LYTIC ACTIVITIES IN RENAL PROTEIN ABSORPTION DROPLETS

An Electron Microscopical Cytochemical Study

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ABSTRACT

The digestive cycle following reabsorption of hemoglobin by cells of the proximal convoluted tubules in mouse kidney and the uptake of ferritin by glomerular mesangial cells in the kidney of normal and nephrotic rats were investigated by electron microscopical histochemical procedures.

Mouse kidneys, sampled at closely spaced time points between 1 to 48 hours after intraperitoneal injection of hemoglobin, and rat (normal and nephrotic) kidneys, sampled at 30 minutes, 2 hours, and 48 hours after intravenous injection of ferritin, were fixed in glutaraldehyde, cut at 50 μ on a freezing microtome, incubated for acid phosphatase and thiol-acetate-esterase, and postfixed in OsO_4. Satisfactory preservation of fine structure permitted the localization of the enzymatic reaction products on cell structures involved in uptake and digestion of exogenous proteins. The latter were identified either by their density (hemoglobin) or their molecular structure (ferritin).

It was found that lysosomal enzymic activities and incorporated exogenous proteins occur together in the same membrane-bounded structures. In the cells of the proximal convolution, lytic activities become demonstrable within 1 hour after hemoglobin injection, appear first in apical vacuoles filled with hemoglobin, and persist in fully formed protein absorption droplets. At the end of the lytic cycle ($\sim$48 hours post injection), the cells have an increased population of polymorphic bodies which exhibit lytic activities. In smaller numbers, identical bodies occur in controls. It is concluded that they represent remnants of previous digestive events.

The means by which the resorptive vacuoles acquire hydrolytic activities remain unknown. Fusion of newly formed vacuoles with residual bodies was not seen, and hemoglobin incorporation into such bodies was only occasionally encountered. Acid phosphatase activity was found sometimes in the Golgi complex, but enzyme transport from the complex to the resorbing vacuoles could not be established. Autolytic vacuoles containing mitochondria or mitochondrial remnants were frequently found during the early stages of hemoglobin resorption, but no definite conclusions about the mechanism involved in the segregation of endogenous material were obtained.

In nephrotic rats ferritin was segregated in membrane-bounded bodies mainly in the mesangial cells and to a lesser extent in epithelial and endothelial cells. Most of these sites were marked by the reaction products of acid phosphatase and organophosphorus-resistant esterase and therefore identified as lysosomes connected with the digestion of incorporated exogenous proteins.

INTRODUCTION

The name lysosomes (*i.e.*, lytic bodies) has been proposed by de Duve *et al.* (1) to designate a group of particles isolated from rat liver which behave essentially like membrane-bounded sacs filled with soluble, acid hydrolases. Under normal conditions, the membrane of the sacs is assumed to prevent the efflux of enzymes as well as the influx of external substrates. To date, about 12 distinct enzymes, capable of hydrolyzing proteins, mucopolysaccharides, and nucleic acids, have been localized in lysosomal fractions (2).

Originally, the identification of lysosomes as structural entities was rendered difficult by their intrinsic polymorphism and by the extensive contamination of lysosomal fractions by other cell components (*cf.* 3); more recently it was facilitated by the widespread use of various acid phosphatase (AcPase) tests for light (4, 5) and electron (6, 7) microscopy. In the interpretation of such tests, it is usually assumed that AcPase activity is a reliable lysosome marker, *i.e.*, it is present only in lysosomes, and is generally accompanied by other hydrolases. Although both these assumptions remain partly open to question, it can be said that AcPase tests have shown lysosomes to be present in a wide variety of cell types in the principal phyla of the animal kingdom (8), and have suggested their frequent association with phagocytosis (endocytosis) (8, 9). As a result of such findings, and of cell fractionation studies carried out on phagocytic cells (10, 11), the original lysosome concept has been adjusted and broadened. At present, according to de Duve (2), four distinct, but functionally related, entities can be recognized among lysosomes: (I) enzyme-storing granules, the predominant form in polymorphonuclear neutrophil leukocytes (10); (II) digestive vacuoles present in many cell types, including those engaged in protein resorption; (III) autolytic pockets separated from the rest of the cytoplasm by a membrane; and (IV) residual bodies containing remnants of the digestion of exogenous or endogenous materials. This systematization is supported by a substantial body of circumstantial evidence, yet the validity of some of its aspects remains to be demonstrated by ascertaining the presence, within the same body or vacuole, of lysosomal enzymes and of recognizable exogenous or endogenous materials. The present study provides such evidence. It identifies as lysosomes of groups II to IV a series of membrane-bounded structures which are known to appear during the experimental uptake of two foreign proteins, *i.e.*, hemoglobin and ferritin, in two different renal cell types, *i.e.*, the epithelial cell of the proximal convolution in the mouse (12) and the mesangial cell in the rat (13). The identification was achieved by demonstrating, at the level of resolution of the electron microscope, the presence of lysosomal enzymic activities and of a foreign protein—or altered subcellular component—within the same membrane-bounded structure.

METHODS

1. *Experimental Hemoglobinuria*

Thirteen female white Swiss mice of The Rockefeller Institute stock, kept on a diet of Purina mouse pellets and weighing 15 to 20 gm, received intraperitoneally 1 ml of a 10 per cent solution of ox hemoglobin in physiological saline and were killed at intervals of 1, 2, 4, 5½, 6, 10, 18, 21, 24, and 48 hours after the injection. Ten animals served as controls.

The hemoglobin was well tolerated; hemoglobinuria appeared 4 to 6 hours after the injection and lasted for ~12 hours.

2. *Ferritin Experiments*

Ferritin, freed of Cd^{++} by dialysis against 0.1 M EDTA and 0.07 M phosphate buffer (pH 7.2) as in reference 14, was injected intravenously under light ether anesthesia into 8 young male Sprague-Dawley rats, of which 2 were normal and 6 nephrotic. The dose administered, *i.e.*, 75 to 150 mg ferritin (1 to 2 ml) per animal, was tolerated without any visible ill effects. Two nephrotics were sacrificed at 30 minutes, two nephrotics and one normal at 2 hours, and an equivalent group at 48 hours after the injection.

3. *Experimental Nephrosis*

Since ferritin uptake by the mesangial and epithelial cells of the renal glomerulus is enhanced in experimental nephrosis (13, 14), young rats weighing originally 155 to 170 gm were rendered nephrotic by 9 daily injections of the aminonucleoside of puromycin at a dose of 1.67 mg/100 gm body weight (*cf.* 14). The total amount given was adjusted daily to compensate for the increase in weight of the animals while under treatment. Ferritin was adminis-

tered, as indicated above, on the 10th day after the beginning of the aminonucleoside treatment.

4. *Techniques for Combined Cytochemistry and Electron Microscopy*

FIXATION: Kidneys, removed under light ether anesthesia from hemoglobin- or ferritin-treated animals, were cut across into thick (2 to 3 mm) slices which were fixed at ~4°C for 2 to 3 hours in 6.25 per cent glutaraldehyde in 0.075 M Na-cacodylate buffer (final pH 7.1) (15).

ACID PHOSPHATASE TEST: Frozen sections ~50 μ thick, cut from the fixed slices on a freezing microtome, were collected in cold Na-cacodylate buffer (pH 7.4) containing 0.33 M sucrose; were rinsed for 20 minutes to 2 hours in one or two cold changes of the same solution; and finally incubated for 15 minutes at 37°C in a Gomori medium. The latter was freshly prepared before each experiment by dissolving 0.12 gm $Pb(NO_3)_2$ in 100 ml 0.05 M Na acetate buffer (pH 5.0) containing 7.5 per cent sucrose (0.22 M) and then adding slowly 10 ml of a 3 per cent solution of Na-β-glycerophosphate. Before use, the mixture was warmed at 60°C for 1 hour, cooled to room temperature, and filtered to eliminate the slight precipitate which usually developed.

After incubation, the sections were rinsed twice for 1 minute in cold 0.05 M Na acetate buffer (pH 5.0) containing 7.5 per cent sucrose and 4 per cent formaldehyde (6), with a short rinse in 2 per cent acetic acid in between the two rinses of acetate-buffered formaldehyde. Controls were run by omitting the substrate from, or by adding 0.01 M NaF to, the incubation medium.

"ESTERASE" TEST: Frozen sections, cut and processed as above, were incubated for 10, 15, 20, 30, and 45 minutes at ~4°C[1] in a modified Crevier-Bélanger medium (16) prepared as follows: 0.25 ml thiolacetic acid was mixed with 0.5 ml each of 0.2 M $MnCl_2$, 0.2 M $CaCl_2$, and 0.2 M $MgCl_2$. The mixture was titrated to pH 5.2 with NaOH (1.0 N and 0.1 N) and the volume brought up to 20 ml with 0.05 M cacodylate buffer (pH 5.2). Five ml of a 0.96 per cent $Pb(NO_3)_2$ solution in 0.05 M cacodylate buffer (pH 5.2) were added and the mixture filtered in the refrigerator, since a slight cloudiness sometimes developed upon $Pb(NO_3)_2$ addition. Such a mixture was stable for only ~2 hours at ~4°C; hence it was freshly prepared for each experiment. In control experiments, the esterase test was carried through on sections preincubated for 1 hour at 37°C in 10 μM and 1 mM E600 (Diethyl-*p*-nitrophenyl-phosphate) dissolved in 0.05 M cacodylate buffer, pH 5.2.

TABLE I

Effect of Glutaraldehyde Fixation upon Renal Acid Phosphatase Activity

Kidney tissue, pooled from two animals in each experiment, was either directly homogenized in 0.25 M sucrose or cut into 5 mm slices, fixed for 2½ hours in glutaraldehyde in cacodylate buffer (pH 7.4), washed 2½ hours in the same buffer (with sucrose added), and finally homogenized (1:20, w:v) in 0.25 M sucrose. Each homogenate was frozen and thawed 6 times before assay. For other experimental details see the text.

Species	Condition of the animals	Condition of the kidney homogenate	Activity	Inhibition
			μgP/mgN/hour	*per cent*
Rat	Normal	Fresh	285.7	—
		Fixed	146.5	48.8
Mouse	Normal	Fresh	250.1	—
		Fixed	125.1	50.0
Mouse	2 hours post hemoglobin injection	Fresh	203.5	—
		Fixed	153.4	24.6
Mouse	12 hours post hemoglobin injection	Fresh	251.5	—
		Fixed	158.8	36.9

5. *Preparative Procedures for Light and Electron Microscopy*

For light microscopy, the product of the AcPase reaction was converted into lead sulfide.

[1] Less satisfactory results were obtained at room temperature.

For electron microscopy, the incubated sections were postfixed at ~4°C for 30 to 60 minutes in 1 per cent OsO_4 in acetate-veronal buffer (pH 7.4) containing 49 mg/ml sucrose, then rapidly dehydrated in ethanol starting at 70 per cent, and finally embedded in Epon 812 (17). From each 50-μ section, the kidney cortex was cut out on a glass slide smeared with Epon, and either placed into a few drops of Epon 812 in a gelatin capsule or fastened with Epon to a flat-topped Epon block. Final polymerization was carried out at 60°C. Embedded 50-μ sections were trimmed to select tubules or glomeruli under the light microscope and sectioned with an LKB or Porter-Blum microtome provided with glass or diamond (du Pont de Nemours) knives.

In each experiment, small tissue blocks were also fixed in 1 per cent OsO_4 buffered to pH 7.4 with 0.1 M K phosphate, and in 6.25 per cent glutaraldehyde in either 0.075 M Na-cacodylate or 0.075 M K phosphate buffer (pH 7.4) with or without postfixation in OsO_4 (15). Thin sections, picked up on grids naked or covered with a carbon film, were stained with 5 per cent aqueous uranyl acetate (18) or lead hydroxide (19), and examined in a Siemens Elmiskop I operated at 80 kv with a double condenser and 50-μ molybdenum apertures in the objective. Micrographs were taken at magnifications ranging from 2,500 to 80,000.

6. *Biochemical Methods*

AcPase activity was assayed on total homogenates prepared with fresh or glutaraldehyde-fixed kidneys of normal rats and mice, and of mice which had received hemoglobin 2 and 12 hours before the experiment. For assaying fixed tissues, the kidneys were cut into 5-mm-thick slices, fixed at ~4°C for 2½ hours in cacodylate-buffered glutaraldehyde, and rinsed at ~4°C for 2½ hours in the same buffer containing 0.33 M sucrose. Homogenates (1:20, w:v) were prepared in 0.25 M sucrose with a tight-fitting teflon pestle, from both fresh and fixed tissues, and stored in the deep freezer. Aliquots were thawed and frozen 6 times in a dry ice-ethanol mixture before assaying the enzyme. Incubation was carried out at 37°C for 1 hour in the presence of Na-β-glycerophosphate at pH 5.0. Liberated inorganic phosphate was determined by the Fiske-Subbarow

General Abbreviations

av, autolytic vacuole	*m*, mitochondrion
bb, brush border	*mb*, microbody
db, dense body	*n*, nucleus
gc, Golgi complex	*rv*, resorbing vacuole
lb, lamellated body	*ti*, tubular invaginations

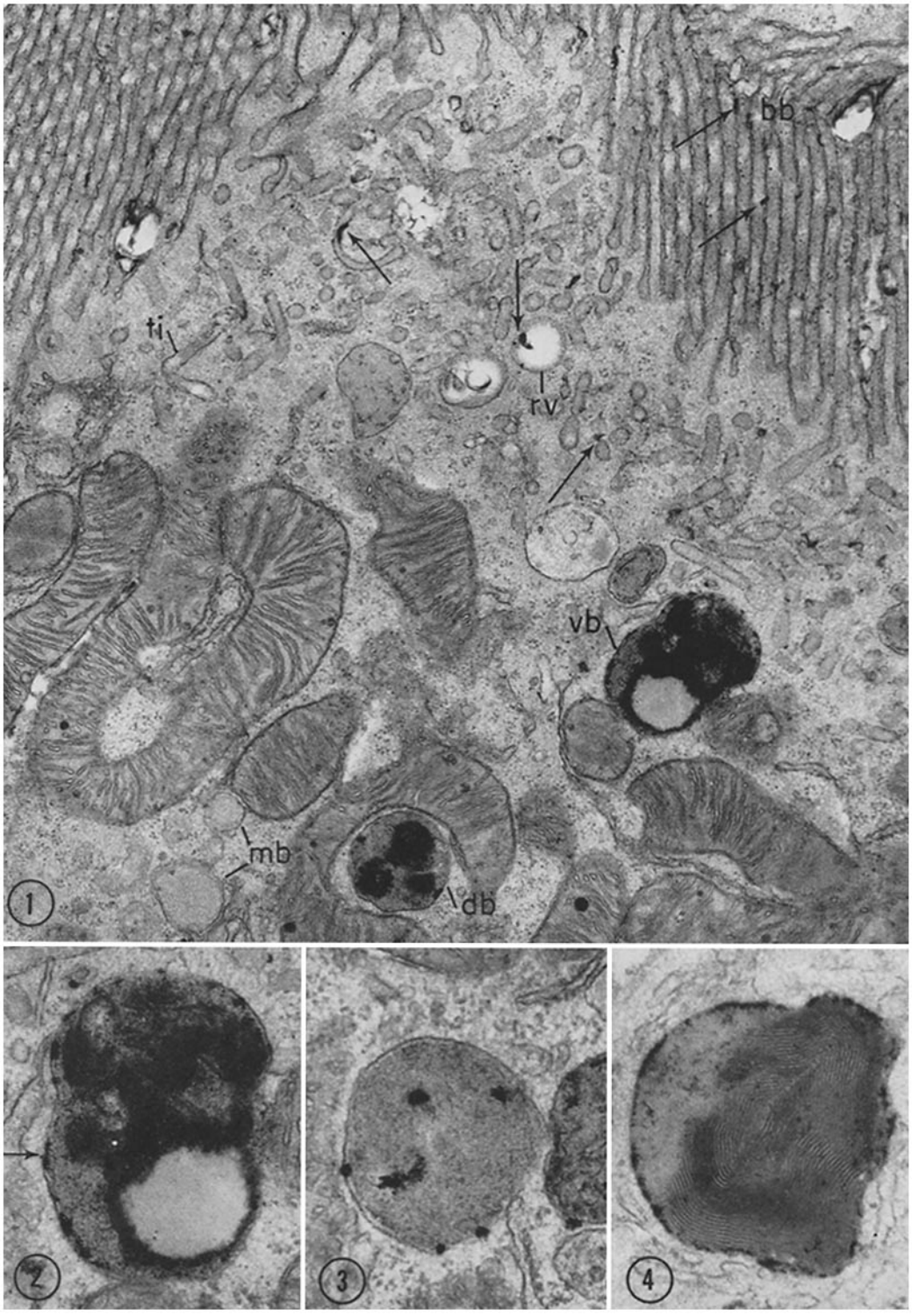
All figures, except Fig. 16, represent frozen sections of glutaraldehyde-fixed material incubated for either acid phosphatase (Figs. 1 to 5, 8 to 15, 17, 18, 20, 23) or thiolacetate-esterase (Figs. 6, 7, 19, 21, 22, 24); postfixed in OsO_4; embedded in Epon 812; sectioned on an LKB microtome; stained with $Pb(OH)_2$ (19) (except Fig. 13); and micrographed in a Siemens Elmskop I.

