EXPERIMENTAL AMYLOIDOSIS

Structural Relationships of Amyloid and Reticulin in Tissue Sections and Isolated Preparations

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The intimate morphologic positioning of amyloid and reticulin in stained tissue sections is well known. Earlier [1] and recent studies [2] with the polarizing microscope indicated that amyloid in certain locations, e.g., hepatic sinusoids, was laid down upon pre-existing reticulin fibers. In many electron microscopic studies where amyloid fibrils were noted within or at the borders of sinusoidal reticuloendothelial (RE) cells, and in relation to basement membranes, the topographic association of amyloid with reticulin (collagen) was either inconspicuous or inapparent.[3-8] Consequently the potential significance of the observations by polarizing microscopy in terms of the pathogenesis of amyloidosis has been minimized.[8] There seem, however, to be sufficient reasons to investigate these relationships further with other techniques. Some of these reasons include: (1) the final formation of amyloid has been attributed by many [8] to the activity of RE cells and strong evidence for this possibility has been reported recently by Cohen, Gross and Shirahama [9]; (2) the formation of those collagen fibers which are impregnated with silver and are PAS-positive (reticulin) and which line sinusoidal channels has been attributed to the RE cells related to these channels [10-13]; (3) agents known to affect collagen formation, e.g., cortisone and ascorbic acid, influence the formation of amyloid under experimental conditions.[8]

In the present study, phase microscopy of trichrome-stained sections from intact and partially hepatectomized mice with amyloidosis, and of hepatic amyloid and reticulin isolated by centrifugation was utilized and revealed amyloid fibers with unusual clarity. The results have confirmed and extended previous observations by polarization microscopy and have shown a rather constant and characteristic topographic relationship be-

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tween amyloid and reticulin fibers. In addition, an apparent structural basis for amyloid "stars" is proposed.

Material and Methods

Tissues were selected from over 100 male mice, $C_{57}BL/10J$ strain, used in previous experiments.[14] Amyloidosis was induced in 60 of these by casein injections. The tissues were fixed for 24 hours in cold (2 to 4° C) 4 per cent formaldehyde in 0.1M phosphate buffer, containing 0.22M sucrose and then washed overnight in cold distilled water. Blocks were either embedded in paraffin or frozen for sectioning at 3 to 6 and 5 to 10 μ, respectively. Selected unfixed tissues were sectioned in a cryostat.

Partial Hepatectomy. During ether anesthesia the median and left lateral lobes of the liver were removed from 6 mice with hepatic amyloidosis. Two mice died after 18 to 24 hours; 2 each were killed at 48 and 72 hours after hepatectomy. All liver remnants were processed for paraffin sections. Four normal mice subjected to the partial hepatectomy served as controls.

Isolation of Hepatic Amyloid and Reticulin. The conditions of isolation were initially established by injecting 0.3 ml 0.5 gm per cent (w/v) Congo red in water intravenously into 2 amyloidotic mice. After 1 hour, the mice were killed and selective Congo red staining of tissue amyloid was noted grossly and confirmed in cryostat sections. This staining served as a "marker" so that the partition of amyloid could be followed visually during centrifugation procedures. When applied to livers of mice not given injections of Congo red, the technique, a modification of the procedure of Krakower and Greenspon[15] for isolating basement membranes, permitted abundant recovery of amyloid and reticulin.

The median and left lateral hepatic lobes were removed and cut into 2 to 3 mm slices; these were taken about 3 mm from the hilar regions to avoid larger vessels. The capsule was removed and the slices were washed briefly in ice cold 0.9 per cent (w/v) saline, and then frozen and cut into sections about 70 to 100 μ thick with a freezing microtome. The sections were transferred to a 50 ml tube containing 40 ml of distilled water with 0.5 per cent (v/v) Triton® X-100. This was kept at 2 to 4° C for 2 to 3 hours, with vigorous shaking at intervals to disrupt the sections, and then centrifuged at 121 × g for 5 minutes. After discarding the supernate, the sediment was re-suspended in the water-Triton® mixture and the procedure was repeated. The sediment was then re-suspended and washed 3 times in distilled water, centrifuging at 400 × g for 10 minutes each time. The final sediment, consisting of loosely packed, grayish-white fiber fragments, was re-suspended in about 5 ml water and small drops were placed on cleaned glass slides. The preparations were either cover-slipped and examined by phase microscopy, or dried overnight and stained.

The staining procedures and tissue preparations used in this study are listed in Table I. The first 5 stains were used routinely on paraffin sections from 70 mice. Serial sections of 6 paraffin blocks containing liver, adrenal and small intestine were examined after similar staining.

In addition to ordinary light microscopy, observations were made by phase microscopy of unstained preparations and of those stained by the trichrome and silver methods. Polarization microscopy was carried out with unstained or Congo red stained preparations mounted with either water or glycerol. Fluorescence microscopy was used after Congo red or thioflavine T staining.

Results

The severity, distribution and staining features of amyloid have been described.[14] The following observations are based on mice showing a moderate to advanced degree of amyloidosis, as well as on normal controls.

Reticulin in Hepatic Sinusoids

The term reticulin applies to those fine branching fibers in hepatic sinusoid lining which were impregnated with silver, showed a weak PAS reaction and stained with light green or aniline blue. Phase microscopy of silver-impregnated and trichrome-stained sections showed the fibers in great detail. In unstained frozen sections (after formaldehyde fixation) mounted with water the fibers showed a positive birefringence in

TABLE I

STAINING REACTIONS APPLIED TO LIVER PREPARATIONS

Stains	Tissue preparations *	Reference
Congo red	P, F, C, I	16
Crystal violet	P, F, C, I	17
Thioflavine T	P, F, C, I	18
Indole (tryptophane)	P, I	19
Periodic acid-cold Schiff	P, F, C, I	20
Toluidine blue	P, F, C	21
Gomori trichrome	P, I	22
Cason trichrome	P	21
Gordon-Sweet's reticulin	P, C, I	23
Sudan black B	P, F, C, I	17
Hemoglobin peroxidase	P	20

* P, paraffin sections; F, formaldehyde-fixed frozen sections; C, unfixed cryostat sections; I, isolated preparations.

polarized light. This was abolished by glycerol mounting.[1,24] These staining properties were retained in isolated hepatic reticulin. New observations on the arrangement of hepatic reticulin fibers and their relationship to the sinusoidal basement membranes will be published separately. The reticulin in adrenal sinusoids and in capillaries of the small intestine exhibited staining features identical to those noted in the liver.

Amyloid in Intact Liver Sections

General features. The amyloid stained consistently with Congo red, thioflavine T, light green and aniline blue, and showed a positive indole reaction as well as alcohol-labile metachromasia with toluidine blue and crystal violet. It was weakly PAS-positive, occasionally sudanophilic and always non-argyrophilic. With polarized light, Congo red-stained amyloid exhibited birefringence and dichroism when mounted in either glycerol or water. Amyloid in other organs stained in an identical manner.

The intensity of staining of hepatic amyloid with most of these dyes or reactions was dependent upon the manner of tissue preparation (Table I), being strongest in unfixed cryostat sections, intermediate in frozen, formaldehyde fixed sections and weakest in paraffin sections. These observations confirm those made by Pearse[17] and others.[25]

The structural characteristics of amyloid fibers were best seen by

phase microscopy of paraffin sections stained by the light green component of the Gomori trichrome method. The following observations were based predominantly on this method of examination.

