NEW FEATURES OF AMYLOID FOUND AFTER DIGESTION WITH TRYSIN

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Since Cohen and Calkins in 1939 (6) described amyloid as composed of fibrils, there have been differing reports about the fibril width and the presence or size of linear or globular subunits in the fibril.

Originally, the width of the human amyloid fibril was estimated at 70 to 140 A. Later estimates of the width varied from 50 to 300 A (1, 2, 4, 5, 7, 12, 19-21, 23, 24). There have been relatively few high resolution studies of the individual fibrils (2, 3, 11, 19, 21-23). In these papers, estimates of the fibril width vary from 50 to 110 A.
The presence of subunit or subunits in amyloid has been suggested by the tissue section study of Ghidoni and Gueft (11), who found that the single fibril had a double line structure. Segmentation or beading of the fibril has also been seen by several investigators (12, 19, 21–22).

The digestion of amyloid with trypsin was undertaken to elucidate further the morphology of amyloid fibrils. Trypsin was used in an attempt to reveal a substructure.

MATERIAL AND METHODS
Crude and refined homogenates of amyloid were prepared from a 7500 g amyloid liver of a tuberculous patient. Paraffin sections stained with hematoxylin and eosin, crystal violet, and Congo red, and examined by light microscopy showed that approximately 50% of the liver was composed of amyloid. All layers of sediments following centrifugation were examined by light microscopy in smears stained with Congo red and crystal violet and in similarly stained thick sections of Epon-embedded material; and by electron microscopy in uranyl acetate-stained thin sections and in negative-contrast preparations (15).

Crude homogenate was prepared by treating 5 g of the liver in a Waring Blender in 100 ml of isotonic saline buffered to pH 7.2 with phosphate. Refined homogenate was prepared by a method similar to that of Cohen and Calkins (8), by treating 500 g of liver in a Waring Blender in 1000 ml of isotonic saline buffered to pH 7.2 with phosphate. This suspension was then centrifuged in an RC-2 refrigerated centrifuge at 18,000 rpm (21,780 g) at 0°C for 20 min, and a thin, creamy top layer of the sediment was collected. Two additional centrifugations yielded lessening amounts of this top layer. The combined weight of the top layers was 50 g or 10% of the weight of the liver used. Following each centrifugation, the larger bottom layer of sediment was brown. The top layers were combined and centrifuged again, yielding about 5 g of a still whiter top layer. The final top layer, which by electron microscopy was observed to be composed of about 85% fibrillar material, is called refined homogenate. The bottom brown layer contained a much smaller quantity of fibrillar material and a large amount of cellular debris. The top layer

![Figure 1](image-url) Figure 1 Crude amyloid, untreated. Amyloid fibrils are seen in clumps. Some are slightly more separated and seen more clearly. PTA staining. × 190,000.

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took the Congo red stain, but the bottom layer did not. Both the crude homogenate and refined homogenate were washed twice by suspending them in distilled water and centrifuging them in a clinical centrifuge at 3500 RPM (1780 g) for 20 min at 5°C. 5% suspensions, by weight, were prepared in Tris buffer, pH 8.0, from the final sediments. Crystalline trypsin (Worthington) was added to aliquots of both the crude and refined amyloid suspensions to make a concentration of 20 mg/ml. These preparations were incubated for 0, 2, 4, 6, 24, and 48 hr at 37°C with constant agitation by a magnetic stirrer. Similar preparations without trypsin constituted controls. These trypsin-treated and control materials were next centrifuged at 3500 RPM (1780 g) in a clinical centrifuge for 20 min at 5°C, washed three times with distilled water as described above, and studied by a negative-contrast technique, utilizing 2% potassium phosphotungstate (PTA) at pH 7.2 (15). A trypsin assay (hydrolysis of p-toluenesulfonyl-L-arginine methyl ester) revealed 158 units/µg of activity (14). Trypsin 20 mg/ml in buffer solution was examined directly by negative-contrast technique. Electron micrographs were recorded with an RCA EMU 3-C microscope at direct magnifications of 27,000 or 50,000 times, on Ilford N 60 plates.

OBSERVATIONS

The amyloid fibrils themselves in crude and refined homogenates had a similar electron microscope appearance. Refined homogenate showed somewhat more separation of fibrils. The fibrils were arranged in dense bundles rarely allowing clear visualization of individual fibrils (Fig. 1). These fibrils were approximately 100 Å wide (96 ± 22 Å). Trypsin digestion caused a loss of nonamyloid material in both the refined and crude homogenates as evaluated by amyloid stains of smears and thick Epon sections. This loss of non-amyloid material was obvious at 2 hr and reached a maximum at 24 hr. Amyloid material itself appeared to be preserved.

