An Analysis of the Close Relationship of Lysosomes to Early Deposits of Amyloid

Ultrastructural Evidence in Experimental Mouse Amyloidosis

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On the basis of recent morpholgic and biochemical studies which suggested the possible involvement of reticuloendothelial (RE) cells and proteolytic enzymes in amyloidogenesis, the present study was undertaken to examine the ultrastructural interrelationship between lysosomes and amyloid fibrils at the sites of very early amyloid deposition. In the spleen, liver and kidney of the experimental mouse model, foci of amyloid deposits were closely associated with the RE cells. The lysosomal enzyme activity, as marked by cytochemical demonstration of acid glycerophosphatase activity, was localized in the primary type lysosomes (as defined by their electron microscopic appearance), in the Golgi complexes, in the small cytoplasmic vesicles and occasionally widespread in the cytoplasm. They showed an intimate relationship to the amyloid fibrils. The findings were interpreted as favoring the hypothesis that the hydrolases play a role in amyloid fibril formation. The enzyme activity was also demonstrated in the secondary type lysosomes which occasionally contained amyloid fibrils that appeared to be phagocytized (Am J Pathol 73:97–114, 1973).

THE CONCEPT that reticuloendothelial (RE) cells are intimately associated with the genesis of amyloid has been generally accepted.[1,2] This notion was initially based on the intimate morphologic relationship existing between amyloid deposits and RE cells,[3] and was verified by a variety of methods including light and electron microscopic autoradiography.[4,5] The chemical nature of amyloid has also been vigorously studied for the past decade. Amino acid sequences of proteins extracted from amyloid have recently been analyzed,[6–10] and one of the major constituent proteins from primary amyloid has

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shown homology in its sequence to the variable segment of immunoglobulin light chain, while most secondary amyloid proteins demonstrated a unique and different sequence. Furthermore, a fibrillar material which had characteristics comparable with those of amyloid fibril was successfully constructed *in vitro* by cleaving Bence Jones proteins with pepsin [11,12] as well as with lysosomal enzymes extracted from human kidney.[13]

Since the prime functions of the RE system are intimately related to lysosomal activity, and since the amyloid chemical composition suggests the possibility that protein fragments may be constituents of some types of amyloid, there has been speculation that lysosomal enzymes might play an important role in the process of amyloid formation. However, no definitive data in support of this concept have so far been presented, although some histochemical studies have described increased hydrolytic enzyme activity in the participant RE cells.[14,15] The present study was undertaken to investigate this possibility in an experimental mouse amyloid model using an electron microscopic cytochemical method.

Materials and Methods

Amyloidosis was induced in 6 to 8-week-old female CBA/J mice (Jackson Laboratory, Bar Harbor, Me) by daily injections of 0.5 ml of 10% casein (Casein Hammersten, Control No. 5320, Nutritional Biochemicals Company, Cleveland, Ohio).[16] Our schedule usually induces amyloidosis in the spleen after 12 to 18 injections, in the liver after 16 to 22 injections and in the kidney after 20 to 26 injections. Animals were sacrificed at various stages of amyloid induction and small blocks (smaller than 2 × 2 × 5 mm) of spleen, liver and kidney were immediately fixed in cold 2.0% formaldehyde–2.5% glutaraldehyde in 0.1 M cacodylate-HCl buffer.[17] Frozen sections cut from other portions of the organs were stained with Congo red and hematoxylin[18] and examined by light and polarization microscopy.[16] By this screening procedure, the tissue blocks from the organs which had minimal amyloid deposits (and so were judged to represent the first or the second day of amyloid induction in the organs) were selected and further processed.

After 4-hour fixation in the aldehyde fixative at 4 C, the tissue blocks were washed in two changes of 0.1 M cacodylate-HCl buffer (pH 7.4) containing 10% sucrose, cut into 30-μ slices on a Sorvall TC-2 tissue sectioner [19] and stored in the same buffer for 12 to 36 hours at 4 C. The tissue slices were then washed once with 0.05 M sodium acetate buffer (pH 5.0) containing 7.5% sucrose and incubated for cytochemical demonstration of acid phosphatase activity in a Gomori medium[20] at 37 C for 20 minutes. The Gomori medium was prepared as described by Miller and Palade.[21] Lead nitrate, $Pb(NO_3)_2$, (0.12 g) was dissolved in 100 ml of 0.05 M sodium acetate buffer (pH 5.0) containing 7.5% sucrose, and then 10 ml of 3% solution of β-glycerophosphate (Grade 1, Sigma Chemical Company, St. Louis, Mo) was slowly added. Before use, the mixture was warmed at 60 C for 1 hour, cooled to room temperature and filtered. Controls were run a) by adding 0.01 M NaF to the incubation medium, b) by omit-

ting the substrate from the incubation medium or c) without incubation. After incubation, the tissue slices were rinsed with two changes of 0.05 M sodium acetate buffer (pH 5.0) containing 7.5% sucrose and postfixed in 2% osmium tetroxide in 0.1 M cacodylate-HCl buffer (pH 7.2) for 1 hour at room temperature. After washing with two changes of 0.05 M sodium acetate (pH 6.5), they were treated with 0.5% uranyl acetate in 0.05 M sodium acetate (final pH about 5.5) for 60 minutes at room temperature.[22] They were then dehydrated in graded ethanols and embedded in Epon.[23]

Aliquots of the tissue blocks fixed in the aldehyde were also processed for electron microscopy in a conventional manner. They were cut into smaller pieces (smaller than 1 × 1 × 1 mm), postfixed with 2% osmium tetroxide in 0.1 M cacodylate-HCl buffer (pH 7.2) for 2 hours at room temperature, dehydrated in graded ethanols and embedded in Epon.

As controls, spleens, livers and kidneys from untreated mice were also prepared in the same fashion as those from amyloidotic mice.

Thin sections were cut on an LKB Ultratome and stained on most occasions with lead citrate[24] for 2 minutes at room temperature; some were not stained or were stained with uranyl acetate[25] and lead citrate. The specimens were examined in a Siemens Elmiskop I at initial magnifications of 1000 to 40,000×.