FIGURE 1 Apical zone of a tubule cell; proximal convolution, control mouse. Dense precipitates of AcPase reaction product mark the periphery of a vacuolated (*vb*) and a dense body (*db*). Spotty deposits of reaction product occur over the microvilli of the brush border, tubular invaginations, and resorbing vacuoles (*rv*) (arrows). Microbodies (*mb*) and mitochondria are free of reaction product. × 24,000.

FIGURE 2 Higher magnification of the vacuolated body in Fig. 1 to show the limiting membrane, the dense content, and the characteristic vacuole of the structure. Deposits of AcPase reaction product (arrow) are seen along the limiting membrane but not in the content and the vacuole of the body. × 45,000.

FIGURE 3 Dense body to show the limiting membrane, the dense granular content, and the deposits of AcPase reaction product along the membrane as well as in the content of the body. × 46,000.

FIGURE 4 Lamellated body showing characteristic internal structure and concentration of AcPase reaction product at its periphery. × 45,000.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procedure (20) and nitrogen by nesslerization of Kjeldahl digests (21).

MATERIALS

Hemoglobin, twice crystallized, and horse spleen ferritin (75 mg protein/ml) were obtained from Pentex Inc., Kankakee, Illinois. Puromycin aminonucleoside was purchased from Nutritional Biochemicals, Cleveland, Ohio; thiolacetic acid from K & K Co., Jamaica, Long Island, New York; and E600 (diethyl-*p*-nitrophenyl-phosphate) from Allbright and Wilson, Ltd., London, England.

RESULTS

I. *Effects of Glutaraldehyde Fixation*

A. On Fine Structure

Fixation in 6.25 per cent glutaraldehyde in 0.075 M cacodylate or phosphate buffer (pH 7.1), followed by postfixation in OsO_4, was tried on a number of tissues and found to give satisfactory results in agreement with the report of Sabatini *et al.* (15). In general, intracellular vesicles and vacuoles tend to be larger and intercellular spaces wider than after isotonic OsO_4 fixation. Since the glutaraldehyde mixture used is definitely hypertonic,[2] such changes are assumed to be osmotic effects. They are more pronounced when glutaraldehyde concentration is increased to 12 and 18 per cent, but not noticeably influenced when the concentration is reduced to 4 per cent. Lower concentrations were not tested.

The rate of glutaraldehyde penetration was not systematically investigated; empirically it was found that 3- to 5-mm-thick slices of the whole kidney were fixed through their entire depth within 2 hours at 4°C and that the duration of fixation over 2 hours was not critical for the preservation of fine structure. For final work, fixation for 2 hours in 6.25 per cent glutaraldehyde was chosen: it gave satisfactory morphological preservation, and enough surviving enzymatic activity. In addition, it permitted the fixation of large tissue blocks which could be easily handled on the freezing microtome. The thorough washing out of glutaraldehyde from either blocks or frozen sections was an essential step for successful postfixation with OsO_4 (*cf.* 15).

Some of the artifacts seen in the final cytochemical preparations are caused by freezing rather than fixation. They consist in further distention of the apical vacuoles, Golgi vesicles, and intercellular spaces (with the exception of occluding zonules (22)); the appearance of whorls of irregularly lamellar, osmiophilic material; and an increase in the frequency of membrane discontinuities. Damage in the form of irregular empty areas, not restricted to any definite structure, was noted in almost every section and was ascribed to ice crystal formation. At variance with recent reports[3] (6, 23) we found no damage definitely ascribable to the low pH of the incubation medium (5.0) or acetic acid rinse (3.0). Hence we were not obliged to carry out the cytochemical tests at pH's higher than optimum. In fact, it was surprising to find out how well the fine structure of the cells withstood freezing and acid treatment after glutaraldehyde fixation.

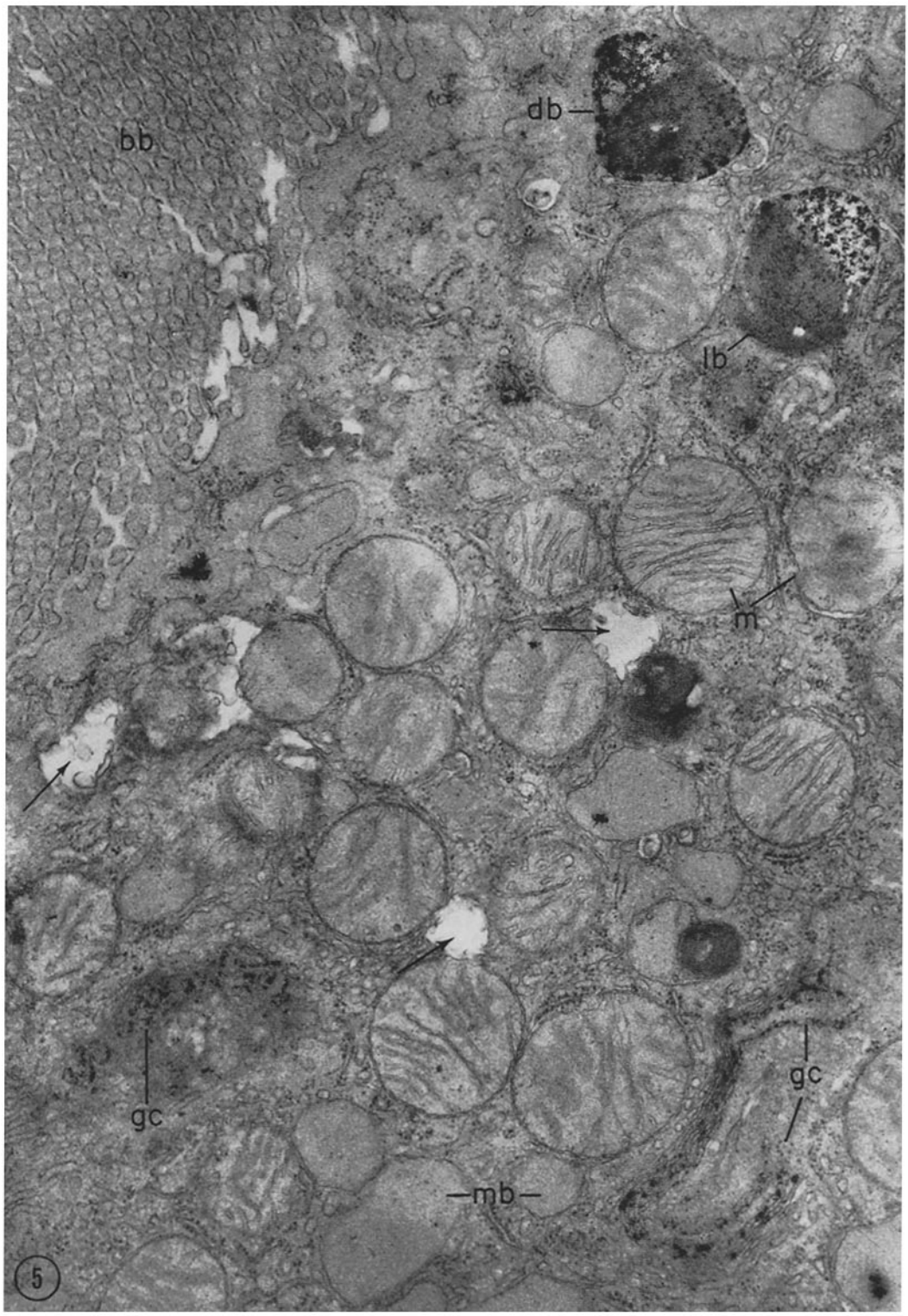
B. On Enzymatic Activity

As shown by the data in Table I, glutaraldehyde fixation resulted in ~50 per cent inhibition of renal AcPase activity. The inhibition was less pronounced in hemoglobin-treated animals. Enzyme activity remained apparently unchanged over the period examined, *i.e.*, 0 to 12 hours after hemoglobin injection.

[2] 6.25 per cent glutaraldehyde in 0.1 M phosphate buffer gave a value of 0.980 milliosmol by the freezing point depression method.

[3] Note, however, that Holt and Hicks used formaldehyde- rather than glutaraldehyde-fixed tissues.

FIGURE 5 Apical and intermediate zone of a tubule cell; proximal convolution, control mouse. AcPase reaction product is neatly localized on the membranes and content of the cisternae of two Golgi complexes (*gc*), one normally (right) and the other obliquely (left) sectioned. Note that the AcPase-positive dense (*db*) and lamellated (*lb*) bodies lie remote from the Golgi complexes. "Empty" areas (arrows) are freezing artifacts. × 29,000.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II. *Intracellular Localization*

Cells of the Proximal Convolution

A. NORMAL MICE: 1. *Acid Phosphatase.* The product of the AcPase reaction (lead phosphate) appeared sharply localized in a small number of membrane-bounded cytoplasmic bodies concentrated in the apical half of the cell, between the base of the brush border and the nucleus (Fig. 1). The morphology of these bodies has been described in detail in a previous paper (12). Those referred to as *vacuolated bodies* have an excentric vacuole partially surrounded by a characteristic crescent-shaped rim of dense material (Fig. 2). Those designated as *dense bodies* have a homogeneous or finely granular content of medium density (Fig. 3), only occasionally marked by a few ferritin molecules.[4] Finally, those described as *lamellated bodies* contain successive, frequently concentric layers of light and dense material with a repeat of ~90 A (Fig. 4). In all these bodies, the reaction product was more concentrated along the limiting membrane than within the dense content. The vacuoles of the vacuolated bodies were generally free of lead phosphate deposits.

The structures involved in the initial steps of protein reabsorption (*cf.* 12), *i.e.*, base of the brush border, tubular invaginations, and associated clear vacuoles, showed no detectable enzyme activity. Only occasionally a slight precipitate formed on the membrane of the microvilli (Figs. 1, 15). The Golgi zone and the basal region of the cells rarely contained AcPase-positive bodies. The Golgi complex proper was found positive only in two cells of one section out of a few hundred examined. In that case, a fine dense precipitate marked the limiting membranes as well as the cavities of the piled cisternae of the complex (Fig. 5). The microbodies (24) appeared swollen (up to 0.8 μ in diameter) and consistently free of detectable AcPase activity (Figs. 5, 14). The nuclei showed a fine precipitate completely removable by an acid rinse and hence considered an artifact.

In normal animals, the number of AcPase-positive sites was limited: it varied from none to a few (average, 0.85) per cell profile, although the cells seem to be active in endocytosis as suggested by their numerous tubular apical invaginations and their relatively frequent (average, 2.2 per cell profile) clear vacuoles.

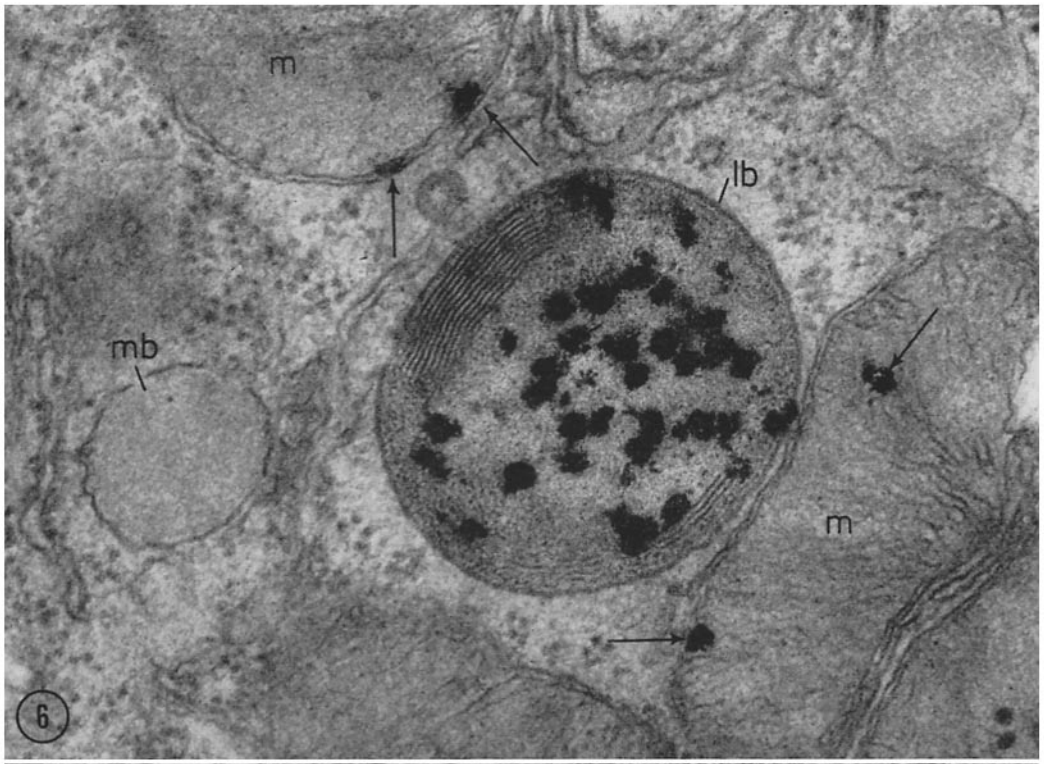
2. *Esterase.* The product of the esterase reaction (lead sulfide) appeared in large aggregates mainly within *vacuolated, dense,* and *lamellated bodies.* It was, however, less sharply localized than the product of the AcPase reaction: there was always a spotty deposition of lead sulfide over the mitochondria (Fig. 6) and the cytoplasmic matrix without a definite association with the endoplasmic reticulum. Preincubation of the tissue in 10 μM E600 suppressed the reaction in the cytoplasmic matrix, in agreement with Wachstein's findings (25); it persisted in the particles, but the number of reacting sites was usually reduced (Figs. 7, 21). Preincubation in 1 mM E600 almost completely abolished the reaction in the particles.

B. HEMOGLOBINURIC MICE: 1. *Acid Phosphatase.* (a) *Variations in the number of active sites:* A systematical survey showed that the number and variety of AcPase-positive sites increases with time after hemoglobin injection. The sites were classified as lytic vacuoles (digestive vacuoles, residual bodies) when they either had the appearances mentioned in the controls or were fully developed AcPase-positive protein absorption droplets (see

[4] Some of the profiles ascribed to these bodies may actually represent sections through the dense crescent of vacuolated bodies.

