THE ISOLATION OF AMYLOID FIBRILS AND A STUDY OF THE EFFECT OF COLLAGENASE AND HYALURONIDASE

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The chemical nature of amyloid has yet to be defined by precise analytic techniques. One explanation for this fact has been the lack of criteria, other than histologic stains, by which the material being analyzed could be identified. In the past several years, it has been demonstrated that in sections of material fixed in osmium tetroxide and embedded in methacrylate amyloid has a fibrous appearance in the electron microscope. This observation, initially made in sections from rabbits with experimentally induced amyloidosis, has been corroborated in studies of amyloid obtained from patients with secondary amyloidosis (kidney), primary amyloidosis (skin), and genetically determined amyloidosis accompanying familial Mediterranean fever (kidney) (1–5). Analogous fibrillar components have not been noted in hyaline deposits of other sorts.

Further studies of the nature of these fibrils and their relationship to amyloid as a whole is predicated upon the development of methods for obtaining the fibrils in relatively pure form. The present report describes a method for isolating the fibrils by differential centrifugation. It also describes the effects of exposure of the fibrils to collagenase and hyaluronidase.

MATERIALS AND METHODS

1. Separation

Amyloid-laden hepatic tissue, obtained at postmortem from a patient with rheumatoid spondylitis, was homogenized in physiologic saline solution with a Potter-Elvehjem homogenizer. One-gram aliquots were macerated in 2 ml of cold saline for 10 minutes, then spun in a Servall RC-2 refrigerated centrifuge (with SS-34 rotor) at 10,000 RPM (12,100 *g*) for 20 minutes at 0°C. The supernatant was decanted, and the sediment was resuspended in saline and respun. This procedure was repeated six times and the final supernatant again decanted. In this fashion, the liver homogenate was divided into three layers, each of which was carefully separated. Aliquots of each layer were diluted, suspended in distilled water on grids, stained with 1 per cent aqueous phosphotungstic acid, and examined in the electron microscope, both before and after shadowing with chromium or platinum-palladium. Subsequently, the three layers were individually washed six times, dialyzed against distilled water, lyophilized, and stored at 0°C. The fractionation scheme is outlined in Fig. 1.

The separatory procedures were repeated on the livers of two patients with secondary amyloidosis and one with primary amyloidosis, as well as on three normal livers obtained from patients who died of other causes. All layers were studied by electron microscopy.

2. Enzyme Studies

(a) COLLAGENASE: Five milligrams of collagenase (Worthington Chemicals) were added to 5 ml of Na_2HPO_4-NaH_2PO_4 buffer, 0.1 M, pH 7.5. To this mixture 2.8 mg of $CaCl_2$ were added, making it 0.005 M with respect to calcium. The collagenase suspension was spun in a refrigerated centrifuge for 60 minutes at 30,000 RPM and the clear supernatant decanted for further use. The activity of the enzyme was assayed by mixing it with known collagen fibrils (courtesy Dr. J. Gross) and incubating at 37°C in a shaking water-bath for 5 hours. A control of buffer

alone was also mixed with collagen and treated in a similar manner. In a separate experiment, amyloid fibrils were pretreated with hyaluronidase (as outlined below) prior to collagenase treatment.

In separate tubes, 2.5 mg of each of the lyophilized layers (3 layers) of the amyloid-laden liver and of the normal liver (two layers) were mixed with 0.25 ml of the collagenase. One tube was incubated for 1 hour, one for 5 hours, one for 12 hours, one for 24 hours, and one for 48 hours, at 37°C, in a shaking water-bath. A small amount of toluene was layered over the 24- and 48-hour digestion mixtures to prevent bacterial contamination. Appropriate controls were also studied, as listed below.

