larger than that of the specific granules. It is interesting to note that a few dense granules are frequently found in the matrix of mitochondria (Figs. 12 and 16). The mitochondrial crests are disposed helically, obliquely, or almost parallel to the long axis.

3. Centriole.—Centrioles have been encountered rarely in the salamander leukocytes (Fig. 5). They display the characteristic locations, size, and structure demonstrated by Bernhard (7), Yamada (65), Porter (45), and Bessis *et al.* (8, 9).

4. Endoplasmic Reticulum (Lamellae).—The endoplasmic reticulum of the leukocytes has been recognized in a paranuclear position, especially in leukocytes in the deeper portion of the perihepatic zone, close to the liver parenchymal cells. The endoplasmic reticulum is represented by stacks of six to ten flattened cisternae similar to those seen in a rat spermatid by Palade (44), those seen in larval *Ambystoma* pancreas by Swift (56), and

those seen in macaque parathyroid gland by Trier (59). Associated with the membranes are dense particles identified as particles of Palade (43); they are presumed to be ribonucleoprotein particles, rich in ribonucleic acid (Fig. 6).

5. Golgi Bodies.—The Golgi bodies have been encountered much less frequently in the neutrophil leukocytes than in the eosinophil leukocyte. The fine structure of the Golgi bodies in the neutrophil leukocyte is essentially as described by Dalton and Felix (11). In general the Golgi bodies can be found in the region of an indentation of the nucleus. This structure consists of dense paired parallel membranes and associated vesicles.

6. Nucleus.—Like human neutrophil leukocytes, most of the nuclear lobes of salamander neutrophils, especially in methacrylate-embedded material, have two typical densities of the nucleoplasm. The denser of the two is subjacent to the nuclear membrane, with the lighter nucleoplasm occupying a more central position (Fig. 5). In a few cases, nucleolar masses may be seen.

B. Leukocytes of Group (b). The Localization of Peroxidase Activity in the Neutrophil Leukocytes of Salamanders is Demonstrated in this Group:

In electron micrographs of the tissues treated with the peroxidase reagent, one can notice a change of density in cytoplasmic components as compared with the normal cytoplasm. The substance which shows relatively low increase in density with the peroxidase reaction is located in the specific granules, especially in the central portion of the granules (Figs. 11, 30 to 33). This change in density is presumably based on accumulation of reaction end-products within the substance of the granules, although morphological details of these products are not obvious. It should be noted that neither mitochondria nor any other recognized cytoplasmic components, nor nucleus, increase their density with the peroxidase reaction.

As shown in Fig. 9, the peroxidase reaction does not occur in all the leukocytes in the liver, but occurs predominantly in the neutrophil leukocytes located in the superficial layer of the perihepatic zone. In other words, the reacting granules decrease in number with increased depth in the perihepatic zone. This may be a consequence of diminishing penetration of the peroxidase reagent into deeper portions of the liver tissue. The leukocytes observed in both control and positively reacting specimens in this study were those located in the superficial layer of the perihepatic zone. It is also noticed that a few of the specific granules (at *x* in Fig. 11) do not increase in density so much as other positively reacting granules. It is presumably due to variations in peroxidase activities among the specific granules.

C. Leukocytes of Groups (c, d, and e). Controls with Single Substrate and Inhibition Test:

It was revealed by the control tests that the specific granules do not change their density if treated with benzidine alone or with hydrogen peroxide alone (Figs. 13, 14, 23 to 26). In other words, the granules change their density only when the leukocytes are treated with the mixture of benzidine (hydrogen donor) and hydrogen peroxide (hydrogen acceptor), namely, the ordinary peroxidase reagent.

Methanol is a powerful inhibitor to the cytochemical peroxidase reaction of leukocyte studied with the light microscope (46). If the osmium-fixed liver tissue is treated with the peroxidase reagent after exposure to methanol, a peroxidase reaction does not occur in the leukocytes in the perihepatic zone. In other words, the reaction of the specific granules in the neutrophil leukocyte is inhibited by methanol, and the granules show no change in density as demonstrated in Figs. 15, 27 to 29. Methanol, however, seemed to be damaging to the cytoarchitecture under these circumstances.

III. Light Microscope Observations

Two μ sections of the osmium-fixed liver tissues treated by the same methods used for electron microscopy show that the brown granules denoting a positive reaction appear distinctly in the neutrophil leukocytes of the tissue treated with the peroxidase reagent (Fig. 7 by paraffin section, Fig. 8 by methacrylate section), while the brown granules do not appear in any of the above mentioned control tests nor in the inhibition test with methanol. However, one can not determine with the light microscope what kind of cytoplasmic component of the neutrophil leukocyte reacts to the peroxidase reagent in these experiments.

DISCUSSION

Four different peroxidases have been prepared in a pure or nearly pure state. Two of them have been crystallized: horse-radish peroxidase and lacto-peroxidase. The other two, cytochrome *c* peroxidase of yeast and verdoperoxidase of leukocytes (58) have not been crystallized.

Peroxidase action of biological materials was first observed in 1855 by Schoenbein (50). He found that plant and animal tissues, just like ferrous salts, were able to activate hydrogen peroxide or other peroxides so as to oxidize guaiac tincture to a blue color. The strong peroxidase activity of pus has long been known. It was observed by Klebs (23) and Struve (53), and shown by Linossier (25) to be caused by a peroxidase. The name "peroxidase" was first applied in 1898 by Linossier (25).

The peroxidase staining of histologic sections has been widely studied by Graham (18), McJunkin (29), Okano (40) and others. They examined the reaction of tissues embedded in paraffin, celloidin, or sectioned after freezing, using mixtures of benzidine and hydrogen peroxide in various concentrations. A review of the literature indicates that the cells which react positively to the benzidine peroxidase reaction are the myeloid leukocytes, Kupffer cells, and endothelial cells lining the sinusoids of liver. The endothelial cells of larger blood vessels and those lining the sinuses of lymph nodes and of spleen do not give a positive reaction.

According to Jordan (20) the cytoplasm of the urodele neutrophil (heterophil) leukocyte in early developmental stages contains a mixture of fine basophilic and oxyphilic granules. As differentiation progresses the granules become smaller—almost dust-like in some instances in the stained films—and change their staining properties to produce a light lilac color in Wright s preparations. He further states that, in the definitive condition, the cytoplasm, except under very high magnification, appears to be practically homogeneous and of a pinkish lilac color. These descriptions of urodele leukocytes are very significant for comparative hematology, but he did not mention a cytochemical peroxidase reaction in the leukocytes. In the studies hitherto reported on comparative hematology (37, 48, 49, 52), the peroxidase reaction is always more intense for human neutrophil leukocytes than for neutrophils of any other kind of animal when benzidine and hydrogen peroxide are used for the substrates of the reaction. According to the studies of Nakamura (39), Sugiyama (55), and Mitsui *et al.* (31), the neutrophil leukocytes of salamanders show an intense peroxidase reaction. Mitsui (35) has already reported an intense peroxidase activity of leukocytes in American salamanders including *Triturus viridescens viridescens*, which was used for the present study.

