Defective Function of Renal Lysosomes in Mice with the Chediak-Higashi Syndrome

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Morphologically abnormal lysosomes demonstrated in individuals with the Chediak-Higashi syndrome (CHS) suggested a defect in the function of these abnormal lysosomes. To gain direct experimental evidence of such a defect, horseradish peroxidase (HRP) was injected intravenously into CHS and control mice, the mice killed at varying intervals and the kidneys studied by ultrastructural cytochemistry. No morophologic difference was observed in the absorption and uptake of HRP by proximal convoluted tubules in the two groups of mice. In CHS mice, however, some of the HRP fused with enlarged lysosomes. By 48 hours after injection, the lysosomes of normal mice had digested all but trace amounts of HRP, whereas large amounts were still present in CHS mice at this time. In CHS mice, moderate amounts were still present at 72 hours and trace amounts 96 hours post injection. This slowed rate of digestion of HRP by lysosomes of the proximal convoluted tubule cells of CHS mice suggests a similar defect in all cells in CHS individuals in which there is a lysosomal degradation of protein or other matter obtained by endocytosis. Such a defect may explain some manifestations of impaired host defense observed in CHS (Am J Pathol 67:227–240, 1972).

THE CHEDIAK-HIGASHI SYNDROME (CHS) is an autosomal recessive disease which has been reported to occur in four species: man,[1] mink,[2] cattle[3] and mice.[4] The homology of the disease in the four species is well established,[5,6] but the basic biochemical defect has not been identified. Chediak-Higashi syndrome is manifested by partial albinism, an increased susceptibility to infectious disease and enlarged anomalous granules in many cell types. The enlarged granules in leukocytes have been shown to be lysosomes,[7] whereas others, such as melanin granules, have not been classified as lysosomes. The manner in which the abnormal granules form in some cell types has been demonstrated. In both leukocytes[8,9] and melanocytes[10] the abnormally enlarged granules are caused by an unregulated fusion of granules.

Although the function of cells with abnormal granules has been

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Supported in part by US Public Health Service, NIH Grants 5TI-GM-414-08, FR-05465 and AI-06591.

Accepted for publication Nov 22, 1971.

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shown to be defective in some respects, such as chemotaxis,[11] killing of bacteria [12,13] and degranulation,[14,15] experimental evidence of a defect in lysosomal function has not been demonstrated directly. In a preliminary report on the catabolism of egg albumin in renal epithelial cells of mice and mink with CHS, evidence was obtained suggesting such a defect.[16] In the present study, horseradish peroxidase was used to study further the functional capacity of morphologically abnormal lysosomes in the renal tubule cells of mice with CHS.

Materials and Methods

Animals

Mice with CHS and the background genes of C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, Maine) as brother–sister pairs of bgbg (homozygous for the beige or CHS trait) and +bg (heterozygous for the beige trait). They were maintained by brother–sister matings of bgbg to +bg for five generations and then were mated at random. The CHS mice used in this study were obtained from these random matings. The control mice were offspring of C57BL/6J mice derived from matings of +bg mice and shown, by test matings, to be free of the beige gene.

Technics

Beige and C57BL/6J young mature female mice were injected in the tail vein with 0.1 ml of saline containing horseradish peroxidase (Sigma Type II, Sigma Chemical Company), 12 mg/100 g body weight. These mice, along with saline-injected beige and C57BL/6J controls, were killed by cervical dislocation at scheduled times from 90 seconds to 96 hours after injection. After the renal veins were incised, the kidneys were perfused with physiologic saline (37 C) injected slowly into the left ventricle of the heart, and the kidneys were subsequently perfused with either 4% glutaraldehyde diluted with physiologic saline or with 1.5% glutaraldehyde in 0.1 M K_2PO_4 buffer to which 1% sucrose (KP-S buffer) pH 7.4 was added. The kidneys were placed in 1.5% glutaraldehyde-KP-S buffer for 2 to 6 hours, washed three times in KP-S buffer and stored at 4 C until sectioned. Kidney cortex was sectioned at 50 μ on a TC-2 Smith Farquhar tissue chopper (Ivan Sorvall, Inc). Cross-sections of cortex were collected, rinsed in KP-S buffer and stained for peroxidase.[17] A saturated solution of 3,3′-diaminobenzidine was prepared by adding 6 mg of 3,3′-diaminobenzidine to 9 ml of 0.05 M Tris-HCl buffer, pH 7.4, and shaking the solution for 30 minutes at room temperature. One milliliter of a 1% solution of hydrogen peroxide was then added, the solution was shaken thoroughly and filtered. Sections of kidney were incubated in 2 to 3 ml of this solution for 10 minutes, washed three times in distilled water, post-fixed for 45 minutes in a 1% solution of osmium tetroxide on ice[18] and *en-block* stained for 2 hours with a 1% aqueous solution of uranyl acetate. The tissues were washed twice with distilled water, dehydrated in graded solutions of ethanol and embedded in Epon-Araldite.[19] Thick and thin sections were cut with glass and diamond knives, respectively, on a Porter-Blum MT-2 ultramicrotome. The thin sections were viewed, either unstained or stained with lead citrate and uranyl acetate, in a Philips 200 electron microscope.