1. Lyophilized top layer of amyloid-laden liver and buffer (control)

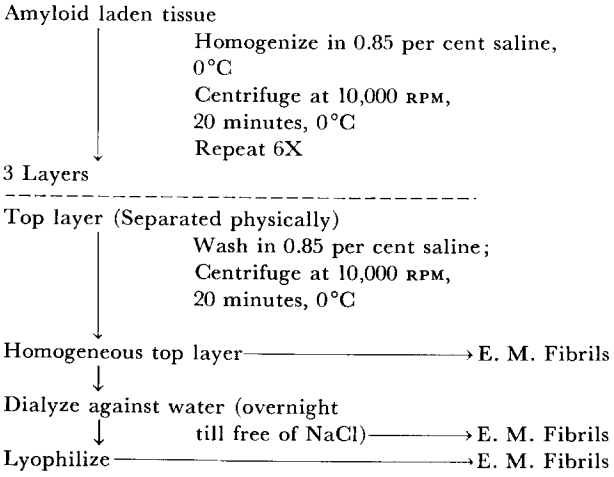

FIGURE 1 Amyloid extraction procedure.

2. Lyophilized top layer of amyloid-laden liver plus collagenase and buffer
3. Collagenase plus buffer alone (control)
4. Lyophilized middle layer of amyloid-laden liver and buffer (control)
5. Lyophilized middle layer of amyloid-laden liver plus collagenase and buffer
6. Lyophilized bottom layer of amyloid-laden liver and buffer (control)
7. Lyophilized bottom layer of amyloid-laden liver plus collagenase and buffer

All tubes were made up in multiple sets: *i.e.*, one set was incubated for 1 hour, one for 5 hours, one for 12 hours, one for 24 hours and one for 48 hours. An additional set of tubes 1 to 3 was also prepared and treated in the same way for use in dry weight determinations.

(b) HYALURONIDASE: Two milligrams of hyaluronidase (Worthington) (300 U.S.P. units/mg) were added to 10 ml of 0.02 M NaH_2PO_4-Na_2HPO_4 buffer that contained 0.45 per cent NaCl at a pH of 7.0. One milliliter of the enzyme solution was added to each of the separated layers of the amyloid-laden liver. Appropriate controls were prepared as in the above experiments: one set of tubes was incubated at 37°C for 30 minutes, one for 45 minutes, one for 60 minutes, and one for 90 minutes. An additional set of tubes 1 to 3 was also prepared in this experiment, again for dry weight determinations.

In both enzyme experiments, after the period of incubation had ended, the tubes were centrifuged at 18,000 RPM for 30 minutes, the supernatant was decanted, and the sediment studied. It was washed and resuspended in either normal saline or distilled water. A drop of the suspension was then placed on a Formvar-coated copper grid for two minutes; the grid was gently dried, and a drop of 1 per cent aqueous phosphotungstic acid was placed on it for 2 minutes. The grid was then washed briskly in distilled water and examined in the electron microscope directly or after chromium or platinum-palladium shadowing.

In order to assess quantitatively the effect of the enzyme treatment on the isolated amyloid fibrils, the dry weight of each suspension in tubes 1 to 3 was determined initially and at a subsequent time interval. After centrifugation and decanting of the supernatant, the residue was washed in distilled water, respun at 18,000 RPM for 30 minutes, and dried in nitrogen (free of oxygen) at 80°C. The residue-containing tubes were then air-evacuated at room temperature for at least 24 hours and weighed on a Mettler Semi-micro balance. Subsequently, the tubes were washed clean, dried, air-evacuated in a similar manner, and weighed. The differences between the 2 weights represented the weights of the residues.