There have been some attempts to isolate substances with peroxidase activity from blood cells by biochemical methods. From leukocytes of horse blood, Neumann (38) prepared a greyish-green substance which gave an intense peroxidase reaction. This substance was presumed to derive from the eosinophil granules because the preparation, on staining with eosin, took on a pink color. Moreover, microscopic examination showed grains with red-stained contours, the interior of the grains being stained yellow-red. In 1941, Agner (1) isolated from leukocyte-rich material (empyema fluid), with his special method, a green colored enzyme which showed characteristic absorption bands and peroxidase activity. He called this material verdoperoxidase or myeloperoxidase, and stated it was possible to demonstrate the presence of verdoperoxidase only in myeloid cells. Furthermore he studied a biological function, especially a detoxicating effect of verdoperoxidase on toxins (2). Wakabayashi (61) also tried to isolate a substance with peroxidase activity from horse blood and demonstrated that this substance showed the same color reaction as did the cytochemical peroxidase reaction of blood cells.

The cytochemical localization of blood cell peroxidase has been studied by many authors because of the usefulness of the method for differentiating lymphoid and myeloid cells in leukemia. As early as 1910, Fischel (15), using a benzidine reaction, found a thermolabile peroxidase in the cytoplasm of eosinophil and neutrophil leukocytes, while the lymphocytes and basophil cells did not give any such reaction. Since then, large numbers of papers on this subject have been published, reporting almost the same results as in the original study by Fischel (15). Among these, the studies of Loele 1912, (27), Goodpasture 1919, (17), and Sato 1922, (47) are the most significant for the development of modern technics for localizing the peroxidase reaction. However, none of these cytochemical methods could demonstrate the definite localization of peroxidase activity in the neutrophil leukocytes, because the cytoplasm is generally stained much more diffusely than by Wright's stain. The limiting factor is that we cannot compare exactly the peroxidase reacting granules with the hematological granules under the light microscope. This comparison becomes more difficult when blood cells are over-stained with the peroxidase reagent, with consequent dense precipitates throughout the cytoplasm.

In the present investigation an attempt has been made to differentiate various particles in the neutrophil leukocytes with the electron microscope and to observe which type of particle contains the substance with peroxidase activity. It was found that large numbers of neutrophil leukocytes occur in the perihepatic region of the salamander liver and the peroxidase reaction of these is not inhibited by fixation for 1 hour with osmium tetroxide. For this reason, salamander liver tissue was fixed with osmium tetroxide before treatment with the peroxidase reagent and sections were examined with the electron microscope.

It is known from this electron microscope study that the localization of peroxidase activity in the neutrophils appeared in the form of dense materials limited to the specific granules. The nucleus, mitochondria, and other cytoplasmic components de-

tected did not increase in density. This result coincides with the data obtained by Takigawa *et al.* (57), who ascertained the localization of oxidase and peroxidase activities within the specific granules by cell fractionation technics, using the light microscope.

On the other hand, in the dehydrogenase reaction of the rat heart muscle fibers reported by Barrnett and Palade (3, 4), reduced tellurite, the end-product of the reaction was visualized in sections as crystals or fine particulate deposits of very high density, localized on or in close relationship to mitochondrial membranes. It should be pointed out, however, that the end-product of the peroxidase reaction demonstrated in this study does not appear as a distinct deposit, but increases only the density of the specific granules in neutrophil leukocytes. For this reason, sectioning and photographic processing were carefully performed and densitometric tracings were made on electron micrographs of both control and positively reacting specimens in order to define the changes in densities.

In the benzidine-hydrogen peroxide reaction, benzidine is thought to be oxidized to diphenoquinone-diimine (after Willstaetter and Piccard, 63, 26) or to polymeric products (13). It is presumed that these water-insoluble substances are produced by the peroxidase activity of the neutrophil granules and are immediately bound to the granules. This could account for the change in density of the granules under the electron microscope.

It is of additional interest that the inhibitory action of methanol on leukocyte peroxidase reaction has been confirmed in the present electron microscopic study. From the hematological point of view, the inhibition by methanol is very significant because methanol has been regarded as one of the best fixatives of blood cells in smear preparations. Regarding the peroxidase reaction of neutrophil leukocytes, it has already been shown by cytochemists working with the light microscope that methanol exerts an inhibitory influence upon the benzidine-H_2O_2 reaction (24, 30, 46, 52, 60), upon the pyrogallol- or guaiacol-H_2O_2 reactions (54), and also upon the orthophenylenediamine-H_2O_2 reaction (33).

It is worthy of special comment that changes in density of the specific granules are evident in electron microscope studies only in very thin sections. A distinction between reaction-positive particles and negative ones is difficult or equivocal in thicker sections (Figs. 5 and 17). For this reason, it is quite helpful to compare the density of a given granule with that of other cellular components by densitometric tracings. Furthermore, it should be noted that, in the present study, the peroxidase itself has not been directly observed under the electron microscope, but has been recognized indirectly by virtue of the binding of the reaction end-product, causing a relatively low increase in density recognizable under the electron microscope.

Although the enzyme technics used here gave an interesting result with cold-osmium-fixed tissues, the following problems still remain unanswered:

1. What kind of results can be obtained when frozen-dried tissues are used?
2. What is the exact nature of the final product that causes increase in density of the specific granules?
3. Do the specific granules isolated from leukocytes homogenate by means of cell fractionation technics give the same information in electron microscopy as described in this paper?
4. Is there any other substrate more effective for demonstration of leukocyte peroxidase in electron microscopy?

It thus becomes desirable, that a clearer understanding of morphology and function of leukocyte peroxidase will be reached in the future, through more detailed cytochemical studies for which the present morphological findings may provide a basis.

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Explanation of Plates

All electron micrographs are of salamander liver tissue fixed with buffered osmium tetroxide solution and embedded in methacrylate except when otherwise indicated.

B = Blood vessel
C = Centriole
CM = Cell in mitosis
E = Endoplasmic reticulum
ES = Endothelial cell of serous membrane
L = Leukocyte
LP = Liver parenchymal cell
M = Mitochondrion
N = Nucleus
Ne = Neutrophil leukocyte
P = Pigment cell
S = Specific granule
Se = Serous membrane

