

## RECONSTITUTION OF AMYLOID FIBRILS FROM ALKALINE EXTRACTS

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### INTRODUCTION

Solubilization and reconstitution is one of the key steps in the purification of fibrous proteins and in the clarification of certain of their physical properties. Since the description of amyloid as a fibrous protein (1-3), several preliminary studies in this direction have been reported for the amyloid fibril. Newcombe and Cohen (4) studied the effect of pH changes on solubility of amyloid and described a precipitate obtained from the alkaline extracts (pH 9.5 in glycine buffer) by reducing the pH to the apparent isoelectric point (pH 4.5). Glenner and Bladen (5) utilized the method of Newcombe and Cohen on a different amyloid extract and succeeded in reconstituting "periodic fibrils" which they believed to be a specific component of amyloid (6) and which have recently been identified in our laboratory as the P (plasma)-component of amyloid (7, 8) by electrophoretic, immunologic, and morphologic means.<sup>1</sup> Benditt and Eriksen (9) also attempted to reconstitute amyloid fibrils from a fraction of a 6-8 M urea extract of amyloidotic tissue, but the nature of the products appears to be difficult to identify.

A crucial problem in the above studies and in the identification of any solubilized and reconstituted macromolecule is an understanding of the subunit structure of the molecule. Recently the present authors have attempted to define precisely the subunit structure of the amyloid fibril and concluded that (10, 11): the amyloid *fibril* consists of a number of 75-80-A wide filaments assembled side-by-side; the amyloid *filament* is made up of several subunits, amyloid protofibrils, which are arranged almost longitudinally to the long axis of the filament and surround a central core 15-20 A in diameter; the amyloid *protofibril* is 25-35 A wide and appears to consist of two to three subunit strands, *subprotofibrillar strands*, helically arranged with a 35-50 A repeat (or less likely is composed of globular subunits aggregated end-to-end). It is

<sup>1</sup> Cathcart, E. S., T. Shirahama, and A. S. Cohen. *Biochim. Biophys. Acta*. In press.

important to note that these amyloid fibrils are structurally quite distinct from the periodic fibrils described by others (5, 6, 9) and that the usual amyloid fibrils form the bulk of the "top layer" (12) as well as the "sucrose separated" material (2).

This paper reports the solubilization of the amyloid fibrils at high alkaline pH, and the reconstitution of the protein to a fibrous form which, insofar as can be determined, is comparable to native amyloid in its tinctorial and ultrastructural properties.

### MATERIALS AND METHODS

The starting materials for the present study were isolated by sucrose density centrifugation (2) from frozen spleens of two patients, one with primary and one with secondary amyloidosis. Although the wet material was used for routine procedures, lyophilized samples were analyzed quantitatively (without any significant difference in the results), and weight hereafter will therefore represent dry weight. 50 mg of the isolated amyloid fibrils were mixed with 20 ml of any one of several alkaline solvents, i.e. ammonium hydroxide, sodium hydroxide, 0.1 M glycine buffer, or 0.1 M phosphate buffer, pH 8.0-13.0. After continuous mild shaking for 12 hr at room temperature, the mixture was centrifuged at 40,000 g for 2 hr and the supernatant was carefully separated. This centrifugation was repeated twice, or occasionally was replaced with centrifugation at 100,000 g for 1 hr. The separated supernatant was filtered through a fine filter paper (Schleicher and Schuell Analytical Filter Paper No. 589 Blue Ribbon). In this fashion a clear extract was obtained.

The pH of the supernatant was then reduced by drop-by-drop addition of 1-2 N hydrochloric acid, and finally was brought to pH 4.5 (delicate adjustment of pH was made by titration of 0.1 N hydrochloric acid and 0.1 N sodium hydroxide). The resultant precipitate was then centrifuged at 10,000 g for 10 min, and the sediment was collected. An aliquot of the sediment as well as of the original starting material was suspended in distilled water and prepared for light and electron microscopy. For light microscopy, the suspension was spread on a gelatinized 1 by 3 inch glass microscope slide, air dried,