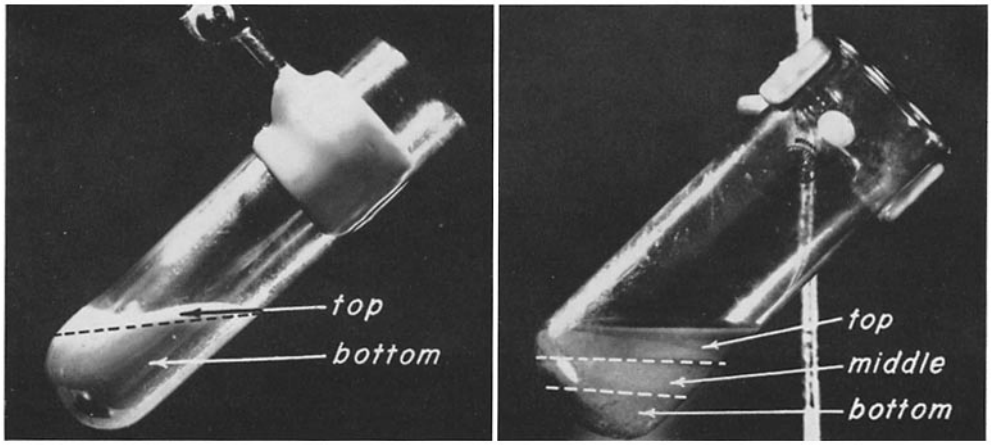

FIGURE 2 *Left:* Normal liver, 2 layers. *Right:* Amyloid-laden liver, 3 layers.
Dotted lines represent approximate division of layers, while the other lines on the photographs represent artifacts (shadows) produced by the centrifugation tubes.

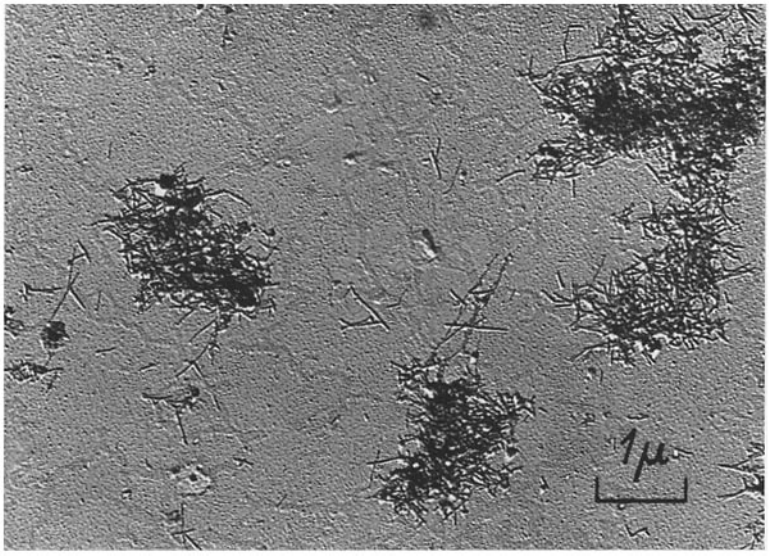

FIGURE 3 Fibrils, shadowed with platinum-palladium, obtained from the top layer of the amyloid-laden liver. ×12,000.

RESULTS

1. As a result of homogenization and centrifugation at 10,000 RPM, amyloid-laden livers could be fractionated into three distinct layers (Fig. 2), as opposed to normal livers in which two layers were demonstrated. The bottom layer of both normal and amyloid-laden livers was coarse and fibrous and had a brownish tinge. The middle layer of the amyloid livers and the top layer of the normal livers were comparable in that they had a granular brownish-orange appearance, while the top layer of the amyloid liver had an orange-khaki hue and a smooth, creamy consistency. A smear of the top layer was placed on a glass slide and was found to consist primarily of material that showed crystal violet metachromasia and stained with Congo red. This was not true of the two layers of normal liver, while the