Results

Horseradish peroxidase (HRP) is endocytized by cells of the renal tubules and when incubated with diaminobenzidine and hydrogen peroxide electron-dense deposits are produced which can serve as ultrastructural markers. None of this cytochemical reaction product was observed in saline-injected control mice of either the CHS or normal group.

Although post-staining with lead citrate and uranyl acetate helped visualize structures in the electron microscope, it was not essential, and electron micrographs were taken of the unstained sections.

In both CHS and black (control) mice killed 90 seconds after the injection of HRP, the reaction product of HRP was present just beneath the brush border in apical portions of the proximal convoluted tubule cells. Little HRP could be demonstrated attached to microvilli of brush borders in either group of mice killed at this or subsequent times. At this early stage of absorption of HRP, there was no demonstrable difference in the distribution or the amount of HRP in the two groups of mice. At 5 minutes after injection, there was no difference in the appearance of HRP in the apical vacuoles of the cells of the two groups (compare Figures 1 and 2), but by this time, some of the HRP had entered deeper parts of the cells and, in the CHS, had fused with enlarged granules in cells of animals with CHS (Figure 2). With mice killed at increasing time intervals after injection, there was an increasing amount of the HRP in phagolysosomes in deeper parts of the cell. In groups of mice killed beyond several hours after injection, there was a concomitant decrease in the amount of HRP present in apical portions of tubule cells in apical vacuoles. By 18 hours after injection, very little HRP was present in apical vacuoles (Figures 3 and 4). At this time, no difference in the amount of HRP in cells of the two groups of mice could be discerned. In individual phagolysosomes, however, because CHS cells had larger but fewer phagolysosomes, there was more HRP in the phagolysosomes of the CHS cells.

There was a gradual decrease in the amount of HRP present in phagolysosomes of both groups of mice killed after 18 hours postinjection. In the control group, however, the rate at which HRP disappeared from the phagolysosomes was more rapid. In the control mice killed 48 hours after injection, only trace amounts of HRP were present (Figure 5). In CHS mice killed at this time a considerable amount of HRP was present (Figure 6), but substantially less than was present in CHS mice killed 18 hours after injection (Figure 4). The amount of HRP in phagolysosomes of CHS mice continued to decrease and, in the group killed at 72 hours, there was a moderate amount of HRP present

(Figure 7), the quantity of which was only slightly less than that present in CHS mice killed at 48 hours (Figure 6). At 72 hours after injection, HRP was present in both enlarged phagolysosomes and in more normal appearing phagolysosomes (Figure 7). At 96 hours after injection, only trace amounts of HRP were present in CHS mice, the amount being roughly comparable to that observed in control mice at 48 hours after injection.

No HRP was evident in the proximal convoluted tubule cells of control mice killed at 72 and 96 hours. Because 96 hours was the longest time interval after injection that mice were killed, it was not ascertained how much longer the trace amounts of HRP persisted in the CHS mice. Because there was a more marked decrease in the amount of demonstrable HRP in the CHS mice between 72 and 96 hours than between 48 and 72 hours, it is assumed that the HRP persisted a relatively short time beyond 96 hours.

Homogeneous electron-lucent bodies were observed in the phagolysosomes of both CHS and control mice. Many of these bodies had a spherical shape, and some appeared to be membrane limited; HRP was not observed in them. In CHS mice, some of these bodies were much larger than those in control mice, but most were approximately the same size (compare Figures 3 and 4). These bodies may represent virgin lysosomes which have fused with the preexisting phagolysosome. On the other hand, they may be simply lipid bodies.

