PHYSICAL, CHEMICAL, AND ULTRASTRUCTURAL STUDIES OF WATER-SOLUBLE HUMAN AMYLOID FIBRILS

Comparative Analyses of Nine Amyloid Preparations*

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Amyloid is a homogeneous, fibrillar, eosinophilic, extracellular protein, first recognized by Rokitansky in 1842 (1). It is generally identified by its characteristic green birefringence when stained with Congo red and examined in the polarizing microscope (2). Several systems of classifying amyloid on the basis of its clinical features are in current use. The one most commonly employed (3), based on the association or lack of association of amyloidosis with certain other disease states, divides the clinical forms of amyloidosis as follows: primary and secondary types, tumor-forming amyloidosis, amyloidosis associated with multiple myeloma, and a large number of rarer forms, most of which appear to be inherited disorders. In general, amyloid deposits are found in characteristic locations in the several types of the disease. Another classification, based largely on histological criteria, divides amyloidosis into the pericollagenous and the perireticular types (4).

It has long been recognized that amyloid deposits in different patients may vary in their appearance when examined with a variety of histochemical stains, such as iodine, Congo red, crystal violet, and methyl violet. However, because of the obvious limitations of the histochemical techniques and the prior lack of either chemical or immunological methods to characterize amyloid, it has not been possible to perform precise comparative studies of amyloids from different individuals. Thus, while all amyloid appears to consist of characteristic fibrils (2), there is no information available to date documenting either similarities or differences among amyloids obtained from individuals having the same clinical type of amyloidosis; nor is it known whether the fibrils associated with

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different clinical or histological forms of amyloid are different or not. The extraction of amyloid fibrils in a high state of purity and in a water-soluble form (5) has made it possible to initiate studies to characterize the fibrils more precisely and to compare amyloid preparations derived from different sources.

In the present article we shall describe attempts to solubilize amyloid from 10 individuals with primary, secondary, and myeloma amyloid as well as present some of the physical, chemical, and ultrastructural characteristics of the amyloid fibrils from 9 of these patients. In addition, the development of a method to obtain smaller fragments by alkaline degradation (6) has permitted preliminary characterization of some of the subunits which make up the amyloid fibrils. The results of this study indicate that all preparations of amyloid examined possess a number of common properties, but that distinct differences between them appear to exist. This conclusion is strongly supported by the immunological studies in the accompanying article (7). Amyloid 1, described in detail previously (5), will be included here primarily for comparison with other preparations and as the prototype for the degradative studies.

Materials and Methods

Sources of Amyloid.—All amyloid-laden organs were of human origin and obtained either at postmortem examination or by surgical procedures. All tissues were frozen soon after autopsy or surgery and thawed only once before preparation. Sections of all tissues were stained intensely with alkaline Congo red (8) and showed the characteristic green birefringence when examined in the polarizing microscope. They were also stained metachromatically by crystal violet.

Amyloid 1: Prepared from the spleen of a 70 yr old male who died suddenly with primary amyloidosis. On postmortem examination, performed by Dr. M. Baden of the New York City Medical Examiner's Office, diffuse amyloidosis without any underlying disease was found.

Amyloid 2: Isolated from the liver of a patient with secondary amyloidosis and obtained from Dr. G. Glenner of the National Institutes of Health, Bethesda, Md.

Amyloid 3: Prepared from a 70 *g* mesenteric lymph node, obtained at laparotomy from a 17 yr old female with unexplained hepatosplenomegaly and with an abdominal mass. Histological study of the node and a liver biopsy specimen showed large amounts of amyloid. Since there was no family history of amyloidosis, no evidence of a plasma cell dyscrasia on bone marrow examination, no sign of a chronic suppurative disease, and normal serum and urine protein electrophoresis, this patient was classified as having primary amyloidosis.

Amyloid 4: Found in the heart of a patient with probable multiple myeloma, who died of congestive heart failure secondary to cardiac amyloidosis. The myeloma protein was typed as γ_1, κ.

Amyloid 5: Prepared from the tongue of a patient with multiple myeloma who produced a $\gamma_1\kappa$ protein. The tongue, resected to prevent airway obstruction, was kindly provided by Dr. A. M. Josephson of the Downstate Medical Center, Brooklyn, N. Y.

Amyloid 6: Prepared from a kidney obtained at postmortem from a patient with multiple myeloma ($\delta\lambda$ protein). The contralateral kidney contained a hypernephroma. This material was also kindly provided by Dr. A. M. Josephson.

