CYTOCHEMICAL OBSERVATIONS ON THE RELATIONSHIP BETWEEN LYSOSOMES AND PHAGOSOMES IN KIDNEY AND LIVER BY COMBINED STAINING FOR ACID PHOSPHATASE AND INTRAVENOUSLY INJECTED HORSERADISH PEROXIDASE

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ABSTRACT

After incubation of formalin-fixed, frozen sections of kidney and liver from peroxidase-treated rats in an azo dye medium for acid phosphatase, and after subsequent incubation of the same sections with benzidine, phagosomes were stained blue and lysosomes were stained red in the same cells. It was observed that newly formed phagosomes were separate from preexisting lysosomes in the tubule cells of the kidney and in the Kupffer cells of the liver at early periods after treatment with peroxidase. At later periods, the color reactions for acid phosphatase and peroxidase occurred in the same granules. The reaction of peroxidase decreased gradually and disappeared from the phago-lysosomes after 2 to 3 days, whereas the reaction for acid phosphatase persisted. In the liver, most of the injected protein was concentrated in large phagosomes located at the periphery of the cells lining the sinusoids. The peribiliary lysosomes showed a relatively weak reaction for peroxidase in the proximity of the portal veins. After pathological changes of permeability, phagosomes and lysosomes lost their normal location and fused, in the interior of many liver cells, to form large vacuoles or spheres. The effects of a reduced load of peroxidase and the effects of the pretreatment with another protein (egg white) on the phago-lysosomes of the kidney were tested. The relationship of the fusion of phagosomes with lysosomes to the size of normal and pathological phago-lysosomes was discussed.

It was reported previously (1, 2) that "droplet" fractions isolated from kidney homogenates of normal rats had an enzymatic composition similar to that of the lysosome fraction isolated from liver homogenates by de Duve *et al.* (3). It was also observed that droplets isolated from the kidney homogenates of rats, which had received an intraperitoneal injection of egg white or horseradish peroxidase, contained, in addition to lysosomal enzymes, relatively high concentrations of the injected foreign proteins (4, 5). These observations suggested for the first time a relationship between lysosomes and the segregation or pinocytosis of foreign proteins. The investigation of these problems was facilitated by the use of horseradish peroxidase as a marker protein. Since peroxidase is not present in most animal cells and since it gives sensitive color reactions, this protein could

be detected *in situ* by its cytochemical reaction with benzidine, and its concentration could be measured in isolated fractions by a simple colorimetric assay. By these procedures, it was possible to demonstrate the occurrence of many "phagosomes" in the cells of the reticuloendothelial system and in the epithelial cells of organs of absorption, secretion, and excretion (6, 7).

In the present investigation, the relationship between lysosomes and phagosomes was studied by combining a cytochemical procedure for acid phosphatase, *i.e.* the azo dye method of Barka and Anderson (8), with the well known cytochemical reaction for peroxidase with benzidine.[1, 2] By applying these reactions successively to the same tissue sections, the lysosomes were stained red and the phagosomes were stained blue in the same cells. It will be shown that at early stages after injection of peroxidase, the lysosomes and phagosomes are separate in cells of the kidney and liver. At later stages, they are combined. In the procedures of other investigators (8, 9), two enzymes, located at *different* cellular sites, were stained in different colors in the same cells. In the present case, the cytochemical reactions for the two enzymes (acid phosphatase and peroxidase) often occurred at the *same* cellular sites, and these sites often represented relatively small granules. This made high demands on precise cytochemical localization, avoidance of artifacts, and good contrast of color of the two enzyme reactions. Novikoff (10) also combined our procedure with injected peroxidase (7, 11–13) and the staining for acid phosphatase according to Barka and Anderson (8). However, in Novikoff's experiments, the fading of the blue product of the peroxidase-benzidine reaction into a brown pigment made it difficult to obtain sufficient contrast of color between the reaction products of the two enzymes.

METHODS AND MATERIALS

Male rats of the Sprague-Dawley strain, each approximately 200 gm in weight, were used. The animals received an injection of 12 mg/100 gm body weight of horseradish peroxidase (type II, Sigma Chemical Company, St. Louis) into the femoral veins.

[1] I am obliged to Dr. Barka for having permitted the use of his acid phosphatase procedure before the method was published.

[2] Some of the present observations were reported at the Ciba Foundation Symposium on Lysosomes in London, February, 1963, and at the Meeting of the Histochemical Society in Washington, April, 1963.