The following description is based on the analysis of more than 5000 micrographs of the present materials and many other micrographs from related studies.

Results

Aspects in Conventional Electron Micrographs

In the conventional electron micrographs, amyloid deposits were closely associated with the RE cells; the fixed reticular cells and the sinus lining cells in spleen, the Kupffer cells in liver, the mesangial cells in kidney and the endothelial cells in all organs (Figures 1–3). At the contact surface, the cytoplasmic processes extended into the amyloid deposit and formed invaginations containing bundles of well-oriented amyloid fibrils. In addition to the above findings which confirmed the previous observations,[1,2,4,5] the following findings were significant in relation to the sites of very early amyloid deposition.

Dense bodies were often predominant in the RE cell cytoplasm on the side contacting the extracellular amyloid, so that they appeared to be polarized towards the amyloid deposit. These dense bodies were predominantly of the primary lysosome type—*ie*, they were relatively small and round to oval, with a smooth outline, and contained a homogeneous matrix of moderate to high electron density.

Small vesicles were frequently found in the RE cell cytoplasm adjacent to the contact surface to amyloid. They were 500 to 1500 Å in diameter, round to tubular in shape and often contained moderately electron-dense homogeneous material. Some of them were attached to the plasma membrane facing the extracellular amyloid.

Small masses of electron-dense homogeneous substance, comparable

to the contents of the primary-lysosome-type dense body, were occasionally found among the bundles of well-oriented amyloid fibrils in the extracellular space adjacent to the RE cell.

General Remarks on Cytochemical Findings

Significant acid phosphatase activity was demonstrated in most of the RE cells (Figures 4–11). When the RE cell was in contact with extracellular amyloid, the enzyme activity was particularly abundant in the peripheral cytoplasm adjacent to the amyloid. The acid phosphatase reaction product was not only confined to the ordinary lysosomal structures and Golgi complexes as anticipated,[26–30] but was also localized in numerous small vesicles which otherwise did not show the ultrastructural features of ordinary lysosomes. Furthermore, RE cells occasionally displayed disseminated deposits of the reaction product throughout the cytoplasm. While the acid phosphatase reaction was generally insignificant on the extracellular amyloid fibrils, on occasion the reaction product was deposited sparsely but definitively on the bundles of well-oriented amyloid fibrils near the cell.

Primary-Type Lysosomes

The primary-lysosome-type dense bodies described above demonstrated acid phosphatase activity as expected (Figures 4 and 5). These lysosomes were predominant in the RE cell cytoplasm adjacent to the extracellular amyloid deposit. They were often localized near the cytoplasmic invaginations that contained well-oriented amyloid fibrils, attached to the plasma membrane and occasionally appeared to open to the extracellular space. In the latter case, a bundle of well-oriented amyloid fibrils could be followed from the extracellular space up to the opening of the lysosome or, on occasion, even into the lysosome. Moreover, significant acid phosphatase reaction product was often found on the extracellular amyloid fibrils in the immediate vicinity of the lysosome opening.

Acid-Phosphatase-Positive Vesicles

The RE cell cytoplasmic vesicles were often acid phosphatase positive (Figures 6–9). As mentioned earlier, they were 500 to 1500 Å in diameter, round to tubular in shape, and contained light to moderately electron-dense material, so that they did not have the structural characteristics of the ordinary lysosomes. Moreover, in most instances they were not associated with the Golgi complex. The acid-phosphatase-positive vesicles were particularly abundant in the peripheral cyto-

plasmic processes abutting the extracellular amyloid. They were often attached to the plasma membrane and occasionally appeared to open directly to or to be connected via narrow channels to the extracellular space near the well-oriented amyloid fibrils. On occasion, vesicles with acid phosphatase reaction were found among the extracellular amyloid fibrils in the immediate vicinity of the RE cell.

Disseminated Acid Phosphatase Reaction and Cytoplasmic Fibrils in the RE Cells

On occasion, the reaction product was present throughout the RE cell cytoplasm, while in nearby cells it had the usual acid phosphatase localization (Figure 10). The cells with the disseminated acid phosphatase reaction rarely showed degenerative changes. In the cytoplasm of these cells, free fibrils of 50 to 100 Å diameter were found frequently in the spleen and occasionally in the liver and the kidney. The cytoplasmic fibrils had ultrastructural features generally comparable with those of the extracellular amyloid fibrils, although they appeared somewhat softer and wavier than the amyloid fibrils.

Secondary-Type Lysosomes Containing Amyloid Fibrils

The RE cells also contained secondary-lysosome-type dense bodies, as defined by their ultrastructural features; they tended to be relatively large in size, spherical but somewhat irregular in shape and had heterogeneous contents (Figure 11). These dense bodies were usually acid phosphatase positive. On rare occasions, they contained fibrillar structures comparable to amyloid fibrils.

Discussion

Gomori's method for the demonstration of acid phosphatase activity is one of the most widely used cytochemical methods, and numerous studies have relied on it. However, any such method has limitations;[31,32] for example: a) only a small portion of the total acid phosphatase activity can be demonstrated by this method, probably depending upon substrate specificity of various acid phosphatase isoenzymes [33–35] and inhibitory effect of lead salt on the enzyme activity. b) In practice, this method visualizes, as a rule, the enzyme activity localizing in the lysosomes and the Golgi complexes but not the microsomal acid phosphatase activity. c) Since diffusion of the enzymes from their original localization could take place during the preparation and because of the affinity of the reaction end product to lipoprotein, etc, false positive localization of the end product could occur; it has often been observed on the nucleus and the membrane system.