FIGURE 6 Small field in the apical zone of a tubule cell; proximal convolution, control mouse. The reaction product (lead sulfide) of a thiolacetate-esterase test appears in a residual body with lamellated and granular content and in two adjacent mitochondria (arrows). × 66,000.

FIGURE 7 Thiolacetate-esterase reaction after preincubation of a similar specimen in 10 μM E600. The reaction product is restricted to the content of a residual body with lamellated and granular content. Dilation of the intercellular spaces (*is*) and local membrane discontinuities are freezing artifacts. × 61,000.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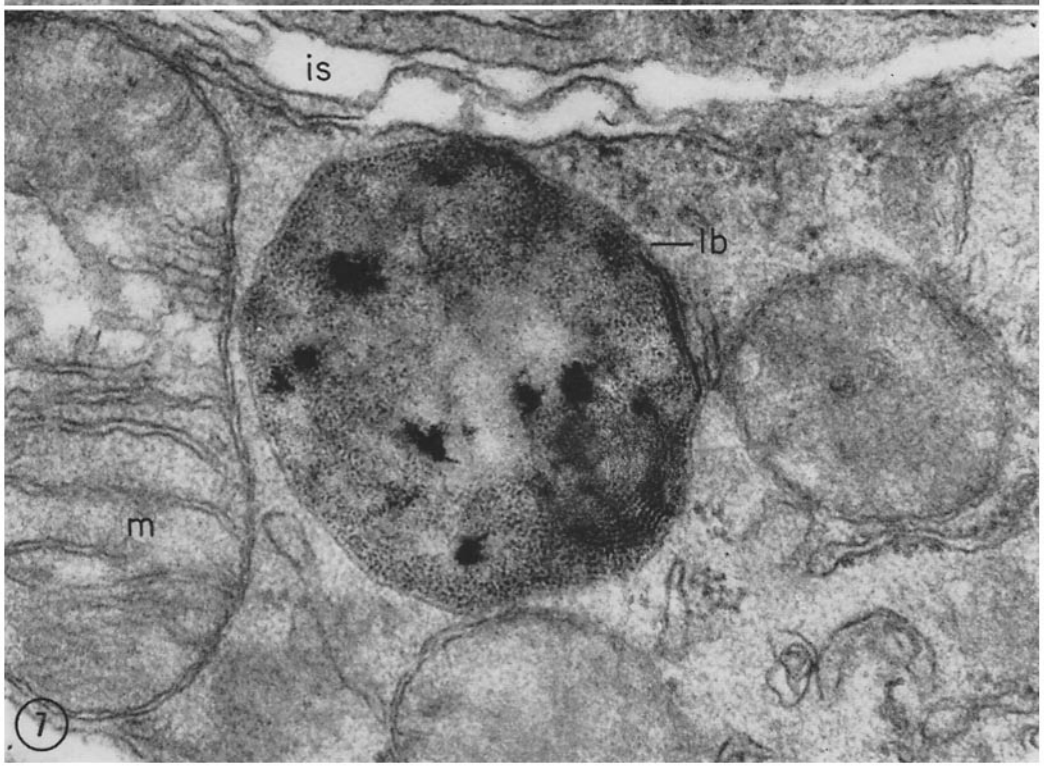
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TABLE II

Average Number of Acid Phosphatase-Positive, Lytic, or Autolytic Vacuoles per Cell Section in Hemoglobinuric Mice

For each time point 50 to 60 cell profiles of the proximal convolution were counted.

Type of lysosome	Hours after hemoglobin injection						
	0	1	2	4	10–18	24	48
Lytic vacuoles*	0.85	2.1	2.1	2.6	2.5	2.7	3.1
Autolytic vacuoles	0	0.2	0.8	0.9	0.9	0.5	0.2

* Including residual bodies (Type II and Type IV of de Duve's classification (2)).

below); and as autolytic vacuoles (cytolysomes (26), autophagic vacuoles (2)) when altered but recognizable cytoplasmic components, most frequently mitochondria, were contained within their limiting membrane. Autolytic vacuoles were absent or only occasionally encountered in normal mice.

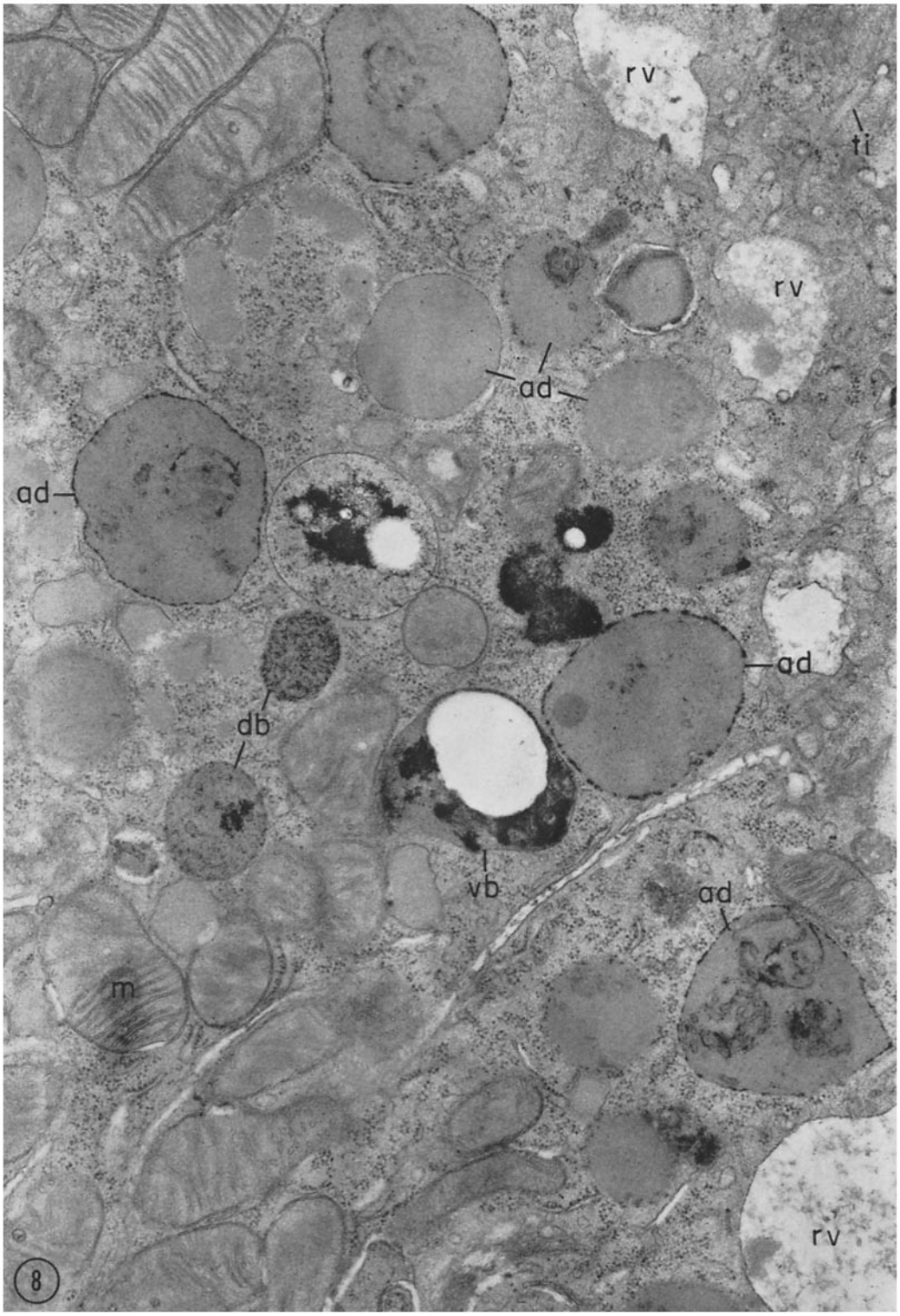
AcPase-positive sites of each type were counted on low magnification micrographs in 50- to-60-cell profiles for each time point. The results (Table II) showed that the number of lytic vacuoles increased continuously over the period investigated, *i.e.* 48 hours, whereas the number of autolytic vacuoles was generally smaller and began to decrease already after ~18 hours.

(b) *Lytic cycles:* The structural and cytochemical modulations which follow hemoglobin incorporation proceed at a relatively slow rate. Differences over short intervals are small and can be conveniently grouped, for the purpose of their description, into early, intermediate, and late events.

Early events (1 to 6 hours after hemoglobin injection).–In many proximal convolutions, a dense homogeneous mass, *i.e.*, hemoglobin in concentrated solution, fills the lumina, the tubular invaginations, and the associated small vesicles at the base of the brush border. The clear vacuoles of the apical region begin early to fill with more or less equal lumps (diameter ~0.1 μ) of similar density, initially disposed along the inner face of the vacuolar membrane. This appearance probably reflects the incorporation in quanta of the hemoglobin concentrated in the lumen (12) and its discharge, still in quanta, into the clear vacuoles (Fig. 8). Within about 1 hour, many vacuoles appear completely filled by a homogeneous mass whose density may exceed that of the hemoglobin in the lumen. In this situation, the vacuoles are usually referred to as protein-absorption droplets. It is in this stage usually that they begin to give an AcPase-positive test[5] (Fig. 8). All these events take place in the apical region of the cell, between the base of the brush border and the nucleus. After the first 2 hours, this region contains a mixed population of partially or entirely filled vacuoles intermingled with a few AcPase-positive residual bodies of the type described in controls. Images which suggest fusion of developing droplets were frequently seen; but images indicating mergence of droplets with residual bodies were not encountered. Occasionally, however, the recognizable content of a vacuolated body was found surrounded by a rim of material similar in density and texture to the incorporated hemoglobin (Fig. 9). Such images suggest that hemoglobin is segregated simultaneously in old residual bodies in

[5] Small deposits of lead phosphate were only occasionally noted in clear vacuoles partially filled with hemoglobin.

FIGURE 8 Apical and intermediate zones of two adjacent tubule cells; proximal convolution, 2 hours after hemoglobin administration. The cells contain a large mixed population of resorbing vacuoles (*rv*), absorption droplets (*ad*) and residual bodies (*db, vb*). Some of the absorption droplets give a clear AcPase-positive reaction, others are negative. Most residual bodies are AcPase-positive. Note that the deposition of reaction product is generally limited to the periphery of the absorption droplets, and that some of the latter contain membranous (mitochondrial?) residues. × 24,000.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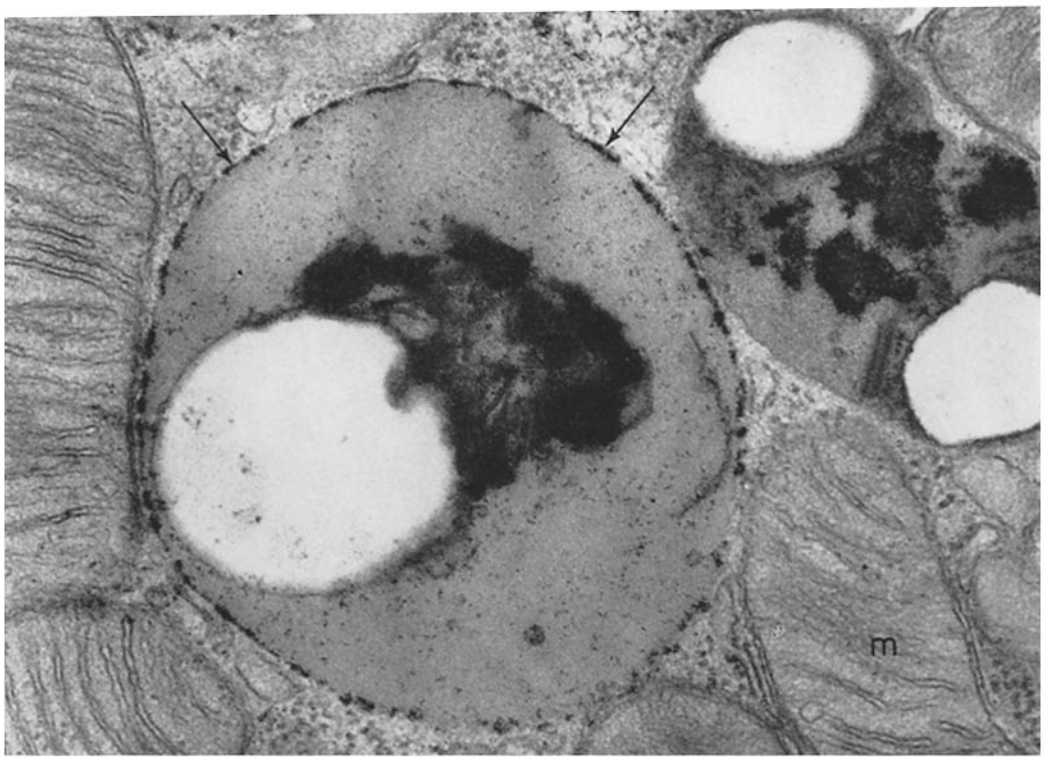
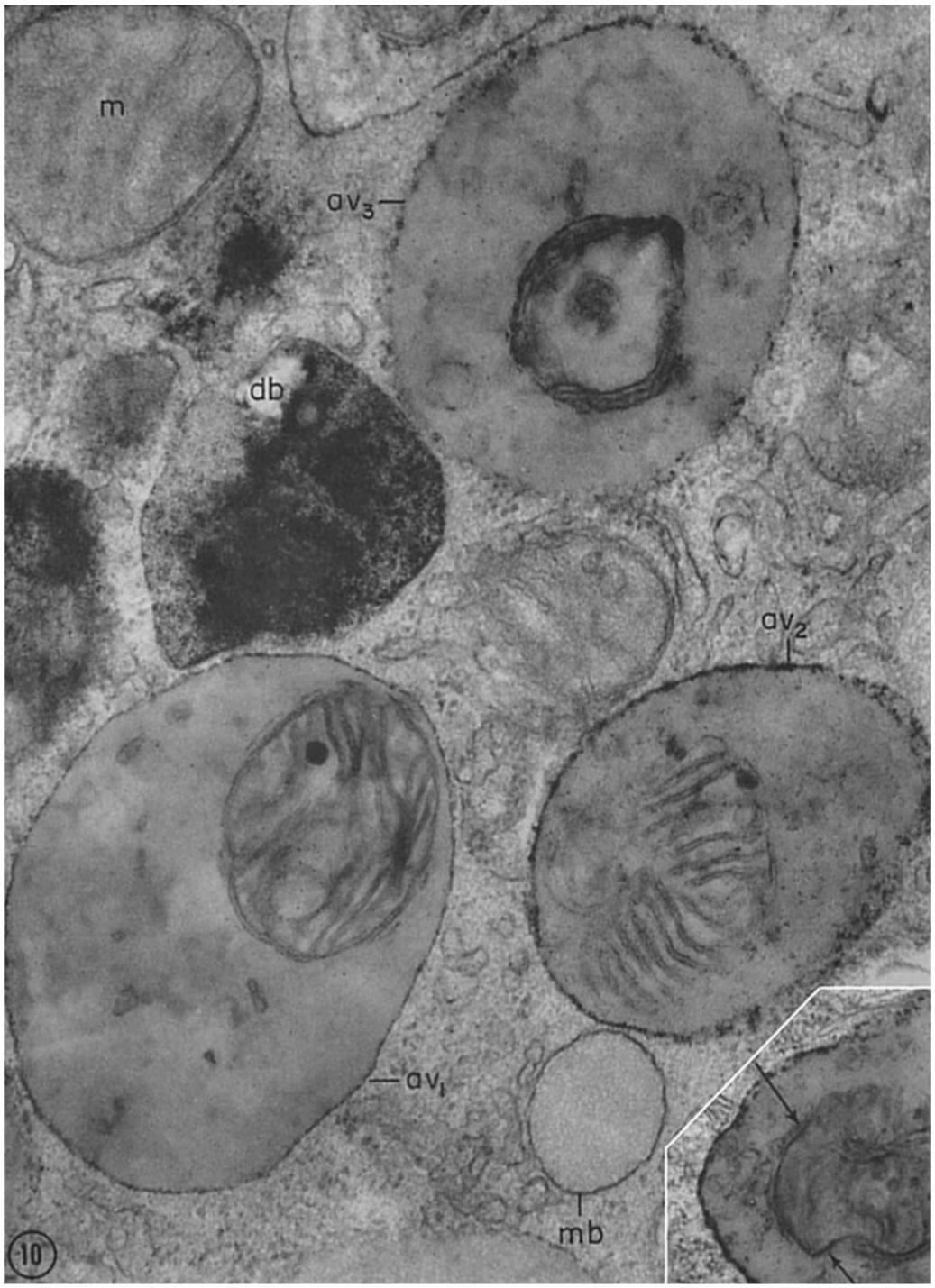


FIGURE 9 Small field in the apical region of a tubule cell; proximal convolution, 2 hours after hemoglobin administration. The dense content and the characteristic vacuoles of two vacuolated bodies appear partially or completely immersed in a matrix that has the same density and texture as the hemoglobin seen in absorption droplets. The membrane of the larger droplet shows discontinuous deposition of AcPase reaction product (arrows). It is assumed that hemoglobin, transported by small vesicles, has been injected between the membrane and the original content of the dense bodies. × 52,000.

addition to newly formed absorption droplets (see note added in proof, page 550). So far, no morphological evidence was obtained about the mechanism involved in this process. Over this first period, there is a progressive increase in number and size of absorption droplets; by 5 or 6 hours they reach diameters of ~1.5 μ and begin to appear in other cell regions, presumably as a result of intracellular migration.

