

# HIGH-RESOLUTION ELECTRON MICROSCOPIC ANALYSIS OF THE AMYLOID FIBRIL

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## ABSTRACT

The ultrastructural organization of the fibrous component of amyloid has been analyzed by means of high resolution electron microscopy of negatively stained isolated amyloid fibrils and of positively stained amyloid fibrils in thin tissue sections. It was found that a number of subunits could be resolved according to their dimensions. The following structural organization is proposed. The amyloid *fibril*, the fibrous component of amyloid as seen in electron microscopy of thin tissue sections, consists of a number of filaments aggregated side-by-side. These amyloid *filaments* are approximately 75–80 Å in diameter and consist of five (or less likely six) subunits (amyloid *protofibrils*) which are arranged parallel to each other, longitudinal or slightly oblique to the long axis of the filament. The filament has often seemed to disperse into several longitudinal rows. The amyloid *protofibril* is about 25–35 Å wide and appears to consist of two or three subunit strands helically arranged with a 35–50-Å repeat (or, less likely, is composed of globular subunits aggregated end-to-end). These amyloid *subprotofibrillar* strands measure approximately 10–15 Å in diameter.

## INTRODUCTION

The striking similarity of the tinctorial properties of amyloid and cellulose, even in the earliest light microscopic studies of amyloid (1), led to the idea that there might be similarity in the steric configuration of the two (2–13). Since the exact dimensions of the amyloid fibril have been far beyond the resolution of light microscopy, amyloid had been generally accepted only as an amorphous structureless substance, although cellulose fibrils were identified in the electron microscope as early as 1948 (14).

In 1959, introducing electron microscopic techniques in the area of amyloid research, Cohen and Calkins first discovered that various types of human and animal amyloid all had a fibrillar ultrastructure (15). Since then, numerous electron microscopic investigations have clearly confirmed that amyloid of all varieties so far examined

possesses a fibrous ultrastructure. The dimensions of the amyloid fibrils in the tissue sections have been reported by most investigators to be comparable in diameter, roughly 100 Å, although some variation in the measurements of the breadth of the amyloid fibrils, i.e. 50–300 Å, has been found in these studies (15–36). Based on the studies of the positively stained amyloid in the tissue sections, delineation of the subunit structure of the amyloid fibrils has also been attempted by a few investigators. In their original paper, Cohen and Calkins suggested that human and animal amyloid possessed a beaded structure (15). After observing tissue sections of mouse amyloid, Gueft and Ghidoni (26) concluded that the fibrils were double and had an interfibrillar space slightly larger than each component. That is, they conceived of the fibril as being composed of two linear compo-

nents, about 25 Å each plus a 25-Å interspace, giving a diameter of 75 Å. In like manner, in their study of human cerebral amyloid in the senile plaque of Alzheimer's disease, Terry et al. (35) suggested that the fibrils were 70–90 Å wide and had a triple density indicating a hollow center. Sorenson (31) has described that after trypsinization and sucrose density centrifugation osmium tetroxide-fixed murine amyloid fibrils have a diameter of 100 Å and a periodicity of 100 Å along the longitudinal axes, and that they have occasionally appeared triple layered.

Such variations in measurements of the dimensions of amyloid fibrils as well as in delineation of their subfibrillar structure reported in electron microscopic studies of amyloid *in sections* are not unexpected, for the following reasons. 1. It is well known that the process of fixation, dehydration, and embedding may introduce shrinkage, swelling, and distortion in the ultrastructure of tissues (37–41). The fixative itself and the chemical composition of the buffer have been demonstrated to be of great importance (42). 2. Since the amyloid fibrils must have been oriented in various directions in the tissue section, it is difficult to be certain that the exact dimensions and clear subunit structure of amyloid fibrils are accurately indicated on the micrographs. 3. Except when highly specialized heavy staining techniques are used (43–45), it is generally believed that biological specimens have a density of about one and, therefore, it may be very difficult to show on an electron micrograph any structure less than 100 Å in size without some treatment to enhance its contrast. However, even intense positive staining that might perhaps double

the weight density would still allow only objects larger than 50 Å to be seen (46–48).

The negative-staining technique (49) has made it possible to visualize smaller biological specimens with the use of the electron microscope than positive staining allowed. With the full resolving power of modern electron microscopes and the recent improvements in techniques for preparing specimens and operating the instruments, high-resolution electron microscopy has become a valuable analytical tool uniquely suited for direct visualization of macromolecular structures often of dimensions as small as 10 Å (43, 47, 48, 50–54).

Reliable techniques for isolating amyloid fibrils from amyloid-laden tissues have also been made available recently (2, 31, 55–61). The isolation method developed by Cohen and Galkins (57) is extremely simple and has given amyloid of fairly high purity in the "top layer," with seemingly little or no ultrastructural change in amyloid fibrils. Despite the hazards of introducing ultrastructural changes by further handling, even purer amyloid fibrils have been collected when the top layer has been subjected to sucrose density centrifugation (2, 56, 59).

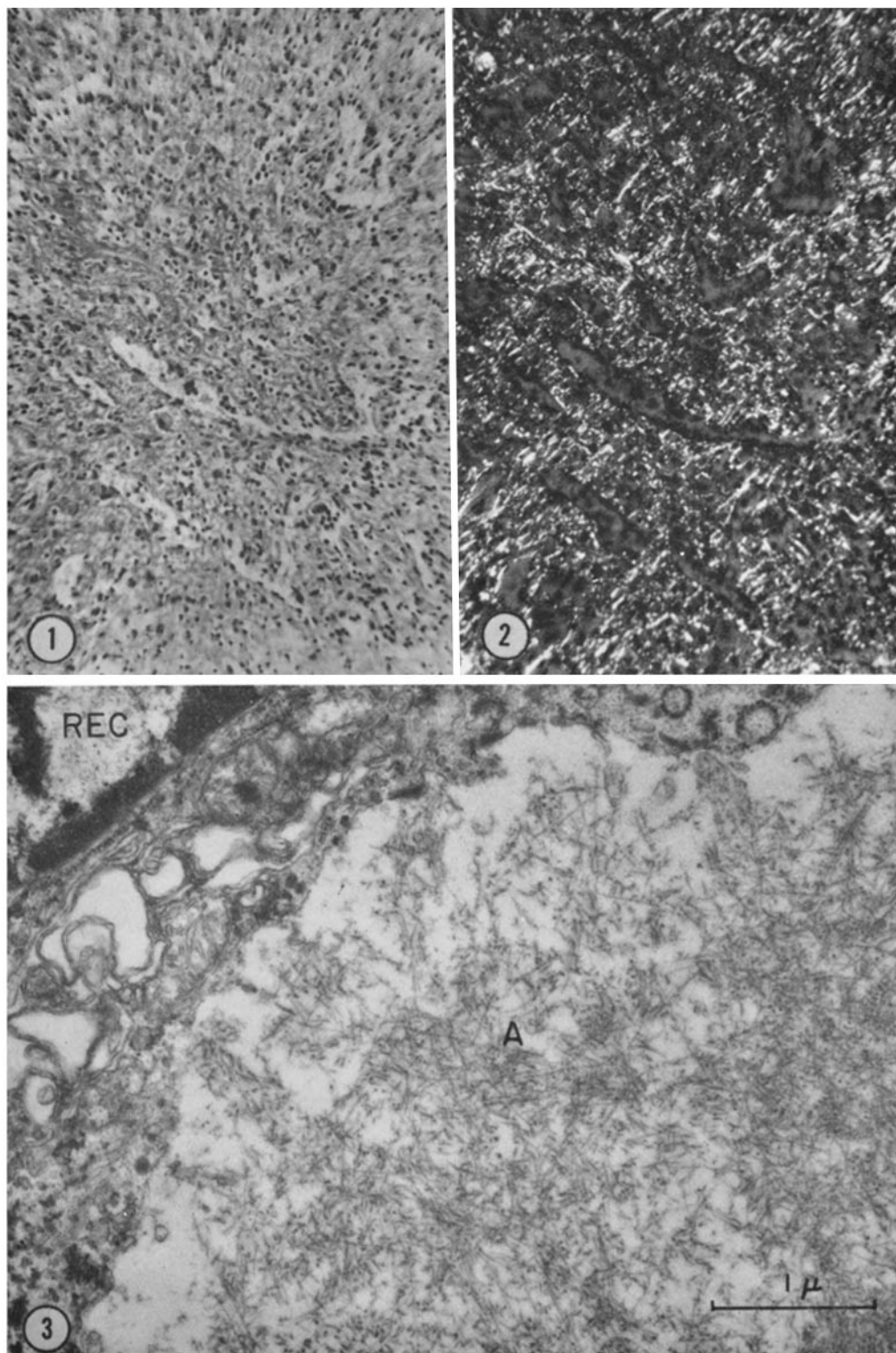
In our previous report on the ultrastructure of isolated amyloid fibrils after negative staining (59), we described in brief the structure of amyloid on the fibril-filament level. An amyloid fibril was shown to consist of a number (1–8) of laterally aggregated filaments. Although some finer subunits of amyloid had been seen, the three-dimensional architecture of the amyloid filament could

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FIGURE 1 A light micrograph of a human amyloid-laden spleen used as starting material for the present study. Marked amyloid infiltration is observed in the red pulp. Stained with Congo red-hematoxylin.  $\times 200$ .

FIGURE 2 Same portion as Fig. 1, photographed through the polarization microscope. Amyloid shows characteristic green birefringence.  $\times 200$ .

FIGURE 3 An electron micrograph of the thin section from a human amyloid-laden spleen. The tissue (48 hr postmortem) was fixed in 4% para-formaldehyde followed by 1% osmium tetroxide, embedded in Epon, stained with uranyl acetate and lead citrate. A portion of a reticuloendothelial cell (*REC*) and the amyloid fibrils (*A*) are present. Amyloid fibrils are long, rigid, nonbranching and roughly 100 Å in diameter. In the reticuloendothelial cell, postmortem changes are seen; i.e. slightly clumped chromatin, distorted mitochondria, largely vesiculated endoplasmic reticulum, and fragile plasma membranes.  $\times 24,000$ .



not be interpreted to our satisfaction from the data available at that time.

Since our previous report, several studies on amyloid ultrastructure have been published from different laboratories (55, 62-64). Boeré et al. (55) isolated fibrils by a process similar to ours and described a 75-A-wide amyloid fibril which they believed to consist of two smaller 25-A strands separated by a 25-A space. Each 25-A strand was said to show about a 40-A periodical beading along the long axis, and a pair of strands (a fibril) twisted with approximately a 400-A periodicity. Emeson et al. (64) studied a trypsinized sample, and described a  $96 \pm 22$ -A-wide amyloid fibril consisting of two filamentous subunits, each 40 A wide with a variable spacing between them of up to 25 A. A filament showed 40-A beading along the long axis, and paired filaments appeared to twist at about 1,000 A to form fibrils.

Bladen and coworkers (62) have described the isolation of a distinctive particle from amyloid preparations, using a completely different technique of separation. These studies have been repeated in part by Benditt and Eriksen (63). Since observations to date do not suggest a direct relationship between the well defined amyloid fibrils, which we and others have described, and this unusual component, this finding will be discussed at greater length only later in this report.

The present report describes the structural organization of the amyloid at what will be defined as the fibril-filament, the filament-protofibril, and the subprotofibrillar levels.

## MATERIALS AND METHODS

### *Materials*

Fresh (unfrozen) amyloid-laden tissues (spleens and livers) were secured from autopsies of two patients with secondary amyloidosis. Frozen amyloid-laden

spleens and livers were also collected from six patients with primary, secondary, and myeloma-associated amyloid. Nonamyloidotic spleens and livers (two of each tissue fresh and another two of each tissue frozen) were used as controls.