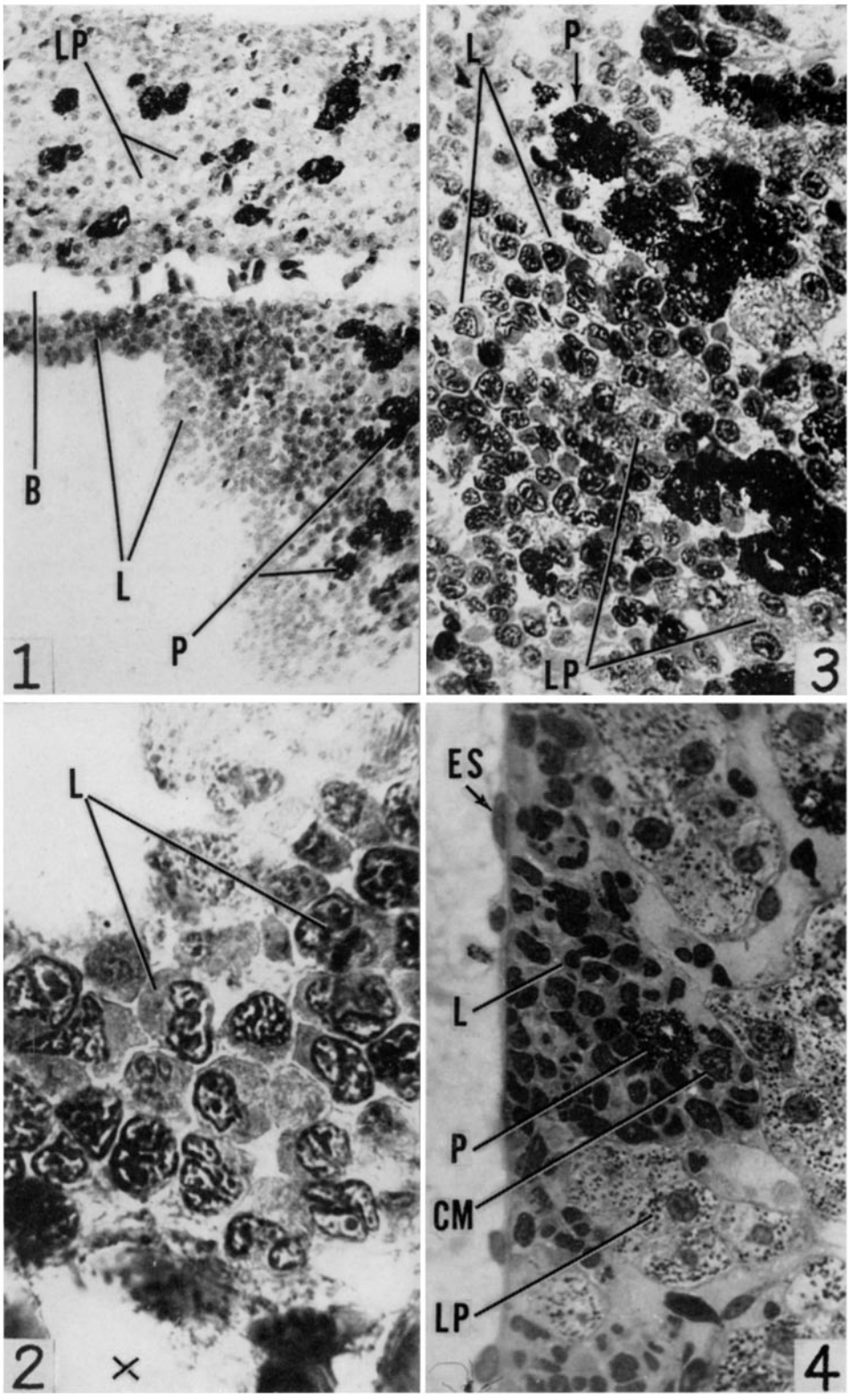
PLATE 121

FIG. 1. Photomicrograph of a 2 μ paraffin section of liver tissue of *Triturus viridescens viridescens*. Stained with Altmann-Mallory method after fixation with Zenker's fluid. Large numbers of leukocytes (*L*) are found in the perihepatic zone. Liver parenchymal cells (*LP*), pigment cells (*P*), and blood vessels (*B*) can also be seen. × 200.

FIG. 2. Photomicrograph of the same specimen as in Fig. 1. A group of neutrophil leukocytes (*L*) can be seen in the perihepatic zone. *X* (Lumen of blood vessel). × 1,200.

FIG. 3. Photomicrograph of a portion of the perihepatic zone of Fig. 1. A group of leukocytes (*L*) mixed with liver parenchymal cells (*LP*), and pigment cells (*P*) can be seen. × 500.

FIG. 4. Photomicrograph of a 2 μ paraffin section of liver tissue of *Triturus viridescens viridescens*. Stained with dilute Giemsa stain after fixation with buffered osmium tetroxide solution. Between the serous membrane and liver parenchymal cells (*LP*) is a layer of leukocytes (*L*) showing a few leukocytes in mitosis (*CM*) and a pigment cell (*P*). *ES* (Endothelial cell of the serous membrane). × 400.