Keeping the above in mind, the present cytochemical findings are best evaluated as follows. The acid phosphatase activity in the classic lysosomal structures may be accepted as is. The localization of the reaction product in the small cytoplasmic vesicles is somewhat unusual, but it seems unlikely that it represents a false positive reaction, for the following reasons: a) the reaction product was confined to the membrane-bound structure, b) the staining conditions of the area were judged to be appropriate from the condition of the other cells in the immediate vicinity, and c) this reaction was not found in control preparations. Moreover, the microsomal localization of acid phosphatase has been revealed by biochemical studies and in some cases by cytochemical methods.[33–35] In addition, the vesicles associated with Golgi complex are often acid phosphatase positive by Gomori's method,[28,31,32,36] and the vesicular system and the vacuolar system to which lysosomes belong are naturally closely related to each other. Thus, it is likely that the reaction product localized in the small vesicles represents true localization of acid phosphatase activity. If so, the acid-glycerophosphatase-positive vesicles should then fall into the broad category of lysosomal particles, even though they are considerably smaller than the classic dense bodies. The finding of disseminated deposition of the reaction product in the RE cell is usually interpreted as the result of diffusion of the enzymes.[31,32] Nevertheless, the considerations that were discussed concerning reaction product localized in the small vesicles may also be applied to this situation, and they seem to favor the interpretation that the finding is not an artifact. Moreover, the animal had had repeated antigenic stimulation and the release of lysosomal enzymes can be produced by various immunologic reactions.[37]

Thus, the present results are consistent with the idea that the primary-type lysosomes and the acid-phosphatase-positive vesicles are in intimate morphologic relationship with the extracellular amyloid fibrils at the site of initial amyloid deposition. Namely, they were localized close to, attached to and often fused with the plasma membrane especially in the portions forming the invaginations containing well-oriented amyloid fibrils. The findings further suggest that the lysosomes released their contents into the extracellular space where the well-oriented amyloid fibrils were near by. These findings may be interpreted as indicating the following possibilities: a) the lysosomal particles participate in amyloid fibril formation, b) they play a role in amyloid fibril resorption or c) the apparent close relation between the lysosomes and the amyloid fibrils is just a coincidental phenome-

non, and lysosomes have no relation to the production or fate of amyloid.

Among these possibilities, the first seems to be best supported by the available data. A number of morphologic studies for the past several decades have presented substantial evidence for an intimate association of the RE cells with the genesis of amyloid.[1-5] By electron microscopy, Gueft and Ghidoni [38] found cytoplasmic invaginations that contained tufts of well-oriented amyloid fibrils at the cell-amyloid interface and designated them as the sites of amyloid formation. This idea has been supported by many investigations,[1,2] including electron microscopic autoradiographic studies,[4,5] and has not yet met any serious challenge. Under the present experimental conditions, the small early amyloid deposits which have been the main targets of the present observations must have been growing quite rapidly. At such a site, amyloid formation should have taken place at much a higher rate than amyloid absorption, if at all. Furthermore, recent biochemical studies have revealed that one of the major proteins of primary amyloid fibril protein extract has an amino acid sequence homologous to the variable segment of immunoglobulin light chain [9] and also that amyloid-like fibrillar material can be constituted *in vitro* by cleaving Bence Jones proteins with proteolytic enzymes.[11-13] These results suggest that cleavage of preexisting proteins by proteolytic enzymes may be a possible mechanism of amyloid fibril formation *in vivo*, at least in some cases. Since lysosomes carry a variety of hydrolytic enzymes,[39,40] their involvement in such a process is possible. In a similar fashion, the ultrastructural interrelation between the ameloblast and the extracellular enamel matrix show a striking similarity with that of the RE cell and amyloid.[41,42] Cytochemical observations of the acid phosphatase activity in the amelogenetic tissue revealed enzyme in the ameloblasts (*ie*, as in the present RE cells), and the results have been interpreted as an evidence for the role of lysosomes in amelogenesis.[41]

Phagocytic activity is widely recognized as one of the important characteristics of the RE cells,[26-30] and lysosomes are closely related to the endocytic function.[26-30,39,40] It seems then reasonable to raise the possibility that the present findings may be related to this function—uptake of amyloid fibrils by the RE cell. However, this possibility has less support. The conditions discussed above favor the role of hydrolases in amyloid fibril formation rather than the reverse. Moreover, the morphologic aspects of the cell–amyloid interaction being discussed here are clearly distinguishable from the patterns of phagocytosis of amyloid fibrils by RE cells which have been studied in detail.[43-46] The lysosomal particles participating in the cell–amyloid interaction are predominantly

the dense bodies characterized morphologically as primary-type lysosomes. Therefore, a possible mechanism by which these lysosomal particles can participate in the uptake of amyloid fibrils by RE cells, if it indeed takes place, may be that these particles release their hydrolytic enzymes into the extracellular space, the hydrolases then alter the amyloid fibrils to some degree and, finally, the cells take up the derivatives, as postulated in collagen absorption.[47] This mechanism has not thus far been tested in the amyloid model.

The third possibility, random coexistence of the lysosomal particles and the amyloid fibrils, is merely a possibility that cannot be excluded at present.

The fact that free cytoplasmic fibrils with structural features comparable with those of the amyloid fibrils were frequently observed in combination with disseminated acid phosphatase reaction may be of interest. Such fibrils have been reported on occasion in amyloidotic tissues.[1,2,48] Since cytoplasmic filaments of comparable features are found commonly in various cell types under nonamyloidogenic conditions,[26,27,29,30,49] the question as to whether such cytoplasmic fibrils are related to amyloid fibrils has been unanswered. If hydrolytic enzymes indeed play a role in amyloid fibril formation, the present findings would favor the view that such fibrils may be amyloid, for it seems reasonable to speculate that amyloid fibrils may be formed in the free cytoplasm when hydrolytic enzymes are released into the cytoplasm and "amyloid precursors" are available there.

In conclusion, a close morphologic relation between the lysosomal particles and the amyloid fibrils at the site of very early amyloid deposition has been reported. The results have been interpreted in favor of the role of the lysosomal particles in amyloid fibril formation. However, the supporting evidence for this interpretation is circumstantial. Finally, if hydrolases indeed participate in amyloid fibril formation, many questions still remain unanswered. What are the source materials? Where are the materials synthesized—locally by the RE cell or elsewhere? Where does the interaction between the source materials and the hydrolases take place—intracellularly or extraceullularly?