### *Isolation of the Samples (Amyloid Fibrils) From Amyloid-Laden Tissues*

Amyloid fibrils were isolated by the procedures reported from this laboratory (2, 56, 57, 59). Approximately 5 g of amyloid-laden tissue were homogenized in about 30 ml of cold physiological saline solution with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 12,500 *g* for 15 min in a Servall RC-2 refrigerated centrifuge at 4°C. The supernatant was decanted, and the sediment was resuspended in 30 ml of saline and recentrifuged under the same conditions. This procedure was repeated at least six times. The sediment was divided into three layers in the following fashion. The top layer of the last centrifugation was carefully physically separated. This sample is called "top layer" sample (2, 56, 57). The top layer was mixed with an approximately equal amount of 75% sucrose, and was allowed to stand in a cold room at 5°C overnight. The following were layered in a centrifuge tube: cold 85% sucrose on the bottom, then cold 75% sucrose, and the mixture of the top layer and 75% sucrose on the top. The centrifuge tube was then spun at 25,000 *g* for 2 hr. The sample which collected in the interphase between the 75% sucrose and the 85% sucrose layers was separated and dialyzed against water. This sample is designated the "sucrose-separated" amyloid (2, 56, 59).

### *Identification of Isolated Material (Especially the Negative-Staining Specimens) as Amyloid*

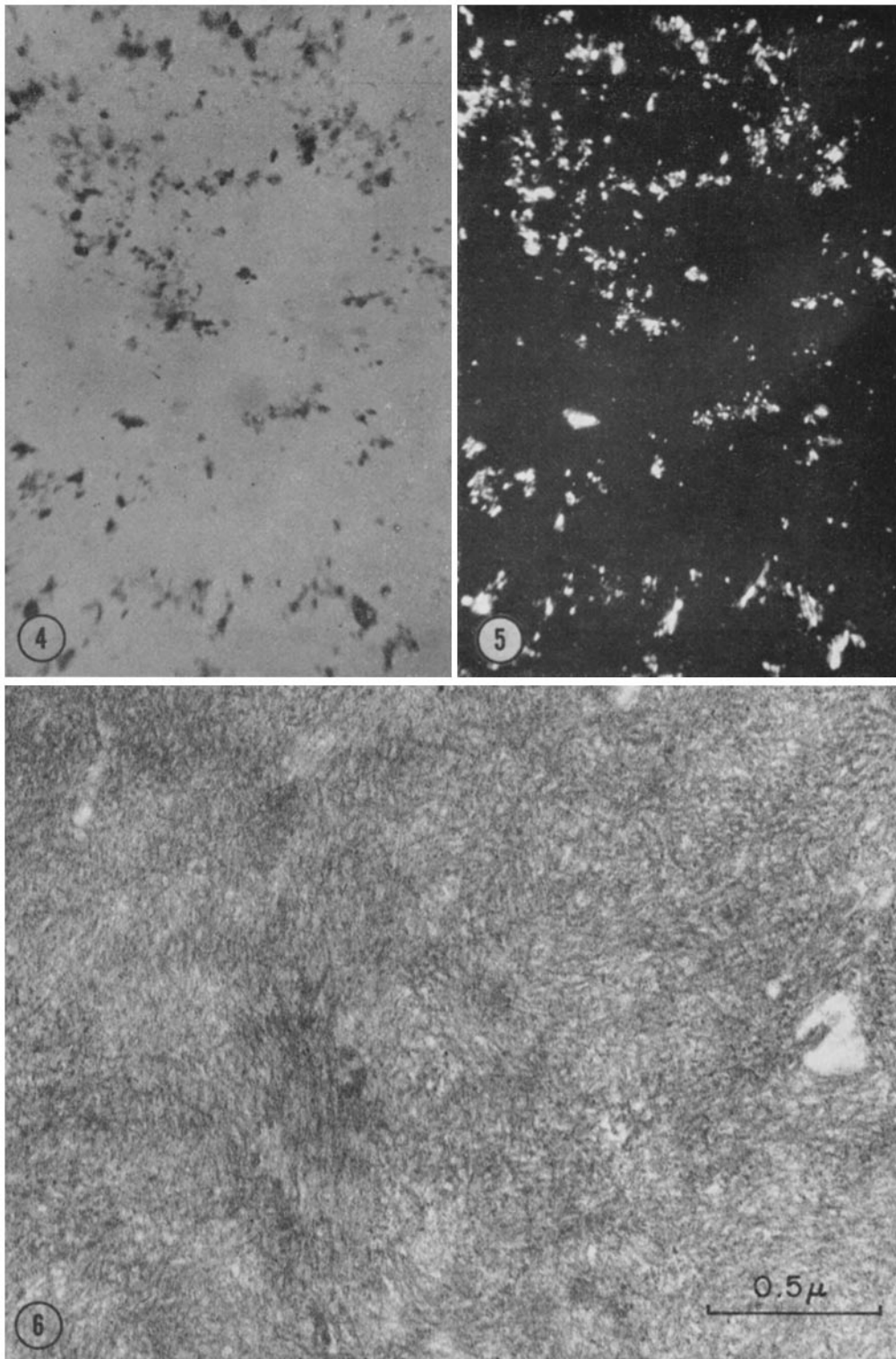
Small pieces of each tissue in the present study were prepared for examination by light and routine electron microscopy. For the light microscopy, the sections were stained with hematoxylin eosin, Congo red, crystal violet, and thioflavine T. Stained sections were observed under the light, the polarization, and the

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FIGURE 4 A light micrograph of a "sucrose-separated" sample appears to be very pure and almost totally amyloid substance. Stained with Congo red.  $\times 200$ .

FIGURE 5 Same portion as Fig. 4, photographed through the polarization microscope. Bulk of the sample shows the characteristic green birefringence.  $\times 200$ .

FIGURE 6 An electron micrograph of a sucrose-separated sample after fixation, embedment in Epon, thin sectioning, and positive staining. Amyloid fibrils are observed to be tightly packed but otherwise identical to those in the conventional tissue sections, and the sample appears to be quite pure.  $\times 50,000$ .



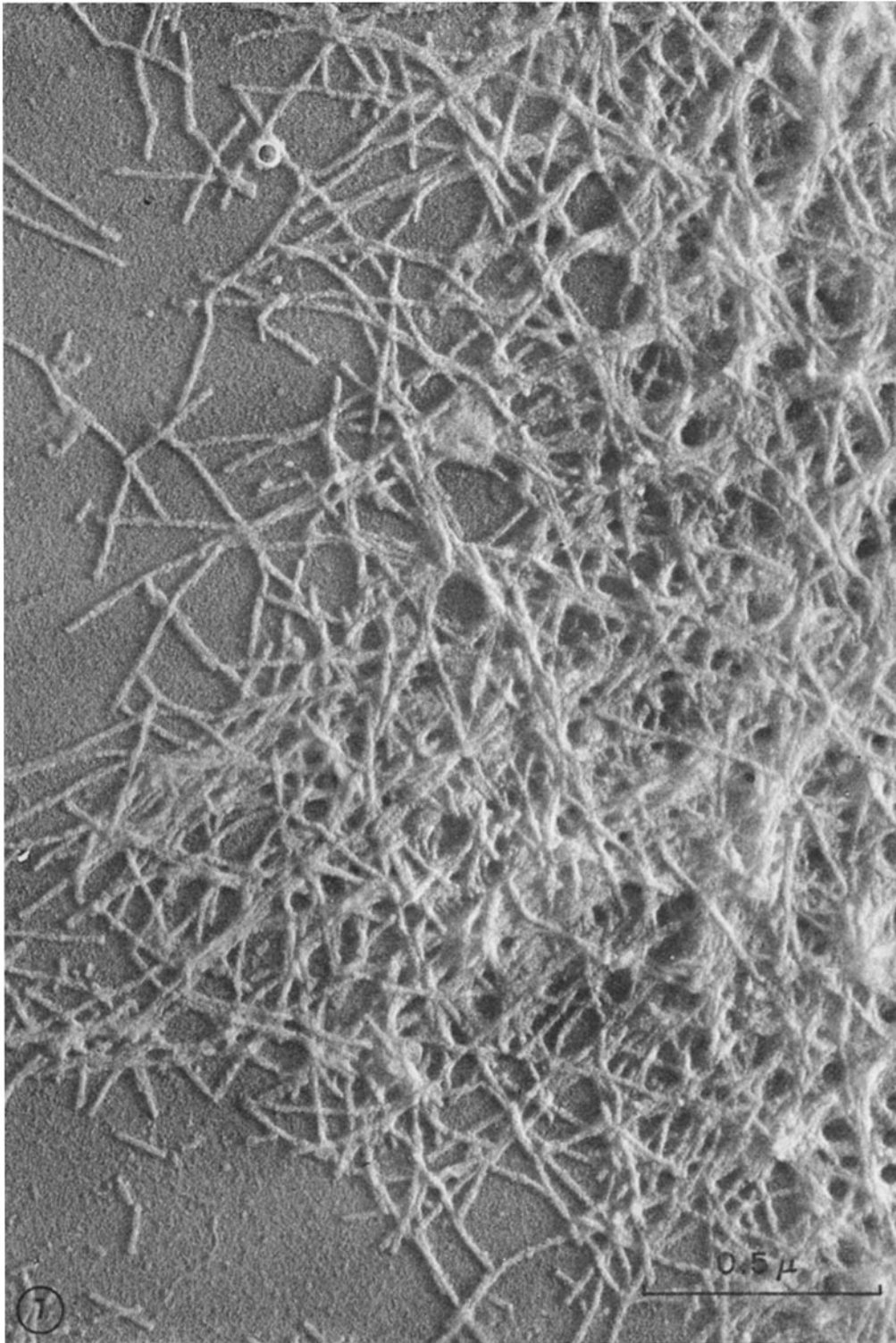


FIGURE 7 A sucrose-separated preparation of amyloid fibrils shadowed with platinum-palladium (see text). Although a small amount of contamination is found among the packed fibrils, the specimen is quite pure and consists almost solely of amyloid fibrils.  $\times 70,000$ .

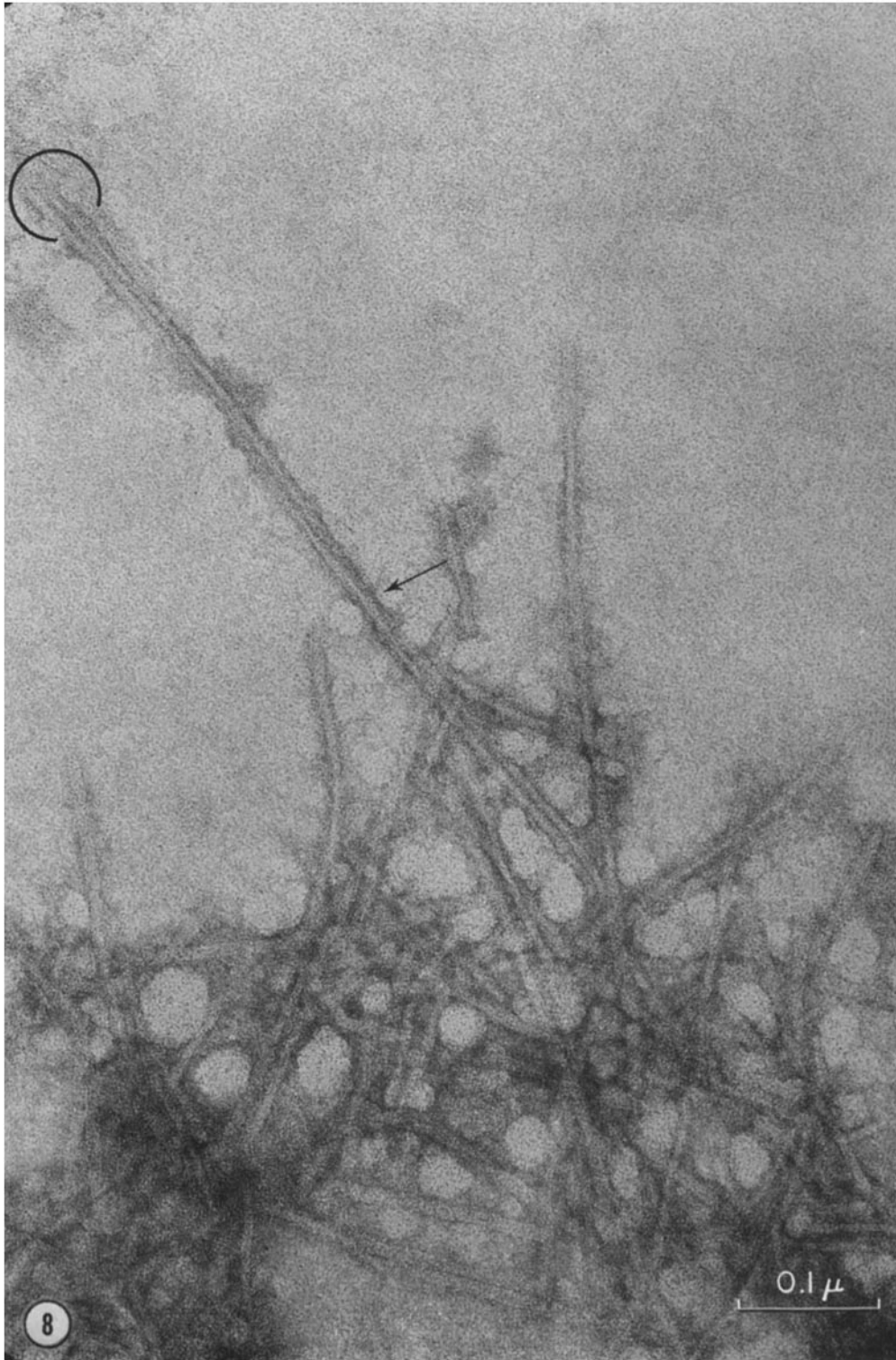


FIGURE 8 A sucrose-separated sample negatively stained with uranyl acetate. The major component of this specimen is fibrous. The amyloid fibrils consist of one or two amyloid filaments aggregated side-by-side. The aggregated filaments (an amyloid fibril) twist occasionally (arrow). The terminations of the filaments of the aggregate are almost equal in length and are smooth and rounded (circle).  $\times 200,000$ .