Amyloid Fibers in Portal Areas. Amyloid formation was initially greatest around portal veins and periportal sinusoids (Fig. 1); after prolonged casein injections all sinusoids showed amyloid involvement (Fig. 1), with widening of the spaces of Disse. In relation to veins, the amyloid fibers, which were much finer than reticulin, were relatively short, uniform and, although haphazardly arranged in many areas, were stacked predominantly in parallel arrays (Figs. 2 and 3). The fibers were orientated perpendicularly or radially, depending upon the plane of sectioning, to the venous reticulin fibers and extended predominantly away from the lumens (Figs. 2 and 3). This relationship to reticulin was confirmed by both conventional and phase microscopy of silver-stained sections and by the disappearance of reticulin birefringence in unstained frozen sections mounted in glycerol.

Amyloid fibers in the periportal sinusoids, were longer and extended into the vascular lumens, sometimes in association with apparently detached endothelial cells and "trapped" red cells. Most, however, traversed widened perisinusoidal spaces (Disse) starting from the sinusoidal reticulin. This arrangement imparted a corona-like appearance to the sinusoids in cross section (Figs. 3 and 4). Not uncommonly, short segments of reticulin appeared as lines with small nodosities, each continuous with an individual amyloid fiber (Fig. 5). Often the fibers were separated from reticulin by a thin "clear" line (Figs. 3 and 4) which, although free from detectable fibers, showed the staining characteristics of amyloid. The involved reticulin was often thickened, split or fragmented (Figs. 2 to 6). The fibers sometimes failed to bind silver and, in addition, focal segments occasionally showed one or more of the following features: a weak indole reaction, metachromasia with toluidine blue, fluorescence with thioflavine T, binding of Congo red, sudanophilia, and a birefringence either after Congo red or in unstained preparations, the latter not always abolished by glycerol mounting.

Intact red cells often enmeshed in amyloid fibers were readily recognizable. Frequently, eosin-staining particles were seen and identified as red cell fragments on the basis of a positive hemoglobin peroxidase reaction.

Sinusoidal Amyloid and Amyloid "Stars". In other zones of the hepatic lobules, the amyloid-reticulin relationships were similar to those described in the periportal sinusoids. Because of the greater branching or anastomosis of sinusoids, however, various amyloid profiles were observed depending upon sectioning planes. Many included the corona-like appearance of amyloid fibers radially orientated to the reticulin ring of

sinusoids (Fig. 3). This circumferential distribution appeared to provide a basis for the star-like arrangement of amyloid fibers first illustrated by Kuczynski[26] and called amyloid "stars" by Jaffé.[27] When a sinusoid was sectioned at a point where it coursed from the plane of sectioning, the amyloid fibers appeared as an incomplete corona or "star" (Fig. 7; compare to Fig. 3). Again the sinusoidal reticulin often showed focal fragmentation. When such an area (Fig. 7) was viewed in cross section by phase microscopy the normal ring of reticulin fibers encircling the sinusoid was inapparent but a ring of fragmented residues persisted (Fig. 8), forming the central focus of the amyloid fibers. The identity of these fragments as reticulin was based on size, binding of aniline blue or light green, a persistent birefringence occasionally abolished by glycerol in unstained sections, and by detection of eventual continuity with intact reticulin fibers by serial sectioning; argyrophilia was inconsistent and could not be relied upon as a marker for reticulin. On occasion such "stars" were unassociated with any detectable reticulin remnants; serial sectioning showed, however, that they merely represented amyloid fibers radially orientated to reticulin but with the level of sectioning falling short of their attachments (Fig. 9; compare to Fig. 7).

There were many smaller "stars" whose structural basis did not depend upon a similar circumferential arrangement of amyloid fibers around the entire sinusoid. When examined with ordinary light after a variety of stains, these "stars" appeared to lie free within sinusoids or in the space of Disse. By phase microscopy, however, single reticulin fibers were found in their centers (Fig. 10). The single fibers appeared to correspond to those normally seen in the spaces of Disse and sometimes referred to as "reinforcement" fibers.

Finally, in other small "stars", reticulin fibers could not be detected even with phase microscopy. When, however, these "stars" were examined in serial section, reticulin fibers were usually found (Figs. 11 to 13).

Amyloid "Stars" in Other Organs

The structural features of amyloid "stars" in other organs were identical to those seen in the liver. In the adrenals, they were located in the zona reticularis (Fig. 14) and, by serial sectioning, consisted of amyloid fibers radially attached to the sinusoidal reticulin (Fig. 15). A similar arrangement was found in capillaries in the villi of the small intestine (Fig. 16), and in the spleen. Here, however, their relationship to reticulin was quite difficult to determine because of the normally complex reticulin patterns.

Amyloid in Hepatic Regeneration

The effect of amyloidosis on the hepatic regeneration rate after partial hepatectomy was not investigated. There were no distinct quantitative

changes in the severity of amyloidosis in the liver remnants as compared to the lobes which had been removed. There were, however, qualitative changes in the amyloid after 48 hours. These consisted of increased intensity of PAS staining, and of a variable argyrophilia not seen in the amyloid of non-hepatectomized mice.

Isolated Hepatic Amyloid

The material isolated (Fig. 17), on microscopic examination, consisted of amyloid, reticulin and segments of veins (or arteries) and was virtually free of hepatic cells or cellular debris. The amyloid retained all of the staining features noted in tissue sections (Fig. 18), although the coloring by light green and aniline blue was decreased slightly.

Orientation with respect to over-all hepatic structure was obviously not possible in the slide preparations. On the basis of reticulin fiber sizes, and the preservation of some structural features, permitted by the use of thick-frozen sections as a starting substance for isolation, comparisons with intact sections were often possible. As in the latter, parallel arrays of amyloid fibers around "spaces" conforming to the size and shape of sinusoids were apparent (Figs. 19 and 20). The perpendicular or radial orientations of parallel amyloid fibers to the reticulin lining these spaces were also obvious, depending upon whether the "spaces" were visualized in longitudinal or circular profile (Figs. 21, 22 and 23a, b). The corona-like image of some of these also indicated that the spaces corresponded to sinusoids cut in cross section (compare Fig. 21 to Fig. 3).

Short amyloid fibers were often clustered near or at the end of single reticulin fibers. Arranged in uneven parallel rows, these amyloid fibers appeared, by multiplanar focusing, to radiate from the entire circumference of a single reticulin fiber, much in the manner of branches from a tree trunk (Fig. 24). They appeared to correspond to the small amyloid "stars" in intact sections (Fig. 13). Focal arrangements of amyloid fibers were noted along the course of single reticulin fibers (Fig. 25), as in the tissue sections (Fig. 26), and where amyloid fibers were separated from the reticulin by a "clear" line.

Discussion

It is clear that amyloid fibers, in mouse tissue sections, show a relatively constant relationship to reticulin in different organs, i.e., a perpendicular or radial orientation of parallel bundles to reticulin fibers. Although previously demonstrated by polarizing microscopy in Congo red-stained amyloid,[1,2] the relationship was seen with greater clarity by the technique reported here. In addition, an apparent structural continuity between the two types of fibers was often visualized. While histologic

orientation of the isolated amyloid and reticulin was difficult, the utilization of thick frozen sections[15] for isolation of fibers did permit some preservation of vascular and sinusoidal structure. In these preparations, the amyloid-reticulin orientation featuring intact sections was retained. Such an orientation also appeared to provide a structural basis for the star-like arrangements of amyloid fibers.[26,27]

While the topographic relationships of amyloid and reticulin are clear, their pathogenetic significance is less apparent. It is highly unlikely, for reasons discussed by Caesar[4] and Cohen,[8] that preformed reticulin is transformed into amyloid.[28] Furthermore to connote, as some have,[29] a cytogenetic relationship of amyloid and reticulin fibers from these static structural associations would appear to be unjustifiable, particularly since ultrastructural and analytical data indicate little or no similarity between the two fiber types.[8] Such dissimilarities in the final fibers, however, need not exclude the possibility that they could arise from the same class of cells. The subsequent discussion will bear upon those features reported here which suggest that the amyloid-reticulin topography may be more than fortuitous. Consideration will be given, moreover, to possible reasons for the differences between light and electron microscopic observations of the association of these fibers.