The basic structure of the phagolysosomes of CHS and control mice was similar. The CHS phagolysosomes were larger, however, and were often more irregular in shape (compare Figures 3 and 4). Although there was often a close association between lysosomes and other cellular organelles, especially mitochondria, the inclusion of these organelles in the lysosomes was not observed in either CHS or normal mice.

Discussion

Although enlarged granules have been observed in many cell types in CHS, evidence from several sources has suggested that it is not a classical storage disease. Cytochemical studies have demonstrated that the enlarged granules in CHS vary from one cell type to another but that the cytochemical reactions are similar to those of the normal granules in a particular cell type. Secondly, it has been demonstrated in leukocytes and melanocytes that the enlarged granules in these cell types arise by fusion of preexisting granules which arise by normal pathways.[8,10] Finally, it has been demonstrated that CHS fibroblasts grown in cultures with fibroblasts from several inherited storage dis-

eases, corrected the cellular accumulation of metachromatic granules in fibroblasts from individuals with those diseases but were not in turn corrected by the fibroblasts of any of the inherited storage diseases.[20]

Most of the studies on CHS involved neutrophils. Although differences have been demonstrated between neutrophils from normal and CHS individuals, attempts to demonstrate a functional defect in the digestive capacity of lysosomes of neutrophils was not obtained. Some of the difficulties associated with the demonstration of such a defect in neutrophils are directly related to the peculiarities of this cell type. Neutrophils produce their lysosomal granules in the bone marrow in a specific stage of maturation. After maturation, the neutrophil is released from the bone marrow and subsequently, either in the blood stream or in the tissues, it phagocytizes material such as bacteria. Upon phagocytosis, the lysosomes fuse with the phagocytized material, digest it and the cell dies.

In contrast to neutrophils, most cells of the body are long-lived, continually producing lysosomes and continually endocytizing and digesting material. To measure the digestive capacity of lysosomes it would be advantageous to use a long-lived cell that is stationary, has a definite orientation and has a primary function in the digestion of nonreplicating material.

The use of the exogenous marker protein horseradish peroxidase (HRP), for functional studies of lysosomes of the renal proximal convoluted tubule cells is well established.[21,22] Proteins with molecular weights of less than 70,000 pass through the glomeruli, with the permeability of glomeruli to smaller protein molecules increasing with decreasing molecular weights.[23] Horseradish peroxidase has a molecular weight of approximately 40,000 and is rapidly cleared by glomeruli.[17,23] It has been shown that this protein accumulates at the base of the brush border of proximal convoluted tubule cells and enters the cells in apical tubular invaginations. The apical vacuoles containing the HRP appear to enlarge either through transport of additional HRP through apical tubules or by fusion with each other.[24] It has been shown that apical vacuoles fuse with the lysosomes and that the endocytized HRP is digested in the resulting phagolysosome.[25] Because there is no endogenous peroxidase in the kidney, the HRP can be positively identified in the aforementioned structures by cytochemical methods with no interference. It was with these advantages in mind that functional studies on lysosomes of proximal convoluted tubule cells of CHS mice and mink were performed using large doses of egg albumin.[16] Although suggestions of a slowed rate of digestion were ob-

tained, good technics were not available to directly identify egg albumin and its exact cellular location.

Thus, in this study, the HRP technic was used not only to identify the endocytized material, but also to reveal rough quantitative differences in the processing of the enzyme. It was concluded that the lysosomes of CHS mice digested the HRP at a slower rate than did normal mice. The present study did not reveal gross differences in the endocytosis of the enzyme, but an abnormality in the uptake of material in CHS has not been suggested by previous studies in the literature.

Although the large granules in the renal tubule cells of the CHS species do resemble nonfunctional residual bodies, the results of this study indicate that they are functional albeit they perform their function of digesting endocytized material more slowly than do phagolysosomes of normal animals.

Whether the slowed rate of digestion is due to a deficiency of a lysosomal enzyme or enzymes is not known. Although one group of investigators has demonstrated a reduced amount of lysosomal enzymes in CHS granulocytes,[26] most investigations have failed to demonstrate such a reduction. In fact, the current opinion is that the defect in CHS involves the membrane of the various granules rather than the content.[14,27] In light of this, it might be speculated that the slowed rate of digestion in lysosomes in CHS is not due to any inherent lysosomal enzyme deficiency but is due, instead, to the steric relationships in enlarged lysosomes. It may take longer for all HRP to come in contact with digestive enzymes within the enlarged lysosomes, compared to the smaller lysosomes of normal cells.