fluorescence microscopes. For electron microscopy, small pieces of tissue were fixed in 3% glutaraldehyde (or 4% paraformaldehyde) in phosphate or cacodylate buffer followed by 1% buffered osmium tetroxide, dehydrated in graded concentrations of ethanol, and embedded in Epon (39, 65-67). Thin sections were cut on an LKB ultratome, stained with lead citrate or uranyl acetate (39, 68, 69), and examined in a Siemens Elmiskop I at initial magnifications of 2,000-40,000. When thin sections were used for high-resolution electron microscopy of the amyloid fibrils, they were stained with uranyl acetate and lead citrate (45) and photographed at initial magnifications of 40,000-160,000.

The isolated samples (top layer and sucrose-separated amyloid fibrils) in the aforementioned procedure were stained with Congo red. In this staining procedure, special caution was taken to eliminate completely the false-positive material caused mainly by stain retention (70) (unpublished data). The stained samples were examined under the light and the polarization microscopes. A part of the pellet was also prepared for electron microscopy. This material was studied in thick sections in the light microscope after being stained with a newly developed Congo red procedure (71), and in thin sections in the electron microscope as described above.

An aliquot of the sample which had been estimated to be very pure amyloid was suspended in distilled water and spread on a carbon-coated grid. Shadow-casting was then applied to the grid under the following conditions: 5-10 mg of platinum-palladium, 10 cm in filament-specimen distance, and 10-15° shadowing angle. The "shadowed" specimen was examined in a Siemens Elmiskop I, and was photographed at initial magnifications of 20,000-40,000.

In the negatively stained sample (see below) which had been estimated to contain quite pure amyloid by the above techniques as well as by the chemical examinations (56) (unpublished data), the structure

which satisfied the following conditions has been designated the amyloid fibril: 1. The structure is that of a long, thin, nonbranching rod. 2. It constitutes the bulk of the sample. 3. It is distinct from all other known fibrous structures (i.e. collagen, elastin, etc.). 4. It has never been found in the controls.

#### *Treatment of the Amyloid Fibrils with Attempt at Partial Disruption*

Since the partial disruption of a polymerized protein (for example, bacterial flagella) has been considered to afford, on occasion, clearer material for interpretation of the substructure of the polymer (i.e. the structure of the monomers) in electron microscopic investigations (48, 51, 72), partial disruption of amyloid fibrils was attempted by use of a variety of methods, including physical, chemical, and enzymatic treatments. For the purpose of the present study, the following two methods have been considered to be most useful: 1. The isolated sample suspended in distilled water was sonicated for 0.5-60 min in the ice-cooled cell of Di Son Tegrator System 40 Sonicator (manufactured by Ultrasonic Industries, Inc., Albertson, L.I., N.Y.). 2. The isolated sample was treated in 1-2 molar urea at 4°C for 1-12 hr, and then was dialyzed against water.

#### *Negative Staining for Electron Microscopy*

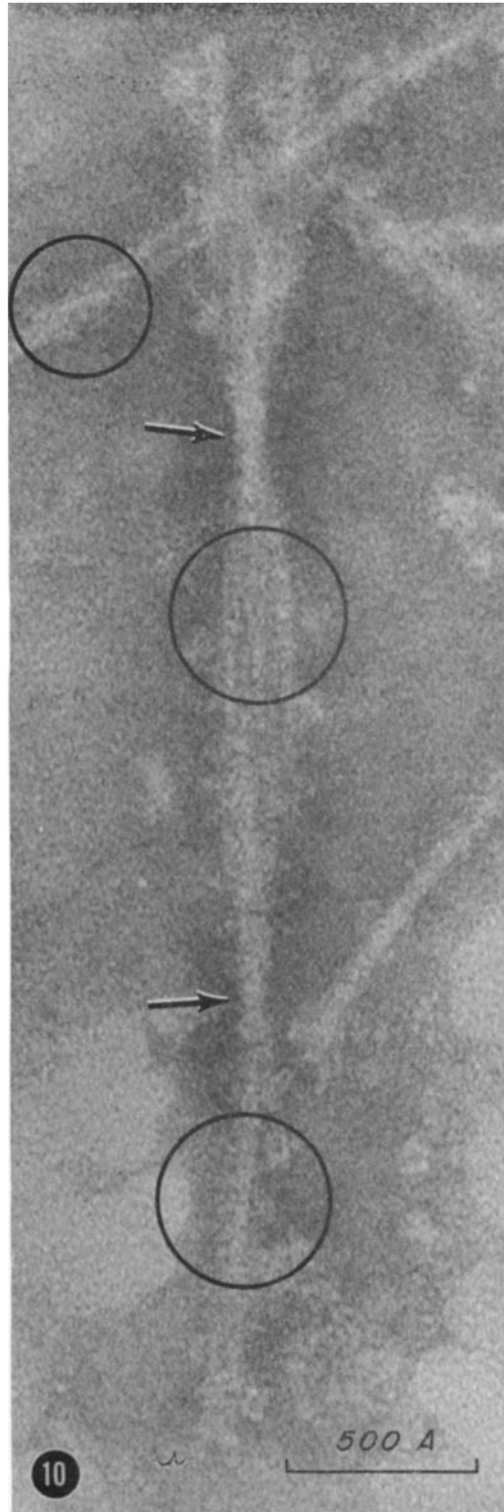
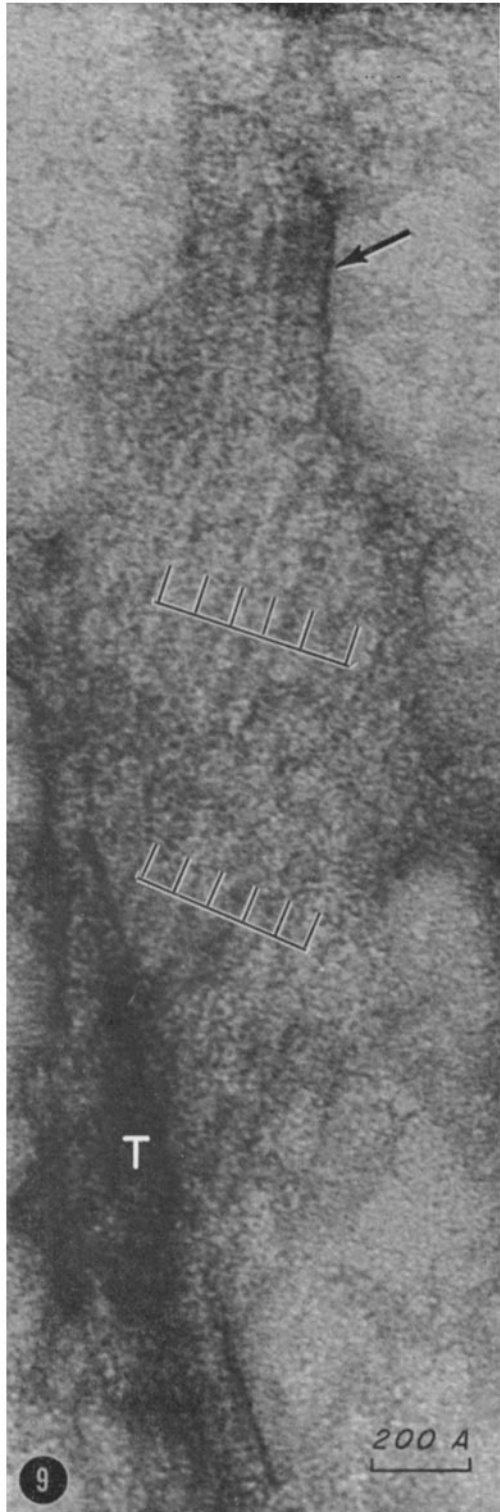
The sample was suspended in distilled water or in 0.4% sucrose to obtain a suitable dilution. One drop of the suspension was applied to a grid coated with a thin carbon film supported by formvar nets, or to a grid coated with a fenestrated formvar film strengthened with a thin carbon coating (39, 73, 74). About 2 min later, the excess was drawn off with a fine pipette. Just before the grid was dried off, a drop of 1-2% phosphotungstic acid adjusted to pH 5.0-6.0 with sodium hydroxide, or 1% uranyl acetate (pH about 4.5) or 1% uranyl acetate adjusted to pH 5.0-6.0

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FIGURE 9 One of the largest aggregates of filaments (about 10) found in the top layer sample. The filaments appear to be assembled side-by-side with no significantly measurable interspace and they appear to twist at one point (*T*). In this aggregate, the center-to-center distance of filaments measured approximately 75 Å as indicated on the micrograph. In a terminal portion, a finer fibrous structure (protofibril), approximately 30 Å wide, may be seen, and its underlying helical structure is suggested (arrow). Negatively stained with uranyl acetate.  $\times 650,000$ .

FIGURE 10 An aggregate of filaments (a fibril) found in the top layer sample after 60-min. sonication. The fibril appears to twist (arrows) and consists of three filaments. Disruption and dissociation can be observed on the structure of each filament. In some portions of the fibrils, 25-35-Å-wide protofibrils arranged longitudinally or in a loose helix along the long axis of the filaments can be demonstrated (circles). Negatively stained with phosphotungstate.  $\times 500,000$ .





with ammonium acetate, was applied to the grid (49, 75). The excess was again drawn off with a fine pipette, and the grid finally was air-dried.

Because histochemical and chemical studies have indicated pH 4.0–5.0 as the isoelectric point of amyloid (76–78), a slightly acidic staining solution was employed (39) and seemed to give excellent results in the negative-staining preparations of these samples.

### *Electron Microscopy of Negatively Stained Specimens*

Grids were examined in a Siemens Elmiskop I. The electron microscope was equipped with a pointed filament and was operated mainly at 80 kv with the double condenser system, with a 200- or a 400- $\mu$  condenser aperture, with or without a 50- $\mu$  objective aperture (48, 54). Before the observation of each grid, the electron microscope was aligned so that astigmatism of the objective lens was less than one step of the fine objective control knob, and the resolution was better than 10 Å by the measurement of the photographic plates of the test specimens (a Fresnel fringe or evaporated platinum particles). Specimens were observed and photographed under the microbeam with low-intensity (less than 5  $\mu$ a) at the initial magnification of 80,000–160,000. To avoid diffraction and phase effects caused mainly by defocus, at least three plates were exposed for recording a particular area with a step focus system in one step by one click of the fine objective control knob or by one or two clicks of the extrafine objective control knob, which was especially installed. “Kodal Projector Slide Plates, Kontrast” (ASA value 12 under standard development) were exposed to the electron beam for 3–10 sec. The exposed plates were developed in D-19 or D-72 developer which was often slightly modified, and their ASA values were often increased about

two to three times by means of the procedure of development (79).

The most satisfactory micrographs have been obtained by means of the combination of the negative-staining preparation with the fenestrated film, electron microscopy without objective aperture, the use of the highest magnification, the photographic recording by shorter exposure, and the enhanced development of the plate.