Shortly after partial hepatectomy in otherwise normal rats, Aterman observed, in the remaining lobes, an increased prominence of sinusoidal cells and of the sinusoidal "basement membranes", the latter by virtue of increased PAS staining.[30] This observation was considered in terms of basement membrane changes accompanying increasing liver mass due to hepatic cell regeneration.[30,31] Others have noted similar features in Kupffer cells and in sinusoidal membranes during repair of hepatic injury accompanied by increased reticulin formation.[32–34] While RE cells have been characterized functionally by their phagocytic capacity, it has been either implied, suggested or stated[10–13,31,33,35,36] that they may assume the role of fibroblasts. These may form collagen, which in certain locations, i.e., "basement membranes", is PAS-positive and argyrophilic by reason of a ground substance and which, in these locations, is termed reticulin (the original reticulum of Mall). In the present study hepatectomy in normal mice substantiated Aterman's observation and in addition revealed increased argyrophilia in the sinusoidal reticulin. Although quantitative changes in amyloid were not detected in regenerating livers with amyloidosis, the amyloid showed an increased intensity of PAS staining and, for the first time with the method employed, variable argyrophilia. The exact meaning of these changes, which will be studied further, is not known but it is suggested that the relationship between amyloid and reticulin may extend beyond topography alone.

An apparent continuity or, at least, a definite abutment of tips of amyloid fibers on reticulin was repeatedly observed. With few exceptions[4,5] this contrasts with many ultrastructural observations[8] which neither include nor emphasize the reticulin relationships either in basement membranes involved by amyloid or in amyloid "stars". Reasons for this contradiction are probably many and extend beyond the question of technical differences. That changes in reticulin during amyloid deposition are sufficient to obscure or mask its ultrastructural recognition might be one reason. The reticulin to which amyloid fibers were attached often stained in a manner identical to that of amyloid. Whether or not this represented an intrinsic change in the reticulin fiber or merely a "coating" cannot be answered. Probably related to either possibility, however, was the loss of argyrophilia and the appearance of a birefringence not always abolished by glycerol in unstained sections. It is necessary to emphasize that this point is raised not to imply that preformed reticulin is transformed to amyloid, but that reticulin alterations may preclude its ultrastructural identification, at least in some amyloid deposits. Another feature which might be related to the apparent obscurity of reticulin in electron microscopic studies is the presence of granular or irregular sudanophilic and osmiophilic material in amyloid deposits. Recent chemical analysis of isolated amyloid fibrils has revealed a 5 per cent total lipid content but the possibility that this represented a contaminant was considered.[37] Chemical analysis of isolated reticulin fibers[38] and polarization studies of tissue sections[39] have also indicated that some normal reticulin fibers contain lipids. Considered together with other reticulin changes, i.e., fragmentation, loss of argyrophilia or normal refraction properties, the granular lipid deposits in amyloid foci may represent breakdown residues from altered reticulin. Other sources for nonspecific lipid deposition in amyloid, however, may also be conjectured. Hyperlipemia as a consequence of the nephrotic syndrome may represent one such source since serum changes compatible with this syndrome have been observed in experimental amyloidosis.[40] Breakdown of membranes of red cells entrapped in amyloid fibers may be another.

The topographic relationship of amyloid to reticulin fibers cannot, of course, be translated into pathogenetic conclusions since amyloid can form in or on the ground substance of basement membranes normally lacking collagen fibrils, e.g., the renal glomerulus. While it is thus possible that the emphasis on reticulin fibers in basement membranes studied is misplaced, the perpendicular orientations of amyloid fibers to these membranes are, nevertheless, of some interest since such an orientation of extracellular fibers to basement membranes is not unique to amyloid. A few electron microscopic studies have demonstrated parallel stacks

of collagen fibrils in the perisinusoidal spaces (of Disse) with their long axes perpendicular, or nearly so, to that of the sinusoidal membrane (Fig. 3, reference 34; Fig. 11, reference 41).

Fine filaments in various extracellular locations have recently been re-examined carefully by Haust.[42] These microfibrils occasionally found arranged in parallel bundles, intermixed with collagen fibrils or associated with fibroblasts, and orientated perpendicular to basement membranes. A role for these microfibrils in collagen formation was postulated. While perhaps sheer coincidence, the microfibrils and amyloid fibrils share certain features: extracellular location, topographic associations with collagen and basement membranes, perpendicular orientation to the latter, small size relative to collagen, and a beaded periodicity. The latter, however, has been inconstant and dependent on the method of tissue preparation. Benditt and Eriksen[43] have also commented upon the ultrastructural similarities of amyloid fibrils to filaments seen in collagen formation during wound healing.[35,44] Further comments on these similarities are merely speculative and pertain to the possibility that amyloid may represent an abnormal accumulation of a normal but ordinarily inconspicuous tissue component.[45] Since attention has been drawn more forcefully to the formation of amyloid by reticuloendothelial cells[9] and since these cells are apparently capable of forming collagen or its precursors, the extent to which "amyloid" and collagen synthesis may be related might deserve further experimental study. Further information about the mechanisms of reticulin (collagen) formation by normal RE cells, however, is obviously required before the elaboration of amyloid by these cells can be related to synthetic pathways for reticulin production, normal or abnormal.

Some investigators have implied or suggested that the final step in amyloid formation consists solely of the precipitation or fibrous conversion of immunoglobulins, with or without antigen[8] and that this occurs freely within the interstitial spaces without local cellular interaction. While polymerization of some globular proteins into fibrous proteins can occur under certain biologic conditions (for example, the conversion of fibrinogen to fibrin) or after manipulation *in vitro*,[7] objections to this theory for amyloid formation have been raised on various grounds,[8] including the failure to demonstrate fiber structure in gamma globulin-anti-gamma globulin precipitates.[6] The relatively constant orientation of amyloid fibers to sinusoidal reticulin, particularly in different organs, although not a serious argument against this theory, is also somewhat difficult to reconcile with it. Even if it is assumed that circulating globulins alone may polymerize into a fibrous protein, thus yielding a crystalline fiber structure, aggregates of such fibers might be expected to show

variations in the ultrastructural character of fibril bundles and of their orientation to surface structures. This anticipated variation would depend largely upon local hemodynamics, much in the same manner the character of an intravascular thrombus is influenced. It is possible, of course, that other local influences, e.g., surface factors or activity of reticuloendothelial cells, could determine the orientation of amyloid fibers arising from such a mechanism.

The method of amyloid isolation reported here is unsuitable for chemical studies of this protein since the samples contained reticulin fibers and vessels. It may be, however, a suitable starting point for further refinements leading to pure amyloid fiber isolation.

