Rapid Cytochemical Identification of Phagosomes in Various Tissues of the Rat and their Differentiation from Mitochondria by the Peroxidase Method*

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(Received for publication, September 15, 1958)

ABSTRACT

1. Granules characterized by their ability to segregate foreign proteins (phagosomes) were identified in the cells of many rat organs after intravenous administration of horseradish peroxidase, by using the conventional test with benzidine for the histochemical detection of peroxidase. The largest numbers of phagosomes were identified in kidney and liver. Considerable numbers were observed cytochemically in pancreas, prostate, epididymis, thymus, spleen, bone marrow, small intestine, heart, pituitary, and mouse mammary carcinoma.

2. The variation in size of the phagosomes ranging from the limit of microscopic visibility up to 5 μ diameter, previously described for kidney, was also observed to occur in many of the other organs. The *average* size of the phagosomes in different organs was also different, the phagosomes of the liver, for example being on the average smaller than those of the kidney, pancreas, and prostate.

3. In squash preparations of kidney and liver, the phagosomes appeared often in curved rows following the course of the cell membranes of epithelial cells. In several other organs, they appeared aggregated in cells located in the vicinity of blood or lymphatic vessels or capillaries.

4. After injection of peroxidase directly into the brain of a rabbit, a striking concentration of peroxidase was observed in phagosomes of endothelial cells of capillaries and vessels, surrounding the site of injection. It was suggested that this localization may offer an explanation for the so called blood-brain barrier.

5. The cytochemical peroxidase method was applied to smears of isolated fractions of kidney and liver. Only the isolated phagosomes, but not the isolated nuclei, mitochondria, and microsomes, reacted with benzidine after administration of peroxidase. The contamination of conventionally prepared nuclear, mitochondrial, and microsomal fractions of kidney and liver with phagosomes of different sizes was observed. By correlating the cytochemical peroxidase test of smears of isolated fractions with the colorimetric determination of peroxidase, acid phosphatase, and cytochrome oxidase in the same fractions, the differentiation of the phagosomes from mitochondria and other cell granules was facilitated.

6. The marked difference in the osmotic properties of phagosomes and mitochondria, detectable after treatment with 70 per cent alcohol, and the difference in their affinities towards basic fuchsin, made it possible to differentiate the phagosomes from the mitochondria. It was found by this simple procedure that kidney cells of normal rats contain a large number of phagosomes ranging in size from 0.5 to 3 μ, whereas liver cells of normal rats contain relatively few phagosomes of this size but many smaller ones (0.2 to 0.5 μ diameter). These increased in size after treatment of the rats with horseradish peroxidase.

* This investigation was supported by research grants from the National Cancer Institute, United States Public Health Service (No. C-2485), and from the New York Heart Association.

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It was pointed out in a previous report (1) that the ability of cells to incorporate protein molecules and other materials of macromolecular or colloidal size (so called pinocytosis and athrocytosis) is probably related to the activity of the cell membrane and certain cytoplasmic granules. Granules of this type ("droplets"), ranging in size from 0.1 to 5 μ diameter, have been isolated from the kidney cells of rats and were found to contain high concentrations of acid phosphatase, acid ribonuclease and deoxyribonuclease, cathepsin, and β-glucuronidase (2, 3). High concentrations of the same enzymes have been discovered in fractions isolated from rat liver by de Duve, Pressman, Gianetto, Wattiaux, and Appelmans (4) who called the granules in which these enzymes are concentrated "lysosomes."

As described (1, 5), the ability of the granules to concentrate proteins can be analyzed by injecting intravenously a few milligrams of a plant enzyme, horseradish peroxidase, as a tracer, and by measuring colorimetrically the concentration of the injected enzyme in homogenates of various tissues or in fractions, isolated from the homogenates by differential centrifugation. By using this method, it was observed that the injected peroxidase was concentrated only in the droplet fractions, but not in the other fractions isolated from a kidney homogenate (5). The largest proportion of the peroxidase administered was present in extracts of kidney and liver; considerable amounts were also taken up by bone marrow, spleen, thymus, pancreas, prostate, epididymis, and small intestine (1). It was pointed out (1) that the analysis of homogenates has to be correlated with the analysis of purified fractions and with histochemical observations before definite conclusions concerning the incidence and properties of these granules in various tissues can be made. The term "phagosomes" has been suggested for these granules characterized by segregating ability (1).

The following report gives an account of a cytochemical modification of the peroxidase method by using the formation of a blue pigment with benzidine for the cytochemical detection of peroxidase. It will be shown that the phagosomes after having taken up the injected peroxidase, can be identified rapidly in squash preparations of various tissues as well as in homogenates and isolated fractions by their reaction with benzidine. By applying the benzidine test to isolated fractions, it was possible to differentiate the phagosomes from the mitochondria and from other cell granules since only the phagosomes showed a positive reaction for peroxidase.

I. Reagents

Benzidine.—A solution of benzidine-dihydrochloride in distilled water was made alkaline with NaOH, the free base was extracted with peroxide-free ether, and the ether was evaporated under reduced pressure. The solutions used contained 0.2, 0.3, and 0.4 per cent of the residue in 70 per cent alcohol. *Hydrogen peroxide.*—1.0 ml. of a 3 per cent solution was added to 100 ml. of the benzidine solution. *Sodium nitroferricyanide.*—1.0 ml. of a 30 per cent solution was added to 100 ml. of the benzidine solution. *Helly's fixative.*—A stock solution containing 5 per cent $K_2Cr_2O_7$, 5 per cent $HgCl_2$, and 1 per cent Na_2SO_4, was diluted 1:4 with distilled water, and 4 ml. of neutral formalin were added to 100 ml. of the diluted solution before use. *Iodine.*—A stock solution containing 0.5 per cent iodine in 50 per cent ethyl alcohol was diluted 2:5 with distilled water. *Sodium thiosulfate.*—A 2 per cent solution was used. *Basic fuchsin.*—A 0.05 per cent solution was used. *Horseradish peroxidase.*—A preparation from the Nutritional Biochemicals Corporation, Cleveland, Ohio, designated by the company "60 units per mg." was used. Since it showed 1685 QDI-red units, corresponding approximately to a purpurogallin number of 325, it had a purity of approximately 27 per cent (1).

II. Preliminary Experiments and Observations

Small pieces of fresh kidney and liver taken from peroxidase-treated and untreated rats were squashed on a microscopic slide with a cover slip, and the reaction with benzidine (formation of a blue pigment by peroxidase) was tried out with this material. All experiments were performed in the cold room (4°C.).

(*a*) *Benzidine Reaction for Peroxidase.*—If squash preparations of kidney and liver from animals injected with peroxidase were treated with an alcoholic solution containing 0.2 to 0.4 per cent benzidine, 0.3 per cent sodium nitroferricyanide, and 0.03 per cent H_2O_2, as indicated by Gomori (6), the phagosomes were found to be stained intensely blue. For reasons mentioned in section *II* (*c*), a 70 per cent alcoholic solution was used as a routine procedure.

(*b*) *Effects of Fixation and Drying of Tissue on the Benzidine Reaction.*—After fixation for 3 minutes in 10 per cent neutral formalin or in diluted Helly's solution, the peroxidase reaction of the phagosomes was abolished. When the squash preparations were treated, prior to the incuba-

tion with benzidine, with 50 to 100 per cent ethyl alcohol for ½ to 1 minute at 4°C., the reaction for peroxidase in the phagosomes was still positive but much attenuated. When the squash preparations were allowed to dry on a microscopic slide and kept for 1 hour in the dried state at 4°C., the reaction was still intense. Even after short flaming in the dried state, the reaction was partially preserved.

(*c*) *Effects of Fixatives and Drying on Preservation of Phagosomes and Mitochondria.*—The phagosomes were observed to swell when squash preparations of kidney and liver were immersed in absolute alcohol, but appeared preserved or only slightly swollen when treated with 70 per cent alcohol. The phagosomes were preserved after fixation with 10 per cent formalin or with diluted Helly's solution.

In contradistinction to the phagosomes, the mitochondria were observed to disintegrate on treatment of the squash preparations with absolute alcohol and to be only partially preserved after treatment with 70 per cent alcohol. They were preserved after fixation with neutral formalin or diluted Helly's solution.

It was found that the mitochondria and phagosomes were preserved when small pieces of fresh kidney or liver were smeared over a microscopic slide without using pressure, by touching each field only once, and by allowing the very thin layer of cell material which adhered to the slide, to dry at 4°C. It is probable that the fast drying (in a matter of seconds) of the very thin layer of cellular material is responsible for the preservation of structure. Most cells were broken up during the procedure and their contents redistributed. Such a preparation, in the case of soft tissues at least, may be compared to a total homogenate which has been dried and fixed immediately after the homogenization of the tissue. The term "touch preparation" is used in the following pages for this type of preparation.

(*d*) *Stability of Blue Benzidine-Peroxidase Pigment towards Reagents Used.*—The blue stain developed by peroxidase in the phagosomes remained stable when the slides with the benzidine-treated tissues were kept for 24 hours in 70 per cent alcohol, diluted Helly's solution, or in 10 per cent formalin. The blue pigment also remained stable when the tissue was treated with 0.2 per cent iodine in 20 per cent ethyl alcohol used to remove Hg after Helly's fixation. However, gradual fading of the pigment within 1 to 2 hours occurred when the tissue was treated with a 5 per cent solution of $Na_2S_2O_3$ used to remove iodine. Only short treatment (½ to 1 minute) with a 2 per cent solution of $Na_2S_2O_3$ was, therefore, applied for removal of iodine after Helly's fixation. The blue pigment was stable when the preparations were embedded in Canada balsam.