Amyloid 7: Was a lymph node biopsy from a patient with multiple myeloma, kindly provided by Dr. C. Lawrence of the Albert Einstein College of Medicine, Bronx, N. Y. Because of insufficient material, no attempt was made to extract the amyloid.

Amyloid 8: Prepared from the spleen and kidney obtained at postmortem from a patient who had a $\gamma_1\lambda$ paraprotein and a type λ Bence Jones protein. It was not possible to make a diagnosis of multiple myeloma on histologic grounds.

Amyloid 9: Obtained from the spleen of a patient with secondary amyloidosis. It was kindly given to us by Drs. Evan Calkins and Paul Bennette of the State University in Buffalo, N. Y.

Amyloid 10: Isolated from the spleen and kidney of a patient with secondary amyloidosis. The patient was an alcoholic with chronic healed tuberculosis.

Amyloid 15: Obtained from the liver of a patient with secondary amyloidosis associated with rheumatoid arthritis.

Extraction.—Amyloid was extracted by the method described earlier (5). After homogenization of the tissue in 0.15 M NaCl in a Virtis homogenizer (Virtis Company, Gardiner, N. Y.), at full speed for 5 min, the homogenate was centrifuged in a No. 30 rotor at 10,000 rpm for 30 min or 15,000 rpm for 15 min in a Spinco Model L ultracentrifuge (Beckman Instruments, Inc., Spinco Division, Fullerton, Calif.). The supernatant was discarded, and the pellet was again homogenized and recentrifuged. After 6 to 12 extractions, most of the soluble contaminating substances, including the rods, "doughnuts," or P component (9–11), were discarded with the supernatant. At the next stage, 300 ml distilled water were added to the residue prior to homogenization. This suspension still contained a sufficiently high concentration of salt so that little or no protein was recovered in the first supernatant after centrifugation at 30,000 rpm for 1 hr. The residue was rehomogenized in distilled water for 10–15 min. During this and subsequent extractions with distilled water, the ionic strength was sufficiently low for the amyloid fibrils to remain in the supernatant after centrifugation at 10,000–20,000 rpm for ½ hr. Water extractions with progressively smaller volumes of water were continued until little additional protein was recovered in the supernatants obtained after centrifugation in a No. 30 rotor at 10,000–20,000 rpm for periods ranging from 15 min to 1 hr.

Electron Microscopy.—Electron microscope studies were performed on amyloid fibrils isolated from specimens 1–3, 5, 6, 8, and 10 and on several embedded tissues obtained from patients 3, 7, 8, and 10.

For comparative ultrastructural studies of isolated amyloid, the negative staining technique was primarily used (12). Without prior fixation, a drop of purified amyloid was placed on a Formvar-covered (Belden Manufacturing Co., Chicago, Ill.) grid and allowed to dry. Subsequently, a drop of 1% phosphotungstic acid (PTA) at pH 5.4 was placed on the grid for 5 min and blotted dry. Alternatively, the amyloid preparation was mixed with an equal volume of 2% PTA or 0.5% uranyl acetate, and a drop of this mixture was placed on a grid. Tissues obtained at surgery or postmortem were cut into 1 mm^3 cubes, fixed with 3% glutaraldehyde for 2–48 hr (13) and postfixed with 2% osmium tetroxide for 1 hr. Since amyloid is of low contrast on electron microscopy, some tissues were washed and resuspended in 0.5% uranyl oxalate in saline for 1 hr. Dehydration and embedding in Epon 812 was accomplished by Luft's procedure (14). Thin sections were contrasted with uranyl oxalate (15) and lead hydroxide (16, 17) and viewed with a Siemens Elmiskop I electron microscope (Siemens America, Inc., New York) at instrument magnifications ranging from 10,000 to 60,000.

Analytical ultracentrifugation was performed (5). Since amyloid was insoluble in normal saline, all ultracentrifugal studies were performed in distilled water. Whenever possible, at least three concentrations of each preparation were examined to permit calculation of an infinite dilution sedimentation coefficient. Because of the previously noted aggregation that occurs with time, all samples except No. 15, which was sent from Israel, were studied within 24 hr of the time of isolation. In a few instances, sufficient material was available to repeat these studies at a later date.