Autolytic vacuoles begin to form during the first hours of hemoglobin absorption. They are similar in size, shape, density of content, and intracellular location to developing or fully formed absorption droplets, except that they also contain

FIGURE 10 Small field in the apical zone of a tubule cell; proximal convolution, 2 hours after hemoglobin administration. The figure shows three autolytic vacuoles containing either a clearly recognizable mitochondrial profile (av_1) or residues of cristae (av_2) or a series of concentric lamellae (av_3) presumably of mitochondrial origin. Note that in all cases the mitochondrial remnants are embedded in a homogeneous matrix, presumably hemoglobin, containing a few vesicular structures, and that all three autolytic vacuoles show a peripheral AcPase-positive reaction. A dense residual body appears at *db*. In the inset, a similar autolytic vacuole contains a decaying mitochondrion whose membranes show local thickenings and densification (arrows). × 57,000; inset, × 37,000.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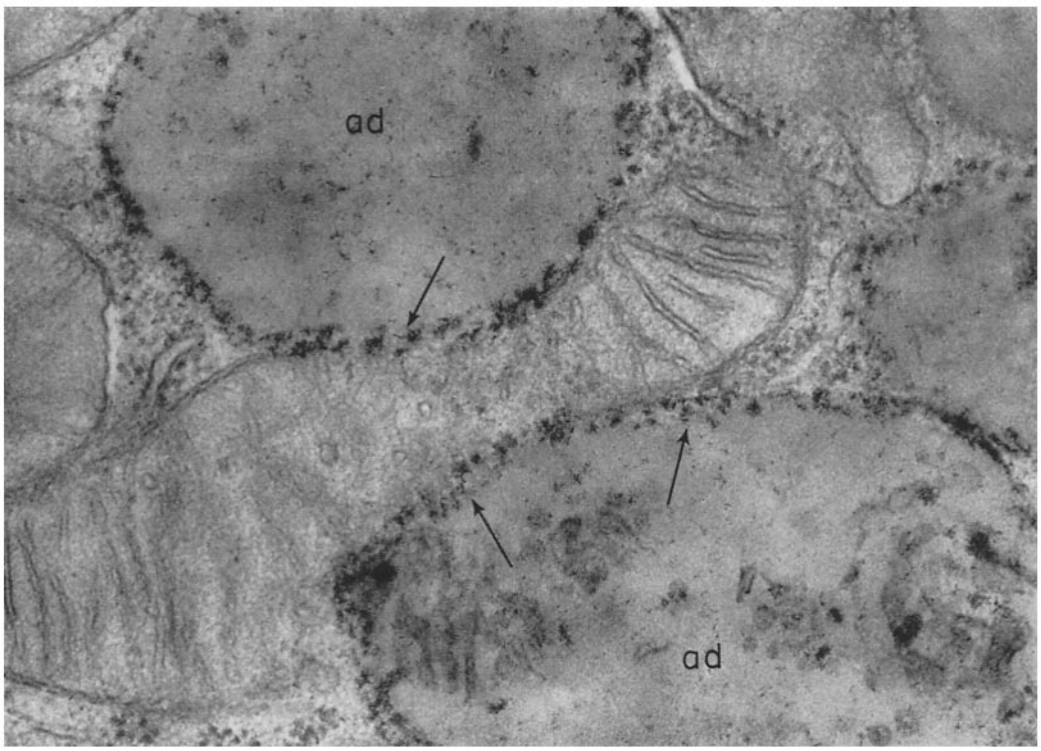


FIGURE 11 Small field in the apical region of a tubule cell; proximal convolution, 2 hours after hemoglobin administration. A mitochondrion appears trapped in between two AcPase-positive absorption droplets (*ad*). The lower one contains a number of membranous residues, presumably mitochondrial remnants. The reaction product consists of aggregates of fine needles (arrows) and is disposed in discontinuous patches at the surface of the droplets. × 56,000.

one to three mitochondrial profiles in various states of preservation and occasionally a few free ribosomes and elements of the endoplasmic reticulum. Some mitochondrial profiles are unaltered, others lack the outer membrane, and still others show thicker and denser membranes disposed in patterns which range from disturbed yet still recognizable mitochondrial arrangements to simple layered structures (Fig. 10). Autolytic vacuoles regularly give a positive AcPase reaction which resembles in every detail that of the absorption droplets. The evidence obtained indicates that most autolytic vacuoles have a heterogeneous content which consists of endogenous and exogenous (hemoglobin) components, but does not show how the former are acquired. Occasionally, mitochondria of normal appearance are found squeezed in between two AcPase-positive absorption droplets (Fig. 11). Images suggesting the incorporation of already altered mitochondria into lytic vacuoles were not encountered.

Intermediate events (10 to 21 hours after hemoglobin injection).—The cells of the proximal convolution appear loaded with large, dense, AcPase-positive absorption droplets (Fig. 12) which are no longer restricted to the apical region, but fill the entire cell. Some apical regions still contain partially filled, AcPase-negative vacuoles, an indication that hemoglobin reabsorption is a slow continuous process in which the participation of individual nephrons or proximal convolution segments is not synchronous.

At 18 and 21 hours after hemoglobin injection, the density of the droplet contents begins to decrease and many acquire a mottled appearance. In addition, ferritin molecules appear randomly scattered both within the content of the droplets and within the surrounding cytoplasmic matrix.

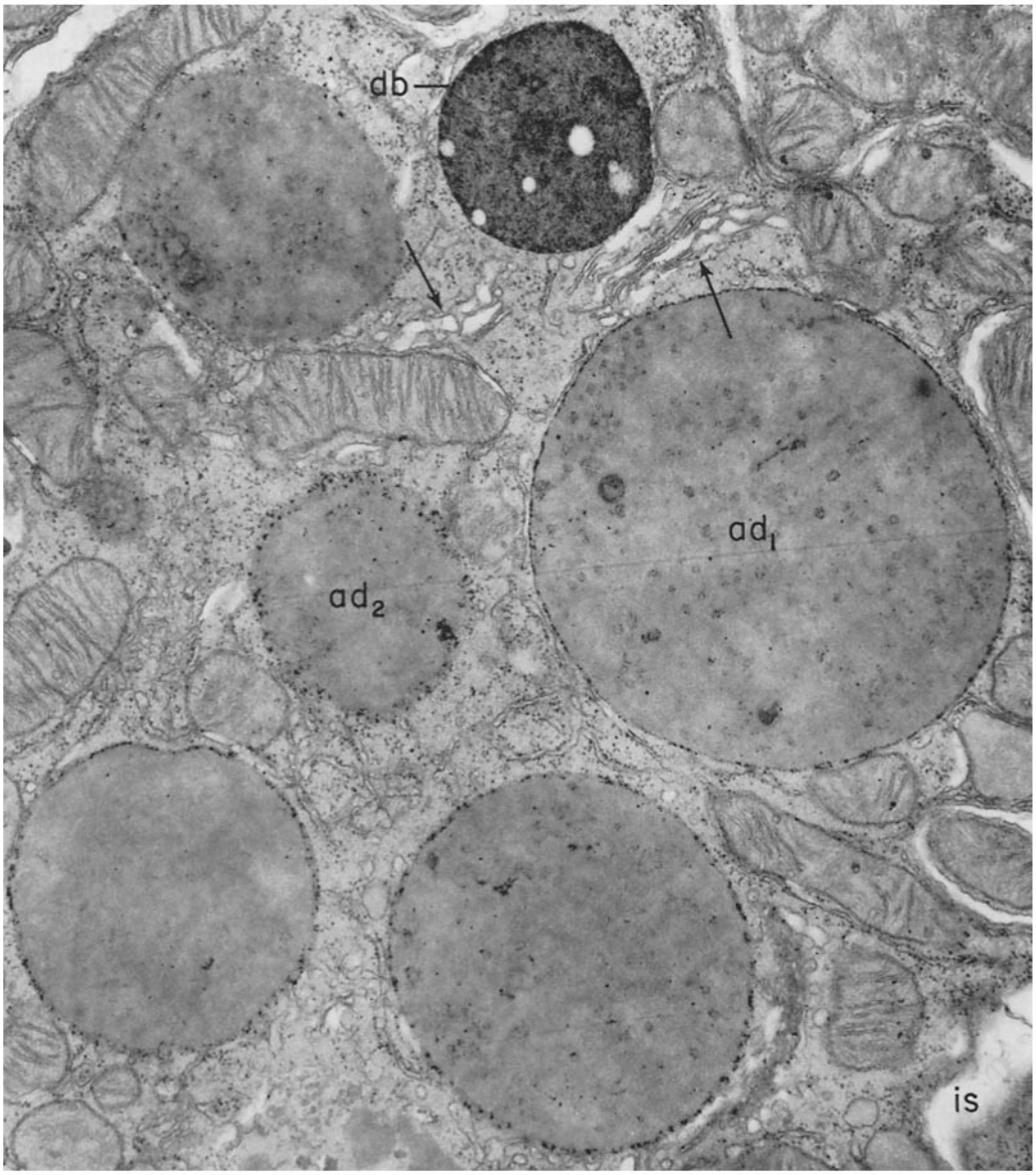


FIGURE 12 Large field in the apical and intermediate zone of a tubule cell; proximal convolution, 18 hours after hemoglobin administration. The micrograph shows five large absorption droplets of which four have distinct deposits of AcPase reaction product at their periphery. The deposits are discontinuous and appear in a fine line in the normally sectioned droplet ad_1, and as a relatively broad band in the obliquely sectioned droplet ad_2. The other droplets are sectioned in an intermediate position. An AcPase-positive residual body appears at *db*. Dilation of the intercellular spaces (*is*) and of the Golgi cisternae (arrows), and local membrane discontinuities are freezing artifacts. × 16,000.

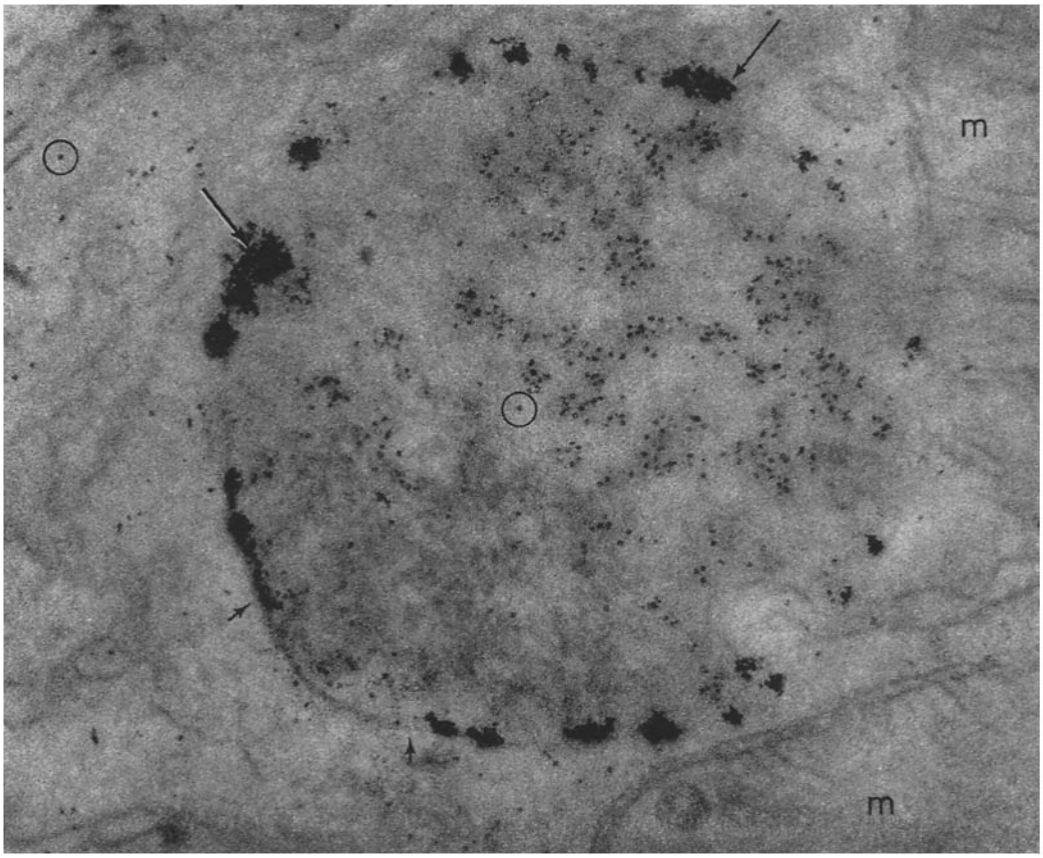
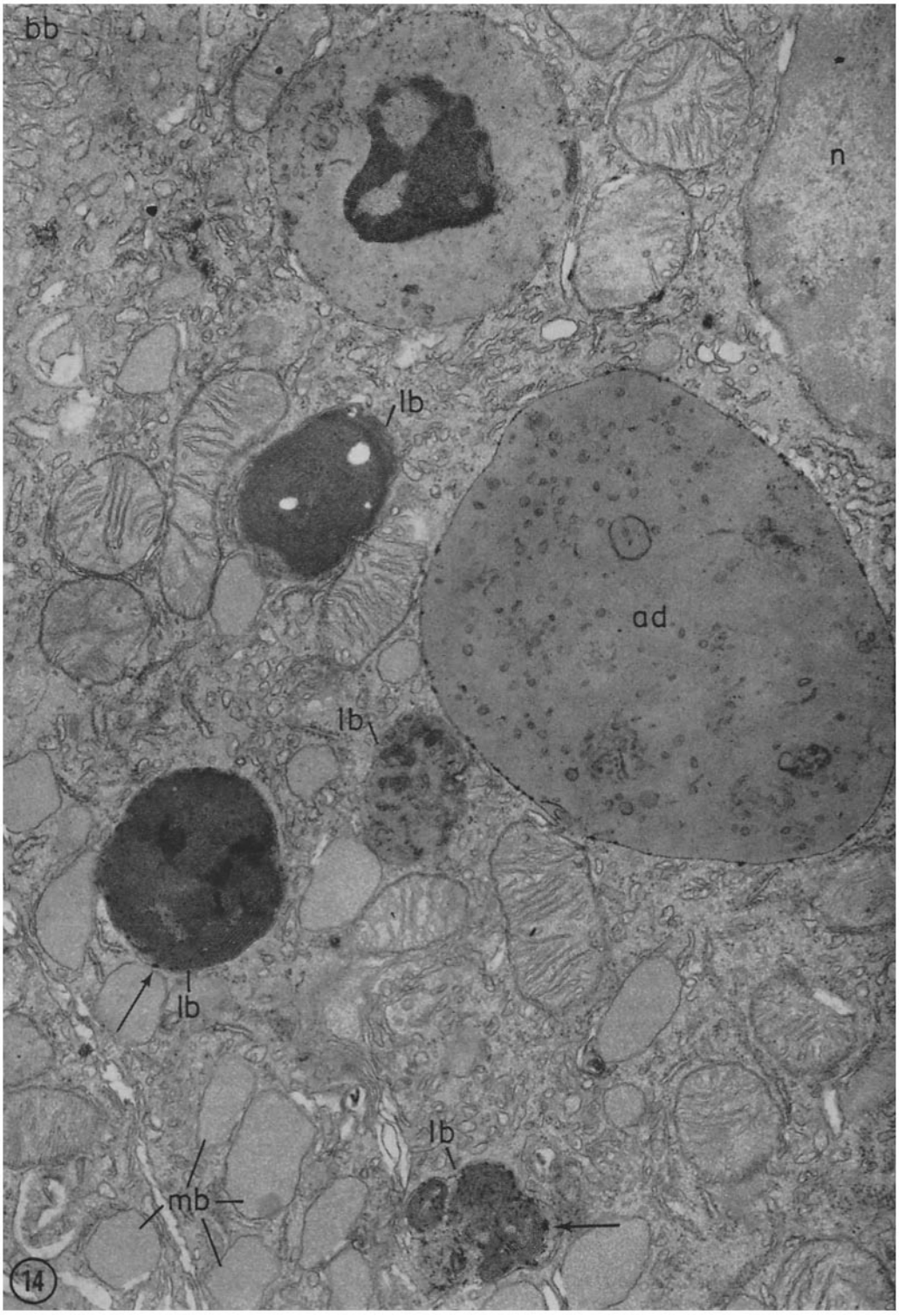