(*e*) *Counterstaining with Basic Fuchsin of Nuclei, Cytoplasm, and Benzidine-Unreacted Phagosomes.*—Basic fuchsin was used in some experiments to counterstain nuclei and cytoplasm to the blue peroxidase stain in the phagosomes, after fixation of the squash or touch preparations with Helly's solution or with 70 per cent alcohol. It was observed that the phagosomes in squash or touch preparations of untreated animals were stained intensely red by this dye, more deeply so than the mitochondria and the rest of the cytoplasm. In tissues of peroxidase-treated animals, those phagosomes which had failed to react with benzidine, could thus be identified (see section *IV* (*a*)).

Since prolonged fixation caused too much granularity of the cytoplasm, only short fixation (½ to 2 hours) with Helly's fluid was performed before staining with basic fuchsin. Fixation in 70 per cent alcohol for 18 to 24 hours (or longer), followed by fixation for 1 hour with diluted Helly's fluid, caused only little granularity of the cytoplasm and facilitated the identification of the phagosomes with basic fuchsin (see section *IV* (*a*)).

III. Staining of Phagosomes after Intravenous Administration of Peroxidase

(*a*) *Routine Procedure.*—In the following paragraphs, the procedure used as routine is summarized and some additional details mentioned.

Small pieces of soft tissues (kidney, liver, etc.) were squashed on a microscopic slide with a coverglass in the cold room (4°C.) where all operations were done. Hard tissues (muscle, skin, etc.) were first reduced to small pieces with the aid of fine dissecting scissors, dissecting needles, or a razor blade, and a few of these pieces were either used as such, or they were squashed on a microscopic slide with another slide. "Touch preparations" of soft tissues were prepared as indicated in section *II* (*c*). In the case of hard tissues, the "brei" obtained by cutting the tissue into fine pieces was smeared over the slide, and the cell or tissue fragments adhering to the glass, were allowed to dry quickly.

The squashed wet tissue or the dry "touch preparation" from a peroxidase-treated animal was placed toward the one end of a microscopic slide where the slide had been labelled with a diamond pencil, and an analogous tissue preparation from an untreated animal

was placed toward the other end of the same slide as control.

The slide was immersed into a 50 ml. staining dish filled with a solution containing 0.2, 0.3, or 0.4 per cent benzidine, respectively, 0.03 per cent H_2O_2, and 0.3 per cent sodium nitroferricyanide in 70 per cent alcohol, and was incubated for ½ to 3 minutes at 4°C. The concentration of benzidine and the time of incubation was varied in relation to the concentration of peroxidase in the tissue under investigation, using the lower concentrations of benzidine and the shorter times of incubation for those tissues in which the concentration of peroxidase was higher, and the higher concentrations of benzidine and the longer times of incubation for those in which it was lower (1). After incubation, the slide was rinsed with cold 70 per cent alcohol, and transferred to a 50 ml. staining dish containing alcohol in the same concentration. The preparations were either examined immediately under the microscope, and then discarded, or they were first fixed more thoroughly.

For immediate observation, the slide was removed from the dish containing 70 per cent alcohol, and the tissue was covered with a coverglass and enclosed with paraffin while still completely immersed in 70 per cent alcohol. If some fragments of tissue were too thick, they were removed before enclosure. Preparations thus obtained were stable for several hours during which time they were used for microscopic observation and photomicrography.

Duplicate preparations to be fixed more thoroughly were either left in the dish containing 70 per cent alcohol for 18 to 24 hours, or were transferred to 10 per cent neutral formalin in 30 per cent alcohol for 1 minute, and from there to diluted Helly's fluid for 1 to 24 hours (see section *II* (*e*)). After fixation in Helly's fluid, the slides were washed with water, transferred to a solution of 0.2 per cent I_2 in 20 per cent alcohol for 1 minute, washed with water, and treated with a 2 per cent solution of $Na_2S_2O_3$ for ½ to 1 minute, and again washed with water. The tissue samples, immersed in water, were then used for the microscopic investigation. In some experiments, they were counterstained with a 0.05 per cent solution of basic fuchsin for ½ to 2 minutes. If desired, air-dried preparations could be mounted in Canada balsam.

(*b*) *Phagosomes in Various Tissues.*—The procedure was applied to various tissues removed 3 to 8 hours after administration of 10 to 15 mg. of the commercial peroxidase preparation into the femoral vein of male rats weighing 300 to 400 gm. In the case of brain, 50 mg. of the peroxidase preparation were injected directly into the brain of a rabbit weighing 11 pounds. As mentioned above, control specimens from untreated animals were processed on the same slide as the tissues from the experimental animals. Most observations were made on squash preparations, immersed in 70 per cent alcohol, and enclosed with paraffin immediately after incubation with benzidine, without counterstaining (Figs. 1 to 3, 5 to 12, 18, and 19). Only the tissues shown in Figs. 4 and 13 were fixed in Helly's fluid for 18 hours, after incubation. In the following comments, the blue staining phagosomes were called "large" when their approximate diameters were from 2 to 6 μ, "intermediate-sized," with diameters from 0.5 to 2 μ, and "small" with diameters from the limit of microscopic visibility up to 0.5 μ.

Kidney.—After incubation with benzidine, the squash preparations were covered with a great number of large, intermediate-sized, and small blue granules (Figs. 1 and 2). They were often arranged in curved rows suggesting a localization in the vicinity of the cell membranes, but some appeared in clumps close to the nucleus of the epithelial cells. In those cases when a glomerulus was seen, only a few blue staining granules were present in the area covered by it (Fig. 2). No blue staining granules were seen in the control tissues from untreated animals.

Liver.—Numerous small and intermediate-sized, and relatively few large blue granules appeared after incubation with benzidine (Figs. 3 to 6). They were often arranged in curved rows or straight lines, and seemed to be located in the vicinity of the cell membranes, probably along bile or blood capillaries. They were only rarely seen over the interior portions of the epithelial cells. The leucocytes which could be identified by their fine peroxidase-positive granules, were increased in number after administration of peroxidase (Figs. 3 and 6). The control tissue from untreated animals showed a few blue granules which seemed to be derived from large granulocytes (Fig. 7 *A*). Other areas of the control tissue were free of blue granules (Fig. 7 *B*).

Pancreas.—Aggregations of large, intermediate-sized, and fewer small blue granules appeared in the neighborhood of blood or lymphatic vessels in the region of the interlobular spaces or of the base of the secretory cells (Fig. 8). The secretory granules remained unstained. No blue granules were seen in the control tissue.

Small Intestine.—After exposing the lumen, the mucosa was washed with saline, scraped off with a spatula, and smeared on a slide. Many intermediate-sized and small granules, and fewer large blue granules were seen in certain areas (Fig. 9). They were often arranged in streaks, perhaps following the course of blood or lymphatic vessels. Many leucocytes were present in certain areas. The control tissue also showed some blue granules in addition to leucocytes.

Prostate.—Aggregations of many large and intermediate-sized and fewer small blue granules appeared in the neighborhood of blood or lymphatic vessels often following the course of the vessels over some distance. They were located either in the endothelial cells, or in cells of the connective tissue surrounding them (Figs. 10 and 11). No blue granules were seen in the control tissue from untreated animals.

Pituitary.—Large, intermediate-sized, and small blue granules were seen in the neighborhood of blood capil-

laries and relatively few over secretory cells. No blue granules were seen in the control tissue.

Epididymis.—Aggregations of intermediate-sized and small blue granules were seen in cells of the connective tissue, probably in the vicinity of blood or lymphatic vessels. There was a tendency toward artifact formation (fine blue graininess of certain regions of the cytoplasm) in the tissues of both the treated and the control animals.

Thymus.—Heavy aggregations of large, intermediate-sized, and small blue granules appeared in certain cells (macrophages ?) of the connective tissue, in the vicinity of blood or lymphatic vessels. Portions of the thymus rich in lymphocytes were free of phagosomes. No blue granules were seen in tissue from untreated animals.

Bone Marrow.—Many large, intermediate-sized, and small blue granules were present in smears of bone marrow, outside of leucocytes. In smears from the control animals, fine blue granules appeared only in leucocytes.

Spleen.—Intermediate-sized and small and fewer large blue granules, the small ones often arranged in rows, were seen outside of leucocytes. The controls also showed some blue granules, in addition to the fine blue granules in leucocytes.

Lung.—Small and intermediate-sized blue granules, in addition to the small ones in leucocytes, were present in certain areas. The controls also showed some blue granules.

Heart.—Numerous large, intermediate-sized, and small blue granules were seen. They were often arranged in lines between bundles of muscle fibers and probably followed the course of capillaries (Fig. 12). No blue granules were seen in the control tissue from untreated animals.

Stomach.—Small pieces of the mucosa, squashed on a slide, showed some large, intermediate-sized, and small blue granules, in addition to those of leucocytes. The control tissue also contained some blue granules both within and outside the leucocytes.

Skeletal Muscle.—No significant numbers of blue granules were seen in muscular tissue proper. Whether they are present in the neighborhood of capillaries, must be decided on microtome sectioning.

Peripheral Nerve.—No blue granules were seen.