Summary

Amyloid, induced in mice by casein injections, was examined in tissue sections and in amyloid-reticulin preparations isolated from the liver by centrifugation. Observations were based on phase microscopy of sections stained with light green which revealed the characteristics of amyloid fibers with unusual clarity. These fibers were arranged in parallel arrays and were orientated toward reticulin fibers of sinusoidal and capillary walls in a fairly constant perpendicular and radial manner. Apparent continuities between the two fibers were observed. These orientations were preserved in unstained isolated preparations examined by phase-contrast microscopy. The star-like configurations of amyloid fibers, described by others, were apparently related to these amyloid-reticulin relationships.

In many amyloid deposits, reticulin fibers were fragmented and devoid of normal argyrophilia and polarizing properties. That these changes could obscure the ultrastructural recognition of reticulin (collagen) was considered as one possible reason why amyloid-reticulin associations have been inconspicuous in previous electron microscopic studies.

Partial hepatectomy was carried out in a small number of mice with hepatic amyloidosis. During early regeneration no increase in amyloid deposits was noted although amyloid fibers showed greater intensity of PAS staining and assumed an argyrophilic property. The similarity of these changes to those in reticulin under similar conditions suggested that the topographic orientation of amyloid and reticulin in amyloidosis may be more than coincidental.

The significance of the striking topography of amyloid and reticulin was discussed in terms of (a) previous proposals that both fiber types are formed by or derived from reticuloendothelial cells; (b) the similarity of these topographic relationships to those of other extracellular filaments or fibrils and basement membranes; and (c) the difficulties of

explaining such topographic orientation by the theory that amyloid forms from a simple precipitation of immunoglobulins in extracellular spaces.

References

1. Missmahl, H. P., and Hartwig, M. Polarisationsoptische Untersuchungen an der Amyloidsubstanz. *Virchow Arch. Path. Anat.*, 1953, **324**, 489–508.
2. Heller, H.; Missmahl, H. P.; Sohar, E., and Gafni, J. Amyloidosis: Its differentiation into peri-reticulin and peri-collagen types. *J. Path. Bact.*, 1964, **88**, 15–34.
3. Cohen, A. S.; Weiss, L., and Calkins, E. Electron microscopic observations of the spleen during the induction of experimental amyloidosis in the rabbit. *Amer. J. Path.*, 1960, **37**, 413–431.
4. Caesar, R. Die Feinstruktur von Milz und Leber bei experimenteller Amyloidose. *Z. Zellforsch.*, 1960, **52**, 653–673.
5. Heefner, W. A., and Sorenson, G. D. Experimental amyloidosis. I. Light and electron microscopic observations of spleen and lymph nodes. *Lab. Invest.*, 1962, **11**, 585–593.
6. Gueft, B., and Ghidoni, J. J. The site of formation and ultrastructure of amyloid. *Amer. J. Path.*, 1963, **43**, 837–854.
7. Sorenson, G. D.; Heefner, W. A., and Kirkpatrick, J. B. Experimental amyloidosis. II. Light and electron microscopic observations of liver. *Amer. J. Path.*, 1964, **44**, 629–644.
8. Cohen, A. S. The Constitution and Genesis of Amyloid. In: International Review of Experimental Pathology. Richter, G. W., and Epstein, M. A. (eds.) Academic Press, New York and London, 1965, Vol. 4, 159–243.
9. Cohen, A. S.; Gross, E., and Shirahama, J. The light and electron microscopic autoradiographic demonstration of local amyloid formation in spleen explants. *Amer. J. Path.*, 1965, **47**, 1079–1111.
10. Popper, H., and Schaffner, F. Liver: Structure and Function. The Blakiston Division, McGraw-Hill Book Co., New York, London and Toronto, 1957, p.156.
11. Rappaport, A. M. In: Diseases of the Liver. Schiff, L. (ed.). J. B. Lippincott Co., Philadelphia, 1963, p.23.
12. Bloom, W., and Fawcett, D. W. A Textbook of Histology. W. B. Saunders Co., Philadelphia and London, 1962, ed. 8, Chapt. 4, pp.85–111; Chapt. 25, p.478.
13. Ham, A. W. Histology. J. B. Lippincott Co., Philadelphia, 1961, ed. 4, Chapt. 17, p.362.
14. Janigan, D. T. Experimental amyloidosis. Studies with a modified casein method, casein hydrolysate and gelatin. *Amer. J. Path.*, 1965, **47**, 159–171.
15. Krakower, C. A., and Greenspon, S. A. The localization of the "nephrotoxic" antigen(s) in extraglomerular tissues. Observations including a measure of its concentration in certain locales. *Arch. Path. (Chicago)*, 1958, **66**, 364–383.
16. Puchtler, H.; Sweat, F., and Levine, M. On the binding of Congo red by amyloid. *J. Histochem. Cytochem.*, 1962, **10**, 355–364.
17. Pearse, A.G.E. Histochemistry, Theoretical and Applied. J.&A. Churchill, Ltd., London, 1960, ed. 2, p.844 and p.850.
18. Vassar, P. S., and Culling, C.F.A. Fluorescent stains, with special reference to amyloid and connective tissues. *Arch. Path. (Chicago)*, 1959, **68**, 487–498.
19. Glenner, G. G., and Lillie, R. D. The histochemical demonstration of indole

derivatives by the post-coupled *p*-dimethylaminobenzylidine reaction. *J. Histochem. Cytochem.*, 1957, 5, 279–296.

20. LILLIE, R. D. Histopathological Technic and Practical Histochemistry. The Blakiston Division, McGraw-Hill Book Co., New York, Toronto, Sydney, London, 1965, ed. 3, pp.270–271 and p.366.
21. MCMANUS, J.F.A., and MOWRY, R. W. Staining Methods, Histologic and Histochemical. Paul B. Hoeber, Inc., Medical Division of Harper & Brothers, New York, 1960, p.132 and p.234.
22. Manual of Histologic and Special Staining Technics. McGraw-Hill Book Co., Inc., New York, 1960, ed. 2, p.66.
23. GORDON, H., and SWEETS, H. H., JR. A simple method for the silver impregnation of reticulum. *Amer. J. Path.*, 1936, 12, 545–552.
24. BREWER, D. B. Differences in the fine structure of collagen and reticulin as revealed by the polarising microscope. *J. Path. Bact.*, 1957, 74, 371–385.
25. MCALPINE, J. C., and FULLER, A. P. Histochemistry of fresh frozen material in localised laryngeal amyloidosis. *J. Path. Bact.*, 1964, 88, 455–462.
26. KUCZYNSKI, M. H. Edwin Goldmanns Untersuchungen über celluläre Vorgänge im Gefolge des Verdauungsprozesses auf Grund nachgelassener Präparate dargestellt und durch neue Versuche ergänzt. *Virchow Arch. Path. Anat.*, 1922, 239, 185–302.
27. JAFFÉ, R. H. Amyloidosis produced by injections of proteins. *Arch. Path. (Chicago)*, 1926, 1, 25–36.
28. JONES, R. S., and FRAZIER, D. B. Primary cardiovascular amyloidosis: Its clinical manifestations, pathology and histogenesis. *Arch. Path. (Chicago)*, 1950, 50, 366–384.
29. CAMERON, G. R. Pathology of the cell. Oliver and Boyd, London, 1952, pp. 334–339.
30. ATERMAN, K. Some factors in the restoration of the rat's liver after partial hepatectomy. I. Glycogen; the golgi apparatus; sinusoidal cells; the basement membrane of the sinusoids. *Arch. Path. (Chicago)*, 1952, 53, 197–208.
31. ATERMAN, K. The Structure of Liver Sinusoids and the Sinusoidal Cells. In: The Liver: Morphology, Biochemistry, Physiology. ROUILLER, C. (ed.). Academic Press, New York and London, 1963, Vol. 1, pp.61–136.
32. POPPER, H.; HUTTERER, F.; KENT, G.; VAN DER NOEN, H. M.; PARENETTO, F.; SCHAFFNER, F.; SINGER, E. J., and ZAK, F. G. Hepatic fibrosis: Pathways and mechanism. *J. Mt. Sinai Hosp.*, 1958, 25, 378–390.
33. POPPER, H.; PARONETTO, F.; SCHAFFNER, F., and PEREZ, V. Studies on hepatic fibrosis. *Lab. Invest.*, 1961, 10, 265–290.
34. POPPER, H.; PARONETTO, F., and BARKA, T. PAS-positive structures of nonglycogenic character in normal and abnormal liver. *Arch. Path. (Chicago)*, 1960, 70, 300–313.
35. ROSS, R., and BENDITT, E. P. Wound healing and collagen formation. I. Sequential changes in components of guinea pig skin wounds observed in the electron microscope. *J. Biophys. & Biochem. Cytol.*, 1961, 11, 677–700.
36. PATRICK, R. S., and KENNEDY, J. S. The synthesis of sulphated mucopolysaccharide at sites of hepatic fibrosis induced by carbon tetrachloride, amyloidosis and the implantation of catgut. *J. Path. Bact.*, 1964, 88, 549–555.
37. KIM, I. C., and COHEN, A. S. An analysis of isolated amyloid fibrils for lipids. (Abstract) *Feb. Proc.*, 1965, 24, 366.
38. WINDRUM, G. M.; KENT, P. W., and EASTOE, J. E. The constitution of human renal reticulin. *Brit. J. Exp. Path.*, 1955, 36, 49–59.