FIGURE 13 Small field in the intermediate region of a tubule cell; proximal convolution, 21 hours after hemoglobin administration. AcPase-reacted, but not $Pb(OH)_2$-stained specimen. The content of the droplet consists of heterogeneous (mottled) material of relatively low density and of ferritin molecules (circle). The limiting membrane of the droplet is visible only between the two short arrows where it is normally sectioned. Heavy discontinuous deposits of AcPase reaction product (long arrows) mark the periphery of the droplet. Ferritin molecules (circle) are also present at lower concentration in the cytoplasmic matrix. × 125,000.

FIGURE 14 Large field in the apical and intermediate zone of a tubule cell; proximal convolution, 24 hours after hemoglobin administration. The field contains only a large absorption droplet (*ad*) filled with hemoglobin and vesicular material and showing discontinuous deposits of AcPase reaction product at its periphery. Four lamellated residual bodies (*lb*) are also present and two of them are marked by AcPase reaction product (arrows). The large body at the top is an absorption droplet with a core of lamellated dense material: possibly the result of a process similar to that described for Fig. 9. Note that the numerous microbodies (*mb*) present in this field are AcPase-negative. × 23,000.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Ferritin-containing droplets are generally AcPase-positive (Fig. 13).

Late events (24 to 48 hours).–At 24 hours the absorption droplets are found decreased in number and content density, while the frequency of residual bodies increases (Fig. 14). At 48 hours, the cells almost regain their normal appearance with the exception of an increased population of small AcPase-positive bodies identical in morphology to the vacuolated, dense, and lamellated bodies of the controls (Fig. 15). The dense and vacuolated bodies invariably contain ferritin sometimes aggregated in relatively large masses (Fig. 15) as previously described (12). In a few residual bodies, mitochondrial remnants are still recognizable. Some lamellated bodies occur in the immediate vicinity of the brush border, and myelin figures with the same periodicity as that of these bodies are occasionally found within tubular invaginations, among the microvilli of the brush border (Fig. 16), or in the nephron lumen.

(c) *General findings:* Some of our findings apply equally well to all time points investigated and as such will be described under this heading.

Enzyme reaction in lytic and autolytic vacuoles.–After an incubation of 15 minutes, the product of the AcPase reaction appears as a dense precipitate precisely localized in most cases along the inner face of the membrane limiting the lytic or autolytic vacuoles. The deposits form a discontinuous layer 100 to 180 A thick, which in grazing sections appears as a succession of patches (Figs. 11, 12), each composed of fine dense needles (~60 to 300 A). There is no deposition of lead phosphate in (by implication no diffusion of enzyme or reaction product to) adjacent structures such as ribosomes (Fig. 17) and membranes of mitochondria (Fig. 18), endoplasmic reticulum and Golgi complex, even when the distances between such structures and the membrane of the reacting vacuoles were not greater than 100 A (Fig. 18). It appears, therefore, that the resolution of the method permits the localization of the reaction product within the dimensions of cellular membranes.

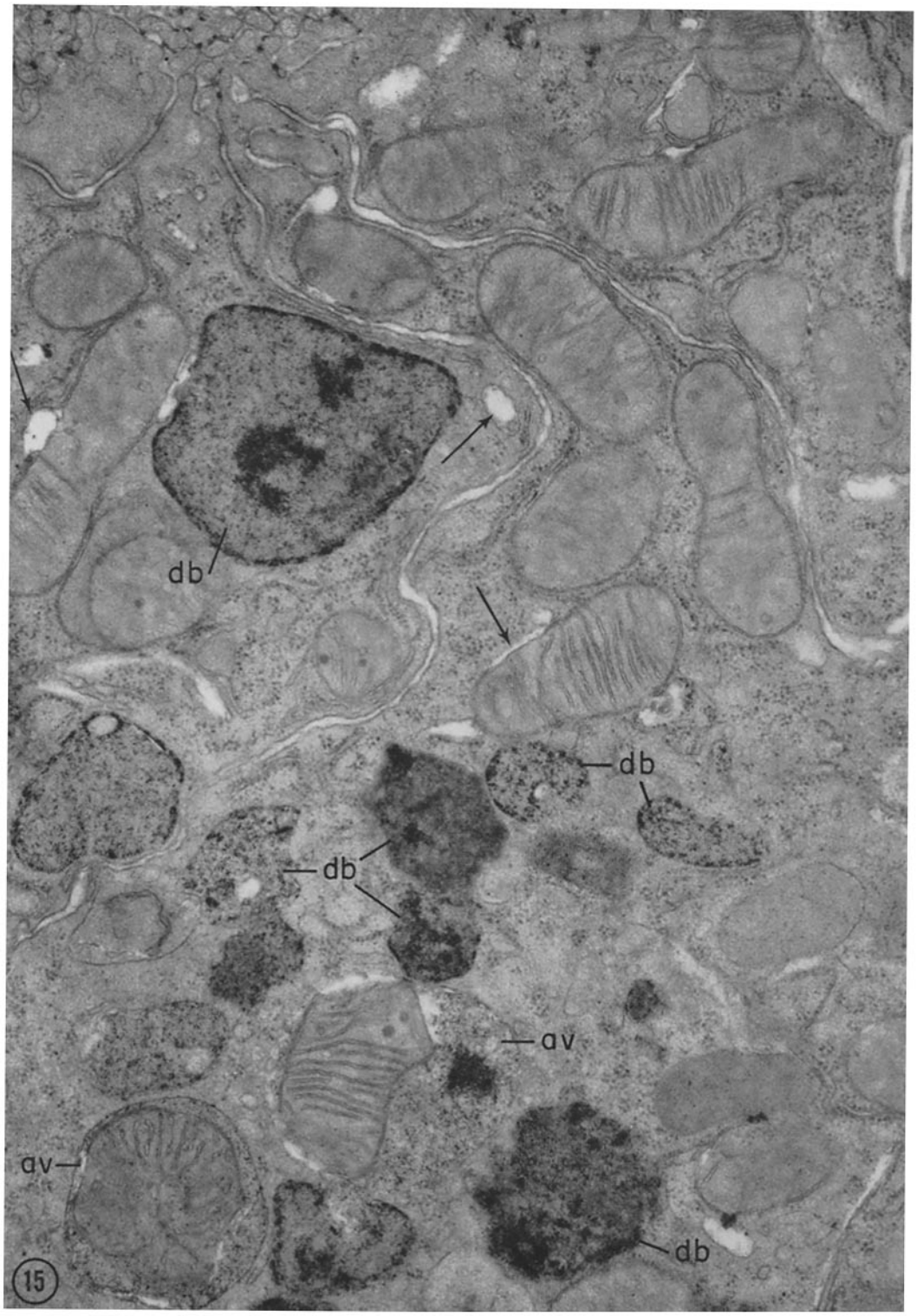
Less frequently, the reaction product is found in the interior of the vacuoles distributed either at random or in clusters. The inner deposits increase in amount when the incubation time is lengthened (30 to 40 minutes at 37°C). AcPase activity in lytic and autolytic vacuoles is completely inhibited by 0.01 M NaF.

Enzyme reaction in other apical structures.–A spotty fine deposition of lead phosphate is occasionally found on the membranes of the microvilli of the brush border (Figs. 1, 15) and on the membranes of some tubular invaginations (Fig. 1). In cross-sections, each microvillous profile is marked by one or two small deposits. The reaction is not more intense than in controls; it does not occur in the absence of exogenous substrate; it is not completely inhibited by 0.01 M NaF; and it is, in general, erratic in the sense that it varies from case to case and may be altogether absent. It may represent the "acid tail" of the characteristic alkaline phosphatase reaction of the brush border (27, 28).

Enzyme reaction in the Golgi complex.–A positive reaction in the Golgi complex was encountered in 7 out of 170 cell sections in which the complex was included. The reaction product marked both the membranes and the content of piled cisternae and adjacent small, smooth-surfaced vesicles. In each case, the reaction was less intense than in absorption droplets. No preferential association was detected between the Golgi complex and the lytic or autolytic vacuoles when the latter were formed, acquired AcPase activity, or were found in close proximity to the Golgi complex during their assumed migration towards the basal regions of the cell.

2. *Esterase Activity.* This activity was investigated only at two time points, namely 2 hours and 12 hours after hemoglobin injection. The reaction

FIGURE 15 Large field in the apical and intermediate zones of a group of tubule cells; proximal convolution, 48 hours after hemoglobin administration. Protein absorption droplets are absent, while the population of residual bodies is greatly increased. Most of them are dense bodies and show an AcPase-positive reaction of varied intensity. Two mitochondrion-containing autolytic vacuoles are marked *av*. Note the fine deposits of reaction product on the cross-sectioned microvilli in the upper left corner. Dilation of the intercellular spaces and empty vesicles (arrows) adjacent to some mitochondria or scattered throughout the cytoplasm are freezing artifacts. × 31,000.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product (lead sulfide) appeared in the form of coarse, dense deposits and was found more diffusely distributed than that of the AcPase reaction. In addition to hemoglobin absorption droplets (Fig. 19) it frequently marked mitochondria and the cytoplasmic matrix. Preincubation in 10 μM E600 inhibited almost completely the reaction at sites other than the absorption droplets.

Renal Glomerulus

A. NORMAL RATS: In specimens examined at 2 and 48 hours after ferritin injection, the distribution of the tracer and the morphology of the mesangial cells were as previously reported (13). Dense, membrane-bounded bodies containing ferritin were found in relatively small numbers in mesangial cells 48 hours after the injection. Some of these bodies gave a positive reaction for AcPase; others were negative. Ferritin-marked bodies also gave a positive reaction for esterase.

Glomerular epithelial cells contained a few dense bodies unmarked by ferritin. Some of them were AcPase-positive. The Golgi complex of a few glomerular epithelial cells was also positive. In some instances this reaction occurred in the absence of ferritin uptake or AcPase-positive dense bodies.

B. NEPHROTIC RATS: At 30 minutes and 2 hours after injection, ferritin molecules were found in large numbers in the spongy areas of the basement membrane and in mesangial cells. At 48 hours, the spongy areas were free of most of the tracer, whereas the mesangial cells contained a large number of membrane-bounded vacuoles and dense bodies filled with ferritin embedded in a dense matrix and concentrated to a varied but generally high degree (*cf.* 13). In those bodies in which the ferritin was less packed, a clear rim 180 to 190 A wide separated the ferritin mass from the limiting membrane (Fig. 20). The rim was less dense than the matrix in which the ferritin was embedded.[6] Some but not all the ferritin-containing bodies of the mesangial cells gave an AcPase-positive reaction which did not vary in intensity over the period examined. The reaction product was preferentially located on the inner side of the limiting membrane of the dense bodies. Fewer deposits occurred in their rim and within their ferritin-marked content (Fig. 20, inset). The same type of bodies gave more regularly a positive esterase reaction (Fig. 21).

The cells of the glomerular epithelium contained numerous dense bodies (*cf.* 14). Some of these bodies, marked by ferritin in small or moderate amounts, were AcPase- and esterase-positive (Fig. 22). In addition, the Golgi complex in a few epithelial cells showed a discrete, and occasionally pronounced AcPase-positive reaction in which the lead phosphate deposits were restricted to the cavities of the piled cisternae and associated smooth-surfaced vesicles (Fig. 23). In such cases, no AcPase-positive lytic vacuoles were found in the vicinity of the complex.

Only occasionally ferritin-containing, AcPase- and esterase-positive (Fig. 24), dense bodies were encountered in endothelial cells.

AcPase reactions were inhibited in all glomerular cells by 0.01 M NaF. Esterase reactions in the same cells were not affected by 10 μM E600.

In general, the frequency of reactive sites and intensity of reactions were considerably lower in the cellular elements of the glomerulus than in the cells of adjacent proximal convolutions in rats, or in the cells of the same nephron segments in hemoglobinuric mice.