At various periods after injection, blocks of kidney and liver were fixed for 18 hours in 10 per cent formaldehyde solutions. Cryostat sections were incubated with AS-TR phosphate (Nutritional Biochemical Corporation, Cleveland) as substrate for acid phosphatase (Burstone, reference 14) and with hexazotized pararosaniline (Chroma 11455, Roboz Surgical Instrument Company, Washington, D. C.) as coupler (Davis and Ornstein, reference 15). The medium recommended by Barka and Anderson (8) was used containing 0.5 mg per ml of AS-TR phosphate (phosphate ester of the anilide of 2-hydroxy-3-naphthoic acid arylamide), 4.8 mM hexazonium pararosaniline, and 5 per cent *N*,*N*-dimethyl-formamide. The reaction was performed at room temperature and at pH 5.0. Subsequently, the same tissue sections were incubated for 5 to 60 seconds with 0.2 per cent solutions of benzidine (purified, Dajac Laboratories, Philadelphia) and 0.015 to 0.03 per cent H_2O_2 until the peroxidase reaction appeared (7, 11–13). As controls, sections from the same block were stained for acid phosphatase alone and for peroxidase alone. Another section was stained for peroxidase after incubation in the medium for acid phosphatase, this medium containing all reagents except the substrate (AS-TR phosphate).

The last mentioned control, compared with the direct staining for peroxidase, showed that the phagosomes had not been damaged significantly during the relatively long incubation for acid phosphatase. In some cases, the double staining produced a more intense blue color at the rims (membranes) of the granules and a more red coloration of the interior (Fig. 4). This artifact facilitated the recognition of the combined staining for the two enzymes in the same granules. The excellent procedure for acid phosphatase by Barka and Anderson (8) provided precise staining and was applied without significant changes. However, the usual method for peroxidase with benzidine showed several deficiencies, such as the fading of the peroxidase-benzidine reaction product from a blue into a brown pigment, and crystallization artifacts of the blue pigment. Therefore, a thorough study was made of some factors affecting the cytochemical reaction of peroxidase with benzidine and the stability of the blue reaction product, and the conventional procedure was modified in certain points. The main improvement concerns a modification of the procedure of Goodpasture (16) for the stabilization of the blue reaction product with nitroferricyanide. The improved procedure which resulted in the stabilization of the blue reaction product for several months, and the influence of other factors, are described in detail elsewhere (17).

When the activity of acid phosphatase was compared before and after fixation of the tissue, it was observed that 50 to 55 per cent of the activity was destroyed by formaldehyde. Holt and Hicks (18)

found inactivation of acid phosphatase by formol of the same order of magnitude. The activity of peroxidase was not reduced significantly by formol treatment of the tissue when the temperature during fixation was held at 0–4°C (17).

CYTOCHEMICAL OBSERVATIONS

Phago-lysosomes in the Cells of the Convoluted Tubules

Following the administration of horseradish peroxidase, 3 stages were distinguished after the entry of peroxidase into the cells of the convoluted tubules.

FIRST STAGE: During the first stage lasting approximately 30 minutes following the injection, phagosomes (peroxidase-positive granules) developed at the base of the brush border or close to the plasma membranes (13). They were separate from preexisting lysosomes (acid phosphatase–positive granules) which were located in the apical and intermediate zones of the cells. Fig. 1 shows this stage in the cells of the proximal portions, and Fig. 2 shows it in the cells of the terminal portions of the proximal convoluted tubules. It may also be seen that the lysosomes are much larger in the cells of the proximal portions of the proximal convoluted tubules (Fig. 1) than in the cells of the terminal portions in the inner cortex (Fig. 2).