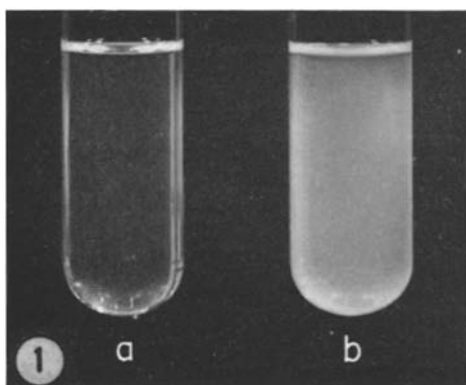


FIGURE 1 (a) Clear amyloid solution produced by fractional extraction (see text) in ammonium hydroxide at pH 11.5. (b) Cloudiness soon after the pH was adjusted down to 4.5.

then fixed in 4% formaldehyde<sup>2</sup>, and stained with Congo red (as well as with other stains) in standard fashion. After thorough rinsing in running water to remove unbound Congo red and subsequent dehydration in ethanol, the slides were examined by light and polarization microscopy. For electron microscopy, the suspension was applied to carbon-coated grids. After shadow casting with platinum-palladium or negative staining with phosphotungstate or uranyl salt (13-15), the grid was photographed in a Siemens Elmiskop I at initial magnifications of 20,000 for the shadowed specimens and 80,000-160,000 for the negatively stained ones. In the shadowed preparations, dimensions were determined after appropriate calibration of the electron microscope and with measured shadowing angles, as previously discussed (11).

## RESULTS

### *Solubilization in Alkali and Reprecipitation*

Precipitates from a variety of extracts were examined by light and polarization microscopy after Congo red staining, as well as by electron microscopy after shadow casting. This examination revealed that both the positive reaction for Congo red (green birefringence on polarization microscopy) and the appearance of a fibrous ultrastructure comparable with native amyloid (3, 11) were usually demonstrated in parallel. The results indicated the following: (a) solubility of the original purified amyloid samples at a variety of pH's was in basic agreement with the results of Newcombe and Cohen (4); (b) the precipitates from

<sup>2</sup> Hayashi, M. Personal communication.

extracts obtained below pH 9.5 showed neither Congo red stainability nor a fibrous ultrastructure; (c) the precipitate from the extracts obtained at pH's 9.5-11.0 demonstrated a faint to moderate reaction for Congo red and a small to moderate amount of fibrous material in the electron microscope; (d) the precipitates from the extracts at pH's higher than 11.0 showed definite positive reaction for Congo red and had a fibrous ultrastructure. These results were generally obtained with all solvents noted above with minor variations.

### *Fractional Extraction and Reprecipitation*

In order to obtain greater purity and to study further the mechanism of solubilization and reconstitution, extraction was performed fractionally in two steps. After the first extraction of the starting material with ammonium hydroxide at pH 10.5 as described above, the resultant residue was extracted with ammonium hydroxide at pH 11.5. By determination of the dry weight of the residues after extraction, it was found that 10-20% of the starting material was first extracted at pH 10.5 and an additional 10-20% with the second extraction at pH 11.5. The supernatant of the second extract after centrifugation and filtering was clear, colorless, and more viscous than the original solvent (Fig. 1 a). During the procedure of reducing the pH (as described above) a visible fine, white cloudiness first appeared at pH 6.0-5.5. The precipitation was most heavy at pH 4.0-5.0 and tended to clump (Fig. 1 b). (If the pH was raised, however, the cloudiness disappeared.) When this material was centrifuged, a cream-colored pellet was obtained which was 5-10% of the weight of the original starting material. This sediment could be dissolved in small amounts of ammonium hydroxide at pH 11.5 to make a concentrated viscous solution. The sediment (material precipitated as above) was clearly stained by Congo red, showed characteristic green birefringence of amyloid under the polarizing light (Figs. 2 and 3), and demonstrated crystal violet metachromasia.

By electron microscopy of the shadowed specimens, the precipitate was found to consist mainly of fibrous structures. Those structures measured 75-80 Å in width, varied in length, and often aggregated side-by-side composing a wider assembly (comparable to native amyloid fibrils). Finer structures roughly 30 Å in diameter, comparable with native amyloid protofibrils (11), were also