### *Observation and Measurement of Micrographs*

The micrographs observed were both original plates and prints which were usually enlarged about three to six times (from original plates with a Simmon Automega model D3 enlarger on Kodabromide F4 or F5 paper) and they were measured directly or with a Bausch and Lomb measuring magnifier. The Observations and Discussion presented here are based on the evaluation of more than 1,000 micrographs of the present study and also of many other micrographs from our previous studies.

## RESULTS

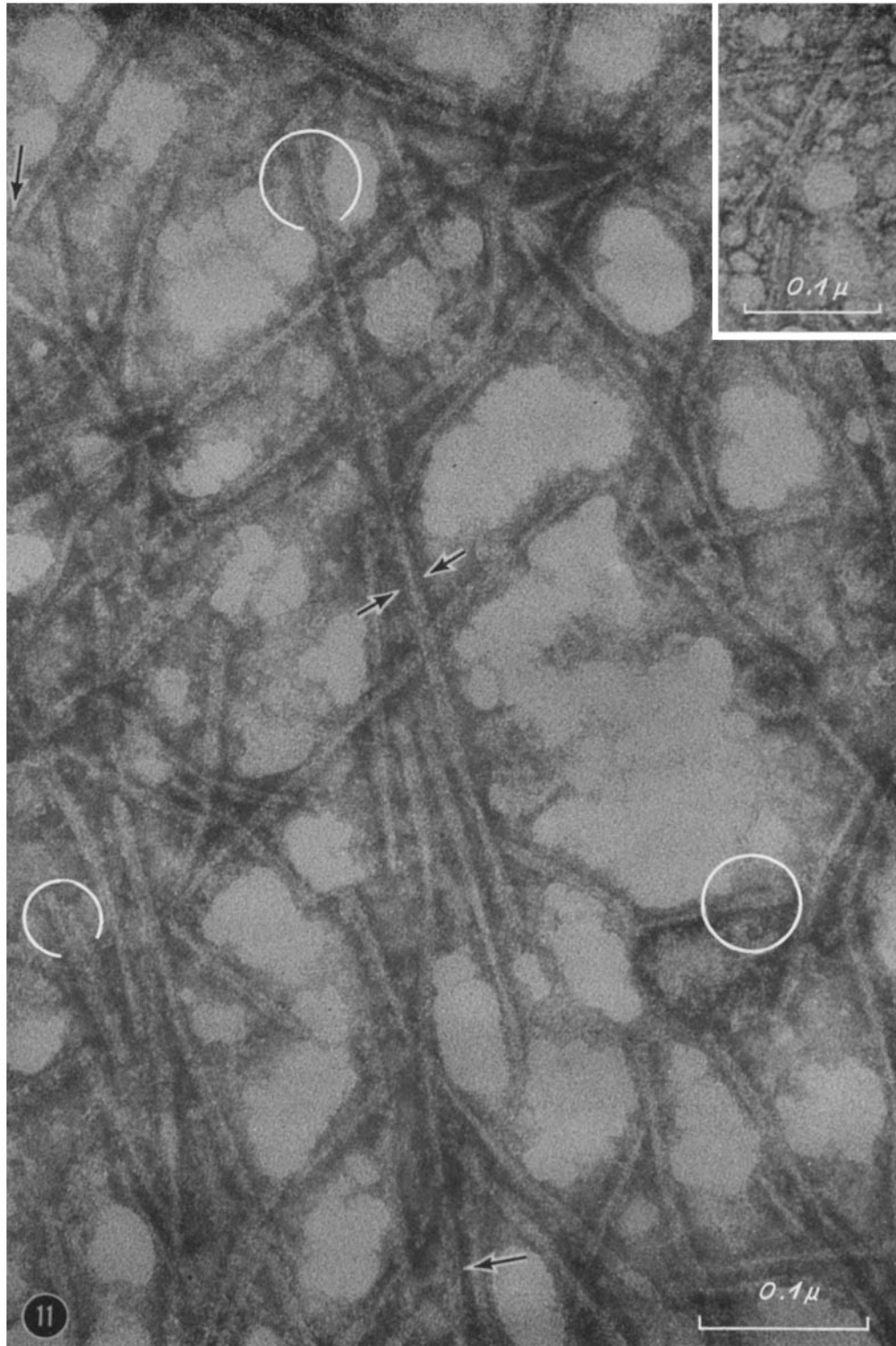
### *Identification of Amyloid Fibrils in the Negatively Stained Specimens*

The tissues used as the starting materials of the present study have all shown large deposits of amorphous eosinophilic material with the characteristic tinctorial properties of amyloid, in contrast to the complete lack of such substances in the control tissues. The amyloid showed crystal violet metachromasia, fluorescence with thioflavine T, and green birefringence after Congo red staining (Figs. 1 and 2). The amyloid deposits have been estimated to make up roughly 50–75% of the

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FIGURE 11 Negatively stained amyloid filaments in a top layer sample. Although the filaments appear to vary in their length, they are found to be uniform in their width and in general proportions. In measurement of their width, some variation (50–110 Å) may be found even in different portions of an individual filament. In some portions, finer linear structures, protofibrils, can be seen beside the filament (arrows) and on the filament (circles). Negatively stained with phosphotungstate.  $\times 250,000$ .

FIGURE 11, insert Amyloid filaments in a top layer sample. In this negatively stained preparation, filaments were suspended in cold 2% sodium phosphotungstate, applied to a grid, and then air dried. (The duration of this procedure was about 5–10 min). In this insert, the majority of the filaments demonstrate an additional feature, i.e. a distinct, dense line about 15–20 Å wide in the center of the filament and running longitudinally through its entire length. This line appears to divide the 75–80-Å-wide filament into the two longitudinal linear structures. This image may represent the existence of a central core in the filament, as would be expected in the model proposed (see text and Fig. 29).  $\times 200,000$ .



tissue mass by means of the light (including the polarization and the fluorescence) microscopic examinations. Indeed, only tissues with such massive deposits of amyloid were selected for the present study. The presence of amyloid fibrils of approximately 100-A diameter has been confirmed by the electron microscopy of the tissue sections in all the specimens used (Fig. 3).

After the isolation procedures, samples were identified as amyloid by the same staining and optical procedures and the content of amyloid was estimated by light and electron microscopy. The major portion of the top layer has shown the characteristic tinctorial properties of amyloid under the light microscope (especially green birefringence after Congo red staining) and also was found to have the characteristic fibrous ultrastructure of amyloid fibrils by the electron microscopic examination of thin sections. The shadow-casted specimens of top layer were estimated to be at least 75–90% amyloid fibrils. The sucrose-separated sample also had all the characteristic tinctorial properties of amyloid (Figs. 4 and 5), and the typical fibrous ultrastructure in the thin sections (Fig. 6). By means of the electron microscopy of the shadow-casted specimens (Fig. 7), as well as by the light and the electron microscopic examinations of sections, it was estimated that this preparation consisted of at least 90–95% pure amyloid fibrils.

Electron microscopy of the negatively stained specimens of the sucrose-separated sample has revealed the fibrous structures as the major component of the sample (Fig. 8). In contrast, no structure of this kind has been found in the specimens from the control tissues. This fibrous component has been designated amyloid, and was subjected to the further investigations outlined below.

#### *Ultrastructure of the Amyloid on the Fibril-Filament Level*

After negative staining, the amyloid fibril has been found to consist of a number of filaments assembled side by side. The number of filaments composing a fibril has commonly been one to four, but on rare occasions, up to ten. The fibril, an aggregate of a number of filaments, has occasionally shown loose random twisting which has no regularity in the direction of the twist (right or left). No regular repeating periodicity or length between twists has been found. The aggregation and twisting have been observed in all specimens from the top layer samples as well as from the sucrose-separated samples (Figs. 8 and 9). The number of filaments most commonly found in a set has varied from case to case: one in a certain case, but two, three, or four in others.

The amyloid fibrils (and filaments as well) have varied widely in length, from 300 to 10,000 A

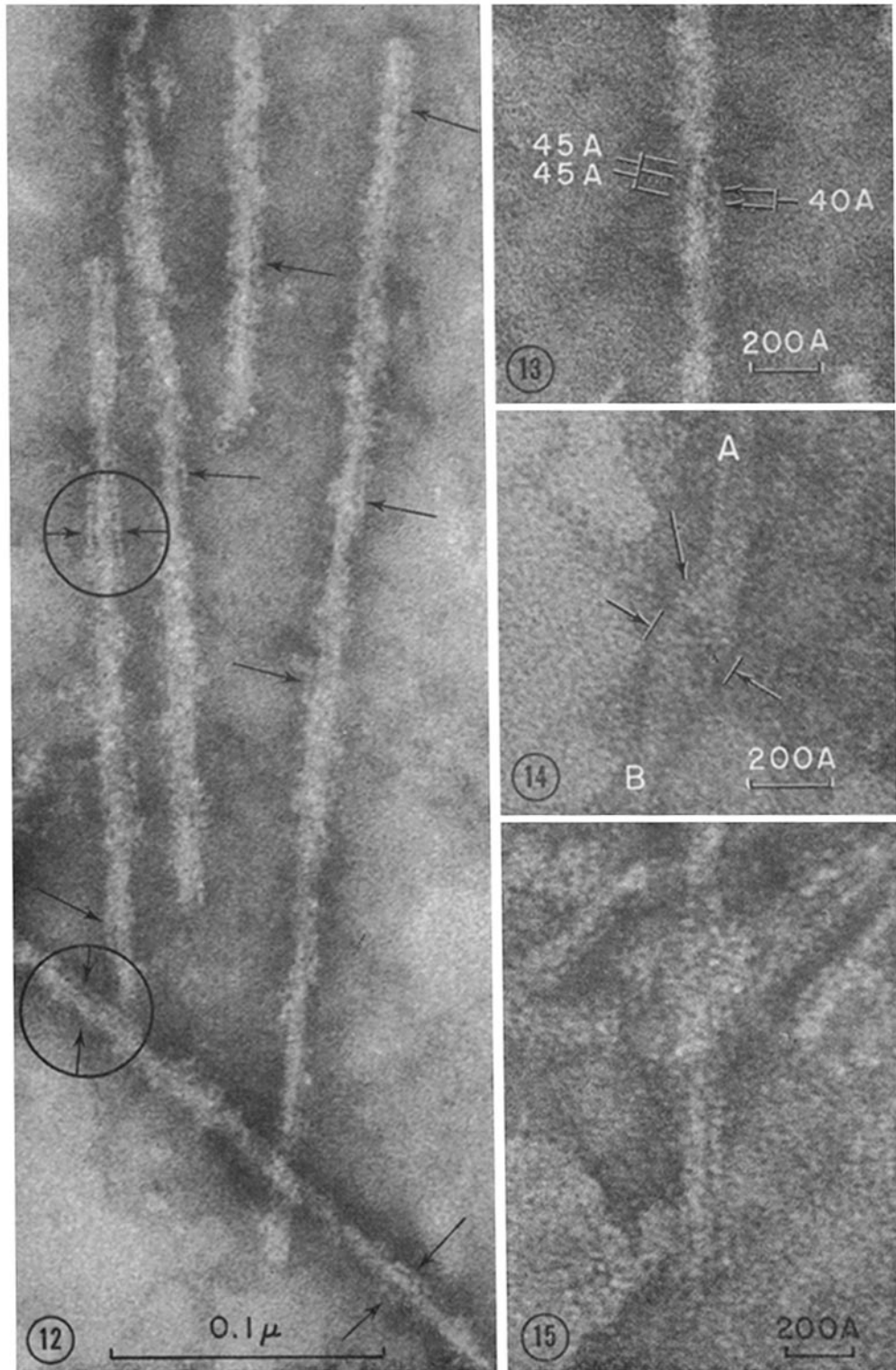
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FIGURE 12 Amyloid filaments sonicated for 3 min. Most portions of the filament measured 100–120 A, suggesting that dissociation and collapse occurred in the ultrastructural organization of the filament. Protofibrils are demonstrated beside the filament (arrows) and on the filament (circles). They are 25–35 A wide and show beading along their length with a 35–50 A repeat. Negatively stained with phosphotungstate.  $\times 400,000$ .

FIGURE 13 An amyloid filament after sonication for 10 min. In the middle portion of the filament, four subunit strands, protofibrils, are seen. They appear to be arranged in a loose helix around the long axis of the filament. They measure 25–35 A in width and 35–50 A in their beading. Negatively stained with phosphotungstate.  $\times 500,000$ .