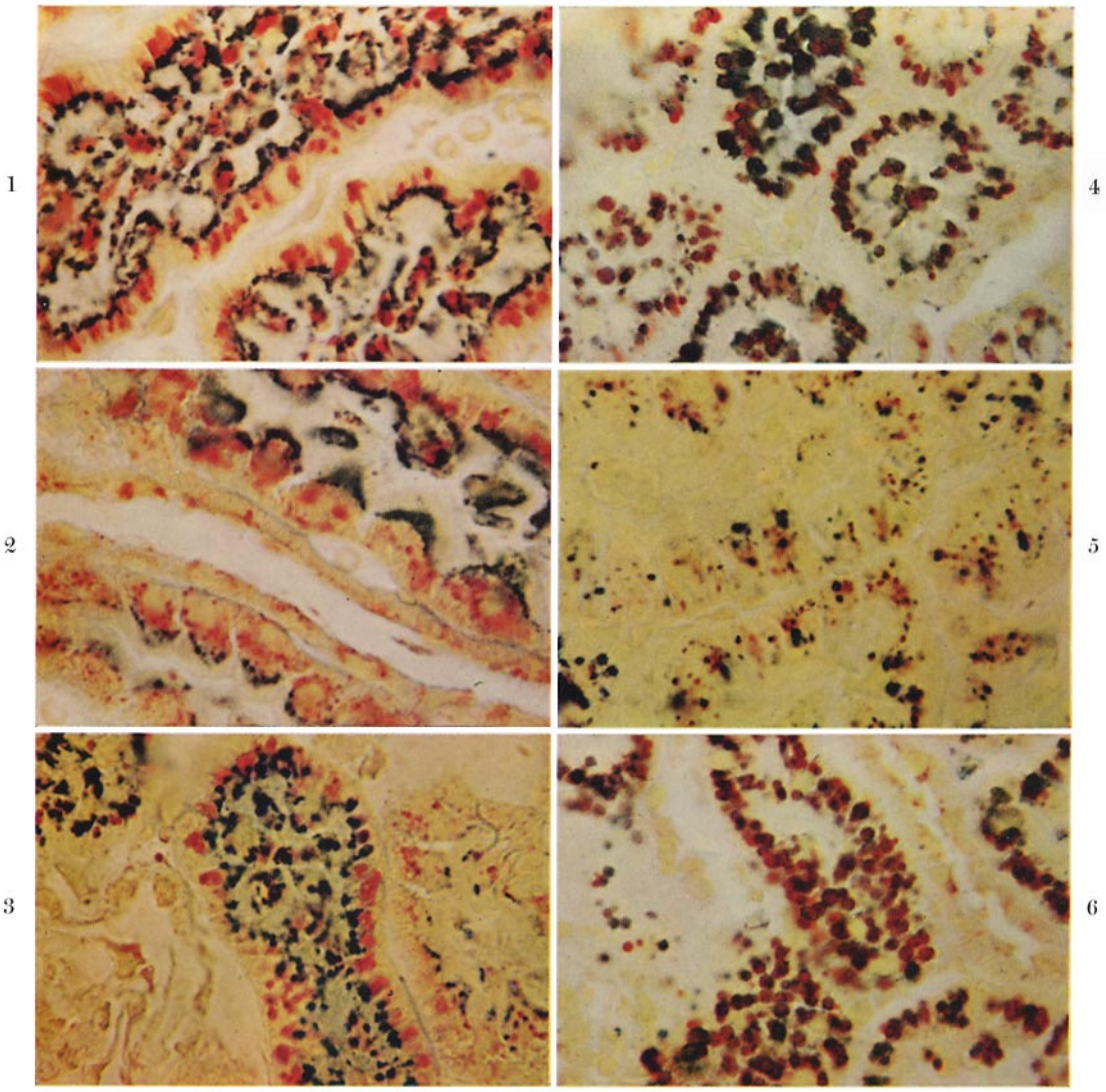
SECOND STAGE: During the second stage lasting from approximately 30 minutes to 1 to 3 days after administration, strong peroxidase activity was detectable in the phago-lysosomes. (For a definition of the term "phago-lysosome," see reference 19). Although the process of fusion between phagosomes and preexisting lysosomes could not be observed on fixed tissue, it was deduced from the following changes. Whereas at 30 minutes after injection of peroxidase many phagosomes and lysosomes were located adjacent to each other (Fig. 3), at 60 minutes after injection (Figs. 4 and 5), at 6 hours after injection (Fig. 6), and for the following 1 to 3 days, most of the stained granules in the cells of the proximal convoluted tubules showed the purple color of the combined reaction for acid phosphatase and for peroxidase. Separate lysosomes and phagosomes in early periods after administration, and combined staining for acid phosphatase and peroxidase in the same granules, later, were also observed in the cells of the thick ascending limbs of Henle's loop, the distal tubules of the outer cortex, the collecting tubules, and in a few cells of the glomerulus. In general, the number of lysosomes, phagosomes, and phago-lysosomes was smaller in the more distally located portions of the nephron than in the proximal convoluted tubules. Macrophages invading the peritubular capillaries after treatment with peroxidase contained very large phagosomes, and they showed a decreased reaction for acid phosphatase during the main uptake of peroxidase (19). The occurrence of phago-lysosomes in different portions of the nephron has been discussed in more detail elsewhere (19) and will be illustrated in a later report.

THIRD STAGE: A third stage was distinguished at which the foreign protein could no longer be detected in the lysosomes. In the cells of the proximal convoluted tubules of the outer cortex, the cytochemical reaction for peroxidase in the phago-lysosomes became negative 2 to 3 days after injection of the standard dose of peroxidase, whereas the reaction for acid phosphatase persisted in the granules. Fig. 7 shows lysosomes in the cells of the proximal convoluted tubules 72 hours after administration when the peroxidase reaction had disappeared from most of the granules. In the more distally located portions of the nephron, the peroxidase activity disappeared, in general, earlier. The uptake and disappearance of peroxidase varied, of course, with the injected dose and with the activity of the commercial preparation. The decrease of peroxidase activity in the phago-lysosomes was reflected in the increasingly longer times of incubation required for the staining of peroxidase, over a period of 1 to 3 days. Since the lysosomes of kidney cells contain cathepsin (2), it may be assumed that peroxidase was digested gradually in the phago-lysosomes.

The present cytochemical observations are in good agreement with previous biochemical experiments in which the decrease of peroxidase activity over a period of several days had been measured in kidney homogenates and in isolated fractions by a quantitative method (20). When these data are compared, one must consider that in some of the present experiments a small part of the peroxidase may have been inactivated by the rise of the temperature above 0–4°C during the fixation with formol (see Methods and Materials).