Regardless of the exact reason for the slowed rate of digestion by lysosomes, the demonstration of this defect has implications in some manifestations of CHS related to impaired host defense. Because of the homology of CHS in the four species, findings in one species can be extrapolated to the other three. It has been shown that mink with CHS have a depressed immune response, especially the anamestic response, to at least two antigens.[28,29] It had been shown previously that macrophages, lymphocytes and plasma cells contain abnormal granules in CHS; and it is thought that these cells participate in some manner in the processing of antigen and the production of antibody. Although the exact role of each of these cells in the immune response is not resolved, there is evidence that the macrophage is necessary for an immune response with some antigens.[30] Although it is hazardous to extrapolate findings between species and cell types, slowed catabolism of HRP in the renal tubule cells in CHS mice may support the hypothe-

sis suggested previously [29] of a slower rate of metabolism of antigen in CHS mink. The application of technics used to study the digestion of peptides by lysosomes of normal macrophages [31] may yield data that will resolve this point if applied to the study of CHS.

Mink with Chediak-Higashi syndrome also have been shown to die more rapidly after infection with Aleutian disease virus than did non-CHS mink.[32] The cause of death in both CHS and non-CHS mink is apparently an immune complex glomerulonephritis.[33] Although no differences have been demonstrated in lesions or in the level of virus in the two groups of mink, the rate at which antigen-antibody complexes accumulate in the glomeruli of CHS mink appears to be increased.[34] Recognizing the role of the glomerular mesangial cell in the digestion of glomerular residues,[23] one may extrapolate findings of the present study to support the suggestion [34] that the increased rate at which immune complexes accumulate may be due to a slowed rate of removal.

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Acknowledgments

We thank Miss Luann Hohenadel and Mrs. Phyllis Hoffman for technical assistance and Mr. Harold Conner for photographic work. We also acknowledge the Veterinary Sciences Research Division of the US Department of Agriculture at Pullman, Washington and its director, Dr. John R. Gorham, for furnishing the electron microscope.

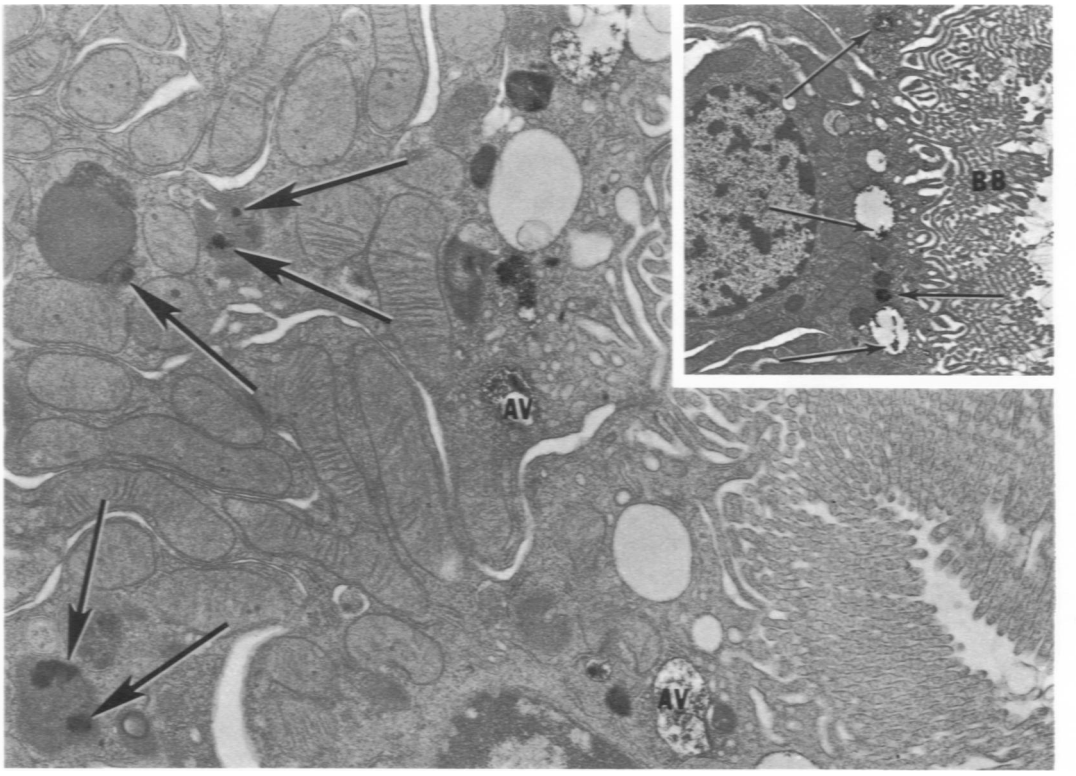
[*Illustrations follow*]

Legends for Figures

All figures are electron micrographs of horseradish peroxidase (HRP) preparations of mouse renal tubule cells. See text for details.