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[Illustrations follow]

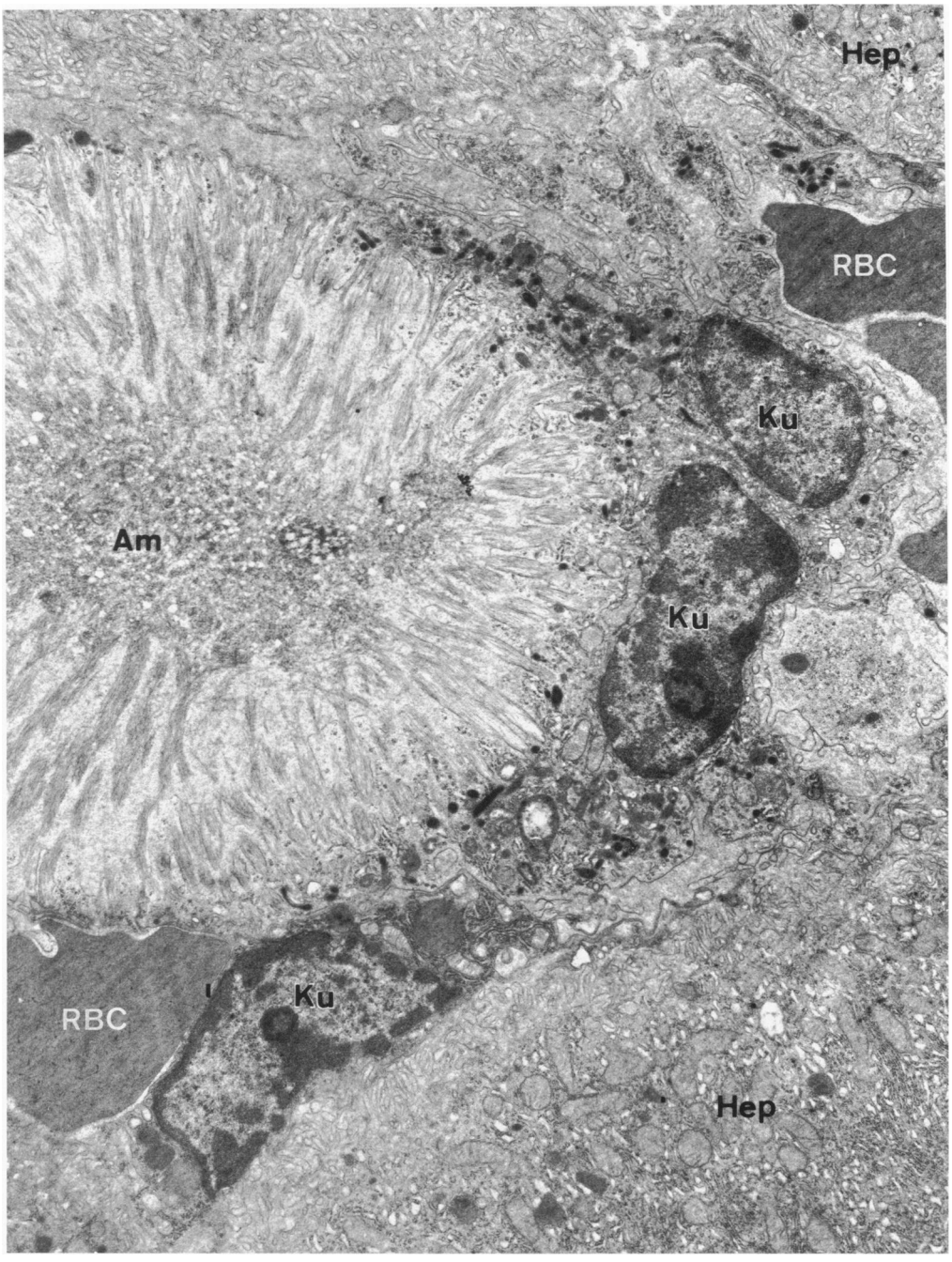
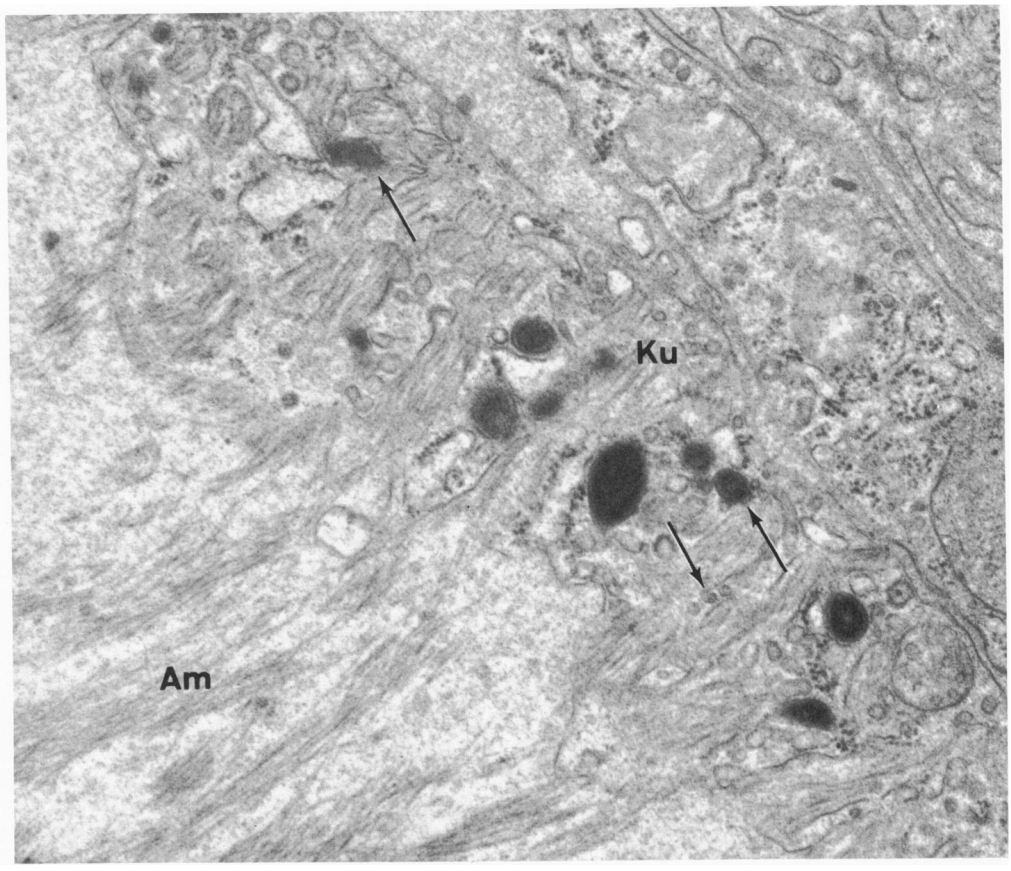