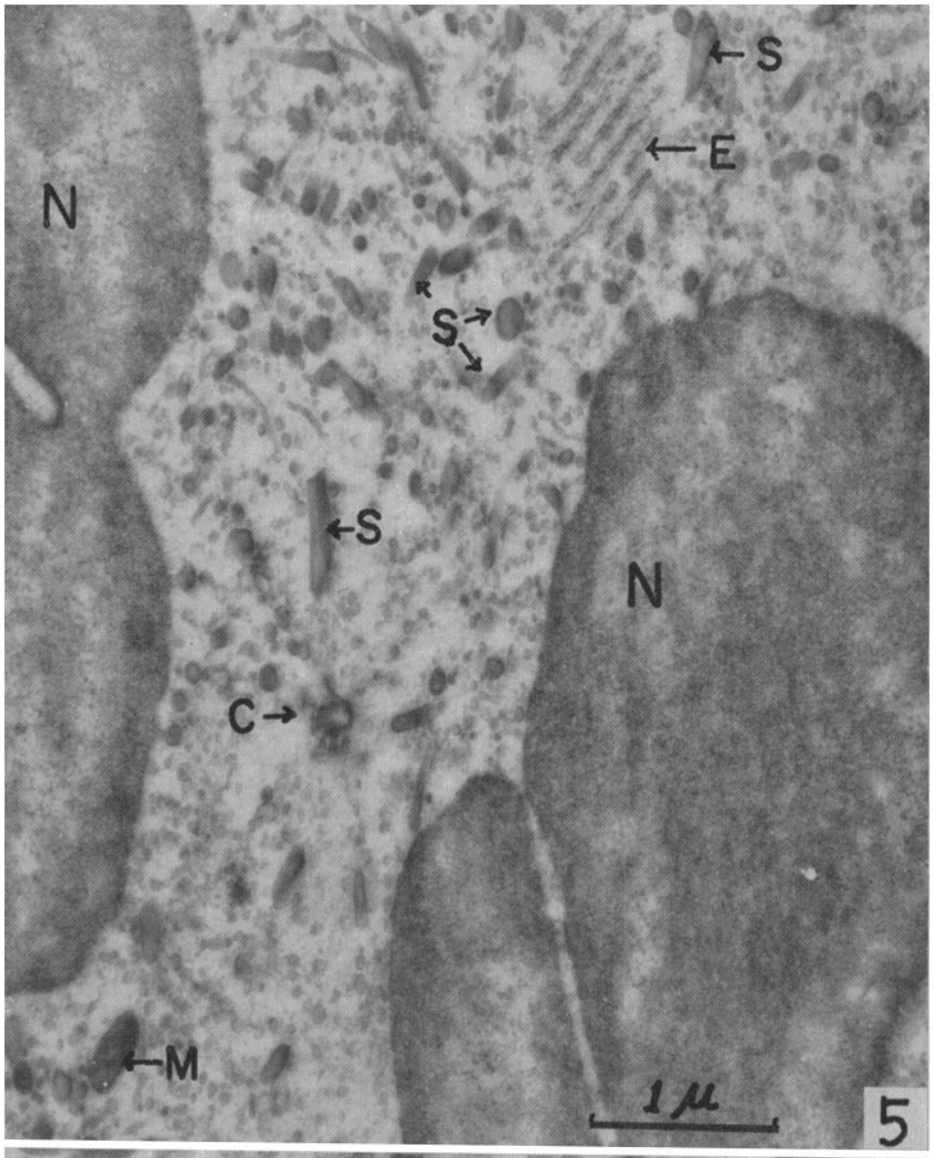


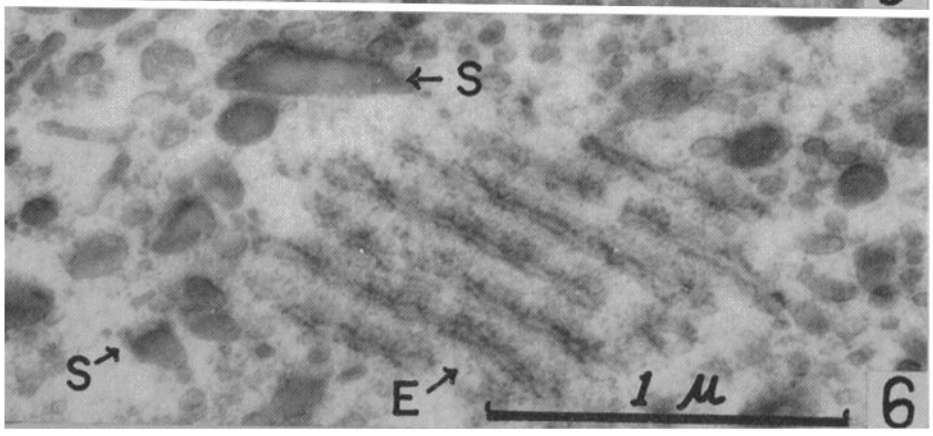
(Mitsui: Peroxidase reaction in leukocytes)

PLATE 122

FIG. 5. Electron micrograph of a polymorphonuclear neutrophil leukocyte found in the perihepatic zone of *Triturus viridescens viridescens*. Various profiles of specific granules (*S*), mitochondria (*M*), centriole (*C*), and endoplasmic reticulum (*E*) with associated particles of Palade can be seen in the cytoplasm. In a comparatively thick section like this, the specific granules appear to be very dark as compared to those in a thinner section (see Figs. 10 and 12). × 23,000.

FIG. 6. Electron micrograph of the endoplasmic reticulum (*E*) with associated particles of Palade and specific granules (*S*) in the same leukocyte as the one shown in Fig. 5. The specific granules appear as round or ellipsoidal profiles, bordered by a denser frame resembling a membrane. × 47,000.





(Mitsui: Peroxidase reaction in leukocytes)

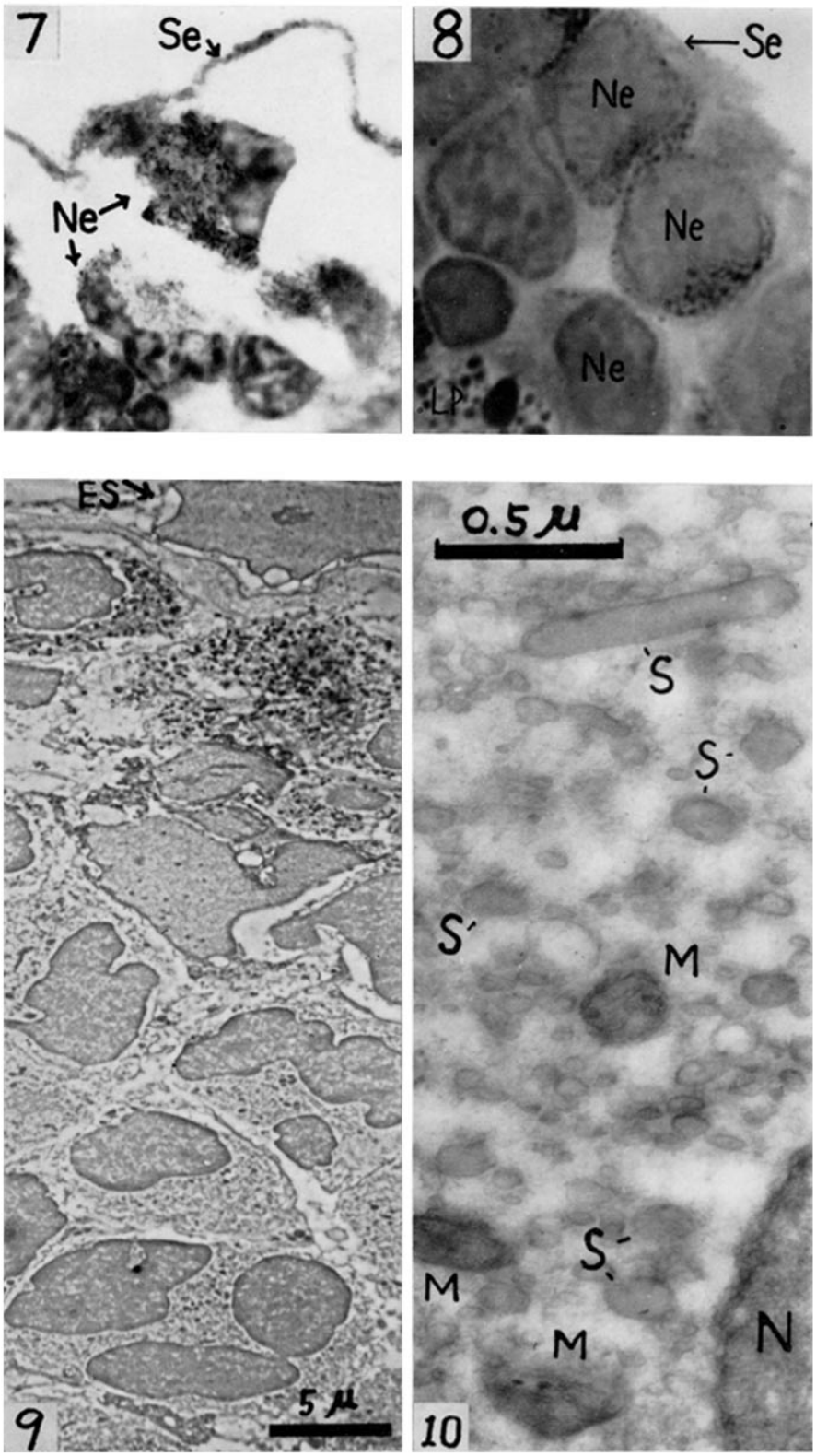
PLATE 123

FIG. 7. Photomicrograph of a 2 μ paraffin section of liver tissue of *Triturus viridescens viridescens*. The tissue was treated with peroxidase reagent after osmium tetroxide fixation. Counterstained with dilute Giemsa stain. The peroxidase reacting granules, which appear as tiny brown dots, can be seen in the cytoplasm of the leukocytes (*Ne*) located under the serous membrane (*Se*). × 1,200.

FIG. 8. Photomicrograph of a 2 μ methacrylate section of liver tissue of *Triturus viridescens viridescens*. The tissue was treated with peroxidase reagent after osmium tetroxide fixation, then embedded in methacrylate by the same method used for electron microscopy. The peroxidase reacting granules, which appear as tiny brown dots, can be seen in the cytoplasm of the leukocytes (*Ne*) located under the serous membrane (*Se*). These brown granules in Figs. 7 and 8 can be clearly seen whether the specimens are counterstained with dilute Giemsa stain or not. *LP* (a portion of a liver parenchymal cell). × 1,500.