Phago-lysosomes in Liver Cells

KUPFFER CELLS: At early stages after treatment with peroxidase, blue-stained phagosomes



FIGS. 1 to 12, × 660.

FIGURE 1 Combined staining for acid phosphatase and injected peroxidase in cells of proximal convoluted tubules of the outer cortex, 15 minutes after treatment. Note that blue-stained phagosomes develop close to the lumen and are separate from red-stained lysosomes. The protrusion of cells into the lumen may be due to an artifact occurring during fixation, or may occur *in vivo*. Experiments are in progress to decide this question.

FIGURE 2 Combined staining for acid phosphatase and peroxidase in cells of the terminal portions of the proximal tubules of the inner cortex, 15 minutes after treatment. Note that phagosomes develop at the base of the brush border, separate from lysosomes, and that the lysosomes are much smaller in this segment of the proximal convoluted tubules than in the outer cortex (see Fig. 1).

FIGURE 3 Combined staining for acid phosphatase and peroxidase in cells of the proximal convoluted tubules of the outer cortex, 30 minutes after administration. Note that many phagosomes and lysosomes lie adjacent to each other.

FIGURE 4 Combined staining for acid phosphatase and peroxidase in the cells of the proximal convoluted tubules of the outer cortex, 1 hour after administration of the protein. Note the blue and red color reactions for both enzymes in the same granules.

FIGURE 5 Combined staining for acid phosphatase and peroxidase in the cells of the proximal convoluted tubules of the inner cortex (terminal portion), 1 hour after the injection of the protein. Note the blue and red color reactions for both enzymes in the same granules, and the smaller size of the granules as compared with those in Fig. 4.

FIGURE 6 Combined staining for acid phosphatase and peroxidase in the cells of the proximal convoluted tubules of the outer cortex, 6 hours after injection of peroxidase. Note the blue and red color reactions for both enzymes in the same granules, and the enlarged size of the phago-lysosomes as compared to the acid phosphatase–positive granules in Figs. 1 and 7.