Fig 1—Low power electron micrograph of amyloidotic mouse liver after 20 casein injections. An "amyloid star" (*Am*) of about 15 μ diameter is surrounded by Kupffer cells (*Ku*). Amyloid fibrils deposit densely and in random array in the central portion of the star, whereas in the peripheral portion they form bundles of amyloid fibrils well oriented perpendicularly to the Kupffer cell line. The Kupffer cells possess many narrow deep cytoplasmic invaginations which contain bundles of amyloid fibrils and show numerous profiles of dense bodies which are particularly abundant in the perpheral cytoplasm of the side contacting with amyloid. *RBC*=red blood cell, *Hep*=hepatocyte (Conventional electron microscopic preparation, stained with uranyl acetate and lead citrate, × 6000).



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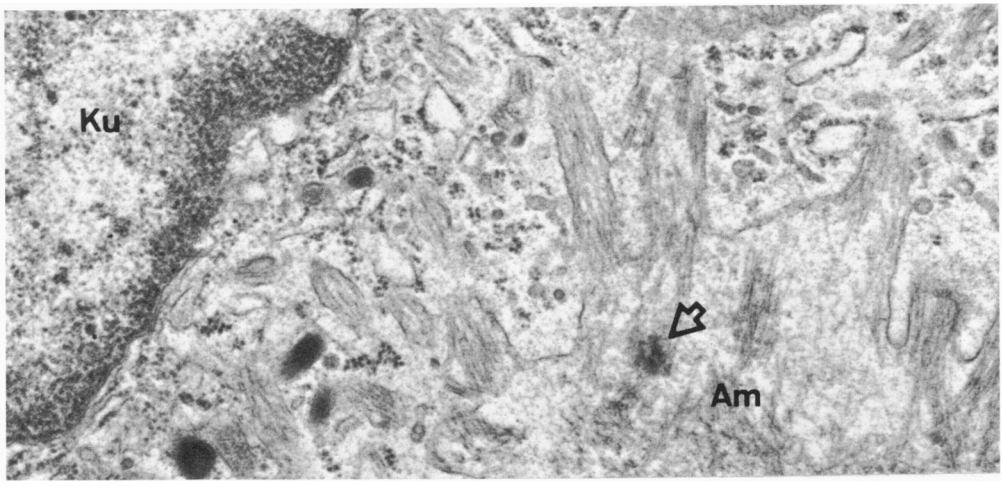

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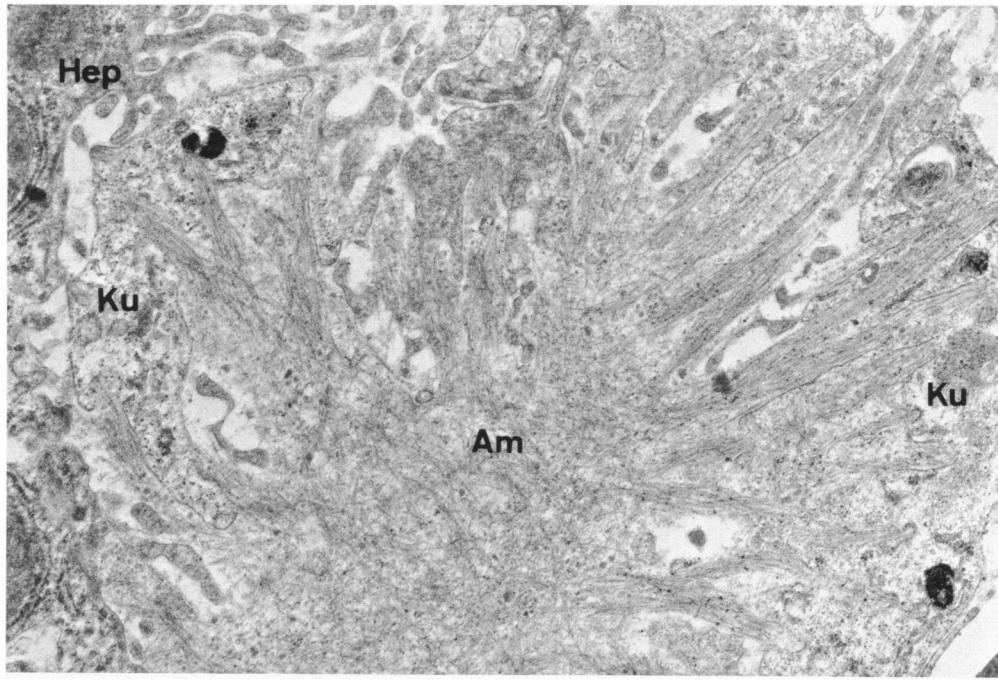
Fig 2—Higher power picture of mouse liver after 20 casein injections. A Kupffer cell (*Ku*) forms many deep invaginations seen in longitudinal or oblique sections and contains bundles of well-oriented amyloid fibrils (*Am*). Many profiles of dense bodies are observed in the Kupffer cell cytoplasm. The dense bodies are relatively small (0.1 to 0.3 μ in diameter) and contain homogeneous matrix of moderate to high electron density. Numerous small vesicles are also in the Kupffer cell cytoplasm and often contain moderately electron-dense material. Some of the dense bodies and the vesicles appear to be attached to or fused with the plasma membrane forming the invaginations (*arrows*) (Conventional electron microscopic preparation, stained with uranyl acetate and lead citrate, × 30,000). **Fig 3**—Similar preparation to that shown in Figure 2. A mass of electron-dense homogeneous substance, which shows characteristics similar to those of the dense body matrix, is observed among the well-oriented amyloid fibrils in the extracellular space (*open arrow*). Findings are otherwise as in Figure 2. *Ku*=Kupffer cell, *Am*=amyloid fibrils (× 30,000).