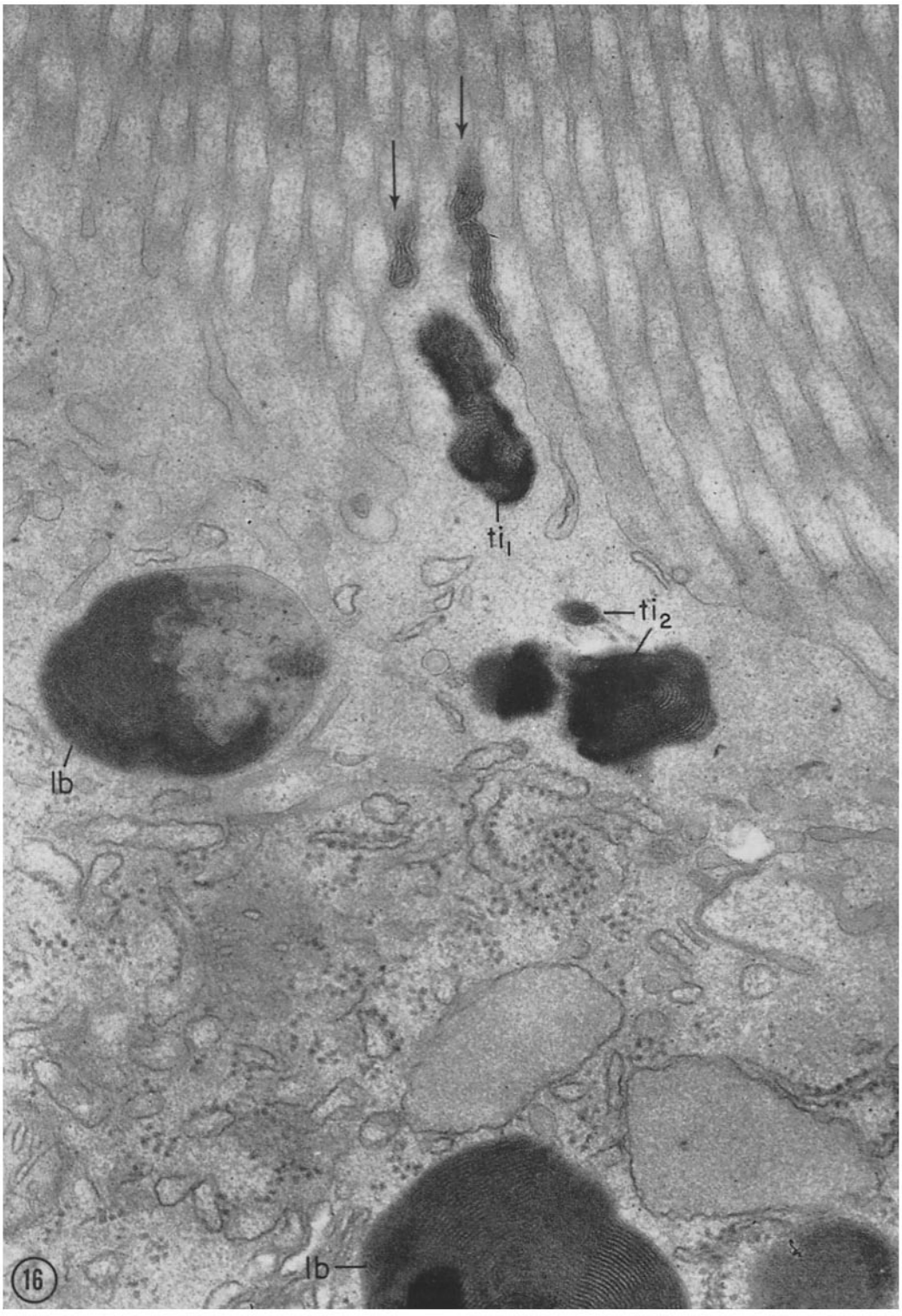
[6] Numerous ferritin molecules appeared free in the cytoplasmic matrix of mesangial cells already at 30 minutes after injection.

DISCUSSION

I. *Identification of Absorption Droplets as Lysosomes*

The salient result of our experiments is the demonstration that lysosomal enzymic activities and recognizable, recently incorporated exogenous proteins occur together in membrane-bounded structures described in the past as absorption droplets or dense bodies in certain cells of the nephron.

FIGURE 16 Apical region of a tubule cell; proximal convolution, 24 hours after hemoglobin administration. Two lamellated residual bodies appear at *lb*; myelin figures fill tubular invaginations at ti_1, and possibly at ti_2; smaller myelin figures (arrows) appear in the clefts between microvilli of the brush border. Specimen not reacted for AcPase or esterase. × 50,000.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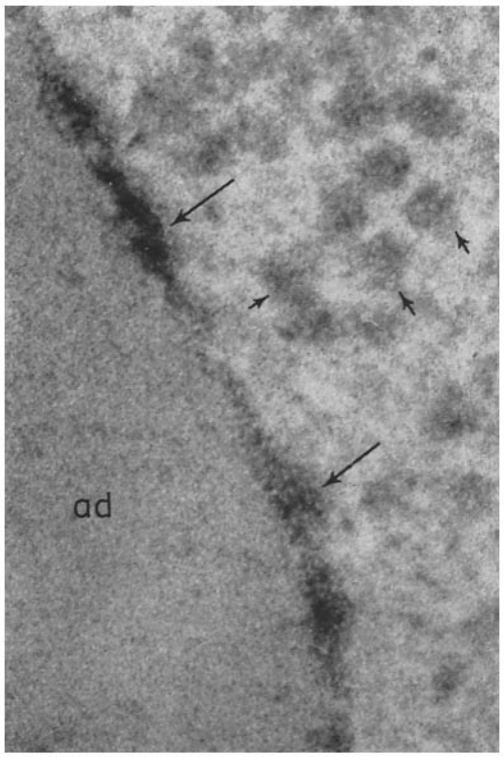


FIGURE 17 Periphery of an absorption droplet (*ad*), 2 hours after hemoglobin administration. The deposits of AcPase reaction product (long arrows) are clearly restricted to the surface of the droplet, and do not mark other structures (ribosomes, short arrows) a few hundred A away. × 290,000.

The demonstration can be considered satisfactory proof of the hypothesis that links the lysosomes to the digestion of proteins and other substances that cells incorporate in bulk from their surrounding medium.

The general conditions of the experiments deserve, however, some comments, since the validity of the demonstration is largely dependent thereon. The work concerns two proteins, *e.g.*, hemoglobin and ferritin; two enzymic activities; and a number of different cell types, namely epithelial cells of the proximal convolution in the case of hemoglobin, and glomerular mesangial, epithelial, and endothelial cells in the case of ferritin. Hence it can be tentatively assumed that the correlation established is of broad rather than limited significance. The work was carried out at a level of resolution better than 20 A on reasonably well preserved material; as such, the identification of the cellular structures involved is entirely reliable, and the possibility of redistribution of exogenous protein during tissue preparation is unlikely. One of these proteins (ferritin) was incontrovertibly identified by the characteristic morphology of its molecules. The identification of the other (hemoglobin) relied on the dense amorphous appearance of its concentrated solutions.

The glutaraldehyde fixation used removed ~50 per cent of the activity of AcPase, the enzyme with which most of our cytochemical observations were concerned. Accordingly, we did not see a complete picture of the activity of this enzyme in our preparations. We assume, however, on the basis of preliminary experiments with cell fractions, that the inactivation is evenly spread among subcellular components and hence that the cytochemical distribution shown is qualitatively valid. A revision of this assumption, which evidently is not excluded, will not affect our main conclusion, *i.e.*, protein absorption droplets are lysosomes, but can substantially alter our interpretation of the early events involved in this process. The corresponding part of the discussion should be read with this limitation in mind.

One of the enzymes studied, AcPase, is generally considered a typical lysosomal enzyme (1, 2, 9) and a reliable lysosomal marker (8, 9), but is not directly involved in the hydrolysis of the proteins used in our experiments. The nature of the other enzyme tested, thiolacetate esterase, is still under discussion. According to Wachstein *et al.* (25) and Hess and Pearse (29), the enzyme involved in this reaction is cathepsin C, especially if, as in our case, its activity is resistant to organophosphorus compounds (*cf.* 25). Yet this interpretation has been contested (30). Hence we cannot ascertain that the test reveals the functionally expected presence of cathepsin in the content of protein absorption droplets. Accordingly, in identifying these droplets as lysosomes we must rely in part on evidence obtained by others on cell fractions. Straus (31–33), for instance, found AcPase, β-glucuronidase, ribonuclease, deoxyribonuclease, and cathepsin in partially purified fractions of "small-," "intermediate-sized-," and "large droplets" isolated from normal rat kidney, and from the kidney of egg white–treated rats. He also followed changes in enzyme amounts and distribution patterns following egg white administration which apparently leads to the transformation of small droplets into

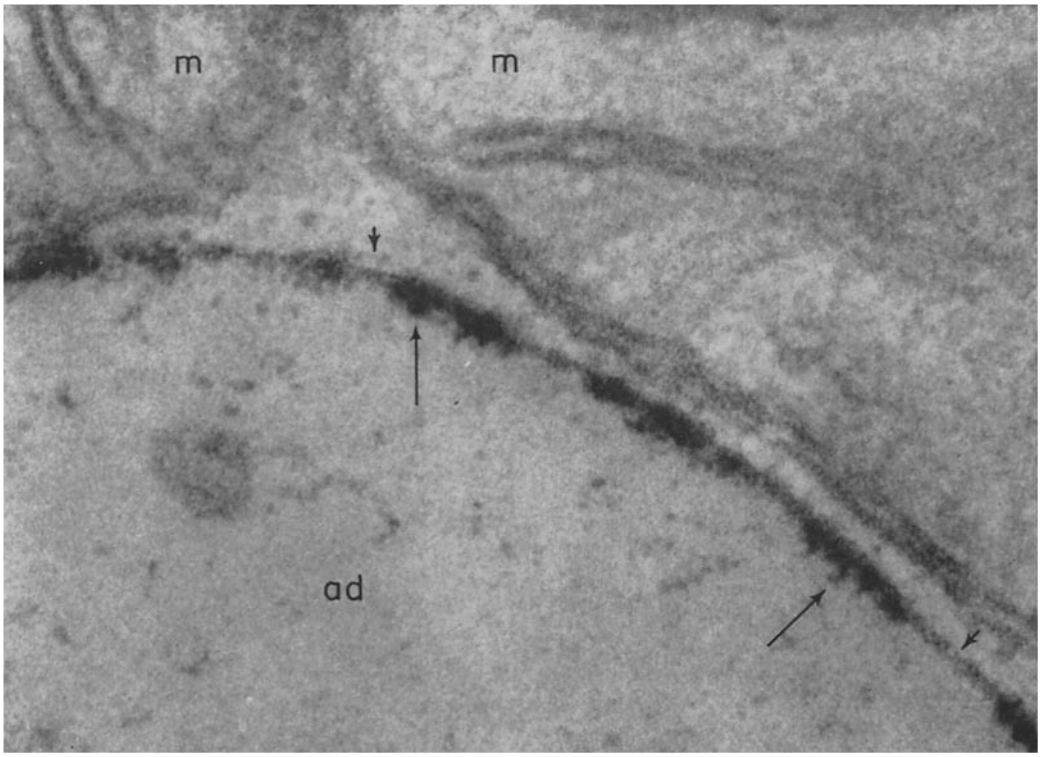


FIGURE 18 Periphery of a large absorption droplet (*ad*), 2 hours after hemoglobin administration. The limiting membrane of the droplet is clearly visible at short arrows. The product of the AcPase reaction forms discontinuous deposits on or immediately inside this membrane (long arrows). Note that the membranes of mitochondria lying at a distance of 100 to 200 A away are free of deposits. Note also that there is a suggestion of fine structure within mitochondrial membranes (see especially the crista in the upper left corner) in this glutaraldehyde-OsO_4 fixed material. × 230,000.

large ones and to an increase in enzyme activity in the final supernate (33). Although his studies reveal some differences in enzyme distribution among these hydrolases, Straus assumed, like de Duve (34), that the lysosomes represent an essentially homogeneous population in terms of enzymic content.

de Duve's evidence concerns hepatic lysosomes and is based mainly on the parallel release of all enzymes upon lysosomal membrane injury. More refined fractionation in continuous density gradients reveals some differences in the distribution patterns of individual enzymes, but shows at least a common pattern for AcPase and cathepsin (34). It can be concluded, therefore, that although the heterogeneity of the lysosomal fraction is not excluded, it is likely that some other enzymes, especially cathepsin, accompany the AcPase demonstrated *in situ* in our experiments.

Finally as far as accuracy of enzyme localization is concerned, it should be pointed out that, in the case of AcPase, there is little or no evidence of diffusion of the enzyme or its reaction product within the cell. The deposits of reaction product most probably exceed in size the actual enzyme(s) site, since they consist of fine crystals and measure up to 300 A; yet they remain confined within and to the membrane of the absorption droplets, leaving unmarked membranous structures at distances of less than 100 A.[7] The situation is quite different in the case of the thiolacetate esterase: the deposits

[7] If we assume that the enzyme is present in the content of the droplets or adsorbed to the inner surface of their limiting membranes, our results indicate that the trapping and precipitation of the reaction product occur within 100 A of the actual site of the enzyme.

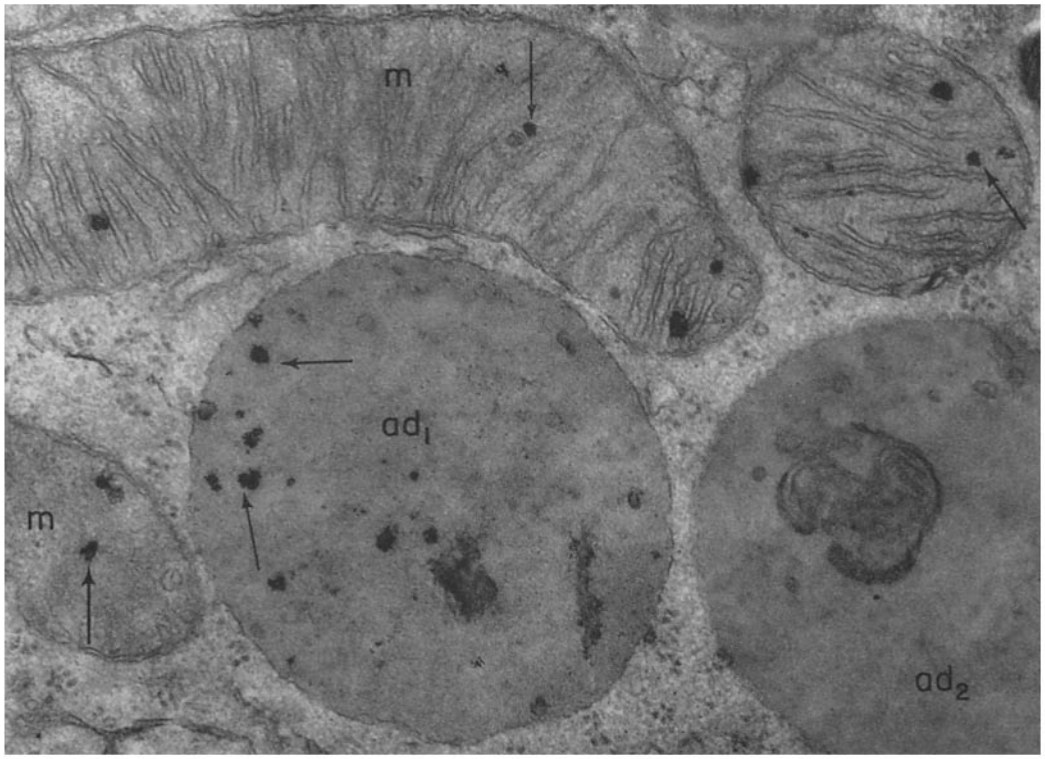
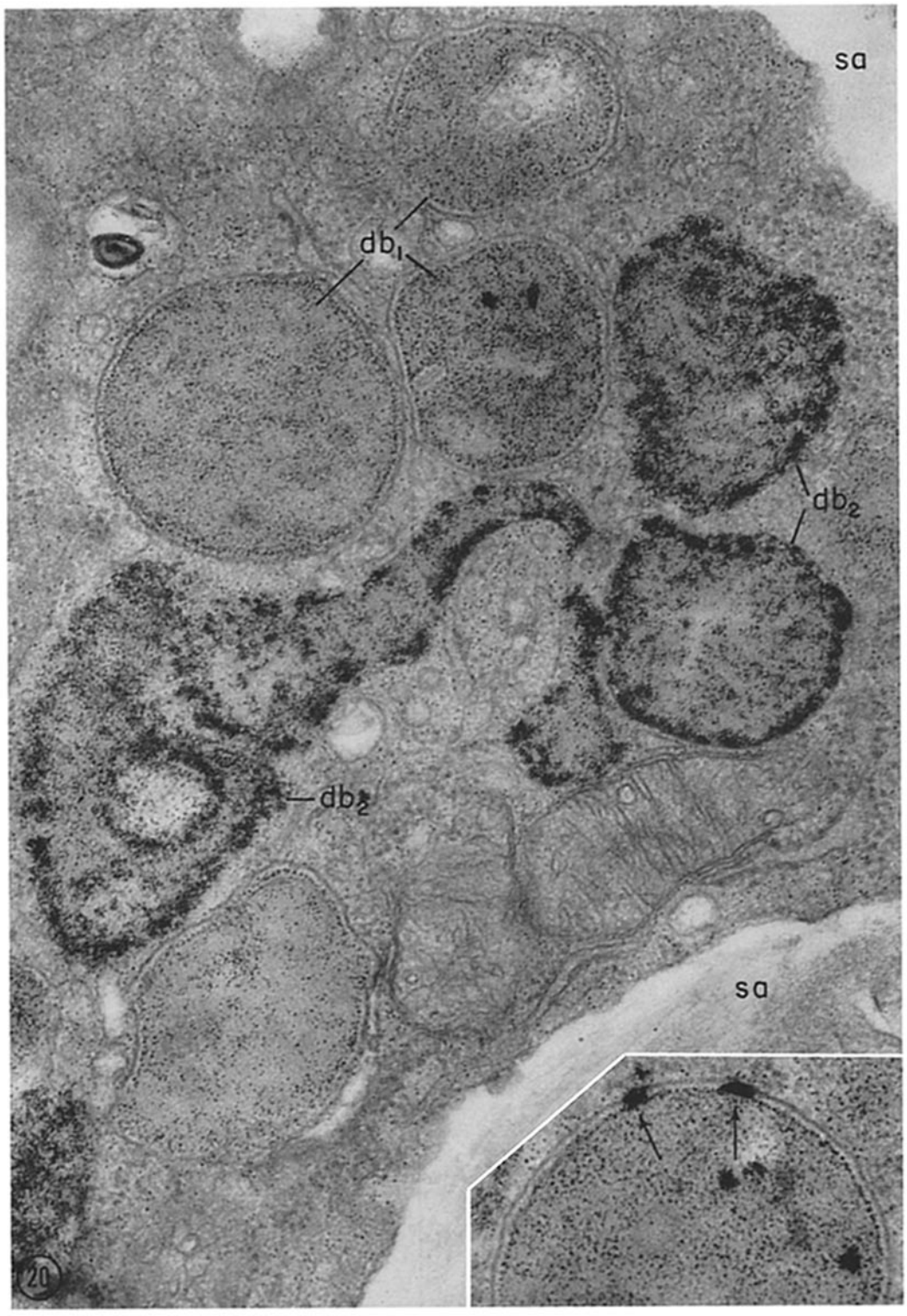