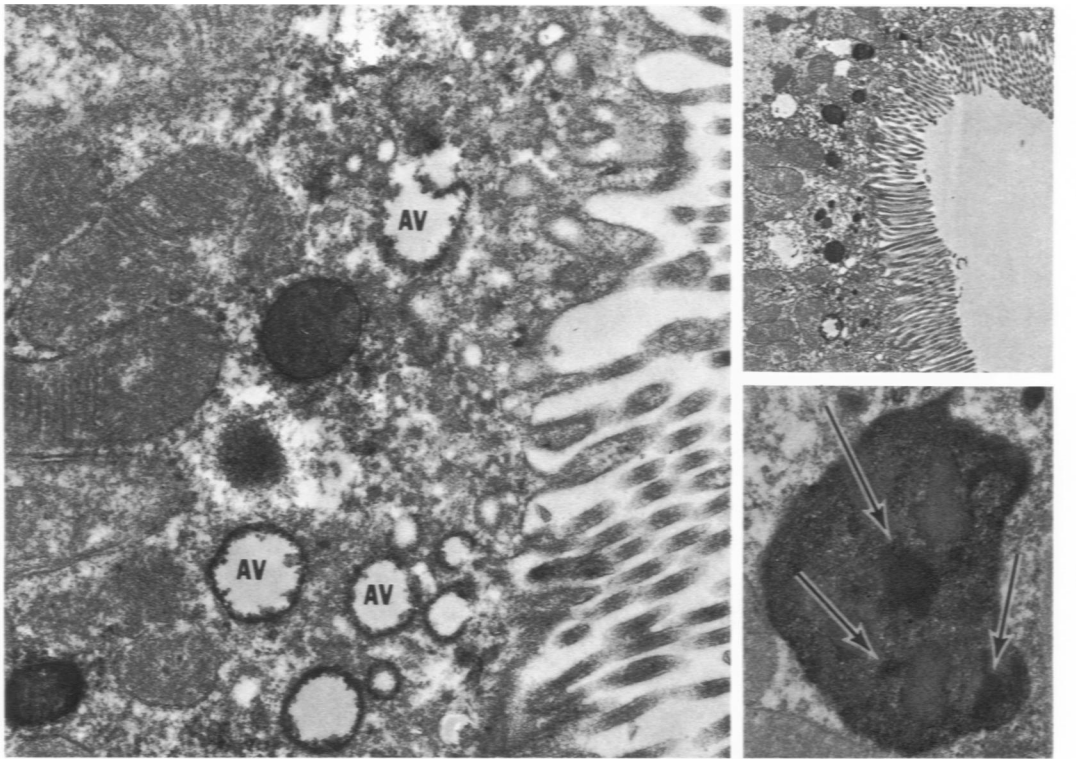
Fig 1—Control mouse kidney 5 minutes after HRP injection. **A**—HRP is present in the apical portion of the cell *(arrows)* beneath the base of the brush border *(BB)*. **B**—In addition to HRP in the apical vacuoles *(AV)*, some has penetrated deeper into the cells and has fused with lysosomes *(arrows)* (A × 4000; B × 14,000).

Fig 2—CHS mouse kidney 5 minutes after HRP injection. **A**—The HRP accumulated in apical vacuoles *(AV)* beneath the brush border *(BB)* in a manner similar to that of the control mouse kidney in Fig 1. **B**—A lower-power view illustrating a similar area. **C**—Particles of HRP *(arrows)* have fused with a lysosome in the deeper part of the cell. (A × 23,000; B × 3500; C × 23,000).