Brain.—No blue granules were found in the brain tissue of rats after intravenous administration of 15 mg. of peroxidase. However, when 50 mg. of the plant enzyme were injected directly into the brain of a rabbit (dorsal region of the thalamus in vicinity of the third ventricle) a striking reaction was seen in the capillaries and small vessels around the area of injection. Numerous large, intermediate-sized, and small blue granules were visible in the endothelial cells or in the glial cells surrounding them (Figs. 13 *A* to *C*).

Connective Tissue.—In subcutaneous connective tissue, certain cells showed many large, intermediate-sized, and small blue granules. The controls showed fine blue granules only in leucocytes.

Skin.—Cells containing blue granules were present but may have been derived from adherent connective tissue.

Mammary Carcinoma of Mouse.—Since such tissue was available through the courtesy of Dr. Steven Mohos, an exploratory experiment was made. Three hours after injection of 5 mg. of the plant enzyme into the tail vein of a mouse bearing a transplanted tumor, the tumor tissue showed many cells with blue granules of large, intermediate, and small size. These cells seemed to be located in the stroma, in the vicinity of vessels and capillaries.

IV. Differentiation between Phagosomes and Mitochondria in Kidney and Liver

Although the following procedures have not yet been applied to tissues other than kidney and liver, they are probably applicable generally.

(*a*) *Staining with Basic Fuchsin after Treatment with 70 per cent Alcohol.*—It was mentioned that the phagosomes are preserved after treatment with 70 per cent alcohol whereas the mitochondria partially disintegrate (section *II* (*c*)), and that the phagosomes stain with basic fuchsin more intensely than the mitochondria (section *II* (*e*)). These properties were observed when fresh squash preparations and "touch preparations" from the same kidney and liver were stained with basic fuchsin after being fixed by: (*a*) diluted Helly's mixture for 1 to 2 hours, (*b*) 70 per cent alcohol, or (*c*) 100 per cent alcohol for 1 to 24 hours.

Fig. 14 shows a "touch preparation" from the kidney of a normal rat, fixed in Helly's solution for 2 hours (section *II* (*e*)), and then stained with basic fuchsin, after the usual treatment with iodine and $Na_2S_2O_3$. It may be seen from Fig. 14 that the phagosomes are much more intensely stained than the mitochondria. As Fig. 15 shows, this differentiation is more accentuated after preliminary treatment with 70 per cent alcohol for 18 hours during which time the mitochondria partially disintegrate whereas the phagosomes are preserved.

In contradistinction to the kidney, a "touch preparation" of the liver of *normal* rats, treated with 70 per cent alcohol and then stained with basic fuchsin, showed only relatively few intensely staining phagosomes within the range of 0.5 to 5 μ diameter. Granules of this type sometimes appeared aggregated around fat droplets (Fig. 16 *A*). Most of the liver granules, charac-

terized by their resistance to 70 per cent alcohol and by their intense staining with basic fuchsin, were very small (0.2 to 0.5 μ diameter), and could only be recognized at high magnification as shown in Fig. 16. After rats had been treated with peroxidase, however, many more granules of intermediate size (0.5 to 2 μ diameter) were present as shown in Fig. 17.

Staining with basic fuchsin after treatment with 70 per cent alcohol, was also applied to smears of cell fractions isolated from kidney and liver homogenates by differential centrifugation. In these smears, the larger phagosomes but not the small ones could often be distinguished by their more intense staining.

(*b*) *Application of Peroxidase Method to Isolated Fractions.*—Since in smears of isolated fractions from peroxidase-treated animals, only the phagosomes but not any of the other, isolated cell organelles reacted with benzidine, the phagosomes could be differentiated from the mitochondria and from the other cytoplasmic granules by this procedure. Air-dried smears from the sediment of isolated fractions or from the original homogenate were submitted to the same treatment as indicated in section *III* (*a*) for squash preparations and "touch preparations."

The sucrose used in the isolation of cytoplasmic fractions (7) did not interfere with the reaction of benzidine with peroxidase. However, in the presence of too much sucrose, the cell material did not always remain fixed to the glass slide. The amount of sucrose was therefore reduced as much as possible by taking only a small amount of the sediment to be investigated on a glass rod, transferring it to a microscopic slide, and smearing it over the slide with the edge of a cover slip. Homogenates in 30 per cent sucrose solution were diluted with water 1:1, and only a very small drop was used for the smear. If the smear dried within a minute, the amount of sucrose had been sufficiently reduced.

Figs. 18 and 19 show the benzidine test with smears of fractions isolated by differential centrifugation from kidney and liver homogenates of peroxidase-treated rats. It should be noted that no attempt was made to purify these fractions; they were only used to try out the procedure and to identify the peroxidase-segregating granules.

(*i*) *Kidney.*—Roughly purified droplet fractions I and combined fractions II + III were isolated (5, 11) from the kidneys of rats, 3 hours after treatment with peroxidase; mitochondria and microsomes were prepared according to Hogeboom, Schneider, and Palade (7). After incubation with benzidine, the smear of droplet fraction I showed many of the large droplets (2 to 5 μ diameter), but not all of them stained intensely blue (Fig. 18 *A*). Droplet fraction II + III, of which no good photograph was made, contained a large number of granules, 0.2 to 1 μ diameter, stained intensely blue, among many colorless granules. The mitochondrial fraction was contaminated by many blue-staining droplets, 0.2 to 1 μ diameter, especially in the lower layer of the sediment. After resuspension and resedimentation of the mitochondria, the contaminating phagosomes were still present, but reduced in number in the upper layer of the mitochondrial sediment (Fig. 18 *B*). The mitochondria themselves remained completely unstained. Although mitochondria partially disintegrated after prolonged treatment with 70 per cent alcohol (sections *II* (*e*) and *IV* (*a*)), they were relatively well preserved after treatment for 1 to 2 minutes with the benzidine reagent in 70 per cent alcohol (Fig. 18 *B*). The upper layer of the microsomal sediment, and more so the lower layer, showed many extremely small blue points, just recognizable in focusing up and down, among an excess of unstained material.

(*ii*) *Liver.*—A series of cytoplasmic fractions were isolated from a liver homogenate in 30 per cent sucrose solution by successive centrifugation, for 15 minutes at each speed, at 2000, 6000, 15000, and 28000 *g*, and then, after diluting the sucrose to 15 per cent, for 90 minutes at 28000 *g*. After incubation with benzidine, the smears of the lowest layers of each sediment contained many intensely blue-staining granules among a great number of colorless granules. Fig. 19 *A* shows a smear of the bottom layer of the sediment obtained at 2000 *g*, with most phagosomes having a diameter of 1 to 2 μ. The phagosomes in the sediment obtained at 6000 *g* were more polydisperse, ranging from 0.5 to 2 μ in diameter (Fig. 19 *B*); those obtained at 15000 *g*, and those present in the small brown layer at the bottom of the microsomal sediment (Fig. 19 *C*), ranged in size from 0.2 to 1 μ diameter. As was the case with the microsomal sediment of the kidney, the upper layer of the microsomal sediment of the liver was also contaminated with many tiny blue granules. The nuclei, mitochondria, and microsomes, in their respective sediments, remained completely unstained. As shown in Fig. 19 *D*, the fat layers floating on the sur-

face and clinging to the walls of the centrifuge tube after high speed centrifugation, were often contaminated by phagosomes ranging in size from 1 to 2 μ diameter.

The extensive overlapping of phagosomes and mitochondria in fractions isolated from the liver of peroxidase-treated rats by conventional methods (7) was also observed when the specific activity of acid phosphatase and cytochrome oxidase was correlated with that of peroxidase (1) in fractions obtained by differential centrifugation at 2000, 6000, 10500, 15000, and 28000 *g*. In two exploratory recovery experiments, it was found that the combined large granule fractions sedimenting at 2000, 6000, 10500, and 15000 *g*, contained 70 to 80 per cent of the cytochrome oxidase, 60 to 70 per cent of the peroxidase, and approximately 40 per cent of the acid phosphatase of the original homogenate. In other exploratory experiments, premitochondrial fractions were separated which showed 3 to 4 times higher peroxidase activity, 2 times higher acid phosphatase activity, and no higher cytochrome oxidase activity than the original homogenate. Postmitochondrial fractions were obtained, showing 4 times higher acid phosphatase, 1 to 2 times higher peroxidase, and no higher cytochrome oxidase activity than the original homogenate. As will be mentioned in the Discussion, the fat layers sometimes showed increased peroxidase and acid phosphatase, and low cytochrome oxidase activity.

DISCUSSION

By using squash preparations and "touch preparations," the phagosomes could be roughly localized and their incidence and size in various tissues could be estimated. However, the structure of the tissues was distorted in the squashing, and unequal penetration of the reagents into the squashed tissue may have caused unequal staining of peroxidase. Microtome sections would be required for a more accurate cytological and histological analysis. The experience gained from using squash preparations may facilitate the application of the peroxidase method to frozen-dried or cryostat sections.

The reliability of the histochemical peroxidase reaction with benzidine for the identification of phagosomes depends on the absence of artifacts and on adequate controls. Artifacts caused by the crystallization of the blue peroxidase-benzidine pigment occurred rarely in our experiments. This may be attributed to the following factor. As mentioned in section *II* (*b*), alcohol weakens the activity of peroxidase within the first minute of incubation. Since the incubation mixture contained 70 per cent alcohol, the enzymatic formation of the blue pigment was slowed down or stopped after the phagosomes had become strongly stained, and before crystallization took place. This process could be regulated by varying the concentration of benzidine between 0.2 and 0.4 per cent, and the time of incubation at 4°C. between 0.5 and 3 minutes.[1] Under these conditions, red blood cells did not hemolyze and hemoglobin did not react with benzidine although it has a weak peroxydatic activity.