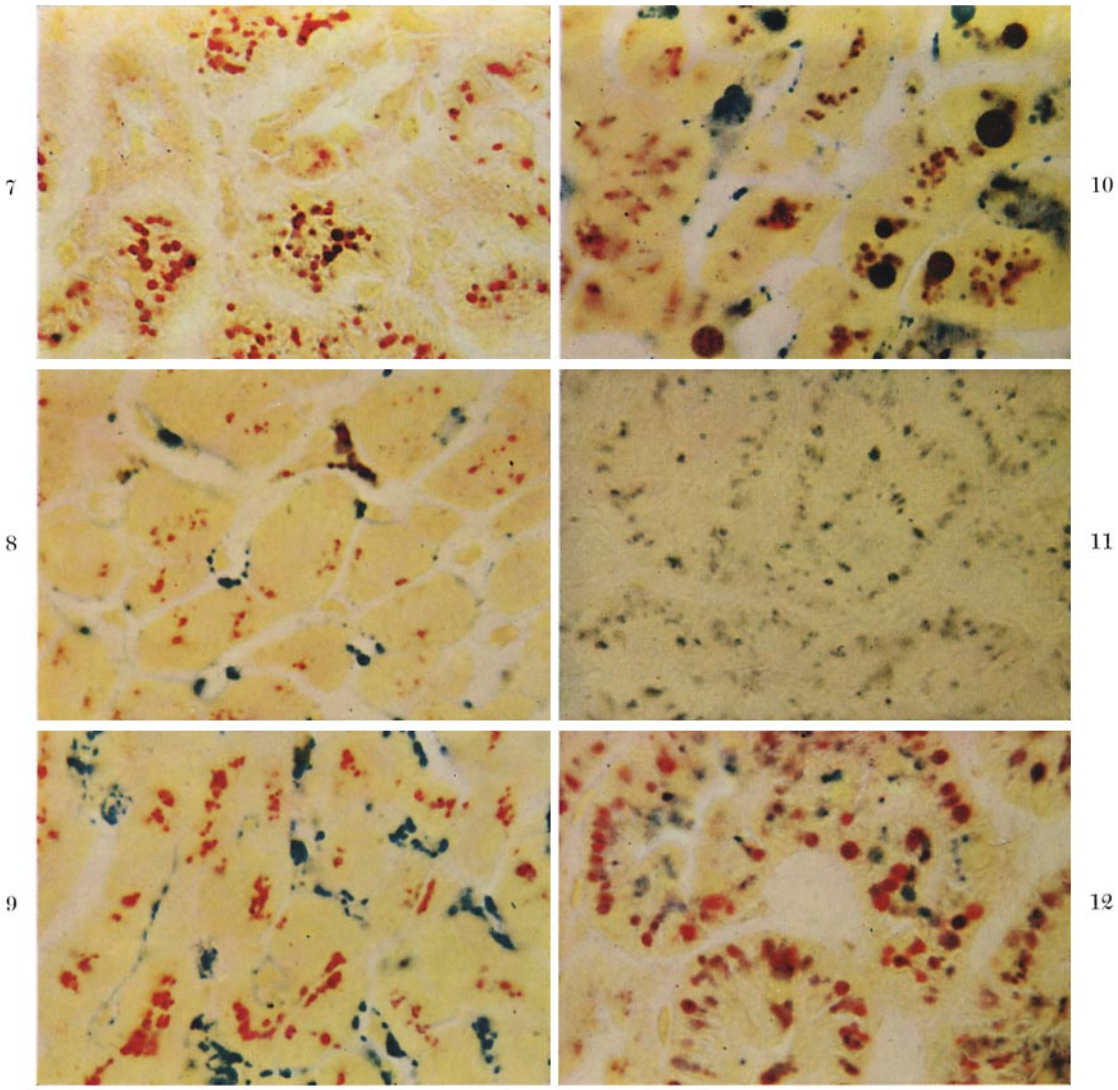


FIGURE 7 Combined staining for acid phosphatase and peroxidase in cells of proximal convoluted tubules, 72 hours after injection of the protein. Note that only a few lysosomes still show the reaction for peroxidase, after prolonged incubation with benzidine.

FIGURE 8 Combined staining for acid phosphatase and peroxidase in liver, 3 hours after injection of the protein. Note the presence of purple-stained phagolysosomes in a Kupffer cell, and the blue-stained phagosomes at the periphery of the cells lining the sinusoids.

FIGURE 9 Combined staining for acid phosphatase and peroxidase in parenchymal cells of liver, 3 hours after injection of the protein. Note the separate location of the blue-stained phagosomes at the periphery of the cells lining the sinusoids, and the red-stained lysosomes along the bile capillaries.

FIGURE 10 Combined staining for acid phosphatase and peroxidase, 3 hours after administration of a toxic preparation of peroxidase. Note the formation of huge vacuoles or spheres containing acid phosphatase and peroxidase, in the interior of the hepatic cells.

FIGURE 11 Staining for peroxidase alone, 1 hour after injection of a dose 11 times lower than the standard dose of peroxidase. Note that a considerable number of phagosomes were formed in the cells of the proximal convoluted tubules, in spite of the low dose, but that they contain much less peroxidase than after a higher dose. Some of the granules are barely visible since their color had faded to brown.

FIGURE 12 Combined staining for acid phosphatase and peroxidase, 90 minutes after injection of the standard dose of peroxidase into an animal which had received, 18 hours earlier, an intraperitoneal injection of a massive dose of egg white. Note that most acid phosphatase-positive granules are very large and show no, or only a slight, reaction for peroxidase, that some of the granules react, with the usual intensity, when stained for peroxidase alone, and that only a few granules react with equal intensity for both enzymes.

appeared at the periphery of the Kupffer cells. From approximately 1 hour to 1 to 2 days following injection of peroxidase, many granules in Kupffer cells showed the purple stain of the combined reaction for acid phosphatase and peroxidase (Fig. 8), thus indicating the fusion of lysosomes and phagosomes.

PARENCHYMAL CELLS AND ENDOTHELIAL CELLS: Most phagosomes in the liver were located at the periphery of cells lining the sinusoids (see Figs. 14 and 15 in reference 13 and reference 19). It was often difficult to distinguish whether these blue-stained granules were located at the periphery of the parenchymal cells themselves or on thin extensions of endothelial cells lining the epithelial cells, or at both sites. The phagosomes at these sites increased in size during the first few hours, then decreased in number, and finally disappeared over a period of 1 day. They may have been extruded into the lumen of the sinusoids. The red-stained lysosomes showed the well known peribiliary location (8). No blue reaction product for peroxidase was detected in the lysosomes under the conditions used as routine (Fig. 9). However, when the concentration of H_2O_2 and benzidine was increased 5 times, the lysosomes (phagolysosomes) in the cells neighboring the portal veins often showed a relatively weak reaction for peroxidase. This could be seen better in preparations stained for peroxidase alone (Fig. 13). (For the routine staining procedure, the concentration of H_2O_2 and benzidine was held as low as possible in order to decrease the risk of artifacts).