Chemical Analyses.—Peptide maps were done on performic acid-oxidized trypsinized sam-

ple according to the method of Katz, Dreyer, and Anfinsen (18), as described previously (19). Chromatography was done first, followed by high voltage electrophoresis. Amino acid analyses were performed on acid hydrolysates by the method of Spackman et al. (20), using a Spinco Model 120 amino acid analyzer. A nitrogen content of 14.3% was assumed for amyloid. The nitrogen content in the sample was determined by micro-Kjeldahl procedure. Carbohydrate analyses were also done (5).

Quantitative Congo Red Binding.—The amount of Congo red bound per milligram protein was estimated (5). In all but one instance (Amyloid 6), the amyloid Congo red complex could be easily sedimented. In the case of Amyloid 6, centrifugation at 10,000 rpm for 30 min was required to sediment the precipitate. A comparison of the amount of Congo red bound by the initial homogenate and the amount bound by the isolated protein or the residue, permitted an accurate estimate of the completeness of recovery (5).

RESULTS

Solubility and Extractability.—Amyloid was extracted from the tissues of all subjects, with the exception of No. 4, with varying degrees of ease and completeness. Only amyloid 4 could not be obtained from the heart under these conditions. Table I lists the yields obtained from the various tissues calculated either by subtracting the amount of Congo red bound by the residue left after extraction from the amount initially bound by the homogenate, or by comparing the amount of Congo red bound by the isolated protein with that bound by the initial homogenate. As can be seen, the per cent recovered ranged from 90% under favorable conditions to about 20% in preparations 6 and 10. Estimates of recoveries are probably too low because of the presence in tissues of other substances which bind small amounts of Congo red.

General Properties.—At concentrations of protein ranging from 1.0 to 5.2 mg/ml seven of the preparations were transparent, yellowish green, serous solutions with only a slight degree of opalescence; only Nos. 3 and 6 were white and much more opalescent. In each instance, most of the protein could be sedimented at 100,000 *g* in 4 hr, and more than 90% could be precipitated by 0.05 M NaCl, $CaCl_2$, or pH 7 0.15 M phosphate buffers. All solutions became increasingly opalescent with time when stored in the cold, and many precipitated spontaneously on standing (5).

Congo Red Binding.—All water-soluble amyloid preparations bound Congo red. Incubation of soluble amyloid with Congo red in 0.15 M NaCl for 1 hr usually yielded a red gelatinous precipitate which could be sedimented at 1,000 *g*. With Aymloid 6, only an increase in opalescence was noted, and a centrifugal force of 10,000 *g* for 30 min was required for sedimentation of the precipitate. Table I lists the amount of Congo red bound per milligram protein in solution. Preparations 1, 2, 5, 8, and one sample of No. 10 bound between 0.26 and 0.35 mg dye/mg protein and No. 15 bound 0.24 mg/mg protein. The two opalescent preparations (Nos. 3 and 6) bound only 0.21 and 0.22 mg. dye respectively; and two other clear solutions (Nos. 9 and 10) bound even less dye. While it seems likely that this variability reflects in large measure chemical

differences between amyloids, the presence of varying amounts of impurities cannot be ruled out. The binding of the dye by protein was accompanied by a

TABLE I

Recovery of Amyolid from Ten Patients and Some of the Properties of the Isolated Protein

No.	Type	Source	Recovery of Congo red binding material*	Mg. Congo red bound	Sedimentation coefficient	
				Mg. protein	Amyloid (H_2O)	DAM (NaOH)
			%			
1	Primary	Spleen	88	0.32	45 (75,110)‡	1.3–2.8
2	Secondary	Liver	78	0.26	40	1.7
3	Primary	Lymph node	N.D.	0.21	7.6§ (13.5)‡	2.1
5	Myeloma	Tongue	37	0.34	74	1.1
6	Myeloma	Kidney	19	0.22	7.9 (9.5)‡	2.5
8	Myeloma	Spleen	92	0.33	41 (70)	2.1
9	Secondary	Liver	70	0.11	150‖	1.3 and 2.4
10	Secondary	Spleen	22	0.13	8.6; 95	2.7
		Kidney	28	0.35	97‖	2.1
15	Secondary rheum. arthritis	Liver	N.D.	0.24	44	2.4
4	Myeloma	Heart	0	N.D.	None recovered	

* $\frac{\text{Mg. Congo red bound by purified protein}}{\text{Mg. Congo red bound by total homogenate}} \times 100$

or $100 - \frac{\text{Mg. Congo red bound by the residue}}{\text{Mg. Congo red bound by the total homogenate}} \times 100.$

‡ Aged.
§ Opalescent.
‖ Concentrated.

shift in the maximal absorption of Congo red from 490 to between 510 and 520 A in all instances.