The processing of control tissues from untreated animals on the same slide as the tissues from the experimental animals showed the presence of some benzidine-positive granules in squash preparations of some organs of normal rats. In general, these were the organs which normally contain a certain number of leucocytes (stomach, intestine, spleen, lung, liver). Since leucocytes contain peroxidase, the benzidine-reactive granules of normal tissues are probably derived from them. Although other types of leucocytes are difficult to disrupt, Fig. 7 *A* suggests that certain types, probably eosinophils, can release peroxidase-containing granules. Neufeld *et al.* (8) have measured recently the peroxidase activity of animal tissues and have found some activity in stomach, intestine, spleen, and lung. These are the same organs which showed some blue granules in the controls of our experiments. Several other organs such as kidney, pancreas, prostate, heart, thymus, containing many phagosomes, did not have any blue granules in specimens taken from untreated animals. By comparing on the same slide the experimental and control tissues, the effect of the peroxidase treatment could, in most experiments, be readily appreciated. In liver, for example, the abundance of phagosomes, and their characteristic localization presented a very different picture from the few blue granules in the control tissue. If in certain experiments, it is difficult to distinguish experimental from control tissue, the contrast between them can be accentuated by

[1] If the formation of the blue pigment was not interrupted early enough, the phagosomes began to show angular outlines as in Figs. 4 and 19 *A*. If the incubation was still continued, crystals began to grow out from the phagosomes.

increasing the dose of injected peroxidase. It must be remembered, however, that the uptake of peroxidase by the phagocytic white blood cells which normally contain peroxidase, cannot be observed by this cytochemical method.

The strong reaction of the phagosomes of various tissues with benzidine after administration of horseradish peroxidase confirms the previous conclusion (5, 9) that these cell granules have the ability to segregate foreign proteins. Since no reaction with benzidine was observed in any of the other cell organelles, this property seems to be characteristic for the phagosomes. Although Coons *et al.* (10) have found a concentration of antigens in the nuclei of certain cells with their fluorescent antibody method, no peroxidase reaction in nuclei was seen during the present experiments.

The cytochemical estimation of the incidence of phagosomes in various tissues (section *III* (*b*)), corroborates in general the colorimetric analysis of peroxidase in extracts of the same tissues reported previously (1). With both procedures, a very high incidence of phagosomes or peroxidase was found in kidney and liver. Considerable uptake was observed also in pancreas, prostate, epididymis, thymus, bone marrow, spleen, and small intestine. More phagosomes were revealed histochemically in heart tissue (Fig. 12) than was expected from the colorimetric analysis of extracts (1). This shows that in the previous experiments (1) the tough heart tissue had not been homogenized sufficiently, and that many phagosomes had probably been discarded with the unhomogenized residue. The presence of a considerable number of phagosomes in the pituitary and in mouse mammary carcinoma, not previously tested colorimetrically, could be ascertained by the histochemical procedure.

The isolation of droplet fractions from the kidneys of normal rats (3) had shown that their size varies from the limit of microscopic visibility up to 5 μ diameter. As in the kidney, the present cytochemical study has indicated a similar spread of size in the phagosomes of various other organs. Although a small amount of swelling may have taken place under the influence of the reagents, large phagosomes (1 to 5 μ diameter) and intermediate-sized phagosomes (0.5 to 1.5 μ diameter) were seen, for example, in the cells of the pancreas, prostate, liver, thymus, connective tissue, and in the vicinity of blood vessels of pituitary and brain. The smallest phagosomes, many probably submicroscopic in size, were present in isolated microsomal fractions of kidney and liver (section *IV* (*b*)). In previous experiments on kidney (11), the administration of egg white had induced a transformation of small into large droplets, connected with a change in the intracellular distribution of the hydrolytic enzymes and their release from a "bound" to a "free" (active) state. The treatment with horseradish peroxidase in the present investigation seems to have caused also an increase in the size of the phagosomes. This may be seen from the experiments shown in Figs. 16 and 17, in which phagosomes in liver of normal and peroxidase-treated rats, identified by their staining with basic fuchsin after treatment with 70 per cent alcohol, showed variation in size. It was observed in other experiments (12) that the injected peroxidase is concentrated in the kidney at first in the small phagosomes (fraction III) and later in the large ones (fraction I). All these observations seem to indicate that the variation in size of the phagosomes is connected with the uptake of proteins or with related metabolic changes. In addition to the variations in size of the phagosomes in the same organs, their *average* size differs also in different organs, the phagosomes of the liver, for example, being on the average smaller than those of the kidney and prostate (compare Figs. 1 and 3, and 15 and 17).

The phagosomes range widely in size from submicroscopic dimensions up to 5 μ diameter. This broad variation explains why it is difficult to isolate cell fractions by differential centrifugation without contamination by phagosomes. This especially is the case of organs very rich in phagosomes such as kidney and liver. The difficulties in preparing homogeneous mitochondrial and microsomal fractions by differential centrifugation have been discussed by Hogeboom, Kuff, and Schneider (13), de Duve (14), and Novikoff *et al.* (15). It is not always realized (16) that nuclear fractions, too, can easily be contaminated with phagosomes, especially from those organs (kidney and pancreas, for example) which contain a considerable number of large phagosomes, and that cathepsin, DNAase RNAase, or acid phosphatase, found in isolated nuclear fractions, may be derived from contaminating large phagosomes. By applying the peroxidase method to smears of isolated fractions from kidney (section *IV* (*b*)), it was observed that many phagosomes remained in the upper

layers of the nuclear, mitochondrial, and microsomal fractions although a great number of them had separated out in the dark brown layers at the bottom of the sediments (3, 11). The activity of hydrolytic enzymes, observed previously in isolated nuclear, mitochondrial and microsomal fractions from the kidneys of normal rats (11), was, therefore, probably derived from these contaminating phagosomes. Many intermediate-sized phagosomes (0.5 to 2 μ diameter) were sedimented together with mitochondria from liver homogenates (Figs. 19 *A* and *B*). They were probably identical with the granules in squash preparations and "touch preparations" showing peroxidase reaction (Figs. 3 to 6) and staining with basic fuchsin (Fig. 17). The same type of large granules may have been present in certain layers of the sediment which Kuff and Schneider (17) obtained by packing of the mitochondrial fraction from liver by high speed centrifugation, and which they found to be rich in DNAase. The contamination by phagosomes seems to be still more pronounced in mitochondrial and microsomal fractions isolated from liver than from kidney, since the average size of the phagosomes in liver is smaller, and since the separation of the phagosomes to the bottom layer of the sediments during differential centrifugation does not seem to take place to the same extent as in kidney.

The presence of a considerable number of phagosomes in the fatty layers floating on the surface of the suspensions or clinging to the wall of the centrifuge tube after high speed centrifugation of liver homogenates (Fig. 19 *D*) requires some comments. At first, it was thought that this was an accidental contamination by occlusion or adsorption and, therefore, an artifact of the experimental procedure. However, two types of observations seem to contradict this interpretation. The first observation refers to a persistent separation of relatively large phagosomes (1 to 2 μ diameter) in the fat layers during high speed differential centrifugation when a comparable concentration of phagosomes of similar size was no longer found in the suspension or in the sediment. The second observation refers to an experiment not described in the text in which a fat layer was isolated from the wall of the centrifuge tube by a procedure used previously for the isolation of lipoprotein structures from plant cells (18), and was found to contain approximately 4 times higher activity of acid phosphatase and peroxidase than the total homogenate. The application of the cytochemical peroxidase test (not yet developed at the time of this experiment) would probably have shown the presence of a great number of phagosomes among the fat droplets of this particular fraction. Two tentative interpretations may be suggested for these observations. It may be assumed either that some phagosomes in liver have a relatively high fat content and, therefore, separate together with fat droplets, or that some phagosomes are bound to fat droplets as shown in Fig. 16 *A*. Reasons have been discussed previously (2, 11) indicating a derivation of the kidney droplets from the Golgi region of the cells. Since Golgi material is known to be rich in lipides, a relatively high lipide content of some phagosomes would not be surprising.

The cytochemical peroxidase method can also be applied, of course, to test the degree of purity of isolated phagosomes themselves. It was mentioned above that the injected peroxidase appears in kidney at first in the small and later in the large phagosomes (12) and that the change in size of the phagosomes is probably related to the uptake of proteins. This may explain why not all of the phagosomes take up peroxidase at the same time and why only a portion of the large droplets had reacted with benzidine 3 hours after administration (Fig. 18 *A*) although practically all of them were stained by basic fuchsin. A more detailed analysis of the changes in the uptake of horseradish peroxidase by kidney phagosomes of different sizes over a period of 30 hours is in progress and will be reported later.