FIG. 9. Electron micrograph of liver tissue of *Ambystoma maculatum* treated with peroxidase reagent after osmium tetroxide fixation. The peroxidase reaction product appears as dense granules in the cytoplasm of neutrophil leukocytes located in the superficial layer of the perihepatic zone. The reacting granules decrease in number with increased depth in the perihepatic zone. This is presumably a consequence of diminishing penetration of the peroxidase reagent into deeper portions of the liver tissue. *ES*, Endothelial cell of serous membrane. × 3,500.

FIG. 10. Electron micrograph of a portion of cytoplasm of normal neutrophil leukocyte in the perihepatic zone of *Triturus viridescens viridescens*. Various profiles of the specific granules (*S*) and of the mitochondria (*M*) can be seen. It is clear in this picture that, in the normal cytoplasm, the specific granules (*S*) do not have a greater density than the mitochondria (*M*). × 56,200.

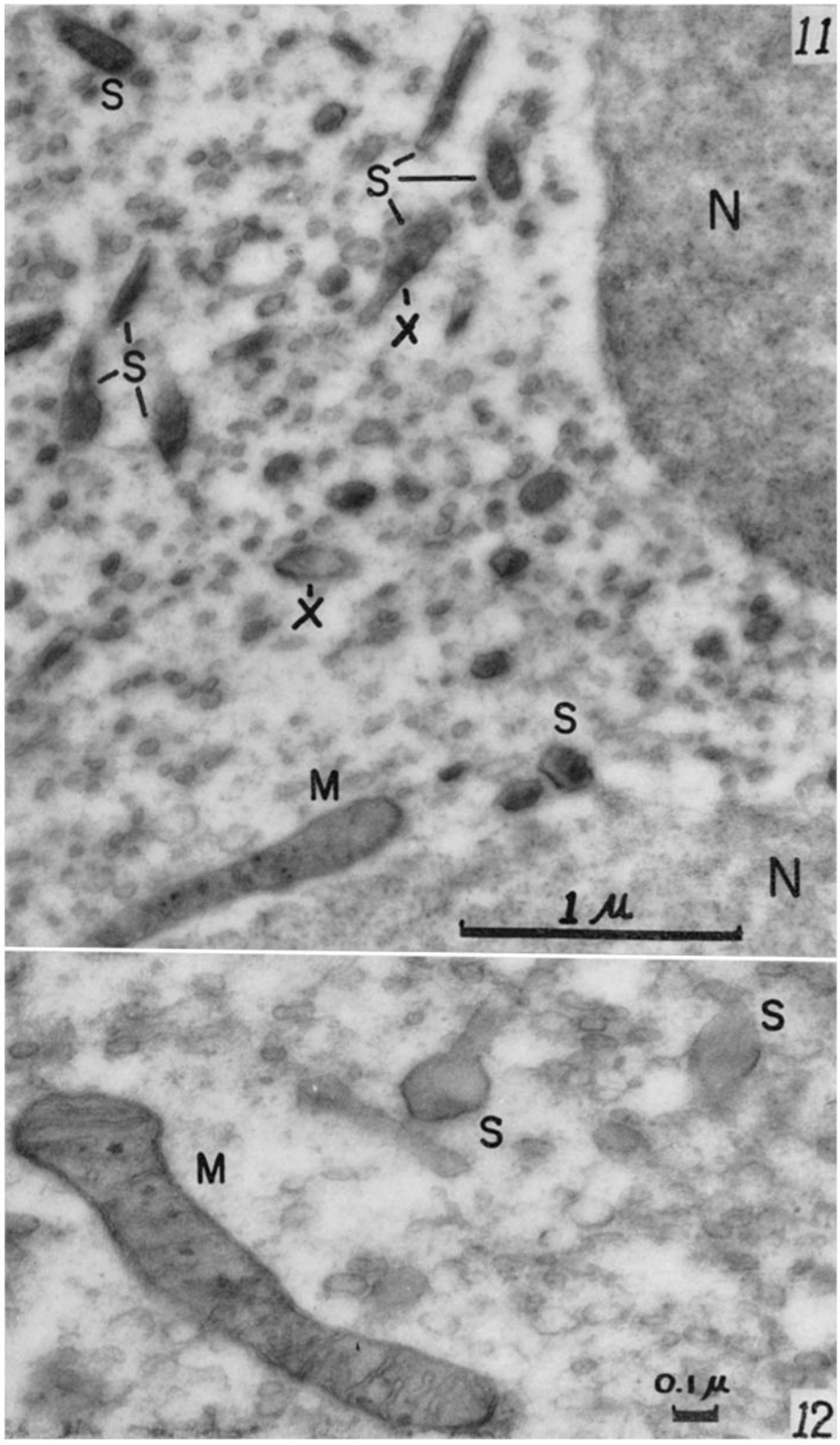


(Mitsui: Peroxidase reaction in leukocytes)

PLATE 124

FIG. 11. Electron micrograph of a neutrophil leukocyte in the perihepatic zone of *Triturus viridescens viridescens*, showing a positive peroxidase reaction limited to the specific granules (*S*). The liver tissue was treated with peroxidase reagent after osmium tetroxide fixation, and embedded in epoxy resin. One can notice that the specific granules (*S*), especially its central portion, increases in density after peroxidase reaction occurs. But, a few of the specific granules with abnormal profiles (at *X*) do not increase in density so much as other positively reacting granules. It is presumably due to variations in peroxidase activities among the specific granules. Compare the density of the specific granules (*S*) and mitochondria (*M*) in Fig. 11 (peroxidase reaction) and Figs. 10 and 12 (untreated control). The result of densitometric tracing for this picture is shown in Figs. 30 to 33. *N* (Nucleus). × 41,000.

FIG. 12. Electron micrograph of a portion of cytoplasm of normal neutrophil leukocyte in the perihepatic zone of *Triturus viridescens viridescens*. Various profiles of the specific granules (*S*) can be seen. It is clear in this picture that, in the normal cytoplasm, the specific granules (*S*) do not have a greater density than the mitochondria (*M*). This finding is important in judging the change in density incurred by the specific granules after the peroxidase reaction. × 59,000.



(Mitsui: Peroxidase reaction in leukocytes)