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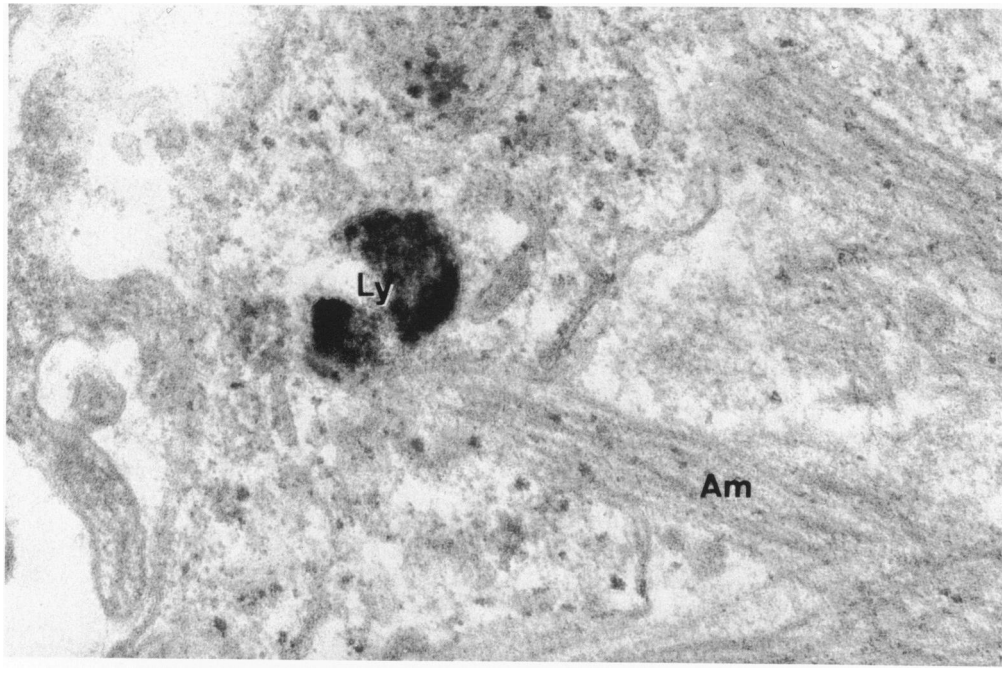

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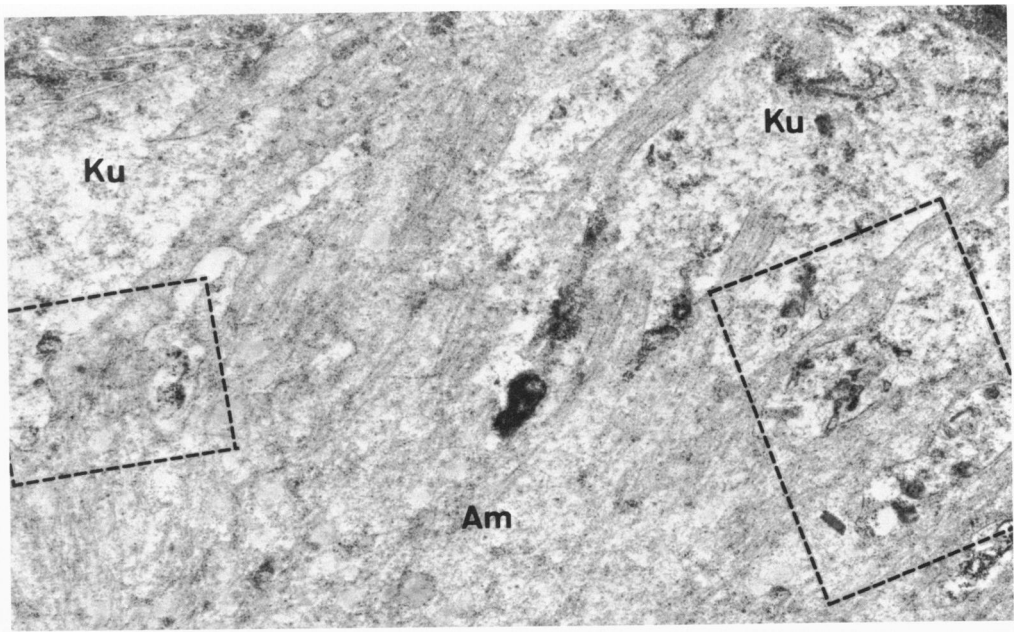
Fig 4—An electron micrograph of mouse liver after 20 casein injections, treated for the demonstration of acid phosphatase activity. An amyloid star (*Am*) of several micron diameter is surrounded by thin Kupffer cell cytoplasm (*Ku*). Several dense bodies seen in this area display deposits of acid phosphatase reaction product. *Hep*=hepatocyte (Stained lightly with lead citrate, × 13,000). **Fig 5**—A higher magnification of a portion of the area shown in Figure 4. A primary-type lysosome with a positive acid phosphatase reaction (*Ly*) is closely associated with a bundle of well-oriented amyloid fibrils (*Am*) (× 80,000).