It was described in section *IV* (*a*) that the phagosomes from kidney and liver can be distinguished from the mitochondria by their greater stability in 70 per cent alcohol, and by their greater affinity toward basic fuchsin.[2] The resistance of the phagosomes but not of the mitochondria toward 70 per cent alcohol points to a marked difference in the osmotic properties of these two types of cell organelles. This may also be inferred from the previous observation (11) that the droplets of kidney cells showed much less swelling than the mitochondria when both were suspended together in 30 per cent sucrose solution at room temperature. The strong affinity of the phagosomes

[2] After completion of this manuscript, a paper by Rather (*Stanford Med. Bull.*, 1948, **6**, 117) was noticed in which this investigator has also distinguished the kidney droplets by their affinity to basic fuchsin.

toward basic fuchsin may be due to the acid groups of mucoproteins, the presence of which is suggested in the droplets of kidney cells by their PAS-reaction (19) and by their stickiness (2).

Several investigators have concluded that the droplets of kidney cells develop from the mitochondria, and that this transformation is induced by the administration of proteins. As was already pointed out by Davies (19), such an interpretation fails to recognize that the kidney cells of normal animals contain a large number of droplets (without injection of proteins), and confuses these droplets with mitochondria. In other animals and age groups (19), the smaller size of the droplets may make their microscopic observation more difficult than in old rats. Since droplets have now been isolated from the kidneys of *normal* rats and have been characterized by their enzymatic properties (2, 3), their differentiation from mitochondria should now be possible. The simple procedure of using 70 per cent alcohol and subsequent staining with basic fuchsin (section *IV* (*a*)) may also facilitate their differentiation. Until now the main difficulty in accepting the hypothesis of the derivation of the droplets from the mitochondria was seen in their very different enzymatic properties (2, 3). Now, a second major difference can be recognized in the inability of the mitochondria to segregate foreign proteins (horseradish peroxidase). It may be argued that the benzidine test as used in this investigation was not sensitive enough to detect cytochemically low concentrations of peroxidase in the mitochondria. However, a quantitative colorimetric analysis reported previously (5) has also shown that the concentration of peroxidase in isolated mitochondrial fractions from kidney was not higher than in the total homogenate and, therefore, probably due to contaminating phagosomes, whereas it was much higher in the fractions containing many phagosomes. It may be assumed, therefore, that only the phagosomes and not the mitochondria have the ability to segregate foreign proteins. The contaminating phagosomes, and not the mitochondria, were probably also responsible for the uptake of proteins labelled with radioactive molecules as described in the experiments by Haurowitz *et al.* (20), and by others.

Although it was not expected that squash preparations of tissues would show much cytological detail, several interesting observations on the localization of phagosomes in the cells of some organs could be made. In kidney and liver, for example, the phagosomes appeared often in curved rows which seemed to follow the course of the cell membranes. This seems to corroborate the close relationship between the cell membrane and the incorporation of proteins (pinocytosis) postulated by several investigators and discussed in a previous report (1). It was surprising that the glomeruli in the squashed kidney tissue appeared practically free of phagosomes, as shown in Fig. 2 and observed on 5 to 6 other glomeruli. Since it is assumed by many investigators (21) that proteins of lower molecular weight pass the glomerulus and are then reabsorbed by the cells of the proximal convoluted tubules, the absence of peroxidase-reactive granules from the glomerulus in our experiments may be due to the failure of the reagents to penetrate there. This question will be reexamined later in microtome sections. The localization of the phagosomes in squashed liver tissue (Figs. 3 to 6) calls to mind electron microscopic observations by Palade and Siekevitz (22), according to which liver cells contain dense bodies in the vicinity of the bile capillaries ("peribiliary bodies"). They are probably identical with the granules which have segregated the plant enzyme in the present experiments. Electron microscopic observations by Rouiller (23) show the existence of direct communications between the bile and blood capillaries. This may explain why the injected peroxidase could be excreted directly through the bile without having to penetrate the interior of the epithelial cells. It is also interesting to note in Rouiller's electron micrographs that the cell membrane shows many infoldings in the region of the bile capillaries and a close relationship with the Golgi zone of the epithelial cells (23).

This cytochemical survey has indicated a striking relationship of the phagosomes to the blood (or lymphatic) vessels and capillaries. In organs such as the pancreas, prostate, pituitary, and thymus, many phagosomes were aggregated in cells in the vicinity of the vessels and capillaries. It could not always be ascertained whether they were contained in the endothelial cells themselves, or in cells of the surrounding connective tissue, or in both. The close relationship to blood vessels could be seen in a very striking manner after injection of a relatively large amount of horseradish peroxidase directly into the brain of a rabbit (Figs. 13 *A* to *C*). It is tempting to hypothesize that the great number of phagosomes present in the endothelial cells of the brain (or in the surrounding glia) is responsible for the so called blood-brain barrier. For it seems plausible to assume that

foreign substances can be segregated by the phagosomes in or around the blood vessels and capillaries, and then digested or detoxified by their enzymes, in agreement with the hypothesis of de Duve *et al.* (4) on the function of the "lysosomes." It is interesting to note that electron microscopic observations by Palade (24), Moore and Ruska (25), and Buck (26) also suggest a marked activity of endothelial cells in pinocytosis. Reference may also be made to observations by Leduc (27), using immunohistochemical methods, on the blood-brain barrier against proteins.

In kidney cells, aggregations of phagosomes appeared quite often close to the nucleus. In many organs, however, it could not be decided whether the relatively few phagosomes apparently within epithelial cells were "smeared" there during the preparation or whether they had been there originally. On the other hand, slow penetration of the reagents into the epithelial cells may have prevented their staining. This question should be investigated on microtome sections.

It may be concluded tentatively from this survey that the phagosomes are concentrated in the cells of the reticulo-endothelial system, and in special regions of epithelial cells, and that they may function in the transport or exchange of substances between epithelial cells of organs of absorption, secretion, and excretion, on the one hand, and the blood and lymph stream, on the other. It is known that the reticulo-endothelial system also contains free cells with phagocytic ability (histiocytes, macrophages) which are often considered to be derived from endothelial cells. The phagosomes of these cells may play an important role in the uptake and transport of substances, and they may protect the tissues by digesting or detoxifying foreign materials (4). By occupying strategic positions close to the cell membranes or to the walls of capillaries or in surrounding connective tissue, and by affecting the penetration of metabolites into cells, or their secretion, excretion, or digestion, the phagosomes may have a regulatory function in the metabolism of cells. As these granules have the ability to concentrate horseradish peroxidase, they may also be able to segregate hormones which are known to function specifically in the regulation of cellular metabolism.

The writer wishes to thank Dr. Steven Mohos for the gift of a mammary-carcinoma–bearing mouse and for interesting discussions; Dr. Gerald Kaufman and Dr. Charles Reiner, for interesting discussions; Dr. Edward Vastola, for injecting peroxidase into the brain of a rabbit; and Dr. Lawrence Herman and Dr. Valentin Yermakow, for permission to use their dark rooms for the processing of the photographic material.

Bibliography

1. Straus, W., *J. Biophysic. and Biochem. Cytol.*, 1958, **4,** 541.
2. Straus, W., *J. Biol. Chem.*, 1954, **207,** 745.
3. Straus, W., *J. Biophysic. and Biochem. Cytol.*, 1956, **2,** 513.
4. de Duve, C., Pressman, B. C., Gianetto, R., Wattiaux, R., and Appelmans, F., *Biochem. J.*, 1955, **60,** 604.
5. Straus, W., *J. Biophysic. and Biochem. Cytol.*, 1957, **3,** 1037.
6. Gomori, G., Microscopic Histochemistry, Chicago, The University of Chicago Press, 1952, 165.
7. Hogeboom, G. H., Schneider, W. C., and Palade, G. E., *J. Biol. Chem.*, 1948, **172,** 619.
8. Neufeld, H. A., Levay, A. N., Lucas, F. V., Martin, A. P., and Stotz, E., *J. Biol. Chem.*, 1958, **233,** 209.
9. Straus, W., and Oliver, J., *J. Exp. Med.*, 1955, **102,** 1.
10. Coons, A. H., Leduc, E. H., and Kaplan, M. H., *J. Exp. Med.*, 1951, **93,** 173.
11. Straus, W., *J. Biophysic. and Biochem. Cytol.*, 1957, **3,** 933.
12. Straus, W., unpublished observations.
13. Hogeboom, G. H., Kuff, E. L., and Schneider, W. C., *Internat. Rev. Cytol.*, 1957, **6,** 425.
14. de Duve, C., *Symp. Soc. Exp. Biol.*, 1957, **10,** 50.
15. Novikoff, A. B., Podber, E., Ryan, J., and Noe, E., *J. Histochem. and Cytochem.*, 1953, **1,** 27.
16. Lang, K., Siebert, G., Baldus, J., and Corbet, A., *Experientia*, 1950, **6,** 59.
17. Kuff, E. L., and Schneider, W. C., *J. Biol. Chem.*, 1954, **206,** 677.
18. Straus, W., *Exp. Cell Research*, 1956, **11,** 289.
19. Davies, J., *Am. J. Anat.*, 1954, **94,** 45.
20. Haurowitz, F., Reller, H. H., and Walter, H., *J. Immunol.*, 1955, **75,** 417.
21. Oliver, J., *J. Mt. Sinai Hosp.*, 1948, **15,** 175.
22. Palade, G. E., and Siekevitz, P., *J. Biophysic. and Biochem. Cytol.*, 1956, **2,** 171.
23. Rouiller, C., *Acta anat.*, 1956, **26,** 94.
24. Palade, G. E., *J. Biophysic. and Biochem. Cytol.*, 1956, **2,** No. 4, suppl., 85.
25. Moore, D. H., and Ruska, H., *J. Biophysic. and Biochem. Cytol.*, 1957, **3,** 457.
26. Buck, R. C., *J. Biophysic. and Biochem. Cytol.*, 1958, **4,** 187.
27. Leduc, E. H., *Anat. Rec.*, 1955, **121,** 328.