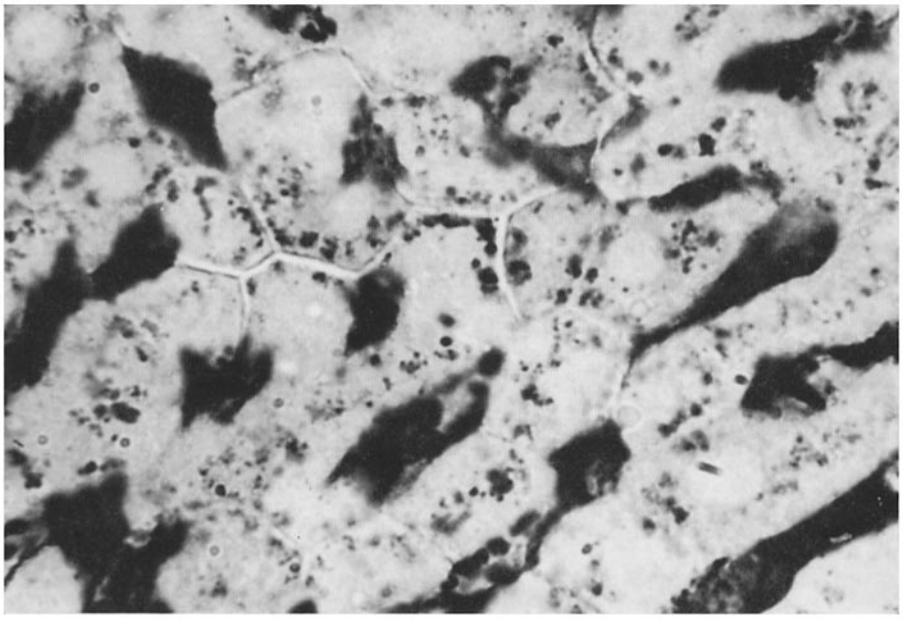


FIGURE 13 Peribiliary lysosomes showing positive staining reaction for peroxidase, 1 hour after injection of the plant enzyme. The usual concentration of benzidine and H_2O_2 had to be increased 5 times to give distinct staining reaction, indicating that the concentration of peroxidase in the lysosomes (phagolysosomes) was relatively low. The reaction occurred only in the periportal areas. The intensely stained phagosomes along the sinusoids had to be underexposed in order to obtain favorable exposure of the peribiliary granules. × 1350.

PARENCHYMAL CELLS AFTER INJURY: The phagosomes and lysosomes lost their normal position at opposite poles of the parenchymal cells after administration of certain commercial preparations of horseradish peroxidase which seemed to be toxic. Phagosomes and lysosomes merged in the interior of the hepatic cells to form large vacuoles or spheres (Fig. 10). The size of these vacuoles or spheres varied from 1 to 15μ in diameter. As will be discussed below, these formations appear to be similar to those observed after injury by other investigators. During the present experiments, the animals became very sick and generally died.

Phago-lysosomes after Low Doses of Peroxidase

In the preceding experiments, relatively high doses, 12 mg peroxidase/100 gm body weight, were injected. With this dose, most lysosomes in the cells of the proximal convoluted tubules had combined with phagosomes. When less peroxidase is injected, the following changes may be envisaged theoretically: (*a*) fewer phago-lysosomes are formed with no reduction of their concentration of peroxidase; (*b*) many phago-lysosomes are formed with their concentration of peroxidase being reduced; (*c*) some cells or nephrons reabsorb less peroxidase than the others.

A dose of 3 mg peroxidase/100 gm body weight was injected, the tissue processed as indicated, and the cryostat sections stained for acid phosphatase together with peroxidase, for acid phosphatase alone, and for peroxidase alone. As judged from microscopic observation, the number of phagosomes and phago-lysosomes in the cells of the proximal convoluted tubules of the outer cortex appeared not to be reduced significantly. However, the peroxidase content of the granules was decreased, as could be detected readily by the much fainter staining with benzidine or by the increased time of incubation required to obtain the usual staining intensity. In addition, some cells or nephrons were stained less intensely than the others. Thus, changes *b* and *c*, mentioned above, seem to represent the reaction of the cells toward a reduced load of protein.

No significant change in the number and staining intensity of the phagosomes in the cells of the liver was observed when the dose was lowered from 12 to 3 mg peroxidase/100 gm body weight. These observations may be correlated with the results of an earlier colorimetric study in which the changes in peroxidase content in relation to dose were compared in homogenates of the kidney and liver (see Table II, reference 20).

In two other experiments, the dose was decreased further to 1.8 and 1.1 mg peroxidase/100 gm body weight, respectively, and the sections were stained for peroxidase alone. Even at the lowest dose, a considerable number of phagosomes were present in many cells of the proximal convoluted tubules of the outer cortex (Fig. 11). However, the staining of these granules was faint and appeared only after prolonged incubation with benzidine. These granules probably represent lysosomes which have combined with a few small phagosomes (micropinocytic vesicles). It should be noted that the dose of 1.1 mg peroxidase/100 gm body weight approached the dose (0.9 mg/100 gm body weight) which was observed in previous experiments with unfixed (squashed) tissue to be limiting for the appearance of phagosomes in the tubule cells of the kidney but not in liver cells (see Table II, reference 20).

Phago-lysosomes after Pretreatment with Egg White

Animals were given an intraperitoneal injection of egg white (4, 20, 21), and, 18 hours later, received an intravenous injection of the standard dose of peroxidase. Ninety minutes later, samples of the kidney and liver were fixed, and the cryostat sections were stained for acid phosphatase and peroxidase combined, and for each of the two enzymes separately.