After 2 hr of trypsinization, both the crude and refined homogenates showed a separation of the fibrils of the dense bundles and a dispersion of fibrils. Often, fibrils 100 Å wide were clearly seen.
to be composed of two smaller strands measuring about 40 A wide (Fig. 2), separated by a space of up to 25 A (Fig. 3). In accordance with Mercer et al. (16) and Filshie et al. (10), we shall call these smaller strands filaments. In some areas, the paired filaments came together regularly in a denser, thin, single line at about 1000-A intervals, an appearance consistent with two twisted filaments (Fig. 4). These values are corrected measurements of our previously reported 135 A wide fibrils with twists at 500 A intervals (9). This pairing was more commonly seen in trypsinated crude homogenate than in refined material. One micrograph showed a fibril that appeared to be made up of four fibrillar elements (Fig. 4); the width of the fibril could not be measured. The trypsinated refined material showed a more random distribution of filaments varying from tangled masses to occasional pairing (Fig. 5). Individual filaments appeared to be composed of round beads, 40 A in diameter, arranged in tandem (Fig. 6). The beading was frequently seen in the trypsinated refined homogenate but rarely seen in the trypsinated crude homogenate.

After 4, 6, 24, and 48 hr of trypsinization of the suspensions, the fibrils became progressively more isolated. The characteristics of the fibrils were similar to those of the fibrils of the 2 hr material. At 48 hr there was no evidence of disintegration of amyloid fibrils. Using techniques identical with those in the experiment, trypsin with buffer was photographed at magnifications of up to 50,000 and could not be visualized.

**DISCUSSION**

The idea of a 100 A fibril composed of two 40 A filaments requires further discussion, especially since previous reports have not agreed upon the width of the amyloid fibril. This disagreement is not surprising if one considers that the various techniques were used on amyloid materials from different sources (2, 3, 12, 19, 21-23). In most studies, estimates of width fall within the range of 90 4- 40 A (1-4, 12, 21-24). More often, the re-
ports of fibril width indicate it to be relatively uniform in a given preparation (3, 4, 12, 21, 23), except for the reports of several studies by one group of investigators (7, 19). In one paper this group estimated the fibril width to be less than 300 Å (7), and in another paper they described the fibril as a structure formed by 1 to 8 side-by-side aggregated filaments each 75 ± 9 Å wide, the four-filament aggregation (300 Å wide) being the most frequently found set (19). This observation was interpreted as indicating a huge variation in fibril width from approximately 80 to 600 Å. Neither 300 Å fibrils nor such a great variation in fibril width have been reported by other investigators. Our observations, that the amyloid fibril is composed of two linear subunits, also finds support from studies of Epon and methacrylate sections in which one fibril was shown to consist of two dense lines (11, 12). The side-by-side arrangement of two filaments with apparent twisting may represent a helical structure of the amyloid fibril. Similar suppositions have been made for the fibrous proteins, keratin, actin, and praying mantis ootheca protein, on the basis of electron micrographs of purified material (10, 13, 17, 18). Fig. 4, in which one fibril appears to be made up of four lines, can be interpreted in two ways. It may merely represent a lateral aggregation of four twisted filaments, but it may also represent two filaments further subdivided into four smaller units.

Beadling or segmentation of amyloid fibrils at 40 Å intervals has been reported in studies in which the techniques of negative contrast (3) and section (12) have been used. A beading of 100 Å has also been found by similar techniques (19). The recent negative-contrast micrographs of Bladen et al. (3) show exceedingly high resolution of 105 Å wide rods segmented at 40 Å intervals, as well as separate 90 Å wide "doughnuts". The "doughnuts", in turn, appear to be made up of five globular units each 20 to 25 Å in diameter. Those authors propose that the rods are composed of these "doughnuts" or toroids stacked side-by-side,
and that the double-line segment images previously described may be lateral views of such arrays. It is difficult at present to reconcile these two sets of images, namely, the paired twisted filaments, and the segmented hollow rods. Several explanations are possible. Since the material we studied was obtained from the sediment and that studied by Bladen et al. was obtained from the supernatant, one explanation would be that fibrils and rods are different structures, both being a part of amyloid. A second possibility is that these rods are the same as the fibrils, but are seen in greater detail because of more favorable orientation in the supernatant. Also, one must consider that the "doughnuts" may be a nonamyloid constituent possibly associated with amyloid production.

These preliminary results indicate that trypsin breaks up dense bundles of amyloid into fibrils. Trypsin plus extensive homogenization and high-speed centrifugation breaks up this material even further, i.e., into filaments. From these observations, it is difficult to evaluate the role of trypsin accurately. It is suggested, however, that trypsin separates and alters fibrils in some fashion so as to bring out the finer details of the fibril. A similar effect was seen by Millard and Rudall (17) and Rudall (18) after trypsination of mantis oöthece protein. The ability of trypsin to separate fibrils suggests the presence of an interfibrillary substance in amyloid.

Summary

Negative-contrast studies of crude and purified human liver amyloid before and after treatment with trypsin have shown that the amyloid fibril is 96 ± 22 A wide, and that it is made up of two filamentous subunits each 40 A wide, with a variable interfilamentous spacing up to about 25 A. The filaments are beaded at 40 A intervals and seem to twist together in pairs at about 1000 A intervals to form the commonly seen fibrils.

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FIGURE 6 Pure amyloid, trypsinated. Individual filaments show beading at about 40-A intervals. A few paired filaments are still present. X 350,000.

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