Explanation of Plates

Plate 86

Fig. 1. Squash preparation of kidney. Peroxidase reaction in phagosomes (droplets) after incubation with benzidine, 4 hours after intravenous injection of horseradish peroxidase. Magnification, 400.

Fig. 2. Squash preparation of kidney. Treatment as in Fig. 1. Note that phagosomes are absent from the glomerulus. Magnification, 170.

Fig. 3. Squash preparation of liver. Peroxidase reaction after incubation with benzidine, 3 hours after administration of horseradish peroxidase. Note that the phagosomes are arranged in curved rows and are smaller than in kidney (Fig. 1). Magnification, 400.

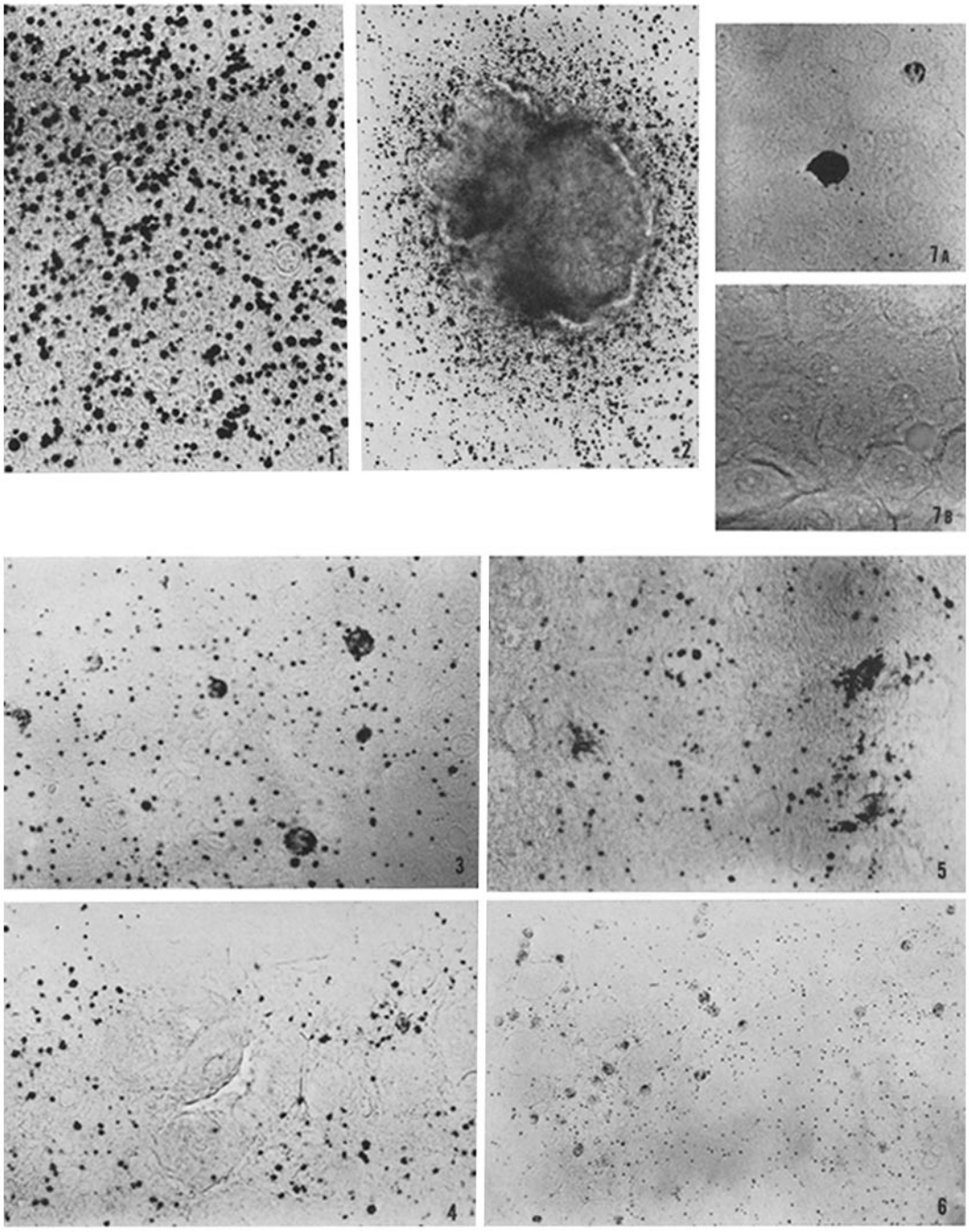
Fig. 4. Squash preparation of liver. Peroxidase reaction in phagosomes, 4 hours after administration of horseradish peroxidase. Note absence of phagosomes from inner portions of epithelial cells. Magnification, 400.

Fig. 5. Squash preparation of liver. Treatment as in Fig. 4. Certain areas, perhaps Kupffer cells, show aggregations of phagosomes. Magnification, 400.

Fig. 6. Squash preparation of liver. Staining for peroxidase with benzidine, 3 hours after administration of horseradish peroxidase. Note arrangement of phagosomes in rows and their absence from inner portions of epithelial cells, and increased numbers of leucocytes. Magnification, 170.

Fig. 7 *A*. Squash preparation of liver of control animal. Incubation with benzidine. Note that peroxidase-positive granules appear to be released from large granulocyte, probably an eosinophil. Magnification, 400.

Fig. 7 *B*. Squash preparation of same liver as in Fig. 7 *A*. Incubation with benzidine. Note that no peroxidase-positive granules are present in this area. Magnification, 400.



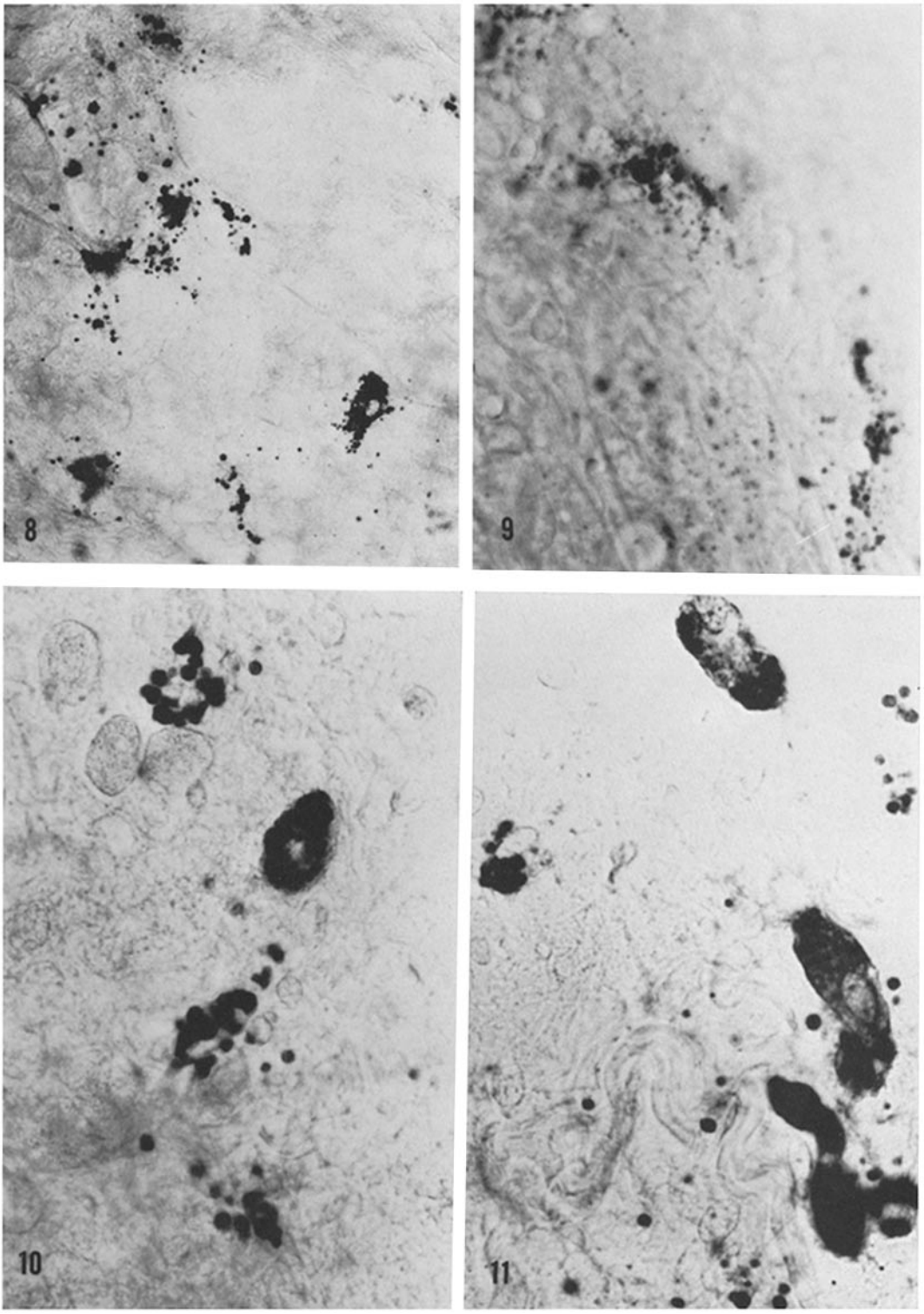
(Straus: Identification of phagosomes)

PLATE 87

FIG. 8. Squash preparation of pancreas. Peroxidase reaction in phagosomes after incubation with benzidine, 5 hours after administration of horseradish peroxidase. Aggregations of phagosomes in vicinity of interlobular spaces. Magnification, 310.