It was observed (Fig. 12) that most of the large "droplets" (phago-lysosomes containing egg white, reference 4) in the cells of the proximal convoluted tubules showed the red color reaction for acid phosphatase. They were stained only faintly blue by benzidine, after prolonged incubation, or they were not stained at all. Relatively few granules in the cells of the proximal convoluted tubules of the outer cortex showed the combined (purple) color reaction for both enzymes with equal intensity. In addition to these granules, the cells contained a number of blue-stained phagosomes showing the usual staining intensity for peroxidase (Fig. 12). In sections stained for peroxidase alone, these newly formed, more intensely stained phagosomes could be distinguished from the pale blue, usually larger phago-lysosomes, probably containing egg white. Thus, the uptake of peroxidase into the "old" phago-lysosomes was depressed, probably because of the preceding accumulation of egg white in the same granules.

In the liver, the number of blue-stained phagosomes at the periphery of the cells lining the sinusoids appeared to be increased rather than decreased after pretreatment with egg white. In the Kupffer cells, the number of purple granules (phago-lysosomes) was decreased, and most granules in these cells either showed the red color of the lysosomes or the blue color of the phagosomes.

These observations may be correlated with earlier quantitative data (20) on the concentration of peroxidase in homogenates and in isolated

fractions showing that there was a 75 to 90 per cent depression of the uptake of peroxidase after pretreatment with egg white in the case of the kidney but not of the liver.

DISCUSSION

As was mentioned in the introduction, earlier biochemical investigations of isolated droplet fractions of kidney cells had shown that foreign proteins (egg white and horseradish peroxidase) are concentrated in granules containing acid phosphatase. The present cytochemical observations have confirmed the earlier data.

It is interesting to compare the fusion of phagosomes and lysosomes in kidney and liver cells (Kupffer cells) with similar processes in other cells. Rose (22) reported the fusion of phagocytic vacuoles with "microkinetospheres," probably representing lysosomes, during pinocytosis in HeLa cells in tissue culture, by phase contrast cinematography. Hirsch (23) observed, by cinematography, a fusion of phagocytic vacuoles with lysosomes in leucocytes during phagocytosis of bacteria. Rosenbaum and Rolon (24) and Müller and Törö (25) studied the appearance of lysosomes (acid phosphatase) in food vacuoles of protozoa. Thus, a fusion of phagosomes (phagocytic vacuoles) with lysosomes seems to take place in many types of cells active in pinocytosis or phagocytosis.

In the present work, three stages were distinguished following the uptake of peroxidase by the kidney cells: the stage of separate phagosomes and lysosomes, the stage of combined phago-lysosomes, and the stage of "old" lysosomes following the complete digestion of peroxidase. It was possible to differentiate these stages because the blue-stained phagosomes, the red-stained lysosomes, and the purple-stained phago-lysosomes showed sufficient contrast of color. During the first period, a close relationship exists between the developing phagosomes and the cell membranes. This was shown in earlier work (11–13, 19) but could not be seen clearly in the present experiments, probably because most of the peroxidase was washed off the cell membranes during the prolonged incubation for acid phosphatase. It may be noted that investigation of pinocytosis and phagocytosis in kidney and other cells by electron microscopy has revealed the following sequence of events: adsorption of proteins (or other macromolecular materials) to the plasma membranes (26), formation of pits or micropinocytic vesicles (27–30), and accumulation of ingested materials in apical vacuoles (28).

The question may be raised as to which structural alteration detectable by electron microscopy in kidney cells (27, 28, 31–34) corresponds to the fusion of phagosomes with lysosomes. Is the transformation of apical vacuoles into dense bodies related to this process? Or is this an indication of the accumulation of ingested materials (proteins) within the vacuoles? The latter process was observed by Miller (28) in his investigation on the reabsorption of hemoglobin by the cells of the proximal convoluted tubules. Miller (28) also described "vacuolated bodies" containing an eccentric accumulation of dense, coarsely granular material. Do the "vacuolated bodies," in which Miller (35) later observed the reaction for acid phosphatase by electron microscopy, represent merging lysosomes and phagosomes? During the present investigation, many phago-lysosomes were seen in the apical regions of the proximal convoluted tubule cells. This is in agreement with the findings of Miller (28) but not those of Novikoff (36). According to Novikoff (36), the phagosomes would acquire acid phosphatase only when they have reached the basal regions of the cells.

The fusion with phagosomes probably is a main cause for the increase in size of lysosomes in various tissues. Biochemical studies of isolated kidney fractions had shown that the number of large "droplets" increased while that of smaller ones decreased after intraperitoneal injection of egg white into rats (21). In the present experiments, the enlarged size of the lysosomes after their fusion with phagosomes could be seen *in situ*. Pathologists have described the appearance of "hyaline droplets" in pathological kidneys in connection with proteinuria (37). In this case, too, the large size of the droplets may be related to the fusion of lysosomes with phagosomes. A similar explanation may account for the smaller size of the lysosomes in the parenchymal cells of the liver as compared to those in the Kupffer cells. If the present observations with injected peroxidase can be generalized, the uptake of larger amounts of ingested materials by the phago-lysosomes of the Kupffer cells as compared with those of the parenchymal cells is responsible for the larger size of the former. These considerations do not exclude the possibility that phagosomes alone may increase in size with the

accumulation of ingested materials, or that "young" lysosomes alone may increase in size with the accumulation of newly formed hydrolytic enzymes. Nor does it exclude the possibility that the phago-lysosomes may *decrease* again in size when they become "older."