Sedimentation Studies.—Because of the solubility properties of amyloid, all studies were done in distilled water. To avoid polymerization, all samples but one (No. 15) were examined within 24 hr after isolation. In several instances

the preparations were studied at a later date or after further concentration. Table I lists the sedimentation coefficients of amyloid preparations from all nine subjects. Six had sedimentation coefficients ranging from 40 to 70S, and one (No. 9) sedimented at 150S. In contrast, amyloid 3 and 6, which were much more opalescent, contained a major component with a sedimentation coefficient of about 8S. After further storage and concentration, these two preparations had sedimentation coefficients of 13.5S and 9.5S respectively. One (No. 10) contained a small 8.6S peak and a large amount of a 95S component; in one experiment, these two components could be extracted separately. None of the three preparations which contained the 8–9S component showed significant numbers of rods in electron microscope studies (9–11), and they did not react with antisera to normal human serum and to the P component.[1] It thus seems unlikely that there was contamination with the P component, an α-globulin present in normal serum which appears to be related to the doughnut or rod-shaped structures that exist in amyloid (9–11). While additional specimens must be studied to permit definitive conclusions, it seems possible that the 8S and 45-50S components may represent different types of amyloid (see below.). The ultracentrifugal behavior does not appear to be related to the etiology of the disease, however, since amyloid from one patient with myeloma and one patient with primary amyloidosis sedimented rapidly while the other two amyloids sedimented slowly. In contrast, the components having sedimentation coefficients ranging from 45 to 150S probably represent different degrees of polymerization of the basic subunit since the higher values were generally found in preparations that required more prolonged handling and more vigorous concentration during the isolation procedure, and these highest values have been noted to appear after storage (5).

Electron Microscopy.—The ultrastructure of isolated negatively stained amyloid fibrils 2, 3, 5, and 8 was similar to that of amyloid 1 (5).

The unit structure seen at moderate magnifications is referred to as the amyloid filament (Fig. 1). It measures 50–80 A in diameter and is of indeterminate length. In "soluble" amyloid preparations such filaments were seen singly or in small clumps, whereas after salt precipitation lateral aggregation of pairs of filaments separated by a 30–100 A wide, dense area was a common finding (Fig. 1). On prolonged standing, when large clumps of intertwined filaments were noted, pairs of parallel filaments could still be resolved at the periphery of such clumps, even when considerable twisting of the fibrils had occurred.

Two preparations, Nos. 6 and 10, differed in appearance. Repeated electron microscopic studies of amyloid 6 failed to reveal any fibrils even following the addition of 0.15 M NaCl which resulted in a grossly visible precipitate. It is assumed that the extraction procedure resulted in a degradation product which

[1] We would like to thank Dr. Alan Cohen for this antiserum.

was no longer fibrillar but was still able to bind Congo red. Unfortunately, there was no opportunity to carry out electron microscopic studies on the intact tissues of this patient to establish whether the amyloid had been in fibrillar form prior to extraction.

Amyloid 10, which contained a small 8.6S peak and a larger 95S component, after ultracentrifugation proved to consist of two different types of filaments when studied by electron microscopy. The smaller type of filament, seen mainly in the fraction containing the 8.6S component, was somewhat narrower than the filament described above and measured 30–50 A in diameter. These filaments had a tendency to twist loosely around each other or to run in parallel pairs (Fig. 2). The second type of filaments, present in greater abundance and found mainly in the major 95S fraction, was strikingly thicker and ranged in width from 80–110 A (Fig. 3). No lateral aggregation of these larger filaments seemed to take place, and a substructure was not easily resolved at moderate magnifications. The different features of the two filaments contained in preparation No. 10 may be appreciated by comparing Figs. 2 and 3. Studies at higher resolution did not provide additional information on the substructure of the smaller filament (Fig. 4), whereas many of the larger filaments could be seen to consist of two or three strands tightly twisted around each other (Fig. 4). At the end of these fibrils, where twisting of the strands appeared to be less tight, a periodic substructure was often suggested. This substructure was much smaller than the periodic rod representing the "P component" of amyloid described by Glenner et al. (9) and should not be confused with it.