39. MISSMAHL, H. P. Experimentelle und Klinische Beobachtungen über wechselnden Lipoidgehalt reticulärer Fasern in Leber, Milz und Niere. *Klin. Wschr.*, 1958, **36**, 29–34.

40. ABRUZZO, J. L.; GROSS, A. F., and CHRISTIAN, C. L. Studies on experimental amyloidosis. *Brit. J. Exp. Path.*, 1966, **47**, 52–59.

41. COSSEL, L. Beitrag zur Ultrastruktur der Blutgewebsgrenze in der Leber. (Elektronenmikroskopische Untersuchungen an Lebern von Mäusen und Leberpunktaten vom Menschen). *Beitr. Path. Anat.*, 1959, **120**, 133–158.

42. HAUST, M. D. Fine fibrils of extracellular space (microfibrils). Their structure and role in connective tissue organization. *Amer. J. Path.*, 1965, **47**, 1113–1137.

43. BENDITT, E. P., and ERIKSEN, N. Amyloid. II. Starch gel electrophoretic analysis of some proteins extracted from amyloid. *Arch. Path.(Chicago)*, 1964, **78**, 325–330.

44. ROSS, R., and BENDITT, E. P. Wound healing and collagen formation. II. Fine structure in experimental scurvy. *J. Cell Biol.*, 1962, **12**, 533–551.

45. BENDITT, E. P., and ERIKSEN, N. Amyloid. III. A protein related to the subunit structure of human amyloid fibrils. *Proc. Nat. Acad. Sci. (USA)*, 1966, **55**, 308–316.

[*Illustrations follow*]

Legends for Figures

Figures 1 to 16 and Figure 26 were prepared from paraffin sections of formalin fixed tissues, stained with Gomori's trichrome and photographed in phase contrast.

Fig. 1. The linear pale areas are amyloid fibers distributed along hepatic portal veins and periportal sinusoids. The round pale foci are amyloid fibers in sinusoids sectioned obliquely or across. × 175.

Fig. 2. A segment of a hepatic portal vein. Reticulin fibers of the vein are thickened and split or reduplicated. Finer amyloid fibers, arranged mostly in parallel arrays, are attached and perpendicularly orientated to the reticulin fiber on the right; this also shows focal discontinuities. × 980.

Fig. 3. Cross section of a small portal vein (or immediate sinusoidal tributary) at the left. The lumen contains red cells and endothelial cells. On either side of the thick encircling reticulin fiber, which is focally disrupted, extend parallel stacks of amyloid fibers. A segment of this vessel is shown in Figure 5 at higher magnification. The structure on right is a sinusoid in cross section. The radial orientation of amyloid fibers (in the markedly widened space of Disse) to the reticulin ring imparts a corona-like image. Amyloid fibers are more haphazardly arranged in the narrowed lumen where some red cells may be seen. × 1,100.

Fig. 4. A higher magnification of the sinusoid on the right in Figure 3. The relationship of the amyloid fibers to the reticulin is shown. A "clear" area is interposed between the two fiber types. × 1,850.

Fig. 5. A higher magnification of the vessel on the left in Figure 3. The nucleus of an endothelial cell (upper arrow) is separated from the parallel rows of amyloid fibers by a "clear" area. A few fibers, however, are in contact with the nucleus. Part of the reticulin encircling the vessel shows small nodosities (lower arrow) which are in alignment or contact with the amyloid fibers extending into the lumen. × 1,850.

Fig. 6. A serial section of the sinusoid shown in Figure 3. At this deeper level the encircling reticulin ring is fragmented with bead-like and linear remnants. The lumen is narrowed by amyloid and contains red cell fragments, identified by positive hemoglobin peroxidase reaction. × 1,100.