A further enlargement of phago-lysosomes was often seen in the cells of the proximal convoluted tubules. Such "composite bodies" (12) showed angular shapes and consisted of an aggregation of *several* lysosomes and *several* phagosomes. It was not always easy to distinguish to what extent the granules were merely aggregated, or had fused. These bodies often seemed to be extruded into the lumen (12). If, in certain cases, these enlarged phago-lysosomes would be segregated together with mitochondria and cell fragments (12), they might be related to the cytolysomes described by Novikoff and Essner (38) and to similar bodies, described by Ashford and Porter (39), in liver cells.

A further drastic increase in the size of bodies formed by merging lysosomes and phagosomes was observed when the normal permeability of the liver cells had been altered by the administration of toxic preparations of peroxidase. The factors responsible for the toxicity are still under investigation. It has been mentioned elsewhere (19) that these huge vacuoles were formed by merging of phagosomes and lysosomes, and are probably related to vacuoles described in injured liver cells by Trowell (40), Doniach and Weinbren (41), Aterman (42), Nairn *et al.* (43), and Anderson *et al.* (44). Anoxia and increased sinusoidal pressure were held responsible for these vacuolar changes (40). Recently, Oudea (45) observed, by electron microscopy, similar formations in anoxic liver after injection of colloidal mercury. Perhaps these structures are also related to vacuoles appearing in liver cells after treatment with dextran as described by Daems (46), and similar vacuoles appearing in kidney cells after injection of dextran or sucrose as observed by Trump and Janigan (47) and by Maunsbach *et al.* (48). Holter (49) mentioned that large vacuoles are also formed in ameba by fusion of lysosomes.

Under physiological conditions, relatively small amounts of peroxidase entered the parenchymal cells of the liver, probably being carried there by very small phagosomes (micropinocytic vesicles). It was suggested (13) that the latter may transport peroxidase from the blood to the bile. Most peroxidase in the liver cells was concentrated in *large* phagosomes which were located at the periphery of the cells lining the sinusoids. It should be clarified by electron microscopy whether these loci represent thin extension of endothelial cells, the cell membranes of the parenchymal cells, or both. The peribiliary lysosomes showed a relatively weak reaction for peroxidase in cells neighboring the portal veins. This indicated that the amounts of the foreign protein entering the hepatic cells varied with the position of the cells in the lobule. Considerable amounts of peroxidase were taken up by Kupffer cells. The uptake of proteins by Kupffer cells has also been reported by Sabin (50), Kruse and McMaster (51), Schiller *et al.* (52), and Roberts and Haurowitz (53). Uptake of proteins by the parenchymal cells was reported by Mayersbach (54).

It was suggested previously (21) that lysosomes are related to secretory granules, and that the changes occurring in kidney cells after injection of horseradish peroxidase represent a combination of the processes of pinocytosis and secretion (11–13). It may be suggested that only young lysosomes, in which newly formed hydrolytic enzymes are stored, represent a type of zymogen granule. These might be the homogeneous-appearing dense bodies with single membranes, described in electron micrographs of kidney cells (28, 31–34). When lysosomes have fused with phagosomes, and when digestive processes have taken place in the granules, the structure will be altered. Such old lysosomes probably correspond to the polymorphous bodies showing lipoprotein membranes and vacuoles seen in electron micrographs of kidney cells (28, 31–34). It would appear from these observations by electron microscopy that most acid phosphatase–containing bodies in kidney cells correspond to such old lysosomes. These residual bodies seem to be able to fuse with newly formed phagosomes. It should be pointed out that, during the first hours after the administration of average or high doses of horseradish peroxidase, only a few bodies remained in the cells of the proximal convoluted tubules which stained for acid phosphatase alone, and that most bodies showed the combined (purple) staining reaction for acid phosphatase and peroxidase. This indicated that most of the preexisting (old) lysosomes had fused with newly formed phagosomes. The question, whether or not a new formation of young lyso-

somes or lysosomal enzymes takes place later after injection of peroxidase, is still under investigation.

Among many questions raised by these experiments, the following may especially be pointed out: Exactly by what process do phagosomes and lysosomes fuse in kidney cells, and where and by which stimuli does the new formation of hydrolytic enzymes take place in the cells, and how are these enzymes brought into the lysosomes or phagolysosomes? The differential staining procedure for injected peroxidase and lysosomal enzymes in the same cells may facilitate further inquiry into these questions.

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