In tissue sections the morphologic variations in amyloid in different areas were even more remarkable. Cursory inspection of the examples depicted in Figs. 5–7 will suffice to point out the differences in size, structure, and orientation of the fibrils. To avoid any possible confusion with other fibrillar proteins, such as elastin, basement membrane material, collagen, or fibrin, we took all sections from regions which were heavily infiltrated with amyloid. In the sections of a well preserved lymph node from patient 7, shown in Fig. 5, the amyloid fibrils were straight and oriented at random; and the amyloid deposits were surrounded by mononuclear and plasma cells. At higher magnification, many fibrils were seen to consist of a parallel pair of filaments. On cross-section, they appeared as irregularly shaped dots in which a radiolucent core was observed only rarely. The amyloid illustrated in Fig. 6 is a thin section of spleen obtained at necropsy from No. 8. Here the amyloid appeared to be tubular since the fibrils, which ran at right angles to the plane of section, were circular (see arrows). No attempt has been made to resolve the hypothetical substructure of these tubules. They appear to be similar to the 80–90 A structures, thought to be made up of five subunits arranged around a radiolucent center, described by Shirahama and Cohen (21). An amyloid-laden area in the spleen, obtained from No. 10 at postmortem, is illustrated in Fig. 7. It demonstrates

parallel orientation of fibrils which is probably responsible for the birefringence of some amyloid-laden tissues when viewed with the polarizing microscope. Though a few areas of parallel-oriented fibrils were seen in all tissues examined, most amyloid consisted of randomly disposed fibrils.

Amino Acid Analyses and Peptide Maps.—Amino acid and N terminal analyses were performed on preparations 1–3, 5, and 6. The results of these studies will not be detailed, however, since the marked variations between different preparations are difficult to interpret in the absence of a precise estimate of the purity of each preparation. Since absolute purity is not a requisite for peptide map studies, it was feasible to subject eight preparations of amyloid to performic acid oxidation and trypsinization, and to subject the resultant peptides to two dimensional chromatography and electrophoresis (18, 19). Peptide maps of amyloids 1 and 2 resembled each other and had from 40–45 dark peptides, many of which were the same in both preparations (Fig. 8*a* and *b*). They did, however, differ in a few peptides that were present in one but not in the other. In the case of amyloid 1, eight peptides gave a positive reaction for histidine and 13 for arginine. Fingerprints of amyloids 3, 5, 6, and 10 always had a large amount of residual material remaining at the origin, and, in general, were of poorer quality so that a precise comparison with the first two proved difficult. The map of amyloid 3, which was faint, had about the same total number of peptides, but differed from Nos. 1 and 2 in the location of at least 10 peptides (Fig. 8*c*). Amyloids 8 and 9 were similar to each other and lacked at least 10 of the peptides present in Nos. 1 and 2 (Fig. 8*d*). Only about 20 dark peptides were seen in the peptide maps of Nos. 5, 6, and 10 (Fig. 8*e*). While these three were quite similar to each other, they differed markedly from the other five.

Degradation Studies.—Because of the large size of the amyloid fibrils, extensive efforts were undertaken to produce smaller structural units. Although it was known that amyloid is resistant to enzymatic degradation (22), a number of enzymes were tested for their effect on the isolated fibrils. Pepsin (pH 2.4 and 4), papain (pH 7), and trypsin (pH 8.2) digestion of amyloid Nos. 1, 8, and 10 for 24 hr at 37°C yielded virtually no dialyzable fragments. When the ability to bind Congo red was measured before and after the treatment, there was no change after pepsin digestion and only a 20% reduction after trypsinization. The only enzyme active on the native protein was pronase, which converted about 80% of the material into dialyzable peptides but did not leave sufficient large molecular weight residue to attempt characterization.

Systematic attempts to produce smaller fragments were carried out in greatest detail with amyloid 1, which proved to be largely insoluble in 6–8 M urea, in 6 M guanidine, and in the presence of 0.1 M mercaptoethanol. Subsequent studies with amyloid 2, 8, and 10 indicated that they remained soluble in urea and guanidine, and that a more slowly sedimenting component could be recovered by chromatography on Sephadex G-200 in reasonable yields. However,

since the major component still appeared rather large, because it was eluted in the void volume, this approach was not studied further. Instead, we investigated in greater depth the effect of sodium hydroxide, which had been shown to yield smaller fragments that were retarded on chromatography on Sephadex G-200 (6).