FIGURE 14 An amyloid filament sonicated for 10 min. Between two relatively intact portions of the filament (A and B), a portion (between small arrows) is widened to about 200 A. In this portion, except for a mere suggestion of a linear structure (arrow), no significant regular structure is indicated. This may suggest that a portion of the filament was dispersed (or dissociated) to very fine structures (at least smaller than the resolution of this micrograph) which might be more strongly adherent in the longitudinal direction than in the lateral direction. Negatively stained with phosphotungstate.  $\times 600,000$ .

FIGURE 15 A terminal portion of a filament sonicated for 10 min. The filament is dispersed into several protofibrils up to 800 A in length. Negatively stained with phosphotungstate.  $\times 500,000$ .



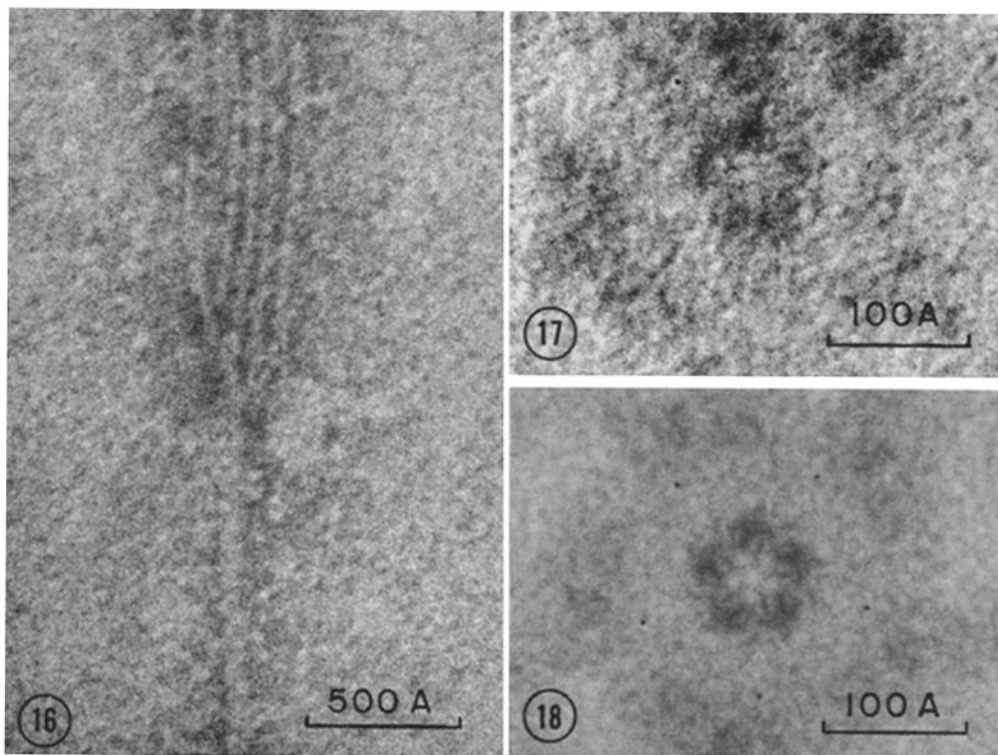


FIGURE 16 An amyloid filament about 75 Å wide (bottom half of the micrograph) is dispersed into several (probably five) protofibrils (upper half of the micrograph), after treatment with urea. Numerous unidentified small structures occur in the background. The individual protofibril is longer than 1,000 Å and is 25–35 Å wide. It has beading along its length with a repeat of 35–50 Å. Negatively stained with phosphotungstate.  $\times 400,000$ .

FIGURE 17 A (probable) cross-section of an amyloid filament in the positively stained tissue section. This structure measures 80–90 Å in diameter, and consists of five subunits of about 30 Å diameter which are pentagonally arranged surrounding an electron-lucent central core of about 20 Å diameter.  $\times 1,840,000$ .

FIGURE 18 Same object as Fig. 17, rotated photographically;  $n = 5$ .  $\times 1,840,000$ .

(Figs. 8, 9, and 11). The width of the amyloid fibrils has also been found to vary from 50 to 300 Å depending upon the number of filaments present (Figs. 8 and 9). The center-to-center distance of the filaments within a fibril has measured approximately 75 Å (as indicated in Fig. 9), and no significantly measurable space separating them has been seen when they have been embedded in an extremely thin stain (sodium phosphotungstate or uranyl acetate) (Figs. 8 and 9). Deposit of the stain, however, has appeared midst the filaments, suggesting the existence of an interfilamentary separation when they have been embedded in a relatively thick stain (59).

The lateral assemblage of filaments is often still found in the specimens after treatment by sonication (even with such relatively severe conditions as 60-min sonication) as well as after treatment with 1–2 molar urea, even when partial disruption could be observed on the ultrastructure of the filaments (Fig. 10). Comparable structures with the above mentioned dimensions and organization at the fibril-filament level have also been revealed on the high-resolution electron micrographs of the positively stained tissue sections of amyloid (Figs. 23–26, and diagram in Fig. 28).

In summary, then, at the moderate magnification of these micrographs, the amyloid filaments

have appeared to be almost uniform in their width and in their general proportions (Figs. 8, 9, and 11), although they have been found to vary greatly from hundreds of Å to thousands of Å in their length. In breadth, they have consistently measured approximately 75 Å with maximum variation of 50–110 Å in diameter. Even on an individual filament, the width has often measured slightly differently at the different portions, but the minimum width has usually not been less than two-thirds of the maximum width on any single filament (Figs. 8 and 11). These data suggest the twisting ribbon-like nature of the amyloid filament or the collaterally aggregated double (or triple) subunit strands with twisting of the filament in some instances (Fig. 11).

#### *Ultrastructure of the Amyloid on the Filament-Protofibril Level*

Observations of the negatively stained filaments have revealed that a 25–30-Å-wide linear structure has often been found immediately beside (on one, or on both sides) the narrowest portion of the filament, i.e. the portion of the ribbon-like structure that appeared to be twisted on over-all view (arrows on Figs. 11 and 12). In addition, one to four similarly fine subunit strands 25–35 Å in diameter, which we shall call amyloid *protofibrils*, arranged almost longitudinally or slightly obliquely to the longitudinal axis of the filament, have been occasionally demonstrated on the untreated filament and more frequently in the partially disrupted filament (Figs. 11 to 13, arrows). Finally, the filament has occasionally appeared to be composed of several smaller strands, protofibrils, in portions of its length, mainly at its end, but at times in the middle. This occurs on rare occasions in the untreated specimens, and more commonly in the specimens sonicated or treated with urea (Figs. 14–16). The treatment with 1–2 molar cold urea was, on occasion, very effective in causing this apparent dispersion or fragmentation of the amyloid filament to several (five or six) long protofibrils which unequivocally connected with the undispersed portion of the filament. Although this observation could not be made on all preparations, the finding seems to confirm the fact that the amyloid filament consists of five (or six) protofibrils (Fig. 16).

Furthermore, a slightly different and probably important image was demonstrable when the staining procedure was modified slightly so as to

give a heavier stain. This occurred when the samples and the stain were mixed before application on a grid, or when a slightly higher stain pH (7.5) was used at a concentration of 2% and the specimen air-dried (Fig. 11, insert). Under these circumstances, the amyloid filament appeared to be divided in a linear fashion by a 15–20-Å wide, dense, clear line running through the center of the filament (often through its entire length). The filament still, however, retained its usual width of 75–80 Å as in the other preparations. Under these circumstances, each of the fine linear images had a width similar to that of the protofibril, but is believed to represent the central core of the five- (or six-) stranded protofibril, for the following reasons. In the first place, this type of image has been demonstrable with the phosphotungstate stain, but never with uranyl stain. Secondly, the procedures used (mixing, prolonged application, higher pH) were calculated to give a denser stain which penetrates to the interstices of the molecule (and apparently did so). Finally, when a filament was stained in this manner, this type of image was observed throughout the entire length of the filament, and was not interrupted as were the protofibrils.

In addition to those data from negatively stained amyloid filaments, the high-resolution electron microscopy of the (probable) cross-section of the filament in the very heavily (45) positively stained thin sections of the amyloid-laden tissue has revealed a structure which is approximately 80–90 Å in diameter and consists of five (or six) pentagonally (or hexagonally) arranged, apparently round 25–35-Å subunits (Fig. 17). The number of the subunits is believed to be five when photographically rotated preparations are studied (80) (Fig. 18). The longitudinal structure of amyloid protofibrils has also been demonstrated on the positively stained filaments in the thin sections of amyloid tissue (Fig. 27).

Thus, although the subfilamentary ultrastructure of amyloid, i.e. the amyloid protofibrils, has been difficult to observe in the untreated specimens, they have been more easily and more clearly demonstrated after the slight sonication. They have been found, on two-dimensional viewing, as finer strands in the filament arranged parallel to each other, and almost longitudinal or slightly oblique to the long axis of the filament. These amyloid protofibrils have measured approximately 25–35 Å in breadth, and approximately 30–40 Å

in the center-to-center distance on the apparently intact filament (Figs. 19–21). Along the length of the protofibril, the appearance of a relatively periodical beading measuring approximately 35–50 Å has been noted (Figs. 9, 12–16, 19–22). In addition, a central core has been observed, lending credence to the proposed model.

#### *Subprotofibril Ultrastructure of Amyloid*

The high-resolution electron microscopy of the slightly sonicated specimens has occasionally given fairly clear configuration of a single protofibril and its substructure when embedded in an extremely thin layer of the stain. The protofibril has measured 25–35 Å in breadth, and has shown a beading appearance along its length with a 35–50-Å repeat as mentioned above. In the central portion of each bead, a small dense spot (about 5–15 Å in diameter) has been observed on most occasions. Therefore, the negatively stained amyloid protofibril appears on the micrograph

most likely as a rope, suggesting the helical nature of the protofibril which consists of two or three strands, 10–15 Å wide, rather than globular, terminally aggregated subunits of the protofibrils (Figs. 19–22).

Thus, the current data indicate that the amyloid fibril, which, in isolated preparations, consists of a number of laterally aggregated filaments about 75–80 Å in diameter, may be constructed in the following manner. 10-Å units (long, thin subprotofibrils) are entwined to form the 25–35-Å protofibril which has a regular 35–50-Å structurally repeating unit along its length. The breadth of the amyloid protofibril appears greater than two of the 10-Å subprotofibrils owing to their twisted nature. The protofibrils then aggregate, probably in groups of five, to form the amyloid filament. This measures about 75–80 Å because of the possible presence of a central core (15–20 Å) and the circular arrangement of the protofibrils (see diagram, Fig. 29).