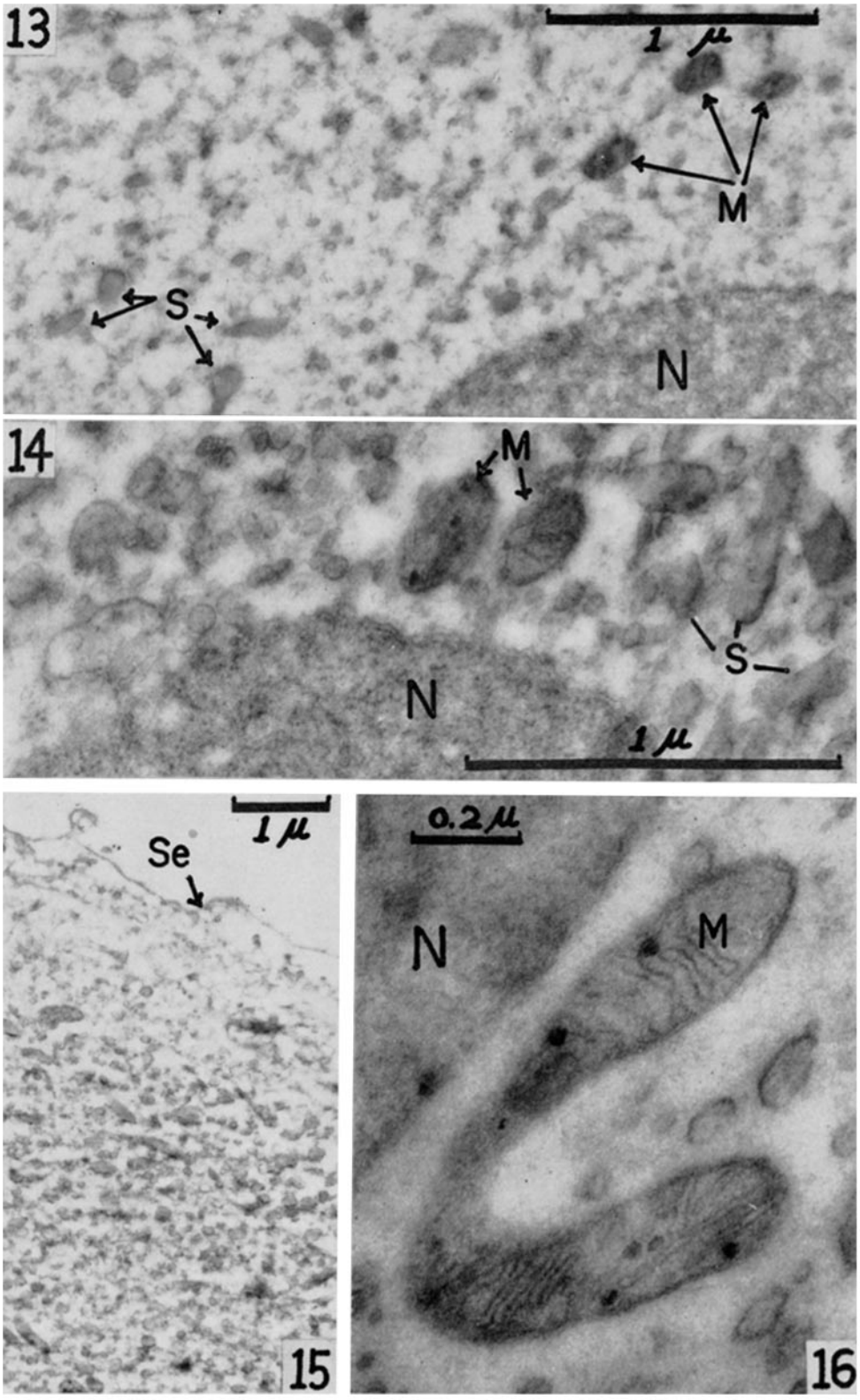
PLATE 125

FIG. 13. Electron micrograph of a control test with hydrogen peroxide in *Triturus viridescens viridescens*. After osmium tetroxide fixation, the tissue was treated with hydrogen peroxide alone. A portion of a neutrophil leukocyte in the perihepatic zone is shown here. The specific granules (*S*) do not increase in density. The result of densitometric tracing for this picture is shown in Figs. 25 and 26. *N* (Nucleus), *M* (Mitochondria). × 41,000.

FIG. 14. Electron micrograph of a control test with benzidine solution in *Triturus viridescens viridescens*. After osmium tetroxide fixation, the tissue was treated with benzidine solution alone. A portion of a neutrophil leukocyte in the perihepatic zone is shown here. Mitochondria (*M*), and specific granules (*S*) showing no increase in density, can be seen. The result of densitometric tracing for this picture is shown in Figs. 23 and 24. *N* (Nucleus). × 56,000.

FIG. 15. Electron micrograph of an inhibition test with methanol in *Triturus viridescens viridescens*. After osmium tetroxide fixation, the tissue was exposed to methanol, washed in water, and treated with peroxidase reagent. A portion of a neutrophil leukocyte and serous membrane (*Se*) are represented here. It is shown here that the specific granules do not change in density, in other words, that the peroxidase reaction of the specific granules is inhibited with methanol as has already been observed under the light microscope. Methanol, however, seems to be damaging to the cytoarchitecture under this circumstance. × 14,000.

FIG. 16. Electron micrograph of a portion of the normal neutrophil leukocyte in *Triturus viridescens viridescens*. The liver tissue was embedded in epoxy resin after osmium tetroxide fixation. A horseshoe-shaped mitochondrial profile (*M*) with intramitochondrial granules can be seen. Cristae mitochondriales are disposed perpendicularly, obliquely, or almost parallel to the long axis. × 86,200.



(Mitsui: Peroxidase reaction in leukocytes)

PLATE 126

FIG. 17. Electron micrograph of a portion of cytoplasm of normal neutrophil leukocyte in the perihepatic zone of *Triturus viridescens viridescens*. This is a comparatively thick section. *S* (Specific granule), *M* (Mitochondria), *N* (Nucleus). × 28,400.

FIG. 18. Densitometric tracing performed along the pathway shown by black line in Fig. 17. Relative value of the light absorption in per cent is shown on the ordinate, and the distance in μ on the electron micrograph is on the abscissa. Higher densities are recorded as upward deflections of the curve.

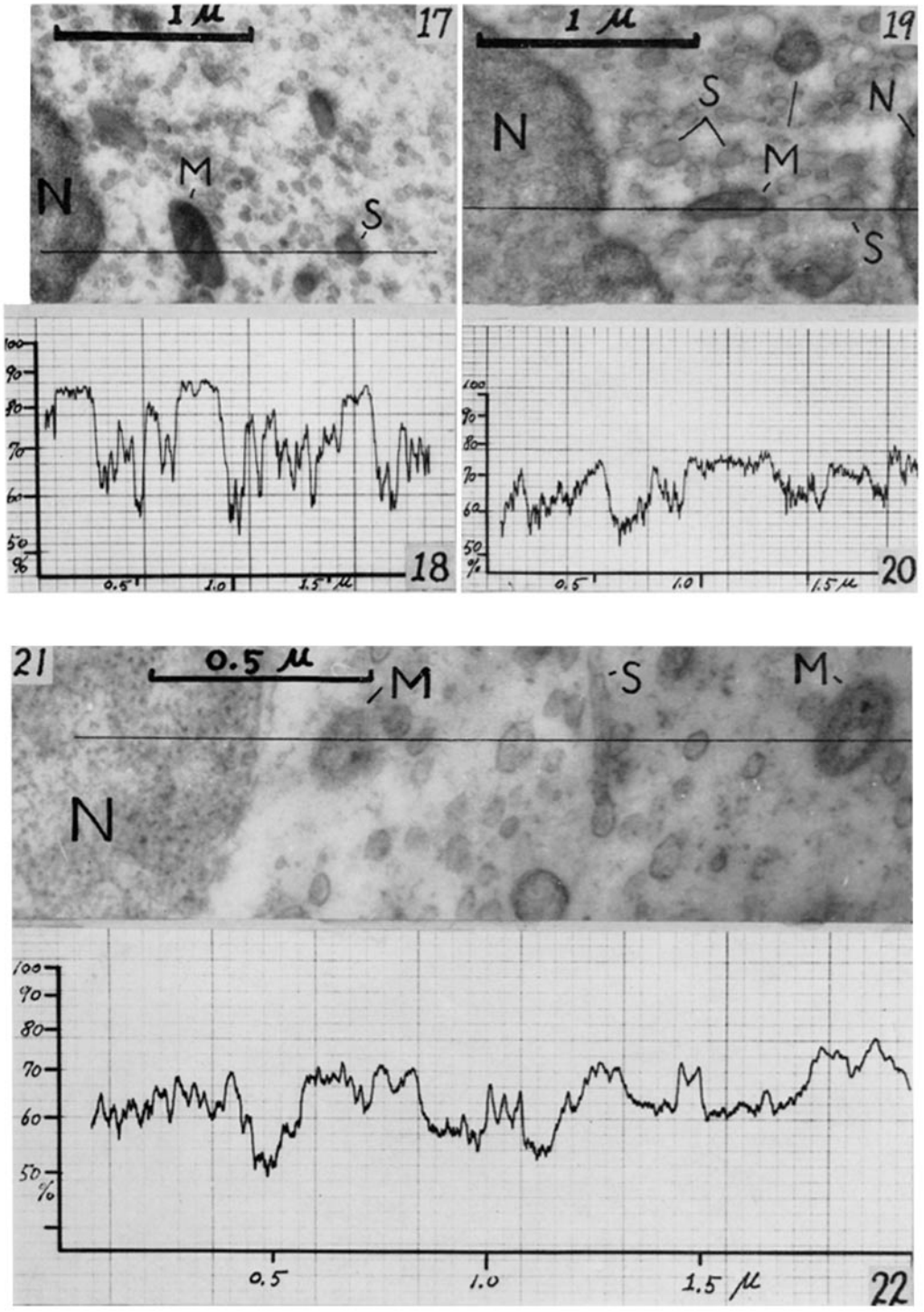
FIG. 19. Electron micrograph of a portion of cytoplasm of normal neutrophil leukocyte in the perihepatic zone of *Triturus viridescens viridescens*. *S* (Specific granule), *M* (Mitochondria), *N* (Nucleus). × 31,800.