FIG. 9. Smear of mucosa scraping of small intestine. Peroxidase reaction in phagosomes, 4 hours after administration of horseradish peroxidase. Magnification, 310.

FIGS. 10 and 11. Squash preparations of prostate. Peroxidase reaction in phagosomes after incubation with benzidine, 5 hours after treatment with horseradish peroxidase. Magnification, 730. Note that Figs. 10 and 11 were taken at higher magnification than Figs. 8 and 9.



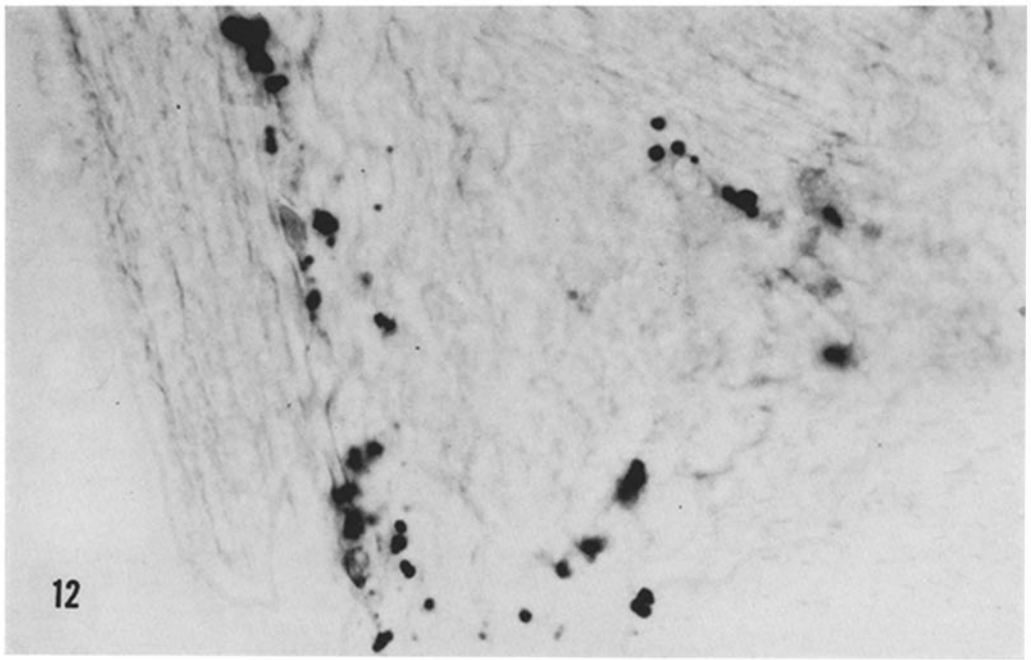
(Straus: Identification of phagosomes)

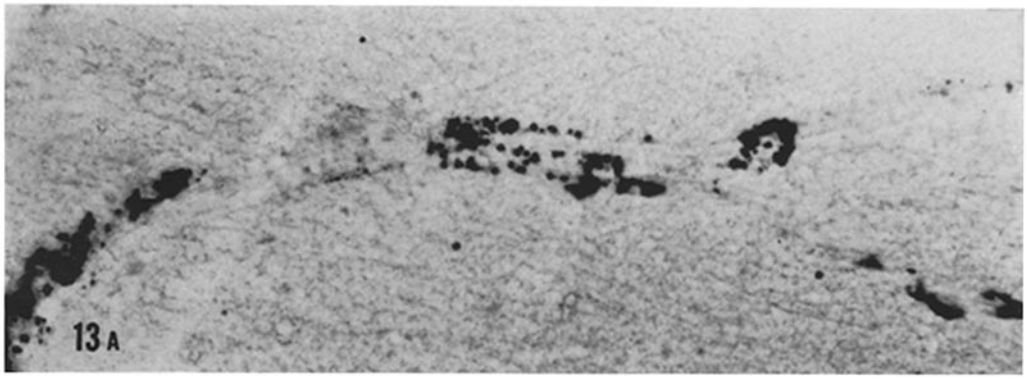
PLATE 88

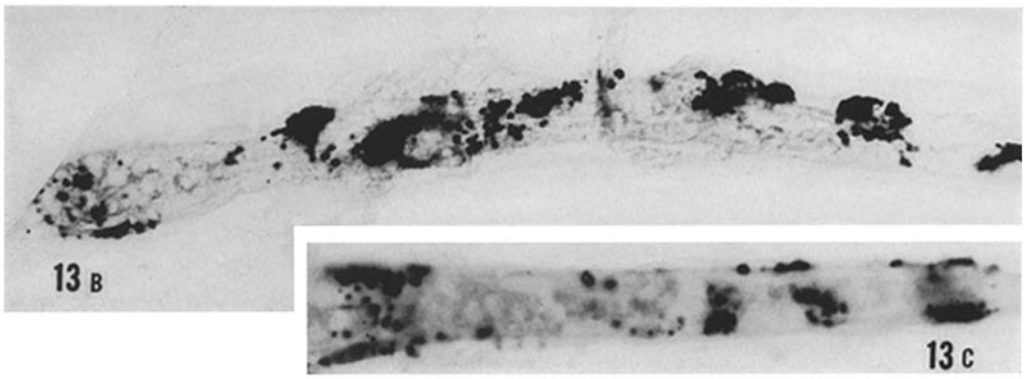
FIG. 12. Squash preparation of heart. Peroxidase reaction in phagosomes after incubation with benzidine, 3 hours after administration of horseradish peroxidase. Magnification, 770.

FIG. 13 *A*. Squash preparation of brain of rabbit, close to site of injection. Peroxidase reaction in phagosomes after incubation with benzidine, 1 hour after injection of horseradish peroxidase into brain. Magnification, 330.

FIGS. 13 *B* and *C*. Same preparation and treatment as in Fig. 13 *A*. Phagosomes in cells of blood vessels which were found accidentally separated from other brain tissue. Magnification, 330. Note that Figs. 13 *A* to *C* were taken at lower magnification than Fig. 12.







(Straus: Identification of phagosomes)

PLATE 89

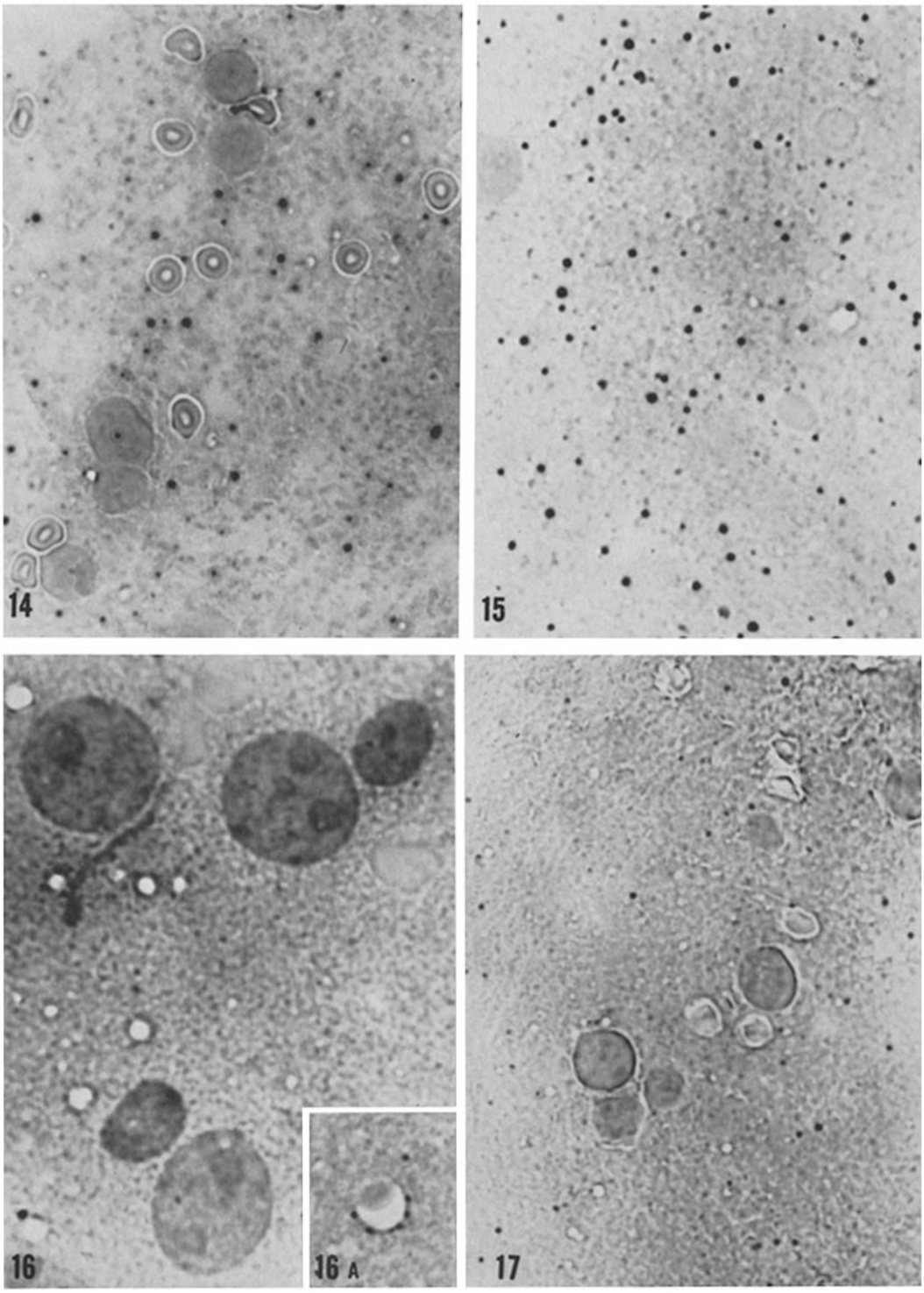
FIG. 14. "Touch preparation" of kidney of normal rat. Preparation fixed in diluted Helly's fluid for 2 hours, then stained with basic fuchsin. Note that the phagosomes are stained more intensely than the mitochondria. Magnification, 770.