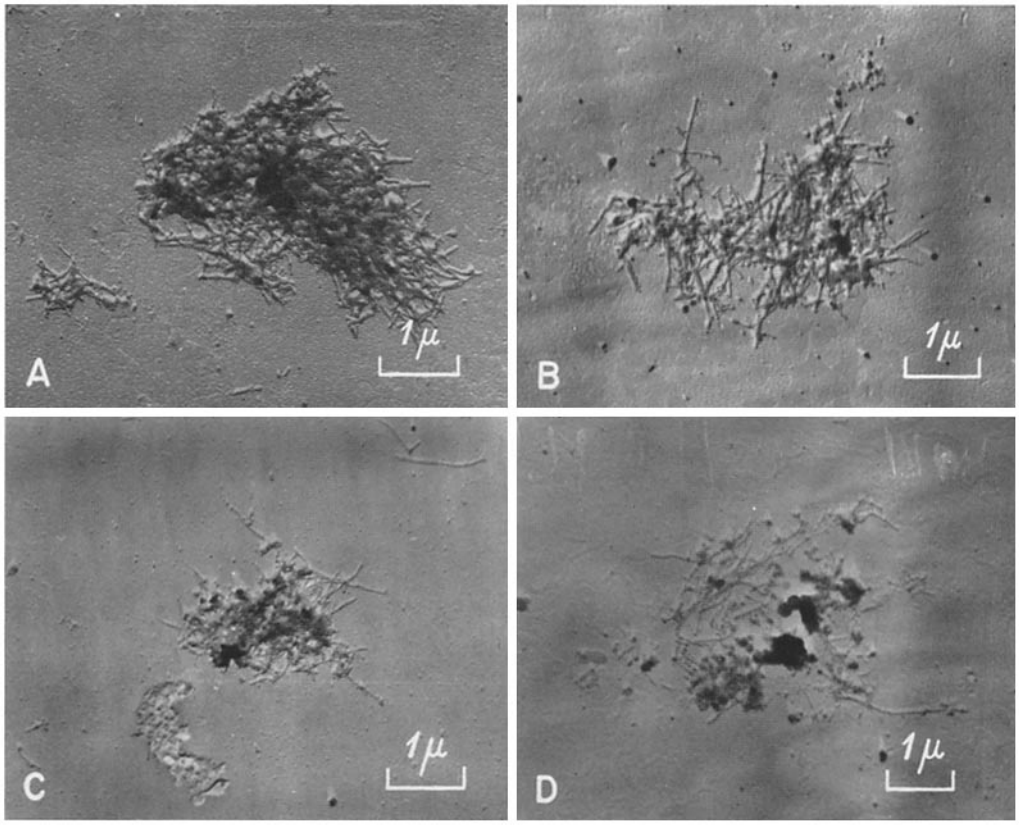

FIGURE 4 Results of collagenase digestion experiments (all preparations shadowed).

A. Control preparation of lyophilized, dialyzed amyloid fibrils incubated with buffer alone for 48 hours. ×10,000.

B. Same preparation as *A*, incubated with collagenase for 12 hours. ×9,800.

C. Same preparation as *A*, incubated with collagenase for 24 hours. ×10,000.

D. Same preparation as *A*, incubated with collagenase for 48 hours. ×8,750.

Note: Fibrils are present in all preparations.

middle layer of the amyloid liver demonstrated minimal amounts of crystal violet metachromasia and the bottom layer almost none.

In the electron microscope the top layer obtained from the amyloid liver was found to consist primarily of fine fibrils (Fig. 3) comparable to those seen in the osmium tetroxide-fixed, methacrylate-embedded amyloid previously described. The middle layer contained a few scattered fibrils and cellular debris, while the bottom layer contained nuclei, cellular debris, and virtually no fibrils.

2. Fibrils in roughly the same quantity were present in the collagenase digests as well as in the controls at the various time intervals (Fig. 4), and in the specimens pretreated with hyaluronidase. The hyaluronidase control material and enzyme-treated preparations also demonstrated abundant fibrils (Fig. 5). Dry weight determinations of the residues of the control and collagenase-treated fibrils at 0 and 5 hours indicated no significant differences in weight after exposure to the enzyme. Similarly, dry weight determinations in the hyaluronidase experiments at 0 and 45 minutes indicated that no digestion took place on exposure to the enzyme.