1A

1B



2A

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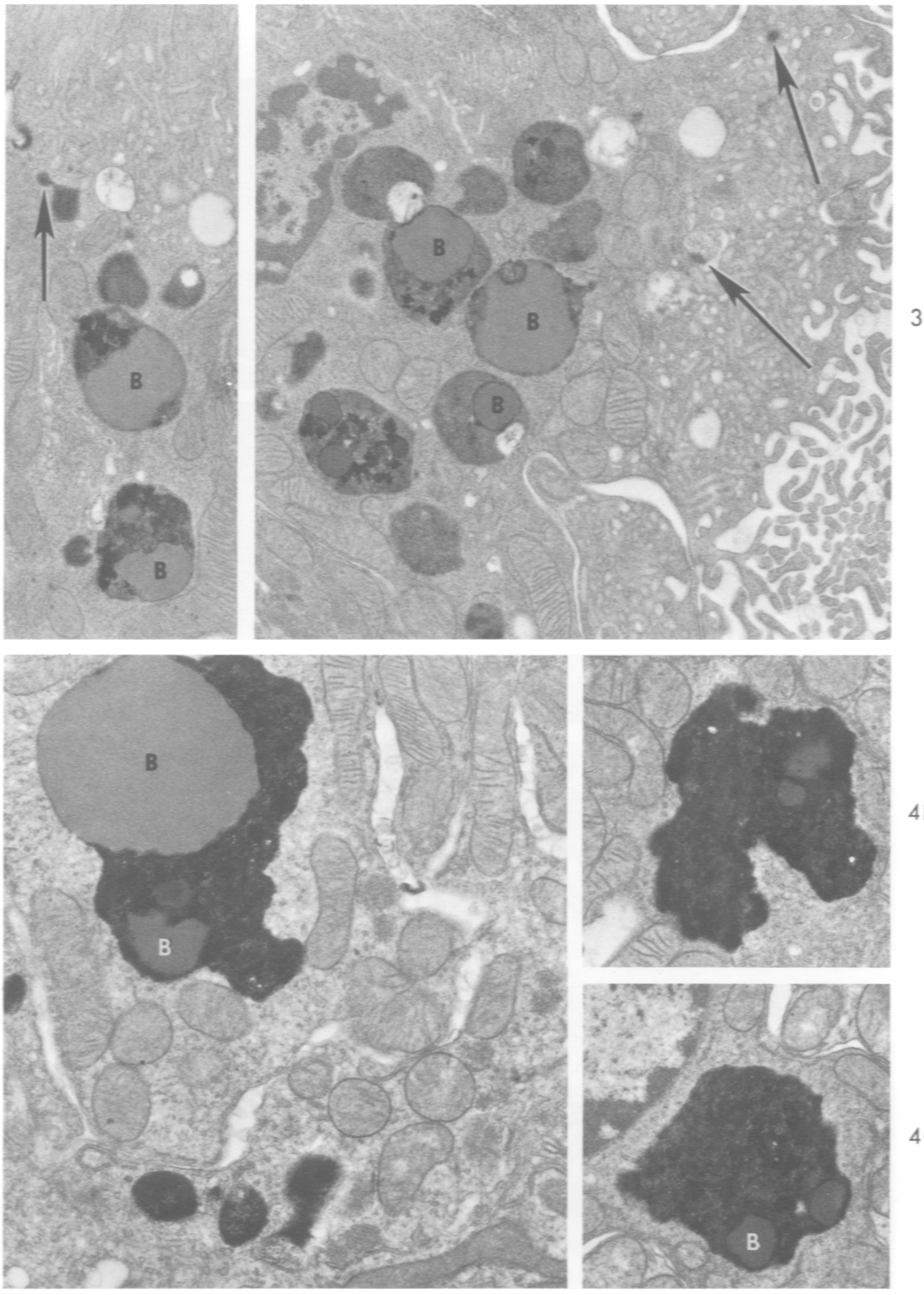
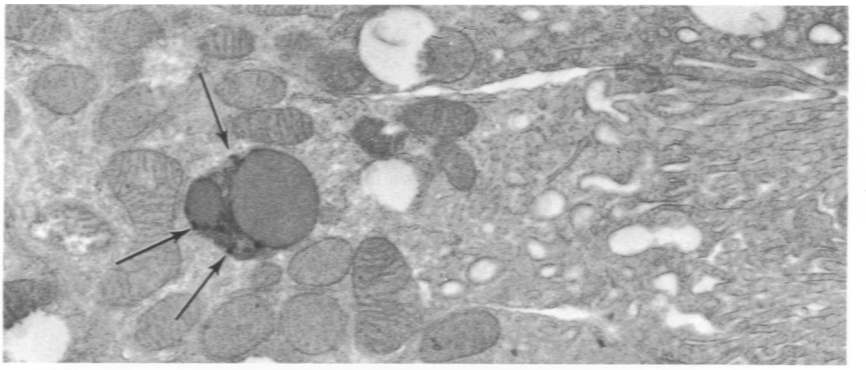
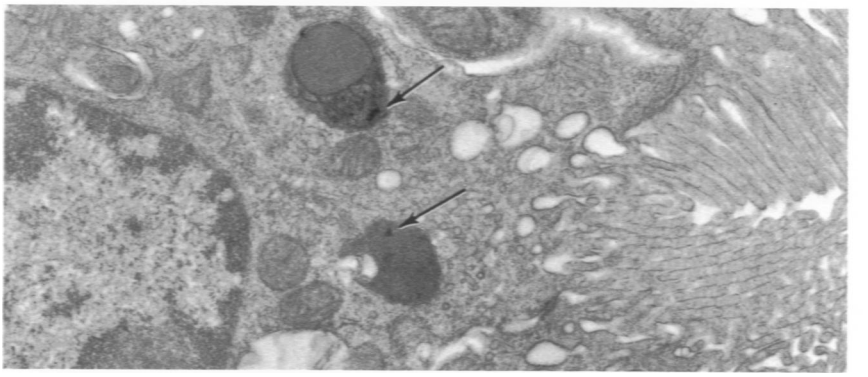