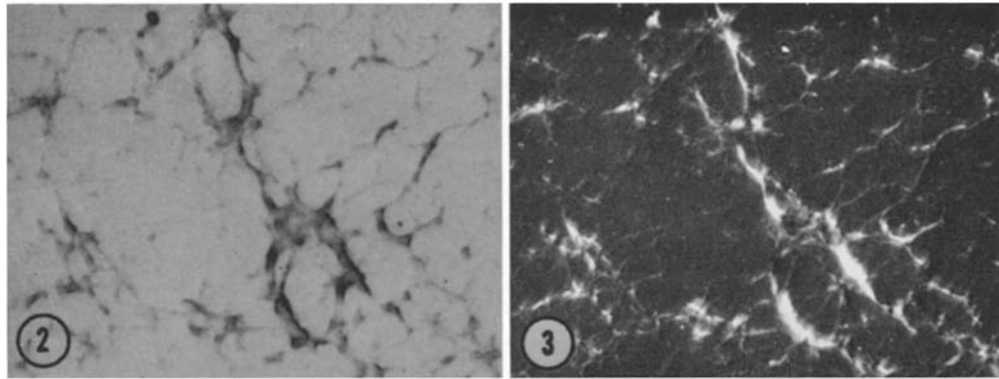


FIGURE 2 A light micrograph of a precipitate from an alkaline fractional extract of amyloid appears to be a very pure preparation of amyloid substance. Stained with Congo red.  $\times 150$ .

FIGURE 3 Same area as Fig. 2, photographed under the polarizing light. The bulk of the precipitate shows the characteristic green birefringence of amyloid.  $\times 150$ .

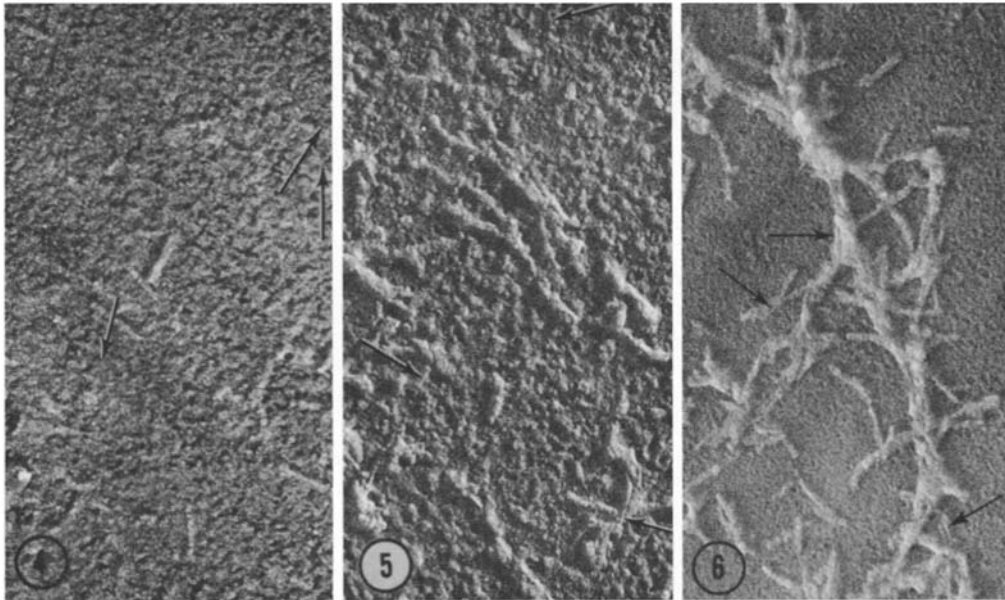


FIGURE 4 An amyloid extract (by a fractional extraction) in ammonium hydroxide at pH 11.5 applied to a carbon-coated grid, dried, and shadow casted. A small number of fibrous structures which are comparable to, but shorter than (less than 2000 Å), amyloid filaments are seen. Unidentified particulate or fibrous (arrows) structures, most with dimensions of less than 30 Å, are numerous in the background.  $\times 50,000$ .

FIGURE 5 The same extract as Fig. 4, but after the pH was adjusted down to 8.5. More fibrous structures and larger aggregates are seen. Their width and length are now comparable to those of the native amyloid filament and fibril. Finer fibrillar structures 25–35 Å in diameter are also present (arrows). The number of unidentified structures in the background have decreased significantly.  $\times 50,000$ .

FIGURE 6 The same extract as Figs. 4 and 5, but after the pH was adjusted down to 4.5. Fibrous structures comparable to native amyloid filaments and fibrils appear fully developed. 25–35-Å wide fibrillar structures are also occasionally evident (arrows). The background is clean.  $\times 50,000$ .