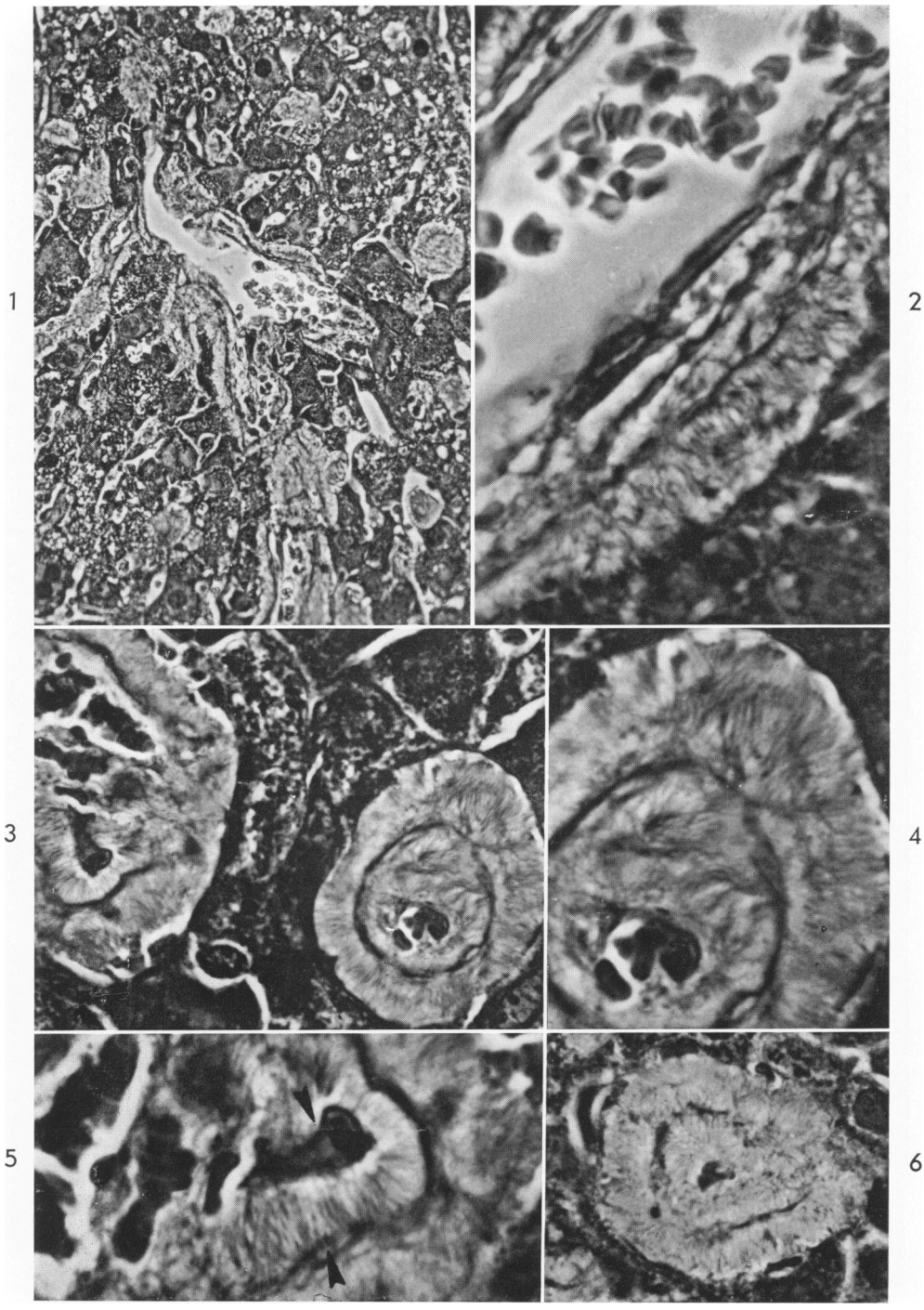
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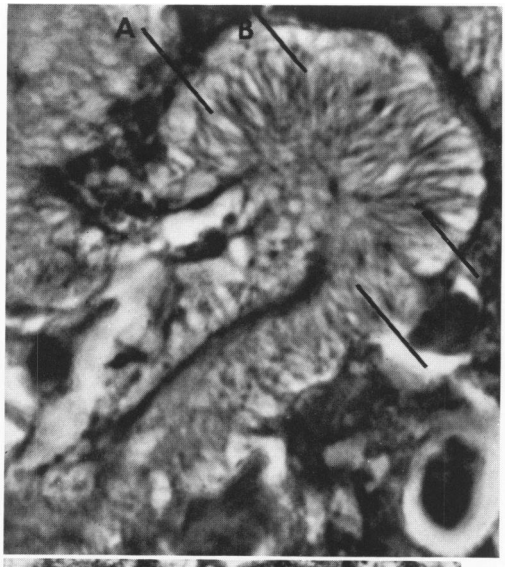
FIG. 7. A mid-zonal hepatic sinusoid sectioned obliquely. At the upper right the sinusoidal reticulin is focally discontinuous and the radially-arranged amyloid fibers in the space of Disse impart an incomplete corona or star-like image. Figure 8 represents a cross section across an area similar to that delineated by line "A" and Figure 9, an area through line "B". × 1,950.

FIG. 8. Amyloid "star". A cross section of a sinusoid where the normal reticulin ring is fragmented (see text). Amyloid fibers exhibit radial or star-like orientation. Two Kupffer cells appear at the left of the "star". × 1,950.

FIG. 9. An amyloid "star" in the space of Disse. The amyloid fibers appear free and to be filling a sinusoid. Serial sections showed, however, that these fibers were sectioned at a level peripheral to their points of attachment to sinusoidal reticulin. Compare to the plane of line "B" in Figure 7. × 1,300.

FIG. 10. A small amyloid "star". A single reticulin fiber in a widened space of Disse forms the core or line of attachments for finer amyloid fibers. To the left of the "star" is the sinusoidal lumen with two Kupffer cells and a red cell. The Kupffer cell to the right of the lumen is in contact with amyloid fibers. × 1,000.

FIGS. 11 to 13. Small amyloid "stars". Serial sections; deeper, right to left, at 36-μ spacings. The apparently separate "stars" in Figure 11 become continuous in Figure 12 as the level of sectioning approaches the middle of the longitudinal axis of a small sinusoid. The lumen of the sinusoid is reached at a deeper level (solid line) in Figure 13 and is filled with amyloid fibers. A fine reticulin fiber is evident in the "star" at the top (arrow) and another "star" may be seen to emerge below and to the left of it, probably arising in a separate sinusoidal tributary. × 1,000.



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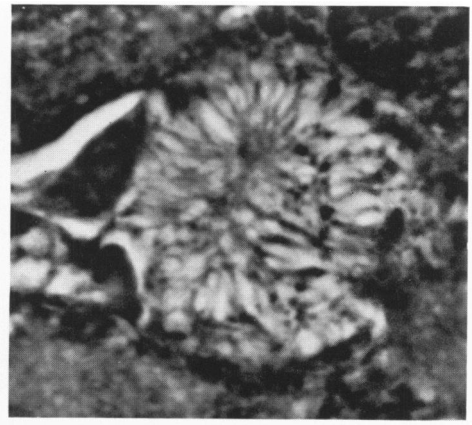

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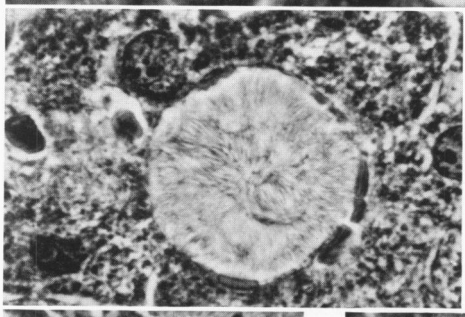

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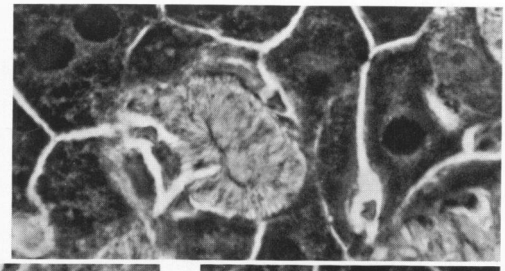

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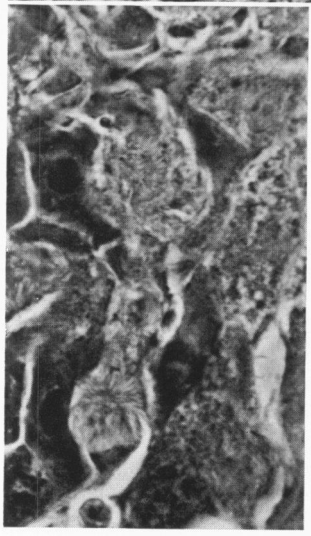

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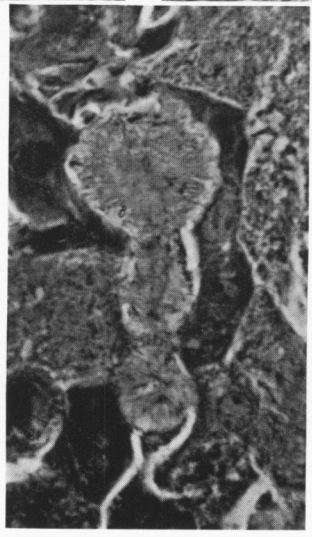

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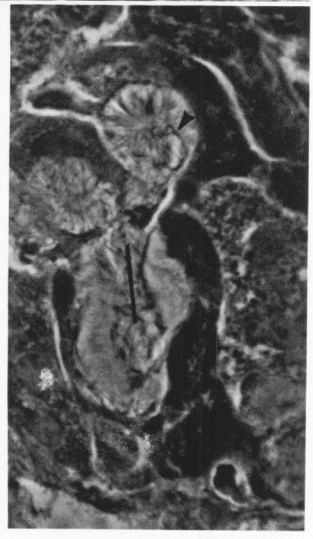

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FIG. 14. An amyloid "star" in the zona reticularis of the adrenal with a small area of medulla at the right edge. The "star" is apparently free in a distended sinusoid. × 800.