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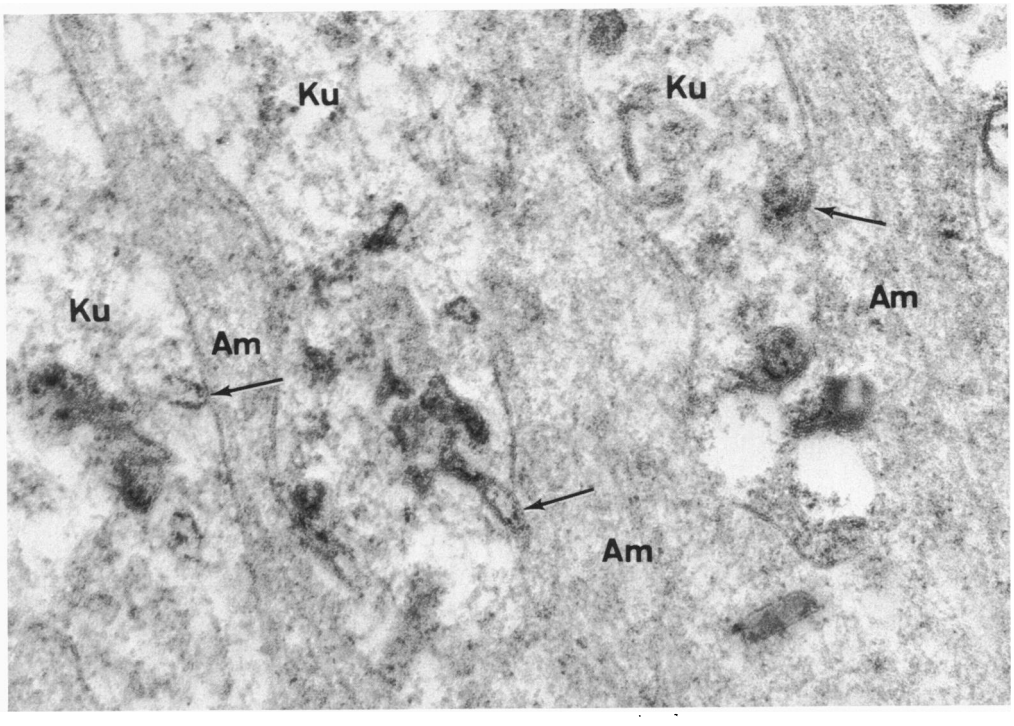

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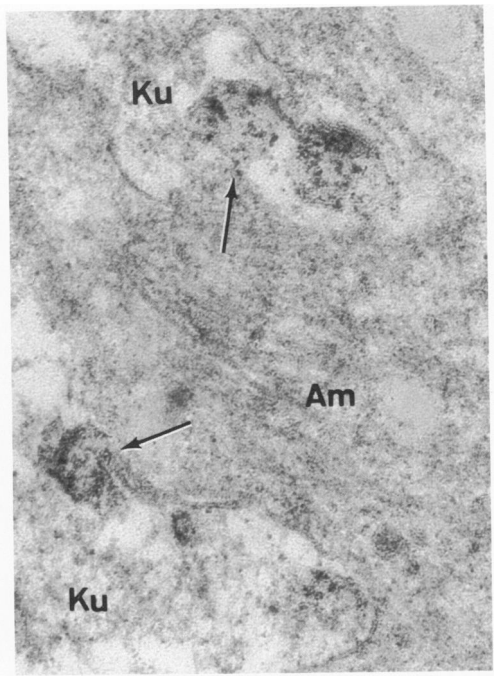
Fig 6—A portion of a Kupffer cell (*Ku*) and amyloid deposit (*Am*) from a mouse liver after 20 casein injections, treated for the demonstration of acid phosphatase activity. The cytoplasmic invaginations of the Kupffer cell are associated with tufts of well-oriented amyloid fibrils. In the peripheral cytoplasm, especially in the cytoplasmic processes, many small vesicles display deposits of acid phosphatase reaction product. Portions of the area surrounded by dotted lines are shown at a higher magnification in Figures 7 and 8 (Stained lightly with lead citrate, × 25,000). **Fig 7**—A higher magnification of a portion of the area shown in Figure 6. Deposits of the enzyme reaction product are fairly well confined to the cytoplasmic vesicles. The cytoplasmic vesicles are 500 to 1500 Å in diameter and scomewhat vary in shape from spherical to tubular. Some of the vesicles appear to be attached to or fused with the plasma membrane (*arrows*) that faces the well-oriented amyloid fibrils (*Am*). *Ku*=Kupffer cell cytoplasm (× 80,000).



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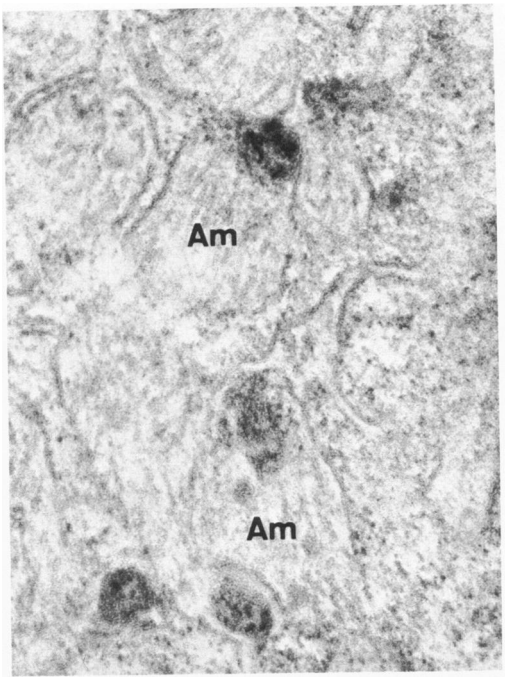