FIGURE 19 Small field in the apical zone of a tubule cell; proximal convolution, 2 hours after hemoglobin administration. Specimen reacted for thiolacetate-esterase without previous incubation in E600. Coarse deposits of reaction product (lead sulfide) mark the content of an absorption droplet (ad_1) and three adjacent mitochondria (arrows). One absorption droplet (ad_2) contains mitochondrial remnants but no reaction product. × 44,000.

are larger and, in addition, less clearly restricted in their distribution. In this case, however, the test may involve more than one enzyme, each of different distribution (*cf.* 30). We can conclude, therefore, that our experiments provide direct, conclusive evidence obtained *in situ* on individual subcellular components of the existence of lysosomes of "digestive vacuole" type. These results confirm and extend our preliminary reports (35, 36). So far the evidence in case has been either indirect, *i.e.* derived from the study of cell fractions (31–33, 37) or incomplete, *i.e.* based on the demonstration *in situ* of either an exogenous protein (38–40) or a lysosomal enzyme (8). Only recently, Straus (41, 42) has succeeded in demonstrating both an exogenous protein (horseradish peroxidase) and an en-

FIGURE 20 Glomerular mesangial cell of nephrotic rat, 30 minutes after ferritin administration. Numerous dense bodies filled with ferritin lie in the cytoplasm which also contains free ferritin molecules. A clear rim, 180 to 190 A wide, separates the bounding membrane of some of these bodies (db_1) from their ferritin-marked content. Heavy deposits of AcPase reaction product appear at the periphery and in the content of the larger irregular dense bodies (db_2). Those provided with a light rim show little reaction product, sometimes restricted to their periphery (inset, arrows). The light region marked *sa* corresponds to the spongy areas of the basement membrane (*cf.* 13). × 54,000; inset, × 68,000.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dogenous enzyme (AcPase) within apparently the same subcellular bodies, by light microscopical histochemical procedures, and similar findings have been reported by Novikoff (9). Our results retain the advantage of demonstrating this association at a higher resolution at which the identification of the cell structures and the localization of the exogenous proteins involved is more reliable. Results comparable to those obtained in our hemoglobin experiments were recently reported by Novikoff (9) after peroxidase injections. In this case, the exogenous protein was tentatively identified by an increase in the density of the amorphous material contained within AcPase-positive droplets. Segregation of exogenous material into digestive vacuoles has been inferred from changes in the density of the lysosomal content by Daems (43) after dextran and iron dextran, and by Wattiaux (44) after Triton WR-1339 administration. Daems succeeded in demonstrating AcPase activity in liver lysosomes presumed to contain dextran, and Wattiaux found by sucrose density centrifugation that the lysosomes changed not only in appearance but also in density after Triton administration.

II. *Lytic Cycle*

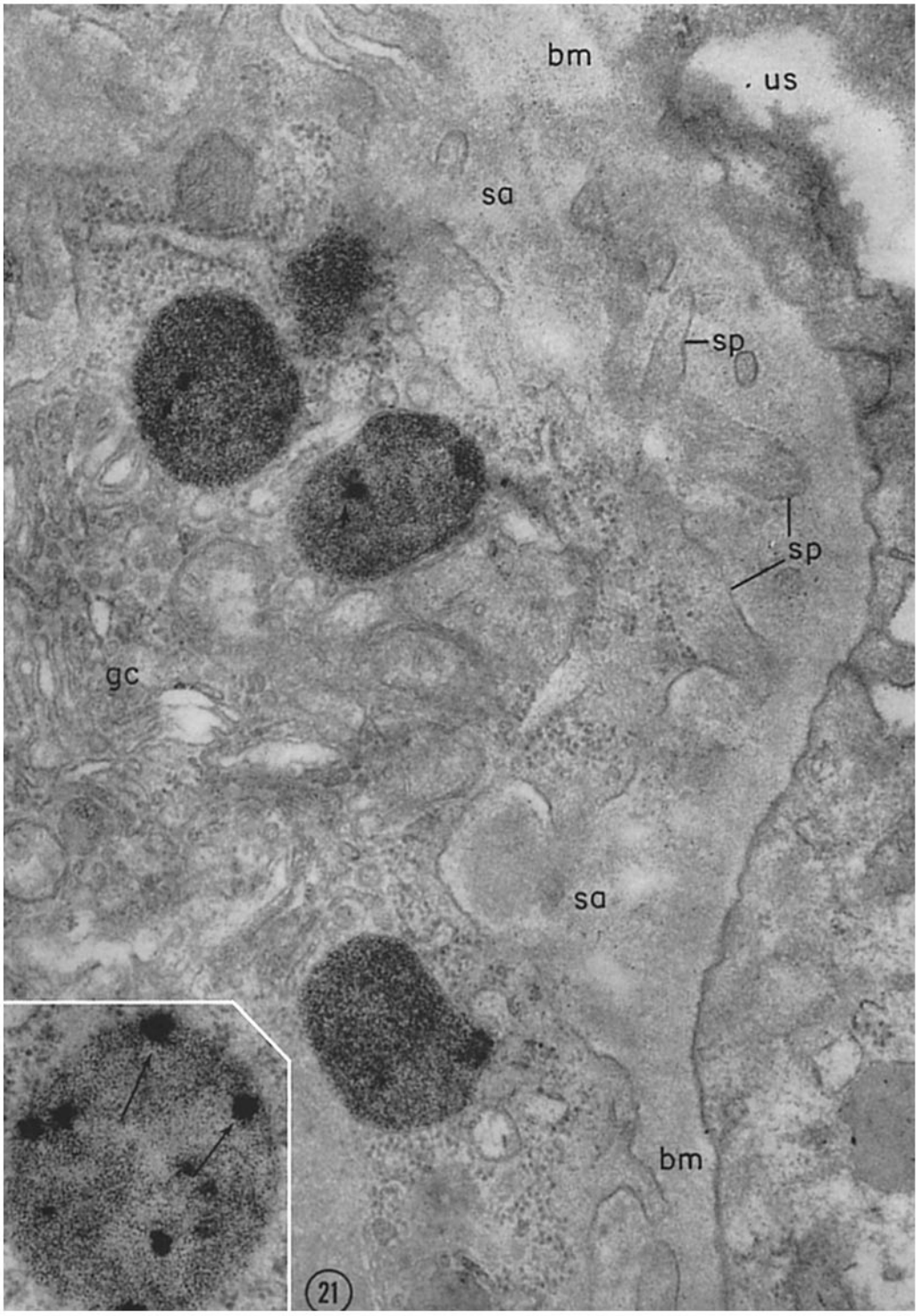
Our previous observations (12, 14, 45) and the present study indicate that the lytic cycle begins with the incorporation in bulk of protein and other substances from the external medium. The uptake is effected by membrane pockets of varied form, and is generally discontinuous, *i.e.* proceeds in quanta. It is followed by fusion of the small absorptive vesicles into or with large vacuoles, and by the subsequent progressive concentration of the initially dilute contents of the latter. In the epithelium of the proximal convolutions, all these operations take less than 1 hour and can proceed in the absence of any detectable AcPase in the absorptive vesicles and vacuoles. The situation is entirely similar to that described by Straus in his experiments with horseradish peroxidase, the various structures identified by us corresponding to his small and large "phagosomes" (46, 47).

At the beginning of the cycle and throughout this first period, AcPase activity is restricted, as in controls, to a few dense, vacuolated and lamellated bodies which, in the light of our results, should be considered as remnants (residual bodies) of previous cycles. AcPase activity begins to be detected in absorptive vacuoles when the concentration of their content is rather advanced, but our evidence on the way in which this activity is acquired is inconclusive and primarily negative. Fusion of absorptive vacuoles with pre-existing AcPase-positive residual bodies was not seen. The residual bodies are also much less numerous than absorptive vacuoles, which, in itself, suggests that they are not the unique or the main source of enzyme. In this respect, our results and interpretations differ from those of Straus (41, 42), who, in light microscope preparations, found extensive superimposition of peroxidase and AcPase reactions presumably as a result of phagosome-lysosome fusions. The systems involved are not entirely similar; yet the difference is greater than expected, and requires further work to be understood. The two sets of results could be explained by assuming that the exogenous protein is fed *via* small apical vesicles into clear vacuoles as well as into residual bodies, a possibility which, with some variations, has also been considered recently by Straus (48).[8]

We did not find in the kidney an equivalent of the storage granules considered as primary lysosomes in polymorphonuclear neutrophil leukocytes (10, 11). Morphologically, the most likely candidates are the microbodies (24), but they are

[8] Dr. W. Straus kindly showed us this reference while still in press.

FIGURE 21 Glomerular mesangial cell of nephrotic rat, 48 hours after ferritin administration. Specimen reacted for thiolacetate-esterase after preincubation in 10 μM E600. Four dense ferritin-containing bodies are seen, two of which are marked by deposits (arrows, inset) of reaction product. The spinous processes of the cell are labeled *sp*, and its Golgi complex *gc*. Spongy areas of the basement membrane (*bm*) can be seen at *sa*. An epithelial cell with an absorption droplet is visible in the lower right corner. The urinary space is marked *us*. A ferritin-containing dense body from a similar specimen, not preincubated in E600, is shown in the inset. The number of reacting sites is higher than in the E600-treated material. × 42,000; inset, × 52,000.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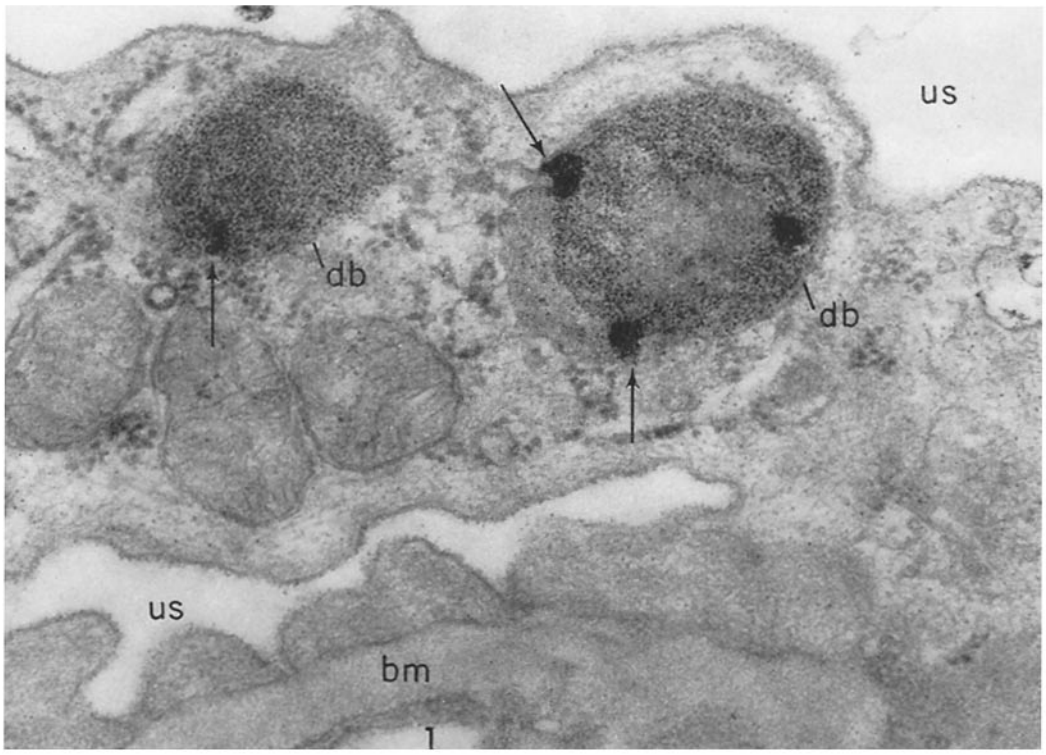
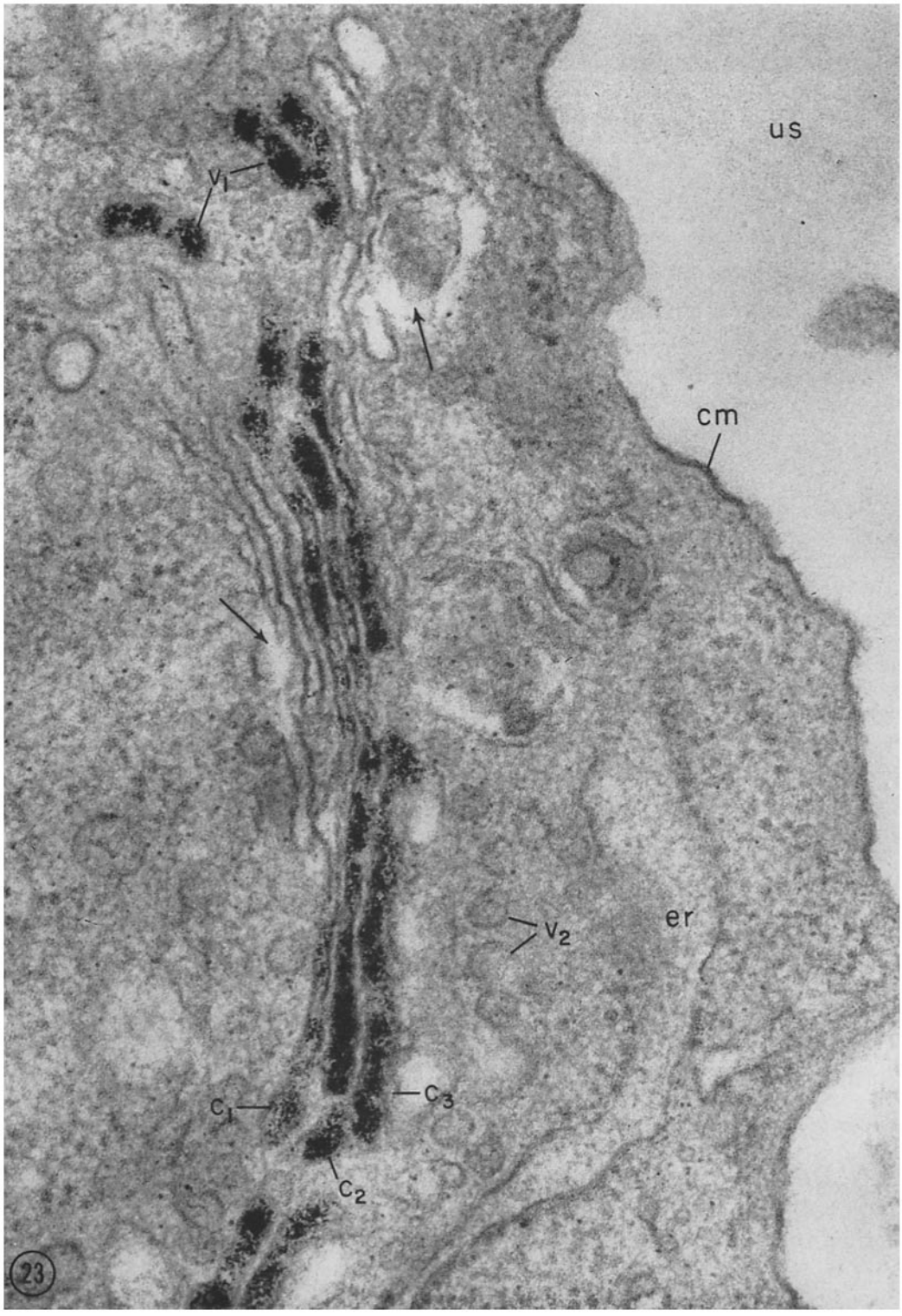