FIG. 15. "Touch preparation" of kidney of normal rat. Preparation treated in 70 per cent alcohol for 18 hours, then in diluted Helly's fluid for 1 hour, and stained with basic fuchsin. Note that the mitochondria have partially disintegrated whereas the phagosomes are preserved. Magnification, 770.

FIG. 16. "Touch preparation" of liver of normal rat. Preparation treated in 70 per cent alcohol for 18 hours, then in diluted Helly's fluid for 1 hour, and stained with basic fuchsin. Note small size (0.2 to 0.5 μ diameter) of intensely stained granules. Magnification, 1450.

FIG. 16 *A*. "Touch preparation" of liver of normal rat. Same treatment as in Fig. 16. Note that some granules are aggregated around fat droplet. Magnification, 1450.

FIG. 17. "Touch preparation" of liver of rat, treated with horseradish peroxidase. Preparation held in 70 per cent alcohol for several days, then stained with basic fuchsin. Note that size of basophilic granules is increased after treatment with peroxidase as compared with liver of normal rat (Fig. 16), and that Fig. 17 was taken at lower magnification than Fig. 16. Magnification, 770.



(Straus: Identification of phagosomes)

PLATE 90

FIGS. 18 and 19. Benzidine test with smears of fractions, isolated from homogenates of the kidney and liver of rats by differential centrifugation, 3 to 4 hours after administration of horseradish peroxidase.

FIG. 18 *A*. Large droplets from kidney, partially purified. Note that not all of the large droplets (2 to 5 μ diameter) are stained and that one of the phagosomes has rod shape. Magnification, 1520.

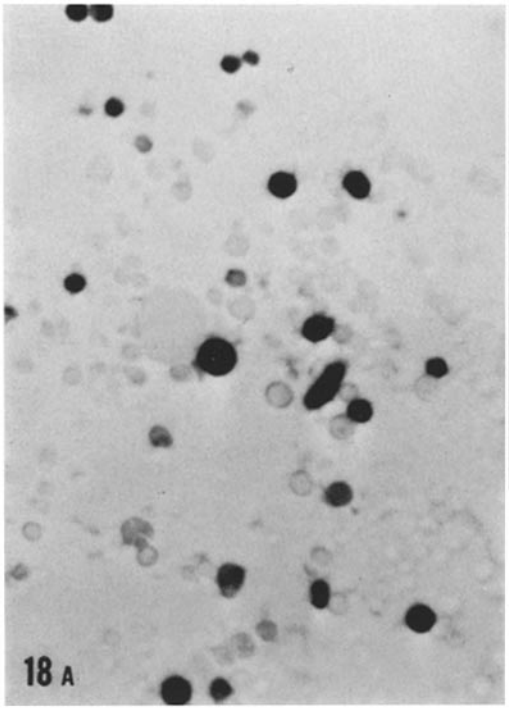
FIG. 18 *B*. Upper layer of mitochondrial fraction, isolated from rat kidney and purified by one resedimentation. Note that the mitochondria are still contaminated by some phagosomes. Magnification, 1520.

FIG. 19 *A*. Smear of lower layer of sediment, obtained from liver homogenate at 2000 *g*. Note that size of most phagosomes is in the range of 1 to 2 μ. Magnification, 800.

FIG. 19 *B*. Smear of lower layer of sediment, obtained from liver homogenate at 7000 *g*. Size of phagosomes from 0.5 to 2 μ diameter. Mitochondria in background were unstained, but appear dark because of lowered condenser. Magnification, 800.

FIG. 19 *C*. Smear of small brown pellet forming the bottom layer of microsomal sediment from liver. Range of size of intensely blue-staining granules 0.2 to 1 μ diameter. Granules in background were unstained. Note that Fig. 19 *C* was taken at higher magnification than Figs. 19 *A*, *B*, and *D*. Magnification, 1520.

FIG. 19 *D*. Smear of fat layer floating on surface of liver homogenate after high speed centrifugation. Phagosomes, 1 to 2 μ diameter, staining intensely blue, among colorless fat droplets. Magnification, 800.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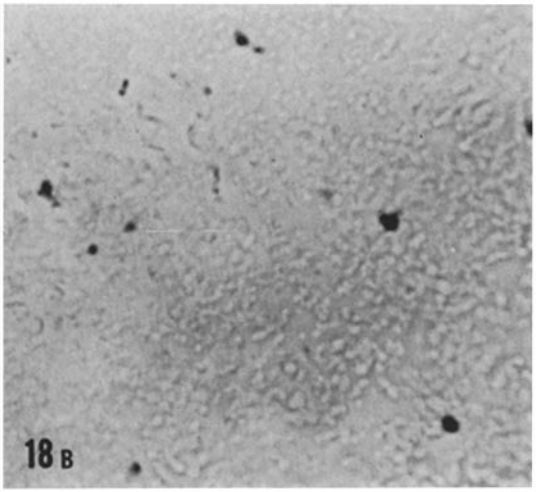
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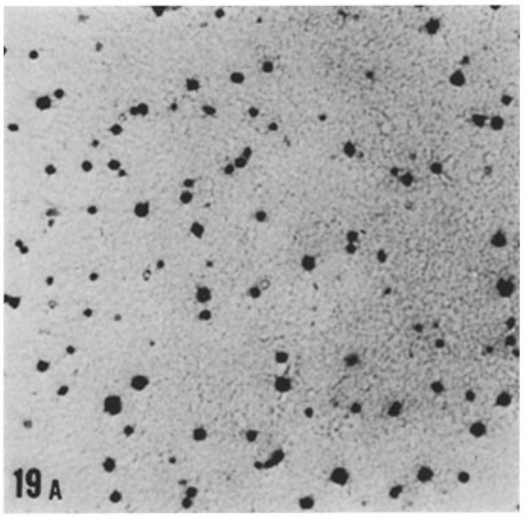
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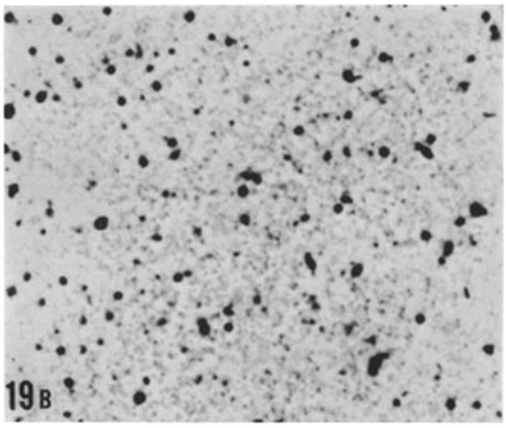
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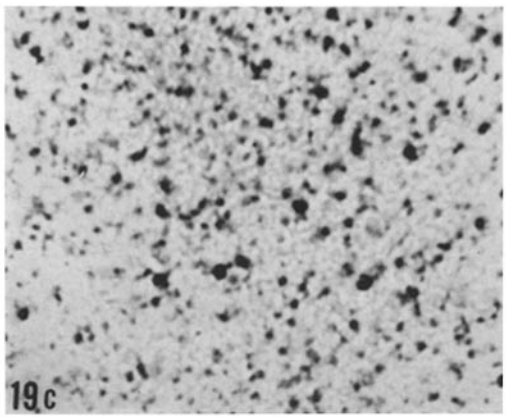
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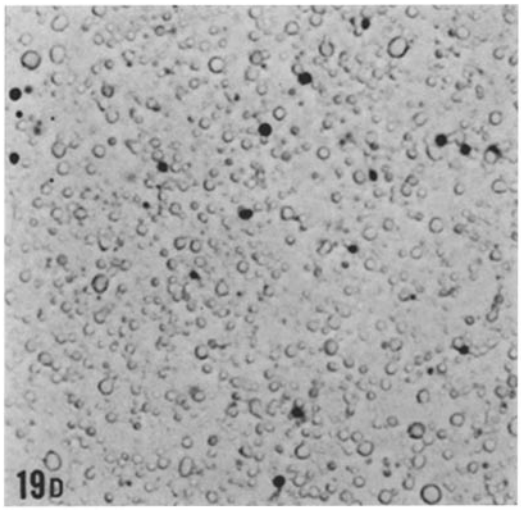
19 D