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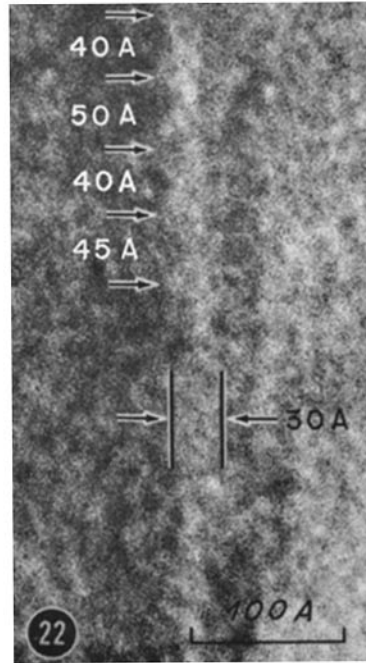
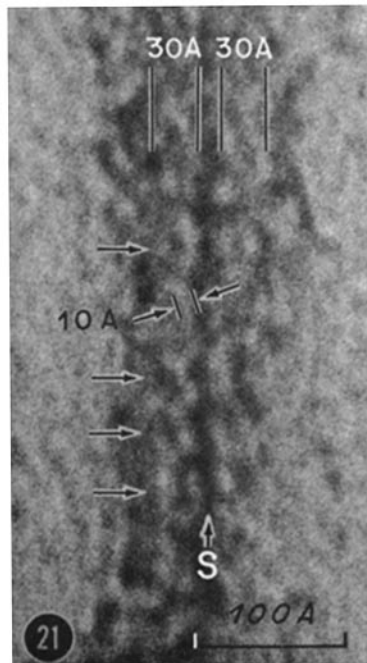
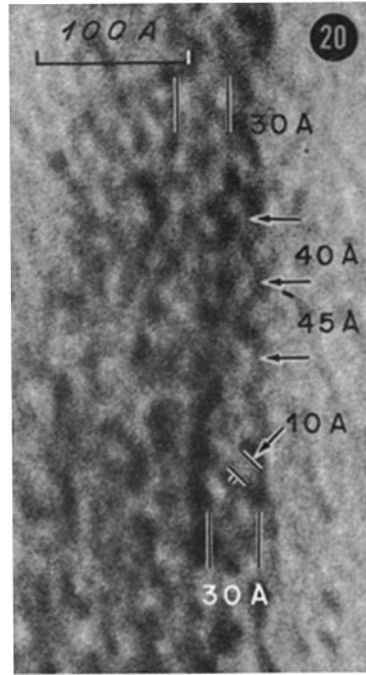
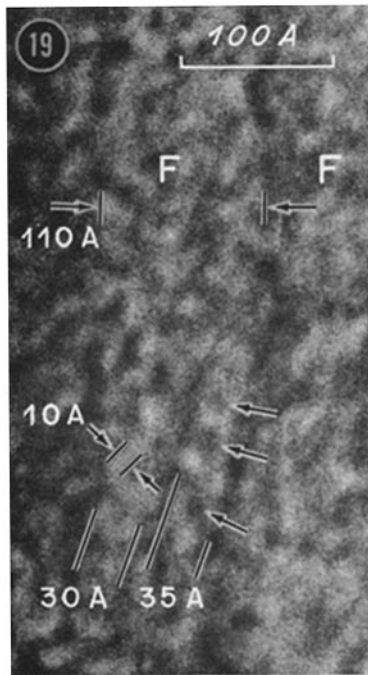
**FIGURE 19** Two filaments (*F*) are presented in this micrograph. Though sonicated for 15 min, they appear to be relatively intact in their filamentary structure. On the filament, in the center of the micrograph, protofibrils appear to be arranged in a loose helix around the long axis of the filament. The width of the protofibrils measured 25–35 Å, and a small, dense deposition of stain (5–15 Å diameter) is indicated on the protofibril with a 35–50-Å repeat along the longitudinal axis of the protofibrils (arrows). These structural findings may represent the two- to three-strand rope of helically arranged subunits (subprotofibrils) of 10–15-Å diameter (as indicated on the micrograph). Negatively stained with uranyl acetate.  $\times 2,000,000$ .

**FIGURE 20** An amyloid filament sonicated for 15 min. It appears to be moderately dissociated, but has the fairly intact ultrastructural organization of the filament. In the right half of the filament, the protofibril structure is seen. In the lower right portion of the filament, the helical nature of 10–15-Å subprotofibrils is also apparent. The width of the protofibrils measured approximately 30 Å and the longitudinal repeat of small dense depositions of stain is 35–50-Å (arrows). Negatively stained with uranyl acetate.  $\times 2,000,000$ .

**FIGURE 21** An amyloid filament after 15-min sonication. Two protofibrils dissociated by the procedure are demonstrated. They are clearly separated by a linear deposition of stain between them (*S*). The protofibrils are approximately 30 Å wide and have a 35–50-Å periodic (arrows) structural repeat along their length. Negatively stained with uranyl acetate.  $\times 2,000,000$ .

**FIGURE 22** A single amyloid protofibril found in a sucrose-separated sample treated by two 15-min sonication procedures separated by a 5-min interval. The protofibril is approximately 30 Å wide and shows the faint but definite structural repeat of 35–50-Å periodicity along its length (arrows). Negatively stained with phosphotungstate.  $\times 2,000,000$ .





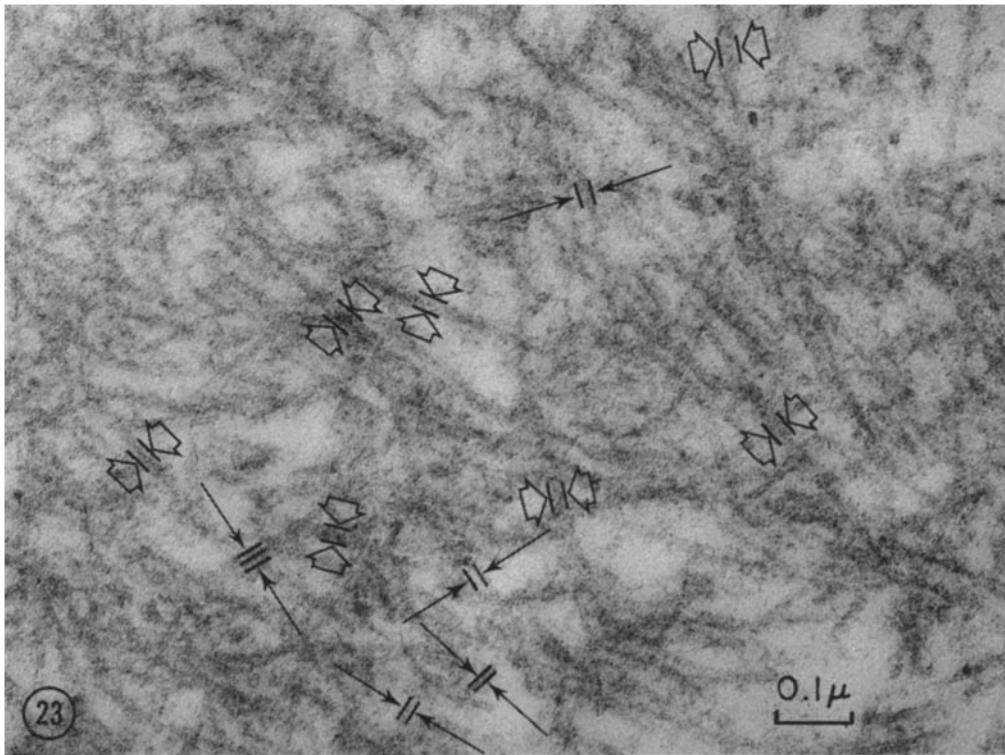


FIGURE 23 Amyloid fibrils in conventional tissue section. The fibrils are arranged in random array and are nonbranching. The measurements of the width of individual fibrils show moderate variation, with the 100-A width being most common (dark arrow), but the 200-300-A width is not unusual (open arrow).

Human amyloidotic spleen (postmortem about 24 hr) fixed in buffered 4% paraformaldehyde followed by 1% osmium tetroxide, embedded in Epon, sectioned on an LKB ultratome. Sections were stained with uranyl acetate and lead citrate and photographed in a Siemens Elmiskop I at initial magnification of 40,000.  $\times 100,000$ .

## DISCUSSION

### *Ultrastructure of Amyloid as Interpreted from Data Obtained in Present Study*

Although numerous studies concerned with chemical and immunological nature of amyloid have been reported (56, 61, 63, 81, 82<sup>1</sup>), there is, as yet, no specific chemical method for the identification of amyloid independent of the histological and electron microscopic criteria. Thus, the original standards for the identification of amyloid in the present study were morphological. It should be emphasized, however, that chemical and immunological examination of the samples used in

the present study have been undertaken and the findings have been consistent with those from the sucrose-separated and the top layer samples as reported elsewhere from this laboratory (2, 56, 61, 81, 82). Those studies as well as the morphological findings have confirmed the high purity of amyloid in the sucrose-separated sample. For example, the material demonstrated crystal violet metachromasia, fluorescence after thioflavine T staining, and green birefringence after Congo red staining. These reactions were consistent in the various purified preparations, whether in sections or smears on slides, and were absent from the controls. In addition, the characteristic contents of nitrogen (about 14.6%), hexose (1.96%), hexosamine (1.77%), sialic acid (0.84%), and uronic acid (0.67%) were demonstrable in the prepara-

<sup>1</sup> References prior to 1964 are available in the recent review of Cohen (2).

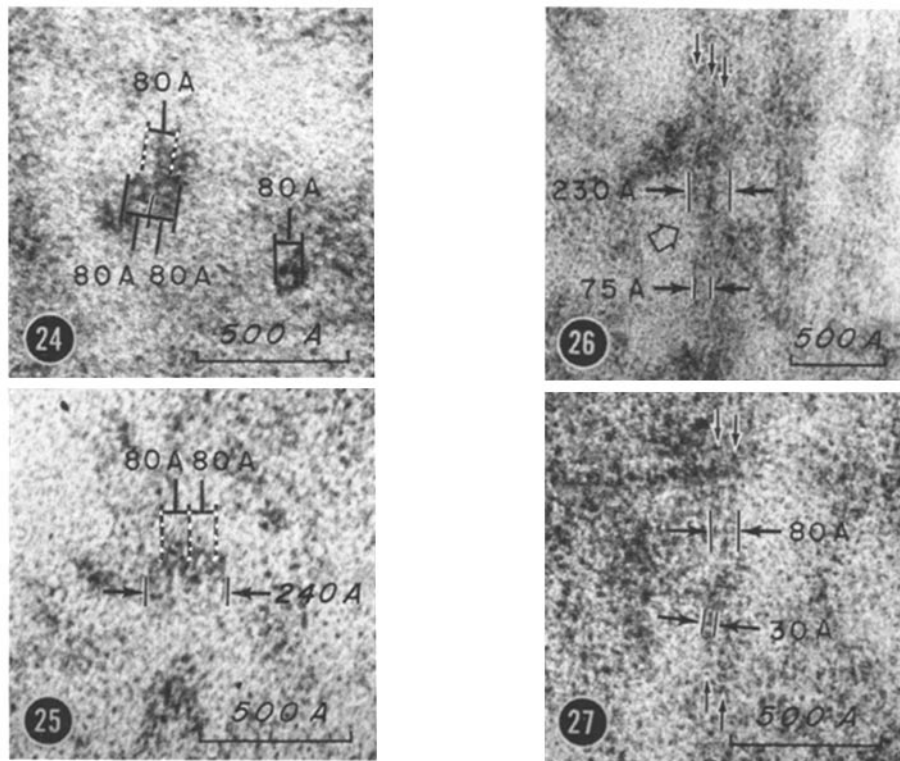


FIGURE 24 Probable cross-sections of amyloid filaments in a positively stained conventionally prepared tissue section. A pair of filaments and a single filament in cross-section are presented. The filaments measure approximately 80 Å in diameter (solid lines), and the center-to-center distance of the paired filaments (a fibril) also measures about 80 Å (dotted lines). Each appears to consist of several subunits of about 30-Å diameter arranged around an electron-lucent central core of about 20-Å diameter. Preparation same as that outlined for Fig. 23.  $\times 400,000$ .

FIGURE 25 Slightly oblique (nearly cross-) section of a set of three filaments (a fibril) in the positively stained tissue section. Their center-to-center distance measured about 80 Å (dotted lines). The total breadth of this aggregate is about 240 Å (solid lines). Preparation same as that outlined for Fig. 23.  $\times 400,000$ .

FIGURE 26 Longitudinal view of an amyloid fibril in the positively stained conventional tissue section. The fibril in the center of the micrograph measures about 230 Å wide. On the fibril, three subunit strands, amyloid filaments about 75 Å wide (arrows), appear to twist loosely (open arrow). Preparation same as that outlined for Fig. 23.  $\times 250,000$ .

FIGURE 27 A single amyloid filament in a positively stained conventional tissue section. The filament measures about 80 Å wide. On the filament, at least three subunit strands, protofibrils about 30 Å wide (arrows) are apparent and seem to be arranged in a loose helix around the length of the filament. Preparation same as that outlined for Fig. 23.  $\times 400,000$ .

tions used as they have been in the past analyses (56). Immunological studies have demonstrated that small amounts of a unique protein (an alpha globulin) could be extracted from the fibril preparations and not from the controls (81, 82).

Thus, we concluded that the fibrous structure under analysis in this study is, indeed, the characteristic amyloid fibril seen in tissue sections and presumably is the most characteristic and major component of amyloid.