Fig 3—Control mouse kidney 18 hours after HRP injection. Most of the HRP is present in phagolysosomes in the deeper parts of the tubule cells. A little, however, is still present in apical vacuoles (*arrows*). Homogeneous bodies (*B*) appearing to be sometimes membrane limited, are present in most of the phagolysosomes and do not contain HRP (A and B, × 14,000). **Fig 4**—CHS mouse kidney 18 hours after HRP injection. The HRP is present in phagolysosomes as was that of the control mice in Fig 3. The phagolysosomes in this figure are larger, which is characteristic of CHS, but in addition some of the homogeneous bodies (*B*) in the phagolysosomes are much larger than those of the control mice (compare with Fig 3) (A, B and C, × 14,000).



5A



5B

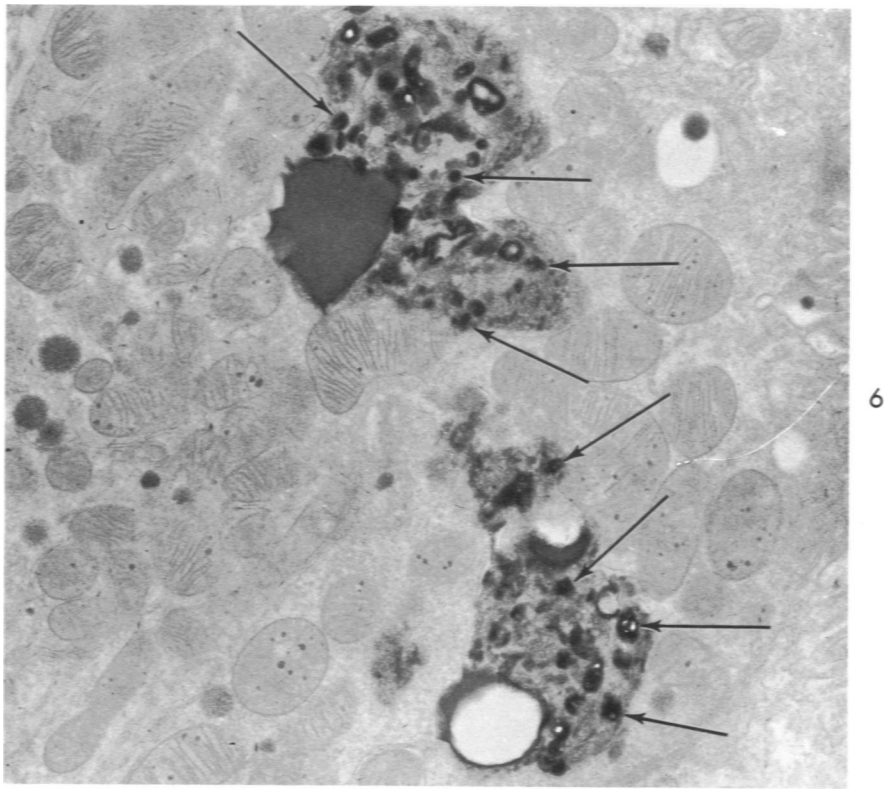


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Fig 5—Control mouse kidney 48 hours after HRP injection. Only trace amounts of HRP are present (*arrows*). The HRP that is present is located entirely within phagolysosomes (A and B, × 14,000). **Fig 6**—CHS mouse kidney 48 hours after HRP injection. Relatively large amounts of HRP (compared to the control in Fig 5) are present in the phagolysosomes (*arrows*) (× 14,000).