DISCUSSION

The structure and composition of amyloid has eluded definite description for well over 100 years. Polarization microscope studies carried out by Divry (6), Missmahl (7), and others (8) indicated

that an organized submicroscopic structure might be present. The suggestion has also been made that the birefringence of amyloid could be based on its deposition on or in relation to collagen and that amyloid might bear an intimate relationship to the collagen fibril. Although the electron microscopic appearance of amyloid as a fibrous moiety superficially supported this concept, it soon became apparent that the amyloid fibril differed significantly in its ultrastructure from collagen. In the present study, the resistance of the fibril to collagenase, under circumstances whereby known collagen fibrils are digested, further emphasizes the distinct differences that exist between these two classes of fibrils. It has also recently been demonstrated in our laboratories that the isolated amyloid fibril contains no hydroxyproline (9), the amino acid unique to collagen, while the glycine and proline contents are about 5.6 and 4.6 gm/100 gm, respectively. The lack of significant amounts of hydroxyproline in amyloid extracts has also been noted (10).

A number of investigators (11, 12) have commented upon the presence of mucopolysaccharide in amyloid, while other workers have indicated its absence on the basis of chemical analysis for uronic acids by a variety of methods (9). Since hyaluronate gels themselves are made up of microfibrils (13), the isolated amyloid fibrils were exposed to hyaluronidase with appropriate controls. The lack of digestion of the fibrils by the enzyme lends further credence to the hypothesis that hyaluronic acid is not a significant component of amyloid fibrils.

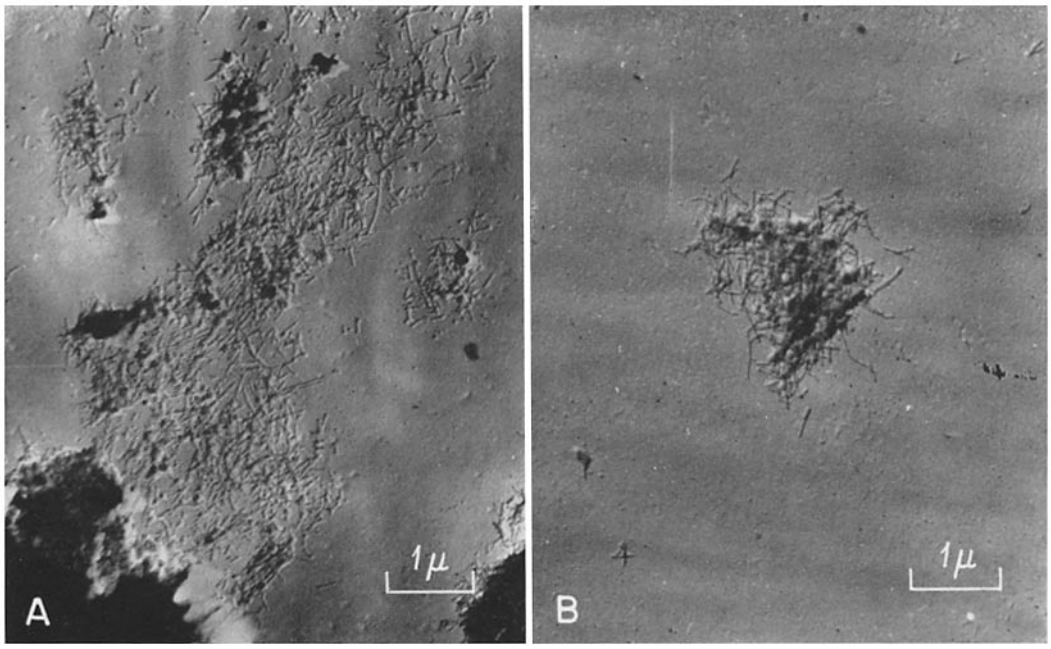

FIGURE 5 Results of hyaluronidase digestion experiments (all preparations shadowed).

A. Control preparation of lyophilized, dialyzed amyloid fibrils incubated without enzyme at 37°C for 45 minutes. ×11,000.

B. Same preparation incubated with hyaluronidase at 37°C for 45 minutes. ×12,000.

Note: Fibrils are present in both preparations.

SUMMARY

A method of isolation of amyloid fibrils from whole tissue is described. It was demonstrated that the enzymes collagenase and hyaluronidase, used separately with appropriate controls, did not affect the structural integrity of the amyloid fibril. This and other evidence indicate that the fibril, a major component of amyloid, is not collagen and does not contain significant amounts of hyaluronic acid.

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