FIGURE 22 Small field in the wall of a glomerular capillary; nephrotic rat, 48 hours after ferritin administration. Specimen reacted for thiolacetate-esterase. Two ferritin-containing dense bodies (*db*) can be seen in the visceral epithelial cell. Both of them are marked by deposits of reaction product (arrows). The urinary spaces appear at *us*, the basement membrane at *bm*, and the capillary lumen at *l*. × 49,000.

AcPase-negative, were not seen to come in contact or fuse with absorptive vacuoles, and, according to evidence obtained on liver, may contain an entirely different group of enzymes. Liver microbodies show a characteristic crystalline inclusion and contain catalase, uricase, and D-amino acid–oxidase (49).

Novikoff and Essner have recently advanced (50) that lysosomes acquire their enzymes from the Golgi complex, an assumption based on the AcPase-positive reaction at this site. In our experiments, however, Golgi elements were only occasionally AcPase-positive and no special relationship or connections were seen between them and the absorptive vacuoles at the time the latter began to show enzyme activity. Moreover, in the absence of any kinetic data, it is impossible to know whether the activity rarely seen in the Golgi complex represents new enzyme on its way to the absorptive vacuoles, or old enzyme still associated with a for-

FIGURE 23 Golgi complex at the periphery of a glomerular epithelial cell; nephrotic rat, 48 hours after ferritin administration. Specimen reacted for AcPase. Heavy deposition of reaction product appears within the cavities of most of the piled cisternae (c_1 to) c_3 of the complex. Some of the structures (v_1) marked by reaction product may represent associated small vesicles or obliquely sectioned cisternal margins. Most of the smooth vesicles (v_2) around the cisternae and the adjacent rough-surfaced elements of the endoplasmic reticulum (*er*) are free of reaction product. The cell membrane is labeled *cm*, and the urinary space *us*. Freezing artifacts can be seen at the arrows. × 89,000.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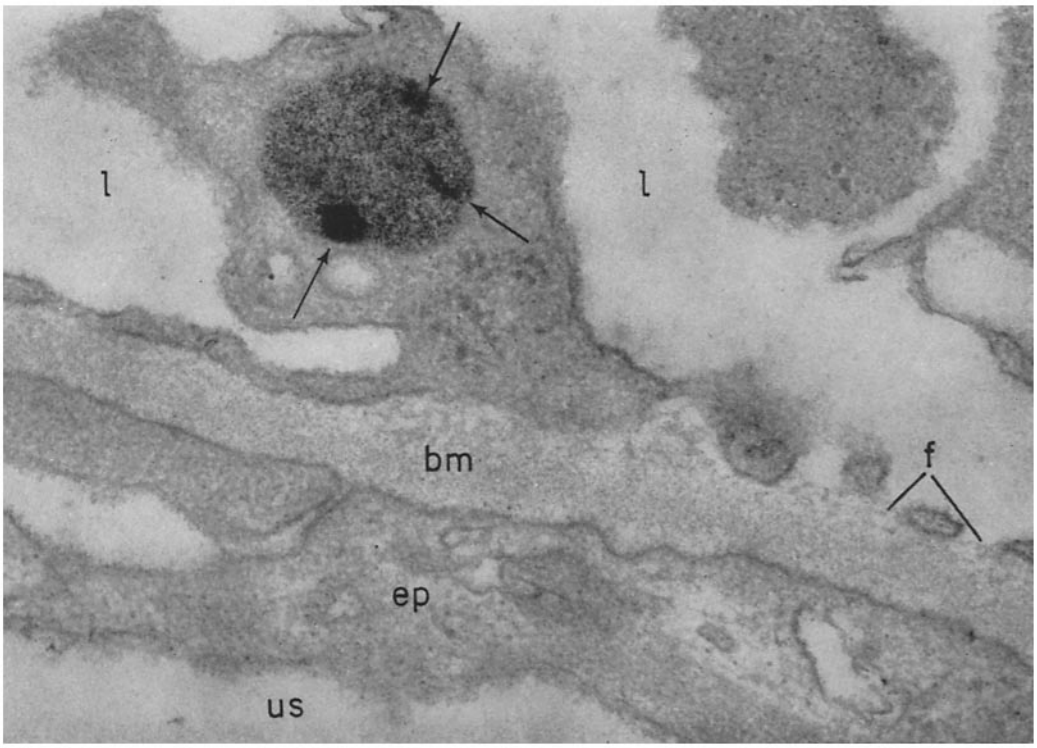

FIGURE 24 Small field in the wall of a glomerular capillary; nephrotic rat, 48 hours after ferritin administration. Specimen reacted for thiolacetate-esterase. A ferritin-containing dense body marked by reaction product (arrows) is seen in the endothelial cytoplasm. The capillary lumen is labeled *l*, the endothelial fenestrae *f*, the basement membrane *bm*, the glomerular epithelium *ep*, and the urinary space *us*. × 68,000.

mer lysosomal membrane which, after the discharge of the corresponding content, has been withdrawn for temporary deposit in the Golgi complex (*cf.* 51, 52). Available evidence indicates that the turnover of lysosomal enzymes in the liver is slow (2).

If we assume that the distribution pattern of AcPase activity is qualitatively valid, that we are not misled by unequal enzyme inactivation or by the presence of the enzyme in an inactive form in certain sites, we must conclude that the nephron epithelia do not store AcPase in primary lysosomes in anticipation of future endocytic activity. The enzyme might be produced, when needed, by a series of operations comparable to those encountered in glandular cells (53, 54) and transported to the absorptive vacuoles by small vesicles at a dilution high enough to escape detection. This assumption implies that the enzyme may well pass through some elements of the Golgi complex. It should be clear, however, that the evidence so far available cannot be considered sufficient proof in case.

As the number of absorption droplets increases, they fill the apical region and subsequently invade the basal half of the cell. In our experimental animals (female mice), this apparently happens only when the absorptive capacity of the cell is taxed. In male rats, however, the basal location is frequent and, according to Novikoff (55), represents the usual situation. This difference may be related to the proteinuria developed by male rats at puberty (56) which leads to active and continuous protein resorption in the proximal convolutions of their nephrons.

As judged by changes in the density of their protein content, digestion within absorption droplets becomes noticeable in about 18 hours and is prac-

tically completed in 48 hours[9] when the appearance of the cells comes back to normal except for an increased frequency of dense, vacuolated, and lamellated bodies. Many of these contain ferritin and piled membranes and, for this reason, can be detected in light microscopy by their positive reaction for iron and phospholipid (12). The late appearance of these entities in the cycle indicates that they are residual bodies. Their characteristic polymorphism probably reflects the varied nature of their original content and the extent to which its digestion has proceeded at the time of fixation. The frequent occurrence of ferritin in these bodies remains unexplained. It may represent ferritin ingested from the lumen and coming from damaged cells up the nephron, but there is little evidence for such damage; or it may be newly synthesized ferritin whose iron is provided by the digested hemoglobin, but in the framework of our current ideas, it is difficult to see how synthesis can occur in a lytic vacuole, or how large protein molecules, synthesized elsewhere in the cell, can find their way within such a vacuole.

The ultimate fate of the residual bodies remains uncertain. Some of them apparently discharge their content into the lumen as suggested by the occasional occurrence of small myelin figures in the tubular invaginations, among the microvilli of the brush border, or in the lumen proper. Such figures have a periodicity similar to that found in the content of the lamellated bodies. The direction in which these figures move is naturally unknown, and in a previous work (12) we assumed that phospholipid molecules (possibly membrane remnants), absorbed from the lumen together with the hemoglobin, were the source of the myelin figures seen in some absorption droplets and in lamellated bodies. With the new evidence on autolytic vacuoles, an endogenous source and a reversed pathway become likely alternatives.

Images suggesting direct discharge of the content of residual bodies were not seen in these experiments, but have been rather frequently encountered in previous work on glomerular epithelia (14, 45). If the digestion of the content is complete and the lytic enzymes survive it, the residual body may become the equivalent of a primary lysosome and re-enter the cycle, but the available evidence suggests that this is not usually the case. Quite often old lysosomes, filled primarily with lipid residues, pile up progressively in certain cell types in which they become wear and tear pigment or lipofuscin granules (*cf.* 2). Some of these granules still contain acid hydrolases (57–60) and have a polymorphic content (61–64) partly reminiscent of that of usual residual bodies.

The preceding reconstruction of the lytic cycle is based primarily on extensive observations carried out on the epithelia of the proximal convolution and is generally supported by the more limited results obtained on glomerular mesangial and epithelial cells. Our findings suggest that during experimentally induced protein uptake lytic processes are stepped up, but that the operations involved therein remain basically the same.

III. *Relation to the Brush Border and the Cell Membrane*

From the beginning to the end of the lytic cycle, the ingested protein is contained within a smooth-surfaced membrane pocket which, in ultimate analysis, is apparently derived from the plasmalemma on the luminal front of the cell. This cell membrane shows characteristic morphological differentiations, in the form of microvilli and absorptive channels, correlated with a distinct enzyme pattern: the part of the membrane covering the brush border gives an intense alkaline phosphatase reaction (27, 28), whereas the part lining the abosptive channels is generally free of such activity.[10] The general arrangement suggests a mosaic in which patches fitted into a continuous membrane have distinct enzyme equipments and subserve different types of transport. Comparable, yet different dispositions have been recorded in other cell membranes such as those of vascular endothelia (65). One may wonder whether such a mosaic represents permanent differentiation or transient display of enzyme activity brought about by configuration changes within a functionally homogeneous membrane. It may be pointed out that the change in activity coincides with the points at which the membrane is infolded within the cell body and at which continuity between in-

[9] This figure agrees with that given by Straus (2 to 4 days) based on the disappearance of the peroxidase positive reaction in "phago-lysosomes" (42, 48).

[10] Micrographs published by Mölbert (27, 28) indicate that the channels give a positive reaction at their introit and are negative deeper in the cell. The AcPase activity occasionally found in the brush border and apical membrane may represent, as already mentioned, the "acid tail" of this intense alkaline phosphatase reaction.

foldings and the rest of the membrane can be interrupted. This situation remains compatible with a functional mosaic, but suggests that the differentiations involved are lasting or permanent.

IV. *Autolytic Vacuoles*

Autolytic vacuoles were frequently encountered in our experimental material. They regularly showed AcPase and esterase activity and contained one or more mitochondrial profiles altered to a varied extent. Other cell components, *e.g.*, elements of the endoplasmic reticulum, ribosomes, etc., were only rarely encountered. The demonstration of lysosomal enzymic activities and recognizable subcellular components within the same membrane-bounded spaces clearly establishes the existence of autolytic vacuoles in the cell types examined (in confirmation of results already published (35, 36)). In hemoglobinuric mice, the evidence suggests that these vacuoles regularly contain hemoglobin. The cell apparently segregates within a common pocket endogenous and exogenous material intended for lysis. Our evidence on the mechanism involved in the segregation of endogenous material is inconclusive and again mainly negative. There is no suggestion that a membrane barrier is progressively built around an already altered subcellular component, but mitochondria are often found squeezed in between two absorptive vacuoles or droplets. This finding suggests that mitochondria may become trapped-in when absorptive vacuoles coalesce, an event which undoubtedly occurs quite often in the apical region of the cell during the first hours of our experiments. In this respect, it should be noted that autolytic vacuoles are absent or only occasionally present in normal animals and that their formation apparently accompanies the uptake of exogenous material. The ultimate fate of the autolytic vacuoles is unknown, but the variety of appearances encountered suggests that they may become in time lamellated residual bodies.

Vacuoles of this type were first demonstrated by Rhodin (24) and subsequently seen by Miller (12) who noted the decay of the entrapped mitochondria. They were also encountered in the nephron epithelium of newborn rats (66); in the proximal convolution of hydronephrotic rats where Novikoff discussed their possible relation to lysosomes (67); in the intestinal epithelium of embryonic (68) and newborn (69) rats; and more recently found in large numbers in rat livers perfused *in vitro* with glucagon (70). In this last situation, Ashford and Porter assumed that the vacuoles were lysosomes (although they did not demonstrate lytic activities therein) and suggested that they "represent portions of cytoplasm set aside for hydrolysis" to provide "breakdown products for a reoriented physiology." AcPase activity in absorption droplets containing recognizable mitochondria was demonstrated by Miller (35) in the nephrons of hemoglobinuric mice and subsequently by Novikoff and Essner (50) in the liver cells of rats treated with a detergent, Triton WR-1339.

Note Added in Proof: Counts of vacuolated bodies, with and without a hemoglobin rim, gave the results tabulated below:

Time	Number of cells counted	Vacuolated bodies	
		Without rim	With rim
0*	51	23‡	0§
1	60	26	5
2	43	35	12
4–6	29	16	20
10	16	37	6
18–21	14	10	0

* Hours after hemoglobin injection.
‡ Per cent of all residual bodies.
§ Per cent of all vacuolated bodies.

The data support the view that newly ingested hemoglobin is fed into old residual bodies, but indicate that the process affects only a relatively small proportion of the latter.

The technical assistance of Mrs. Lee Adinolfi is gratefully acknowledged.

This investigation was supported by a research grant (HE-05648) from the National Institutes of Health, United States Public Health Service.

Received for publication, April 1, 1964.

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