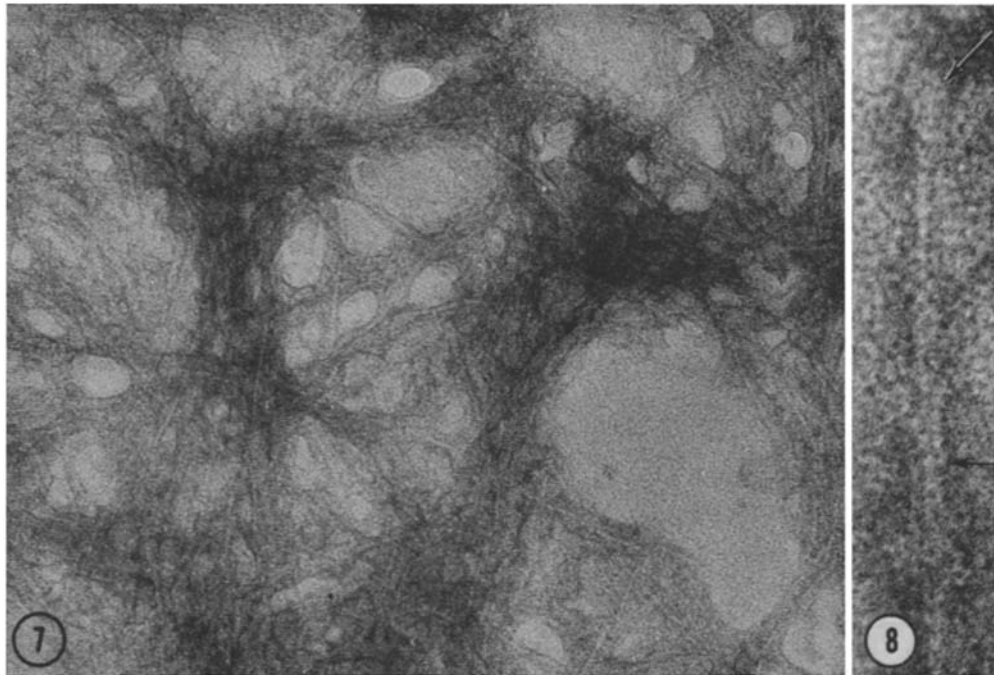


FIGURE 7 Precipitated amyloid. 25–35-Å wide fibrillar structures are clearly observed. These fibrillar structures, which are comparable to amyloid protofibrils, tend to aggregate side-by-side in a 70–100-Å wide assemblage. Negatively stained with phosphotungstate.  $\times 160,000$ .

FIGURE 8 A reconstituted amyloid filament. At least three amyloid protofibrils (25–35-Å wide fibrillar structures with a beaded appearance) can be seen in a portion of the filament (on one side view) (lower arrow). These protofibrils appear to be arranged in a loose helix around the long axis of the filament. The terminal structure of the filament (upper arrow) is similar to that of the native amyloid filament (11). Negatively stained with phosphotungstate.  $\times 500,000$ .

seen in these specimens (Fig. 6). Electron microscopy of the negatively stained specimens revealed that the bulk of the precipitated material consisted of 25–35-Å wide fibrous structures of varying length (up to microns), which were again identical with the amyloid protofibrils (11). They tended to aggregate side-by-side, and often assembled in 70–100-Å wide units (Figs. 7 and 8) as amyloid filaments. No significant difference in the results was found between the amyloids isolated from primary and secondary amyloid disease. Similar results were obtained with the different solvents mentioned earlier. Light and electron microscopy both demonstrated the high purity of these fibrous components in the precipitate (Figs. 2, 3, 6, and 7). Moreover, in a control study, material solubilized then precipitated in the same manner from a non-amyloidotic spleen has shown neither the tinctorial

nor the ultrastructural properties characteristic of amyloid.

#### *Development of Fibrils in the Extract During the Reduction of the pH*

The electron micrographs of the shadowed samples which were collected at various stages during the reduction of the pH of the extract (applied directly on the carbon-coated grids and air dried) have been informative with regard to the polymerization of the molecule. The shadowed specimen from the second *extract itself* (pH 11.5) showed that the bulk of the specimen consisted of unidentified, very fine particulate and fibrous structures (with dimensions less than 30 Å) and a small number of fibrous structures somewhat comparable to the amyloid filament but usually quite short

(less than 2,000 Å) (Fig. 4). After gradual reduction in pH (by adding 1.0 N hydrochloric acid, one drop per 5 min), the shadowed specimen from the extract at pH 8.5 suggested that the unidentified background structures had decreased in number, while the fibrous structures increased in number as well as in their width and length (Fig. 5). Finally, the specimen from the extract at pH 4.5 in which the precipitate had already appeared showed that the bulk of the specimen consisted of well-grown, fibrous components, most of which were comparable to native amyloid fibrils or filaments in their width and length and that the background was almost clear (Fig. 6).