FIG. 20. Densitometric tracing along the black line in Fig. 19.

FIG. 21. Electron micrograph of a portion of cytoplasm of normal neutrophil leukocyte of *Triturus viridescens viridescens*. The tissue was embedded in epoxy resin. *S* (Specific granule), *M* (Mitochondria), *N* (Nucleus). × 63,600.

FIG. 22. Densitometric tracing along the black line of Fig. 21.

The densitometric tracings in Figs. 17 to 22 of the normal neutrophil leukocyte (untreated controls) reveal that the density of the specific granules (*S*) is much the same or slightly less than that of the mitochondria (*M*), and that both the cristae mitochondriales and the limiting membrane (the frame resembling a membrane) of the specific granules have higher densities.



(Mitsui: Peroxidase reaction in leukocytes)

PLATE 127

FIG. 23. Electron micrograph of a control test with benzidine solution, showing a portion of cytoplasm of neutrophil leukocyte of *Triturus viridescens viridescens* (a portion of Fig. 14). *S* (Specific granule), *M* (Mitochondria), *N* (Nucleus). × 40,900.

FIG. 24. Densitometric tracing along the black line in Fig. 23. Ordinate (relative value of the light absorption in per cent), abscissa (μ on the electron micrograph).

FIG. 25. Electron micrograph of a control test with hydrogen peroxide solution, showing a portion of cytoplasm of neutrophil leukocyte of *Triturus viridescens viridescens* (a portion of Fig. 13). *S* (Specific granule), *M* (Mitochondria), *E* (Endoplasmic reticulum), *N* (Nucleus). × 40,900.

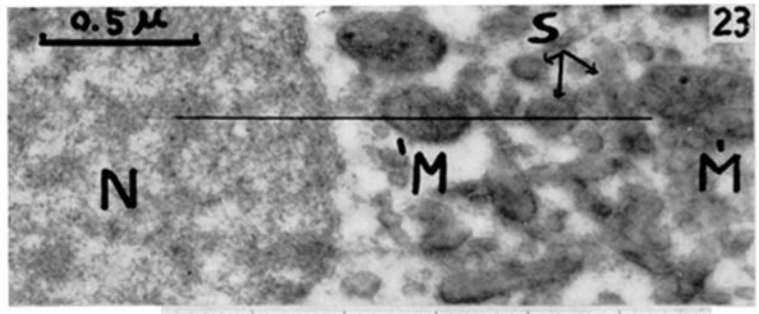
FIG. 26. Densitometric tracing along the black line in Fig. 25.

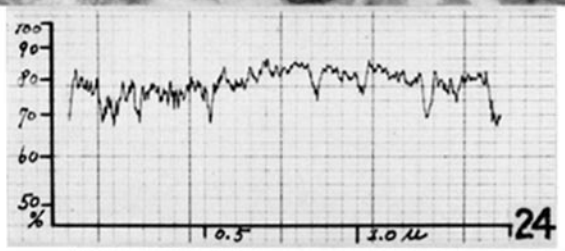
FIG. 27. Electron micrograph of an inhibition test with methanol, showing a portion of neutrophil leukocyte found in the perihepatic zone of *Triturus viridescens viridescens*. Methanol, however, seems to be damaging to the cytoarchitecture. *S* (Specific granule), *M* (Mitochondria), *N* (Nucleus). × 23,900.

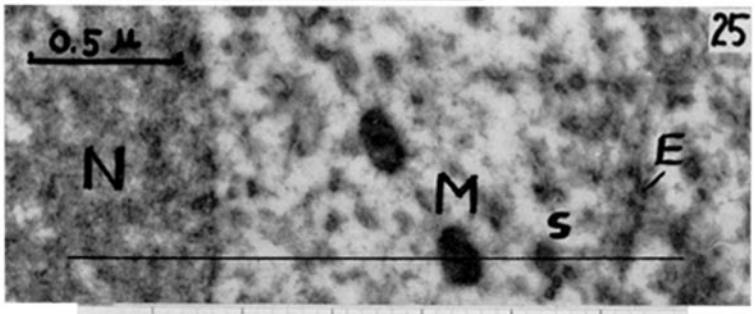
FIG. 28. Enlargement of the area outlined in Fig. 27, showing specific granule (*S*), mitochondria (*M*), and a portion of nucleus (*N*). × 31,800.

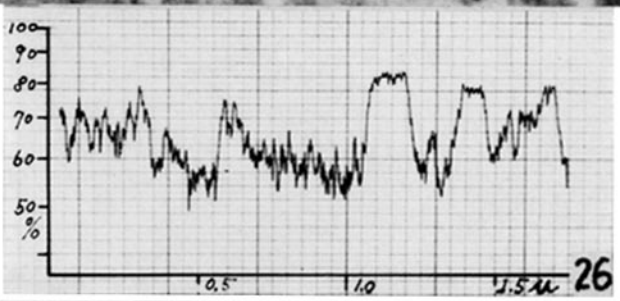
FIG. 29. Densitometric tracing along the black line in Fig. 28.