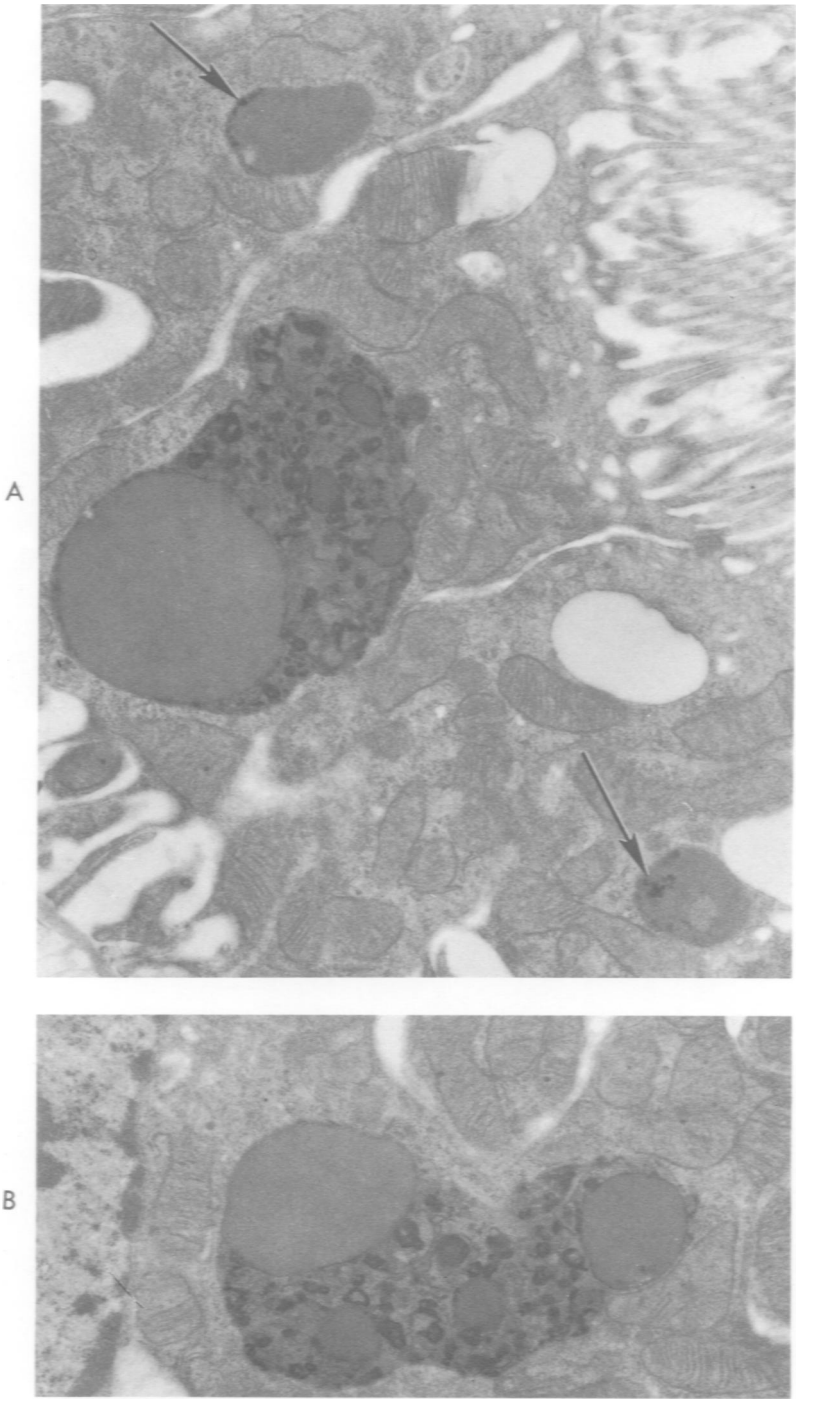

Fig 7—CHS mouse kidney 72 hours after HRP injection. Moderate amounts of HRP are present in the large phagolysosomes and some HRP is present (*arrows*) in what may be a normal-sized phagolysosome or possibly the sectioned end of a large phagolysosome (A and B, × 14,000).