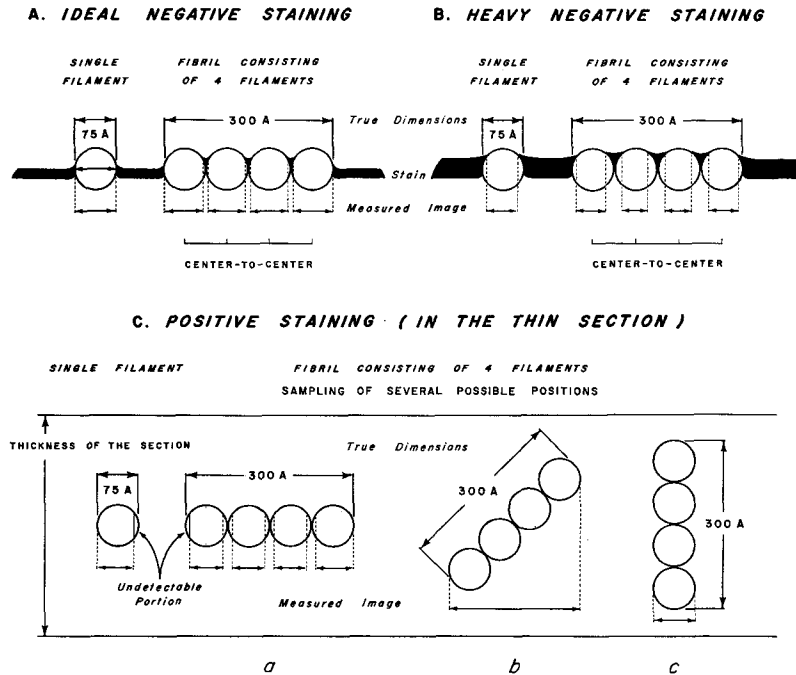


FIGURE 28 A diagram showing possible different electron micrographic images of the amyloid fibril under a variety of conditions of staining and positioning. Fibrils consisting of a single filament and a fibril consisting of four filaments are used as examples. The wide variation in their appearance, depending upon the type of staining procedure (positive or negative), the angle of placement on the grid or in the stain, the number of filaments in the fibril, are apparent.

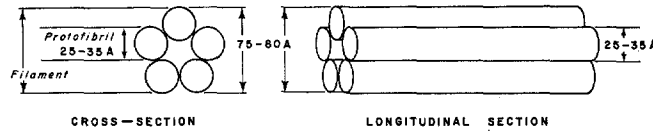
In the previous section and for the subsequent discussion, the fine structure of the fibrous components of amyloid has been classified into the following four moieties by their dimensions. Where possible currently used terms (amyloid fibril and amyloid filament) are used as in previous reports. 1. *The amyloid fibril* is defined as a side-by-side aggregate of a number of filaments and is the structure commonly seen on electron microscopy of tissue sections. 2. *The amyloid filament* is approximately 75–80 A wide and consists of five (or less likely six) subunits, called protofibrils. 3. *The amyloid protofibril* is about 25–35 A wide and probably consists of two or three helical subunits (or, less likely, is composed of globular subunits assembled end-to-end), each of which is approximately 10–15 A wide, i.e. *the amyloid subprotofibrillar strands*.

The term “amyloid fibril” has been most commonly used as the name of the fibrous component of amyloid since its first description (15). On the basis of the electron microscopic studies of the isolated fibrous component of amyloid, we have

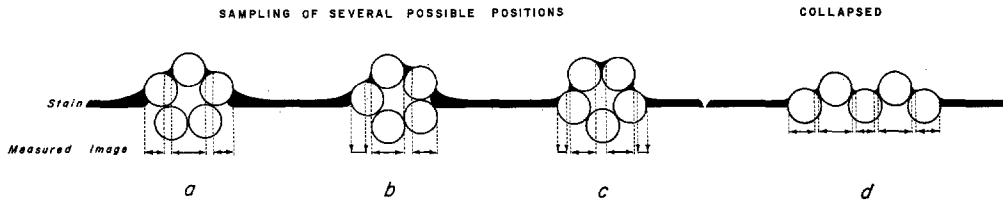
proposed that the term amyloid fibril be retained as the name for an aggregate of a number of approximately 75-A “amyloid filaments” (2, 59). Although the possibility exists that the aggregation of the filaments may be an accidental product of the isolation procedure, the following data from the present study suggest that the aggregation is not an artifact but a native condition. First, the aggregation has been found in all specimens, no matter how prepared. Secondly, the aggregated filaments are equal in length and also the termination of the filaments has been consistently unbroken or smooth, suggesting that those filaments might be synthesized or polymerized together in a set (Figs. 8 and 9) (83). Third, the aggregation is still present after treatment by sonication or with urea. Fourth, the aggregation has seemed to be very tight (without any, or with extremely little space separating filaments) and the side-by-side arrangement of the filaments has not changed even when they are twisted.

The dimension of roughly 100 A has been accepted as the diameter of the amyloid fibrils in-

**A. MODEL FILAMENT AND PROTOFIBRILS**



**B. FILAMENT AND PROTOFIBRILS UNDER IDEAL EMBEDDING FOR NEGATIVE STAINING**



**C. FILAMENT AND PROTOFIBRILS WITH EXCESSIVELY THICK EMBEDDING FOR NEGATIVE STAINING**

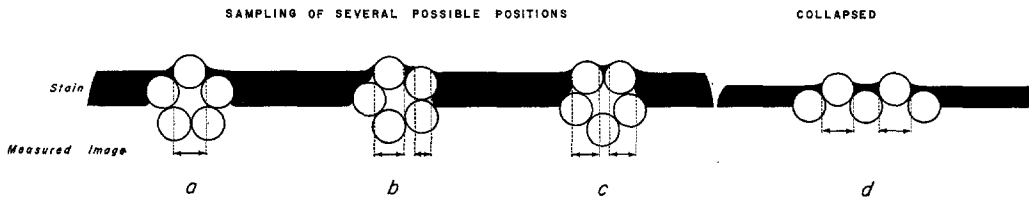


FIGURE 29 Diagram showing a proposed model of the amyloid filament, composed of protofibrils (*A*), and the possible varying images that might be demonstrated on the micrograph after negative staining (*B* and *C*). The amyloid filament of 75–80 Å diameter consists of five protofibrils 25–35 Å wide arranged parallel to each other and almost longitudinal to the long axis of the filament. On the cross-sectional plane of the filament the protofibrils are arranged pentagonally with a 15–20-Å central core. This structure may be demonstrated differently, depending on its position as well as on the embedding thickness of the stain. As can be seen in the diagram (*B*, *a–d*), some variation in measured dimensions is to be expected even under ideal conditions. When an excessively heavy staining procedure is used (*C*), the image dimension may significantly underestimate the true diameter of the structure under study. In actual preparations, the central core may be stained to some degree and may, in addition, cause more complicated shades of the amyloid filament image.

tissue sections by most investigators, including the present authors. Owing to this fact, as well as their observation on trypsinized, negatively stained amyloid fibrils, Emeson et al. (64) have criticized the results of our previous report of filaments (59) in the belief that aggregates are not seen in tissue sections: "neither 300-Å fibrils nor such a great variation in fibril width have been reported by other investigators." However some investigators from other laboratories as well as ours have, in fact, reported such a relatively large measurement (200–300 Å) for the diameter of amyloid fibrils.

Also, in our electron micrographs of the tissue sections, as well as in most published electron micrographs, 200–300-Å-wide fibrils, each not apparently resolved into two or three finer strands, are not unusually found in a certain percentage of the fibrils (Figs. 23–26). There is little doubt, however, that the 100-Å diameter is the most commonly observed form in conventional tissue sections (2, 15–36). Indeed, in our report on amyloid filaments (59) the large aggregate of filaments was uncommonly observed when 500 such aggregates were measured.

It should also be noted that generally, in electron microscopic studies, if the object was expected to be uniform, the smallest dimensions demonstrated on the micrographs were used to interpret the true dimensions of the object, and the larger dimensions were thought of as the accidental aggregation or the overlapping of the images. Since the results of our observations strongly suggest that the assemblage of the amyloid filaments is native, we believe that these elements should be identified separately. We still recommend the terms "amyloid fibril" and "amyloid filament," for the term "filament" (though occasionally used almost synonymously with "fibril") is more often used for the fibrous structure which has a dimension between that of a "fibril" and that of a "micro-proto-fibril" (84, 85). The interrelation of the fibril and filament in tissue section and on negative staining is diagrammatically illustrated in Fig. 28. It is our belief that the minor variations reported on negative-staining experiments on amyloid (55) are due to technical considerations, such as excessive or too little use of phosphotungstic acid, as illustrated and discussed further on.

The amyloid filaments have appeared to be relatively uniform in their width as well as in their general proportion at the moderate magnifications of the micrographs. Careful analysis does indicate that a range in diameter of 50–110 Å exists even in different portions of an individual filament. The variation in these values and the other aspects of the amyloid filaments as described in this report may be quite reasonably interpreted, if the following interpretation of the three-dimensional architecture of amyloid on the filament-protofibril level is accepted (see diagram in Fig. 29). On the negatively stained filament (on one side view of the filament), one to four 25–35-Å-wide subunit strands arranged parallel to each other and longitudinal or slightly oblique to the long axis of the filament have been observed. When the filament has been treated and partially disrupted, it has been found to be dispersed into five (or less likely six) protofibrils 25–35 Å wide. The protofibrils have been occasionally followed up to 1,000 Å in length on one side of the filament, and no regularity in the direction of twist or in their twisting periodicity has been found when they have been arranged oblique to the longitudinal axis of the filament. The above evidence suggests that the amyloid filament consists of five (or less likely six) subunit strands (protofibrils) 25–35 Å wide which

are arranged parallel to each other and almost longitudinal to or in a loose helix around the long axis of the filament, and that on the cross-sectional plane of the filament the protofibrils are arranged pentagonally (or hexagonally) with a 15–20-Å central core. This structure has been also supported by the (probable) cross-sectional view of the filament in the positively stained tissue section (Figs. 17, 18, 24).

The use of the term "amyloid protofibril" to denote the subunit strand of approximately 25–35 Å width is in accord with the terminology used for other fibrous proteins of comparable dimensions (84, 85). When the filament, as defined above, has been negatively stained, the width and the configuration may be demonstrated differently depending on its position as well as on the embedding thickness of the stain as shown in the diagram (Fig. 29).

Taking into consideration the above data on the occasional variation in filament diameter and the suggested protofibril subunit, how can we now more accurately define the diameter of the amyloid filament? Approximately 75–80 Å has been indicated as the average value of the measurements in the present study as well as in our previous study, and the variation of 50–110 Å has been noted. This range probably includes the measurement of filaments embedded too thickly in the stain, and possibly of the "collapsed" filament. It is likely that the most reliable data are obtained with measurements on the bundles of filaments, for the following reasons: 1. They have seemed to aggregate with minimal or no interspace; 2. In the aggregate, the filament would be most protected from damage during preparation; 3. If the filament represents a native form, the dimension of the filament in the aggregate may have "functional" or "structural" value as it might have in the living tissue. Thus, the consistent 75-Å dimension has been indicated as the center-to-center distance of the filaments in the aggregate, with unexpected but good agreement with the mean value of the direct measurement of individual isolated filaments.

The studies of the protofibril indicate a relative periodic beading with a 35–50-Å repeat along its length. These beads may represent as helically arranged subunit strands or end-to-end aggregated globular subunits. The data from the protofibrils studied at the highest magnifications may well

correspond to a ropelike structure which consists of two or three subunit strands. On the micrographs, those subunit strands (*amyloid subprotofibrils*) have been measured as 10–15 Å in diameter, and found to coil with an approximate repeat of 35–50 Å (Figs. 19–22). Thus, if the amyloid protofibril is a two-strand rope, the helical periodicity of the subunit strand should be 70–100 Å, and if the protofibril is a three-strand rope, the helical periodicity should be 100–150 Å. This interpretation for the subprotofibrillar ultrastructure of amyloid is supported by the results of the experiments in which partial disruption of the amyloid filament was induced. The filament has seemed to disperse to the protofibrils relatively easily, and the protofibrils which dispersed from the filament have retained their fibrous nature.