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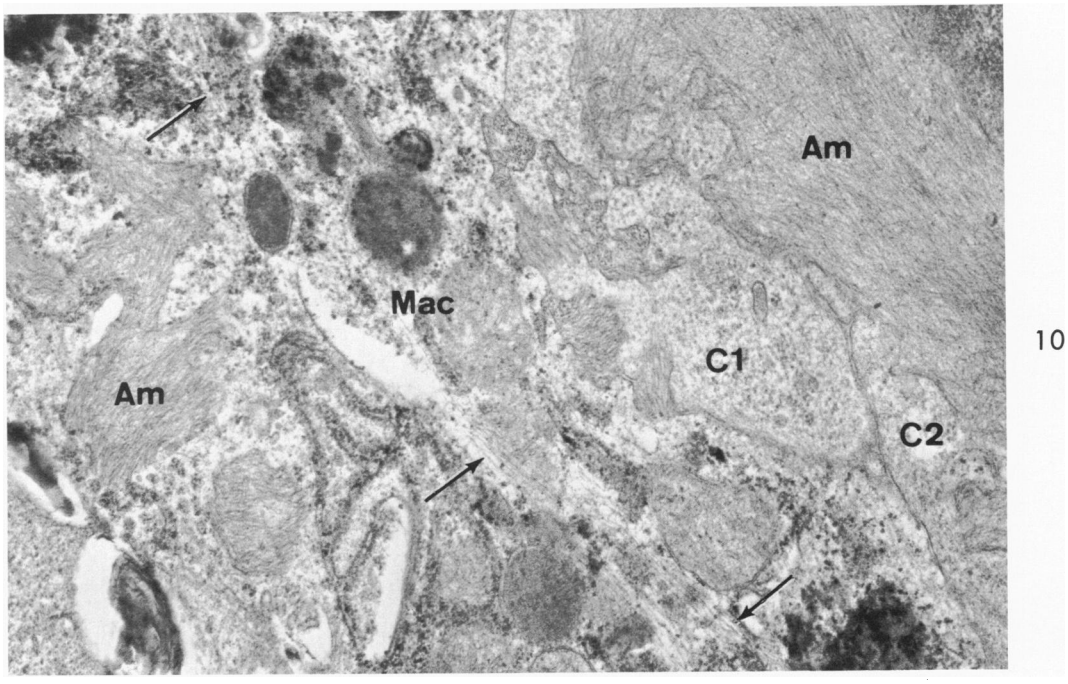

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Fig 8—A higher power electron micrograph of a portion of Figure 6. Cytoplasmic vesicles with the reaction product are in the Kupffer cell cytoplasmic processes (*Ku*), and some are apparently open to the extracellular space (*arrows*) (× 80,000). **Fig 9**—A preparation similar to that shown in Figures 6–8. In this area, the cytoplasmic invaginations of a Kupffer cell which contain the well-oriented amyloid fibrils (*Am*) are seen almost in cross section. Several acid-phosphatase-positive vesicles are closely associated with the amyloid-containing invaginations (× 80,000). **Fig 10**—A portion of amyloidotic mouse spleen after 16 casein injections, treated for the demonstration of acid phosphatase activity. A macrophage (*Mac*) exhibits disseminated deposits of the enzyme reaction product throughout the cytoplasm, although the deposits tend to be heavier on the lysosomal structures. In the cytoplasm, fibrils (*arrows*) are observed without being surrounded by any membrane and are structurally comparable with the extracellular amyloid fibrils (*Am*). Other cells near by (*C1, C2*) are virtually free of the reaction product (Stained lightly with lead citrate, × 20,000).

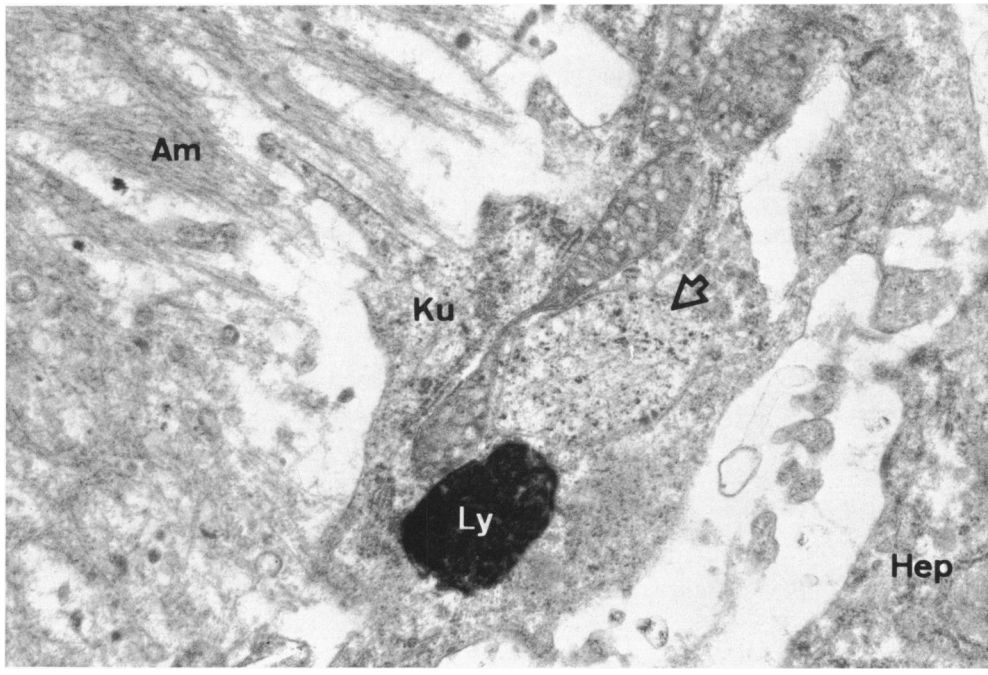

Fig 11—A portion of mouse liver after 25 casein injections, treated for the demonstration of acid phosphatase activity. A phagocytic vacuole (*open arrow*) which contains fibrils comparable to amyloid fibrils and demonstrates sparse deposits of the enzyme reaction product is seen in the Kupffer cell cytoplasm (*Ku*). A dense body (*Ly*) with heavy enzyme reaction is also present. *Am*=amyloid fibrils, *Hep*=hepatocyte (Stained lightly with lead citrate, × 20,000).