FIG. 15. A deeper serial section of Figure 14 shows an empty sinusoidal lumen (inserted arrow) delineated by a ring of reticulin with radially orientated amyloid fibers. The sinusoid is sectioned slightly obliquely and the amyloid fibers at the upper right of the lumen arise from a different level. There is an apparent continuity of reticulin and amyloid fibers near the tip of the arrow. × 800.

FIG. 16. Jejunal villus. Amyloid fibers exhibit a radial orientation to the wall of a small vessel in the villus stroma. Red cells appear in the lumen. Tangential sections of the amyloid fibers fall short of the vessel lumen, resulting in the appearance of amyloid "stars". × 900.

FIG. 17. Tube on left contains amyloid fibers isolated from the liver of an amyloidotic mouse; tube on right contains reticulin fibers isolated from the liver of a normal mouse.

FIG. 18. Isolated amyloid. The preparation was dried on a slide and stained with Congo red and by the Gordon-Sweet silver technique. Thick, dark areas are amyloid fibers distributed along reticulin fibers, many of which are inconspicuous at this magnification. The longitudinal profiles correspond to the long axis of the sinusoids. Thick reticulin fibers are relatively free of amyloid. Approx. × 150.

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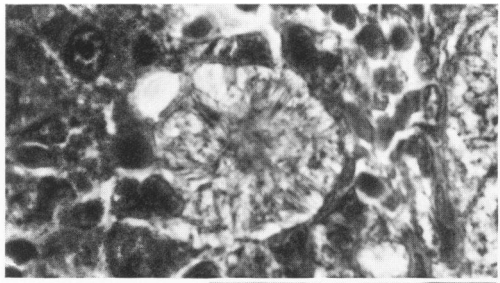

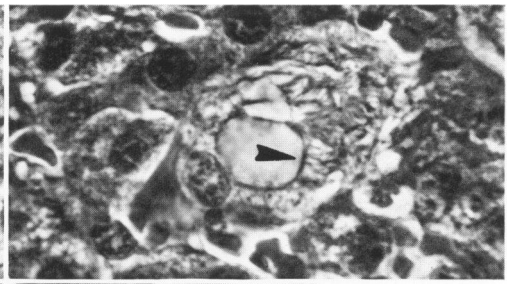

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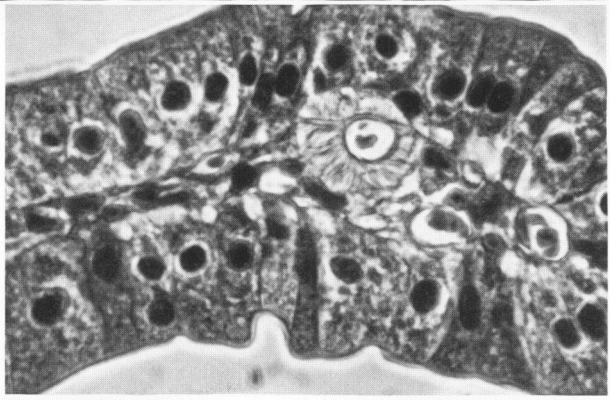

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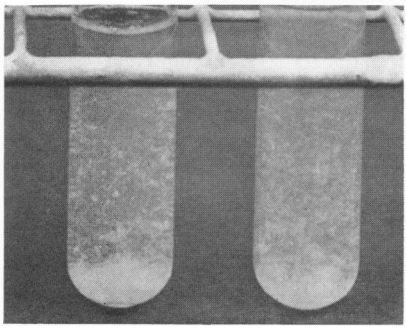

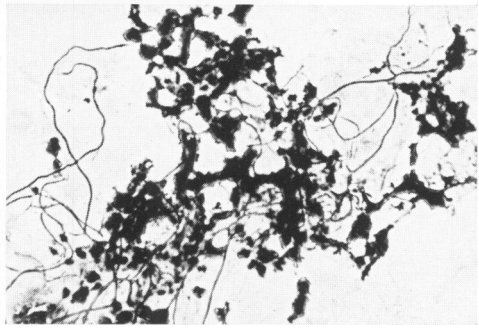

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Figures 19 to 25 were prepared from isolated amyloid, mounted unstained on slides with water and photographed by phase contrast.

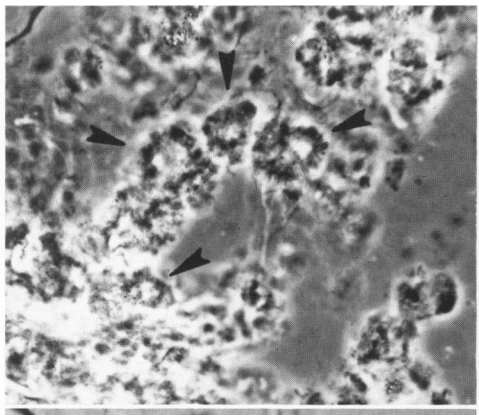
FIG. 19. Amyloid fibers exhibit radial orientation around circular "spaces" (arrows) which are interpreted as cross sections through sinusoids (compare with Fig. 21). Other collections of amyloid are in a slightly different plane of focus. Fine and thick reticulin fibers are present and there is a segment of a vein occupying the lower left corner. × 450.

FIG. 20. Features are similar to those described in Figure 19 but there is also orientation of the amyloid fibers to the longitudinal "spaces" (arrows) interpreted as sinusoids (compare with Fig. 22). × 450.

FIG. 21. Framed within a mesh of thick reticulin fibers is a circular profile of amyloid fibers radially orientated to a central "space". Lining a segment of this space is part of a reticulin fiber (arrow). By focusing, the entire space was found to be lined by this reticulin; the structure is considered to be a sinusoid in cross section. (See Figs. 23 a, b). × 1,070.

FIG. 22. The structure in the middle is composed of two spaced reticulin fibers running parallel. Associated with these fibers are parallel and haphazard arrays of amyloid fibers. This structure is felt to represent a portion of a sinusoid sectioned longitudinally. A vein occupies the lower part of the illustration. × 1,070.

FIG. 23. (a) One end of the reticulin fiber, running diagonally from top left to center, forms a dipper-like curve where amyloid fibers are attached. At this level of focusing the reticulin fiber (arrows) is only partially visible. (b) The same field as that shown in (a) but at a different plane of focusing. The diagonal portion of the reticulin fiber is out of the plane of focus but the curved portion (arrows) and its relationship to the amyloid fibers are more clearly evident. × 1,070.