However, the interpretation of the ultrastructure of such extremely small dimensions must be made very cautiously. Although several reports have already described biological ultrastructure of about 10-Å dimensions after negative staining and high resolution electron microscopy (50–53), and although a subunit of such dimensions has been apparently demonstrated on our present micrographs, the unit is so small and the technical problems and potential variations due to stain and alignment are of such nature that we prefer only to suggest this subprotofibril structure as a possibility. In order to detect a unit of 10 Å or less in these studies, a relatively large or no objective aperture has been employed, instead of a relatively small aperture (48, 54). Under these conditions, the contrast of the image may be produced mainly as the phase-contrast (54). In this case, the structure of 10-Å dimensions may not represent the exact size and the true shape on the image of the electron micrograph. In addition, “background noise” at this resolution can be confused with the true image, despite all precautions. Therefore, even though our interpretations are based upon study of many micrographs which gave consistent results, the subprotofibrillar structure of amyloid should be interpreted conservatively, leaving open, as yet, several possibilities concerning its construction and dimensions. This is particularly necessary at the present time, when no reliable x-ray diffraction data of this substance are available. Thus, even the remote possibility that the amyloid protofibril may consist of globular subunits attached end-to-end still exists.

### *Comparison of Fine Structure of Amyloid with that of Other Fibrous Proteins*

The ultrastructural organization of the amyloid fibril as interpreted in the present study bears a certain resemblance to that of other fibrous proteins, especially keratin (51, 85–96). It also has features in common with the ultrastructure of flagella (especially to type B; see references 99, 100) (48, 72, 83, 96–101), myofilaments (myosin and actin) (53, 102–107), collagen (84, 108, 109), and elastin (110–114), as well as the carbohydrate, cellulose (14, 115–118).

The ultrastructure of keratin has been well studied by means of electron microscopy as well as x-ray diffraction (51, 85–96). According to Fraser et al. (90), the alpha-keratin microfibril consists of a circular ring of nine protofibrils with an additional center pair. The centers of the protofibrils of the outer ring lie on a circle 60 Å in diameter (i.e. the diameter of the microfibril is 80 Å). Individual protofibrils are 20 Å in diameter. Each consists of a three-stranded alpha helix rope. Each strand is regularly divided into a repeating sequence of three distinct portions of similar length. Using the negative-staining and high-resolution electron microscopy, Dobb (51) has recently demonstrated the consistency of the keratin protofibril substructure with the data obtained from x-ray diffraction studies. When the structure of the amyloid filament is compared to the structure of the alpha-keratin microfibril, it is apparent that both of them have an approximately similar (75–80 Å) diameter, and consist of a number of protofibrils arranged almost longitudinally. Nine circularly arranged keratin protofibrils with a center pair (i.e. 11 protofibrils in all) compose the keratin microfibril, and five pentagonally arranged amyloid protofibrils compose the amyloid filament. The keratin protofibril has a diameter of approximately 20 Å, and amyloid protofibril has a diameter of approximately 30 Å. The alpha-keratin protofibril consists of a three-strand rope of alpha-helices, and the amyloid protofibril consists of a two- to three-strand rope of coiled, 10–15-Å-wide subprotofibrillar elements.

It is also interesting that cellulose microfibrils have been shown to have dimensions very similar to those of the amyloid protofibrils after negative-staining and high-resolution electron microscopy (115, 116). Although some variation was found among various kinds of cellulose, the width of

cotton cellulose microfibrils has been reported to be about 35 A and the center-to-center distance of the microfibrils to be about 40 A. Cellulose microfibrils are also arranged almost parallel to each other in the tissue sections.

*Comparison of Present Data with Those of Other High-Resolution Studies of Amyloid*

The results of our previous report (59) have been confirmed for the most part in the present study. However, the beaded appearance along the length of the filament with about a 100-A periodicity, which was described in the previous report, has not been clear on the filaments stained and photographed by the techniques currently employed. On the other hand, when the aggregated filaments have been embedded in the relatively thick stain layer and photographed with the small objective aperture and at the relatively low initial magnifications such as 30,000–40,000, about a 100-A (and occasionally 50-A) periodical beading along the length of the filament has often been demonstrable as previously. The beaded appearance seems to us now to be an image of some underlying, as yet undefined, repeating structure enhanced by diffraction and phase effect. However, we could not clarify in the present study the reason for the two different images caused by the different techniques. The observations of Boéré et al. (55) are comparable to those in the present studies. Their measurements of the dimensions of the fibrous components of amyloid (75 A on negative staining) correspond well with the dimensions at the filament-protofibril level in the present study. As is apparent from the diagram (Fig. 29), if the filaments were embedded moderately deeply in the stain, and if the central core of the filament were somewhat stained, the filament might appear as they described. We have not observed the 400-A twisting periodicity, described in their report, either at the fibril-filament level or at the filament-protofibril level. However, the possibility exists that the amyloid fibril or the amyloid filament may assume a regular twisting under certain preparatory conditions.

The results of Emeson et al. (64) are not directly comparable with the results of the present study, for a variety of reasons. First, the trypsinization might cause unexpected changes in the fibril ultrastructure. Second, a review of their published micrographs indicates that the fibrils seem to be quite deeply embedded in the stain in their prepa-

ration, in contrast to the thin embedding employed in the present study. It might be, however, that their observations were actually made on the structures at the fibril-filament level of the present study. For example, as it apparent from Fig. 28 in this report as well as from the figures in our previous report (59), the filament which is embedded deeply in the stain may give a value for diameter even as low as 30–50 A. Thus, it is possible that their "fibril" consisted of two filaments with an apparent 30–40-A width and a 30–40-A space between them.

On the basis of the study of the samples collected from amyloid-laden tissues by a differential centrifugation technique, unique structures have been described by Bladen et al. (62). Their technique differed in that they did not study the sediment containing the bulk of the amyloid fibrils but rather a small pellet obtained from the supernatant washing of amyloid after high-speed centrifugation. Those authors described two types of particles, one a rod about 100 A wide, the other a small pentagonal structure about 90 A in diameter. Narrow electron-opaque bands divided the rods at regular intervals into smaller segments so that they have appeared cross-striated with periodicity of approximately 40 A. Frequently, however, the dense bands have varied in width, suggesting that the segments have been easily disunited. The pentagonal structures have seemed to be single segments lying on their flat side, thus affording an end view of the amyloid rod. The pentagonal structures consisted of five 20–25-A globular sections surrounding a dense core, indicating to those authors that the rods were of hollow structure, possibly consisting of an array of globular units. Subsequently, following the method of Bladen et al., Benditt and Eriksen (63) accepted these dimensions as representing the subunit structure of amyloid.

These structures described by Bladen et al. and designated as amyloid by them have shown very clear structural regularity at the level of 30–40-A units. Although structures of 30–40-A dimensions should not be difficult to demonstrate in negatively-stained preparations, we have never seen such structures on the fibrils which had been carefully identified as amyloid in our laboratory and which, it must be remembered, constitute at least 90–95% of the protein studied (i.e., the preparation of Bladen et al. is comparable to a microsomal fraction). Several important questions,



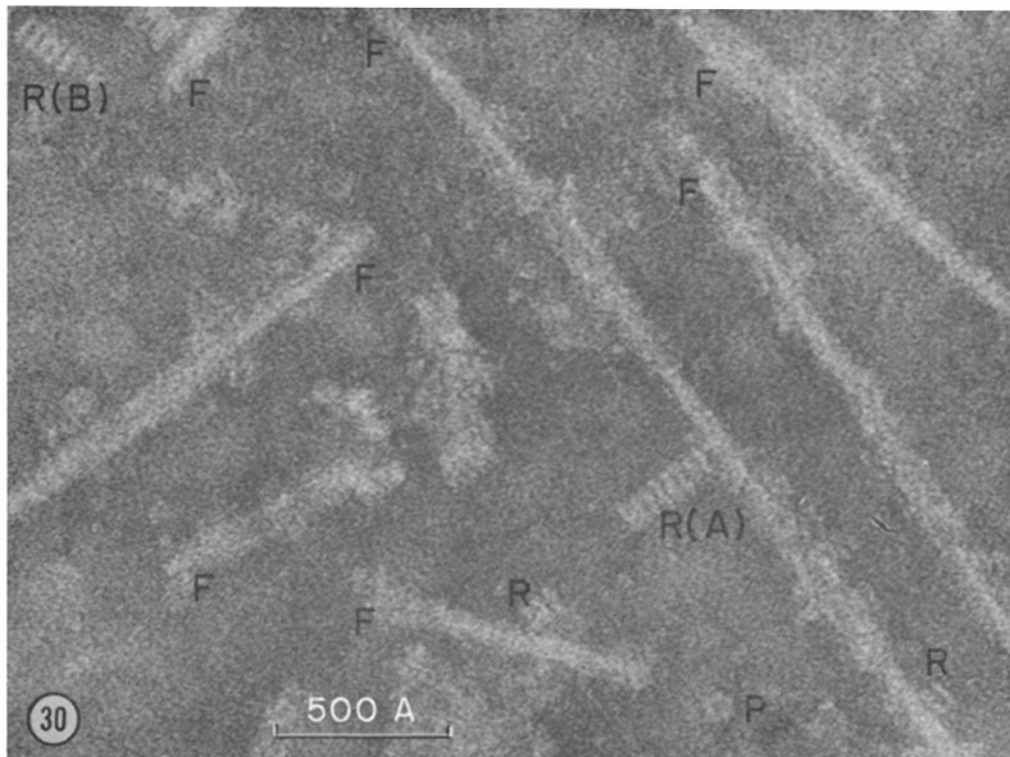


FIGURE 30 A top layer sample negatively stained with phosphotungstic acid after 5-min sonication. At least three different structures can be observed: amyloid filaments (*F*), rods (*R*), and a particle (*P*). See text for detailed explanation. The filaments have the structure already described in this article. The rods are distinct from the filaments, and measure about 100 Å in width. The particle measures about 100 Å. These latter structures (rods and particles) are comparable to those previously described (62) and constitute only a small fraction of our current preparations.  $\times 450,000$ .

*Note:* The 40-Å banded rod is clearly demonstrated in two positions (*A* and *B*) perpendicular to each other on the micrograph. This suggests that on this micrograph an analysis of dimensions can be made in any direction.

therefore, must be raised: 1. Is the object described by Bladen et al. amyloid? 2. Is the object the same as the one we studied, or a different component of amyloid, if it is amyloid at all? 3. Why do the objects appear so completely different from each other in ultrastructure if they are the same? Soon after the results of Bladen et al. were presented, we reviewed our previous electron micrographs, and found very rarely the two types of structures described by those authors, but these structures were seen in our preparations quite distinct from the fibrils which we designated as amyloid.

During the present study, the particles of approximately 100-Å diameter with a central dense core have been found in various specimens. The

rod structures approximately 100 Å wide with about a 40-Å periodic banding have also been found occasionally, especially in the sonicated top layer samples. However, the distribution of the rodlike structures in our specimens has been estimated to a few per cent at most, and the length of the rod has never measured more than 800 Å. The rodlike structures have been clearly differentiated from the filament we described (Fig. 30), as follows: 1. The rod has the same width along its entire length even when embedded relatively deeply in the stain, in contrast to the moderate variation in width on the more common filament. 2. The rod has shown a very clear and regular banding with a periodicity of approximately 40 Å,

as opposed to the longitudinal (or slightly oblique) linear subunit structures seen on the most carefully prepared filaments. 3. The end structure of the rod has been observed to be clear cut with no evidence of dispersion into finer longitudinal rows, as opposed to the termination of filament which is irregular, obscure, and often dispersed.

Bladen et al. have also stated that their rods "differed radically, however, from these fibrous proteins (keratin fibrils and some flagella) as well as from collagen and elastin in their tendency to fragment across their long axis rather than slitting lengthwise into microfibrils or protofibrils. If the rods were composed of several three-stranded ropes of helices as has been suggested for keratin, it is difficult to envision that they should break so easily at regular intervals." In contrast, the results of the present study have supported the great similarity between the biophysical nature of the amyloid fibrils and that of those protein fibrils.

The questions presented above as to the nature of these new elements still remain. Whether these new elements are other components of amyloid or even nonamyloid substances must be considered. Their ultrastructural appearance (and method of isolation) are quite reminiscent of lipid structures derived from cell walls (50, 119-123) as well as of other small intracellular components (124-127).

The authors wish to thank Professor A. G. Matoltsy for his critical reading of the manuscript. The skillful photographic and technical assistance of David Feigenbaum and Orville Rodgers is also gratefully acknowledged.

Grants in support of these investigations have been received from the United States Public Health Service, National Institute of Arthritis and Metabolic Diseases, Grant Nos. AM-04599 and T1-AM-5285, and the Arthritis Foundation.

Received for publication 21 October 1966.

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