## DISCUSSION

Prior to the identification of amyloid as a specific fibrous protein a wide variety of analyses of its composition had been carried out. These included several attempts at extraction at varying pH levels and were recently reviewed in detail (3). In those studies, several workers employed alkaline extraction methods (Hass and Schulz, reference 16; Wagner, reference 17; Larsén, reference 18) which they believed solubilized the amyloid, but the results were not conclusive since the underlying nature of the amyloid was not clearly understood.

The present data demonstrate that amyloid fibrils can be at least partially solubilized by alkaline buffers at pH's above 11.0, and that from the solubilized fraction a material can be reprecipitated which is comparable to native amyloid in its tinctorial and ultrastructural appearance. However, the procedure alone may not directly serve for the purification of amyloid fibrils since the solvent and the reprecipitating method are not selective for amyloid, but could apply to other tissue proteins as well. For example, the starting material may contain small amounts of contaminating protein, and this too can be identified in the reprecipitated material. In addition, ammonium hydroxide at pH 11.5 will dissolve over 50% of control normal spleen tissue, and, although one cannot recover amyloid fibrils on reprecipitation, this procedure demonstrates that impurities could be carried along if the above method alone was used.

Purification of protein by means of fractional extraction has been used by others (19, 20), and our results demonstrate that the method may serve for purification of the amyloid fibrils. Indeed, the precipitate resulting from the fractional ex-

traction has been found to be a purer preparation of amyloid.

The clearer demonstration of the structure of amyloid protofibrils on the electron micrograph suggests that interprotofibrillar bonding in the reconstituted amyloid filament is not so tight as in the native one (21, 22), and also suggests that protofibril is an important unit in the process of solubilization and reconstitution of the amyloid fibrils. In addition, the tendency of the amyloid filament to disperse into the protofibrils has been demonstrated in our previous study (11). It is possible that this structure represents the unit structure of amyloid comparable to the tropocollagen molecule of collagen, but further study will be needed to verify this point.

The results of the final experiment (the third section in Results) may be interpreted in various ways. The fibrous structure found in the preparation from solution at pH 11.5 may represent an incompletely polymerized amyloid molecule appearing when the solution was dried out (22), fragments of native amyloid fibrils suspended in the solvent, or a structure related to a single molecule (23-25) of amyloid. At any rate, the reduction of unidentified structures in the background and the sequential growth of the fibrous structures are noteworthy.

The present results would not as yet be enough to clarify completely the following questions. Are the amyloid fibrils all of single molecule in the extract? What is the structure of the single molecule of amyloid? How are the molecules polymerized to form the fibrils? Does any relationship exist between the usual amyloid fibrils and the periodic fibrils (or the P-component)? For the perfect purification of the derivative, further procedures are necessary even after the fractional extraction. These problems are currently under investigation, and the preliminary results<sup>3</sup> are in support of the interpretation of the present report. For example, a pilot analytical ultracentrifugation of the fractionally extracted sample in the glycine buffer at pH 11.5 showed a major peak which was calculated as approximately 44S. This suggests the uniformity of the solubilized amyloid fibrils in the extract (most probably in the state of monomer though less possibly of dimer or polymer), and strongly supports the concept of *reconstitution* in the interpretation of the present results.

<sup>3</sup> Cohen, A. S., E. S. Cathcart, and T. Shirahama. Unpublished data.

## SUMMARY

Amyloid fibrils were extracted with ammonium hydroxide and other solvents at high alkaline pH (pH 11.5), and then precipitated by reducing the pH to 4-5 with 1-2 N hydrochloric acid. The precipitate showed tinctorial and ultrastructural properties identical with those of native amyloid. Attempts were also made to obtain this derivative in purer form by using a fractional extraction scheme. The data suggest that these methods are

useful in the purification and reconstitution of the amyloid fibril and its subunits.

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