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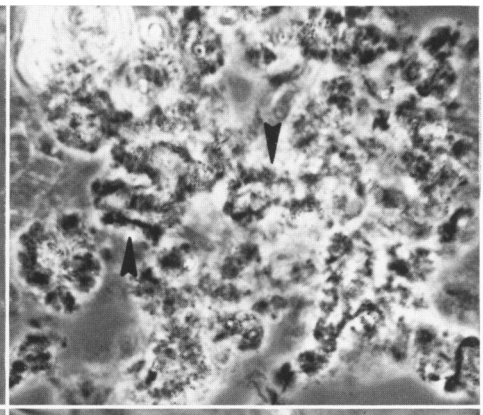

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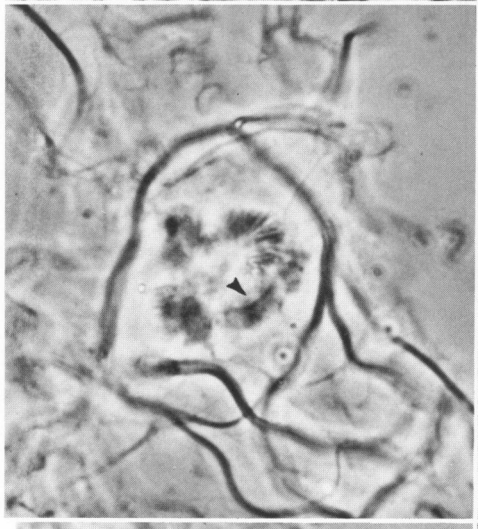

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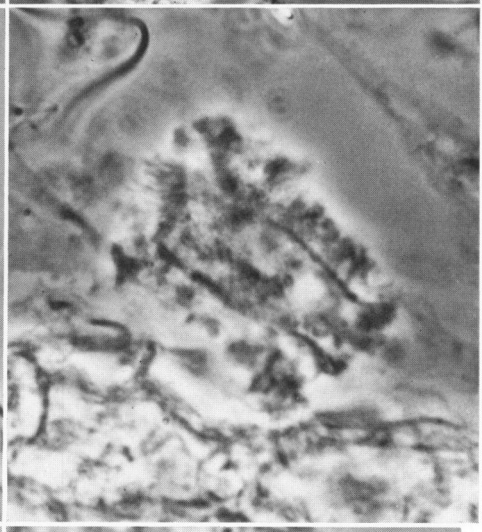

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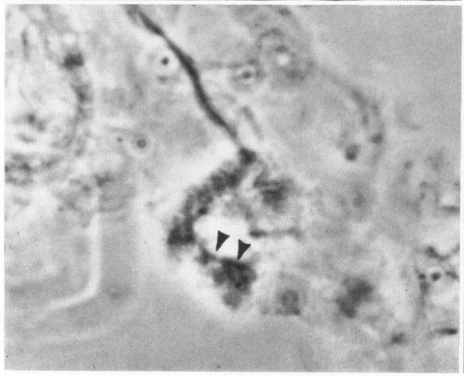

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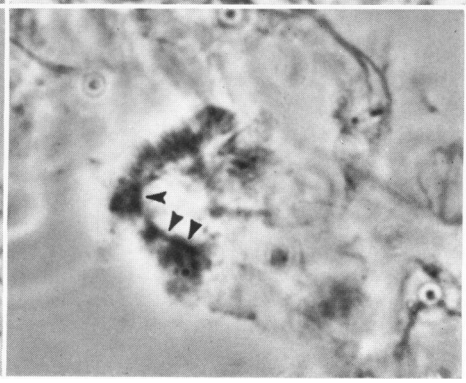

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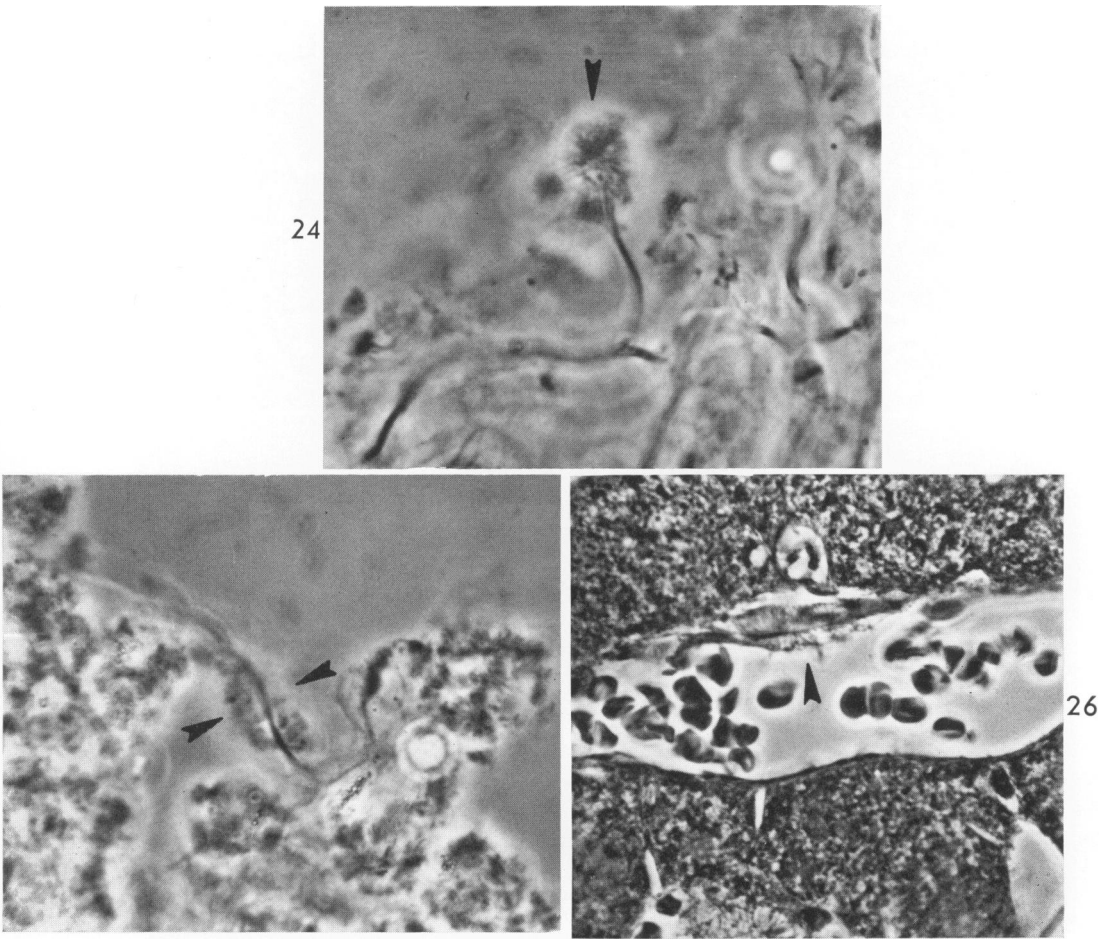

FIG. 24. Near or at the tip of a reticulin fiber is a star-shaped cluster of amyloid fibers (arrow). Compare to Figure 13. × 1,070.

FIG. 25. In the center is a reticulin fiber bordered on either side by amyloid fibers (arrows). The amyloid fibers are focally separated from the former by a "clear" line. The rest of the field is occupied by amyloid slightly out of focus. Compare with Figure 26. × 1,070.

FIG. 26. An intact liver section shows a periportal sinusoid. Parallel stacks of amyloid fibers are orientated on both sides of a segment of the sinusoidal reticulin (arrow). Compare to Figure 25 which shows similar features in an isolated preparation. × 900.