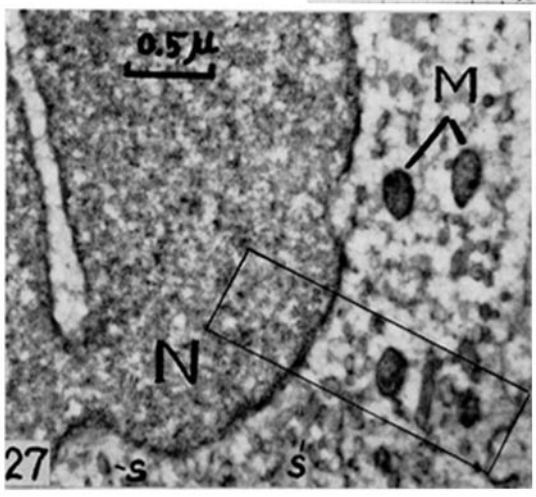
It is known from Figs. 23 to 29 that, in the controls and inhibition tests, the density of the specific granules is much the same or slightly less than that of the mitochondria.

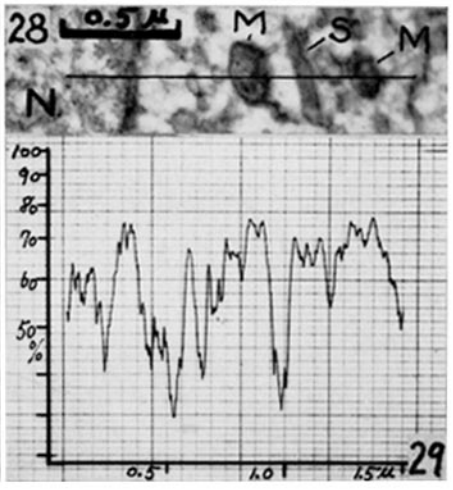












(Mitsui: Peroxidase reaction in leukocytes)

PLATE 128

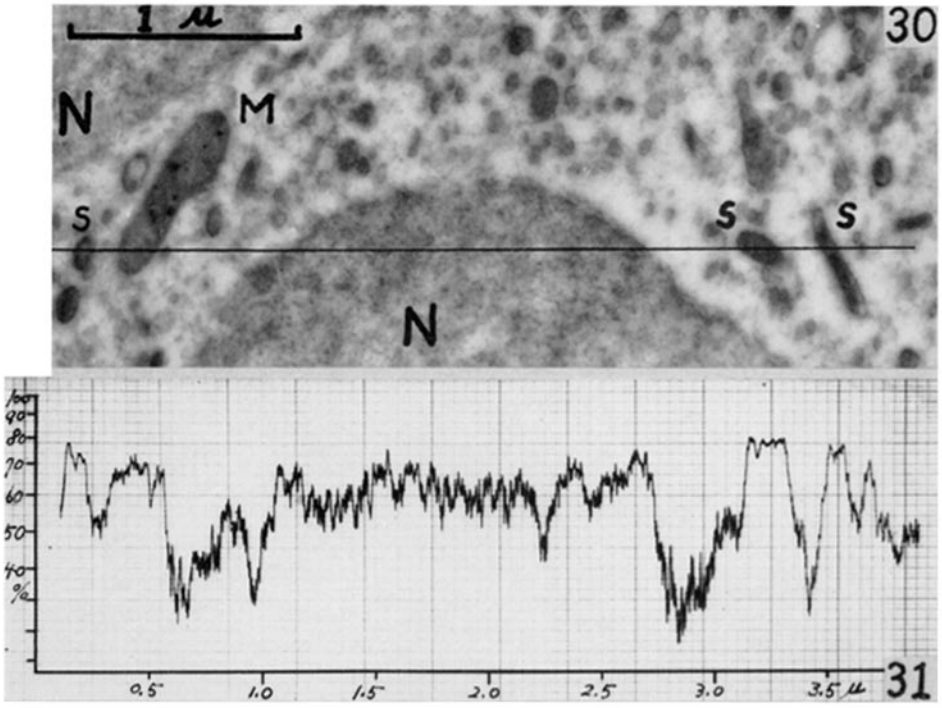
FIG. 30. Electron micrograph of a portion of a neutrophil leukocyte in the perihepatic zone of *Triturus viridescens viridescens*, showing a positive peroxidase reaction in the specific granules (*S*). This picture corresponds to the right portion of Fig. 11. *M* (Mitochondria), *N* (Nucleus). × 30,700.

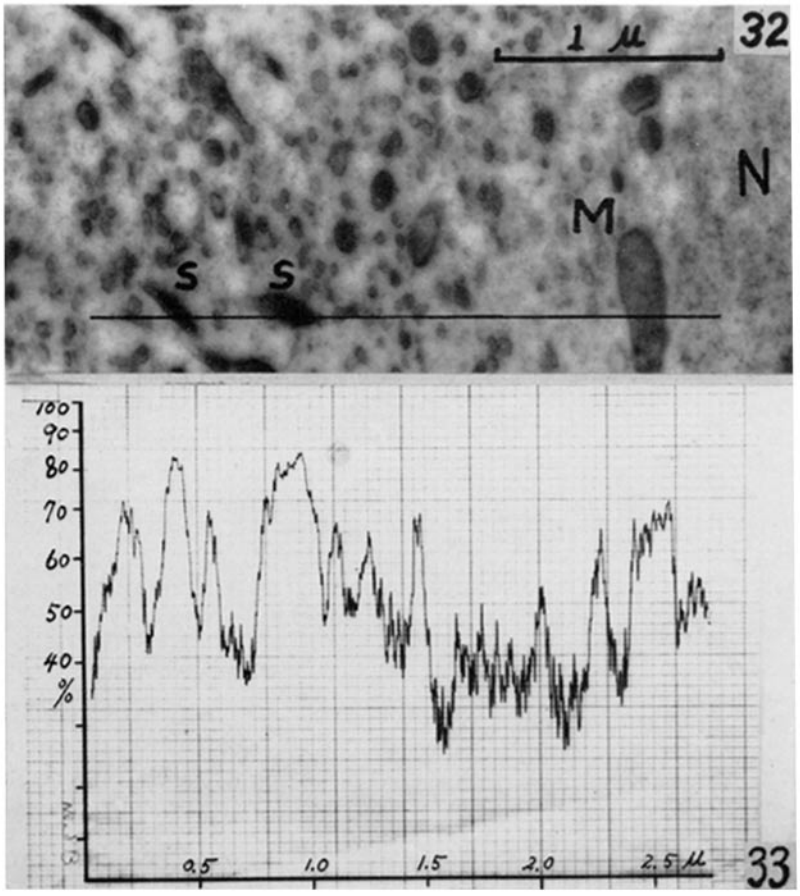
FIG. 31. Densitometric tracing along the black line in Fig. 30. Ordinate (relative value of the light absorption in per cent), abscissa (μ on the electron micrograph).

FIG. 32. Electron micrograph of a portion of a neutrophil leukocyte in the perihepatic zone of *Triturus viridescens viridescens*, showing a positive peroxidase reaction in the specific granules (*S*). This picture corresponds to the left portion of Fig. 11. *M* (Mitochondria), *N* (Nucleus). × 30,700.

FIG. 33. Densitometric tracing along the black line in Fig. 32. The sensitivity of the photometer was increased 1.7 times as compared to Fig. 31. Ordinate (relative value of the light absorption in per cent), abscissa (μ on the electron micrograph).

It is clear from these results of densitometric tracings that the specific granules in test-incubated cells have the greatest density among the cellular components, and that the granules show a relatively low increase in density as compared to those in controls and inhibition tests.





(Mitsui: Peroxidase reaction in leukocytes)