Of all the methods attempted, treatment of amyloid with 0.1 M NaOH proved to be most useful; it was therefore applied to all preparations. Addition of 0.1–1 M NaOH to each of the amyloid solutions listed in Table I resulted in the instantaneous clearing of the opalescence that was often present in stored solutions. These solutions remained clear as long as they were in sodium hydroxide. The following procedure was developed to prepare alkaline degraded amyloid (DAM). The amyloid solution was made 0.1 M in NaOH and allowed to stand for 1–3 hr at room temperature. Following dialysis against distilled water, less than 10% of the material was lost. Neutralization to pH 7 with hydrochloric acid resulted in a small precipitate. The supernatant was used as such or was centrifuged at 40,000 rpm for 1 hr. Following centrifugation, about 40–60% of the starting material was recovered in the supernatant and was called supernatant DAM (Super-DAM). In general, the yield of DAM was greatest when fresh preparations of amyloid were used; it was somewhat lower when stored samples or when material that had previously been precipitated by the addition of sodium chloride were subjected to alkaline degradation. The yield of Super-DAM could be increased further by more prolonged (48–72 hr) alkaline treatment and brought up to 90% by first heating amyloid at 100°C. Boiling alone resulted in some clearing of opalescence. In one instance, a boiled sample was subjected to analytical ultracentrifugation; the sedimentation coefficient had decreased from 45 to 3.7S following this procedure. The mode of action of alkali on amlyoid has not yet been elucidated and is currently under study.

General Properties of DAM.—The solubility of DAM in a variety of aqueous solvents was significantly greater than the solubility of amyloid. While amyloid was completely insoluble in solutions of salt more concentrated than 0.02 M NaCl, the alkaline degraded amyloid did not precipitate completely in solutions containing as much as 1 M NaCl. In contrast to amyloid, DAM was not precipitated by 0.1 M HCl and by 0.1 M acetic acid, but was completely precipitable by 20% trichloracetic acid and by 50% saturated solution of ammonium sulfate. Like amyloid, DAM was rather resistant to digestion with a number of proteolytic enzymes.

Size.—A rough estimate of the size of amyloid and DAM was attempted by comparing their ability to pass through Millipore filters (Millipore Filter Corp., Bedford, Mass.) of various sizes. DAM appeared to be significantly smaller since water-soluble amyloids 1 and 2 failed to pass through a 0.220 μ Millipore filter while DAM 1 and 2 passed through a 0.010 μ filter.

Metachromasia and Congo Red Binding.—Degradation of the amyloid by

sodium hydroxide did not significantly change the metachromatic capacity of amyloid, nor did it appreciably diminish its ability to bind Congo red. However, the Congo red seemed to be less firmly bound by DAM since the complex was dissociated when applied to a Sephadex G-200 column in 0.1 M NaOH. An extensive study of the metachromasia of amyloid has been published separately (23).

Sedimentation.—When examined in 0.1 M NaOH, DAM sedimented as a single, fairly homogeneous peak with a sedimentation coefficient ranging from 1.1 to 2.8S (Table I). When the sodium hydroxide was removed by dialysis against pH 7-buffered saline or distilled water, an asymmetric peak appeared. This probably reflected various degrees of polymerization of the product upon

TABLE II

Carbohydrate Content of Amyloid and DAM Per Cent of Dry Weight

	Amyloid	Alkaline degraded amyloid
Hexoses	1.3	2
Hexosamines	0.3	0.6
Uronic acid	0.3	0.44
Sialic acid	0.1	0.13

removal of the dissociating agent. The main component of this heterogeneous component in water or 0.15 M NaCl has a sedimentation coefficient ranging from 1.7 to 4.5S.

Chemical Analyses.—Amino acid compositions of four preparations of DAM were strikingly similar to those of the native amyloid and indicated no selective loss of any amino acid. The only major difference was the complete absence of cysteine in the NaOH-treated DAM sample. This is probably the result of an elimination reaction known to occur on exposure to alkali (24). As was the case with native amyloid, the results will not be listed in detail because of the possible presence of impurities. The peptide maps of four of the DAM preparations were strikingly similar to the native amyloid (Fig. 8*f*). In general, the position of the peptides appeared to be identical, although a few appeared to be lacking in one instance. Detailed studies comparing the carbohydrate composition of amyloid and DAM were done only with preparation No. 1. The results are listed in Table II. It would appear from this single study that no carbohydrate was lost during the alkaline treatment. Additional studies will be required to determine whether the apparent slight increase in each of the carbohydrate moieties is significant.

DISCUSSION

Amyloid, a fibrillar protein, accumulates extracellularly. It is identified by its homogeneous appearance under light microscopy and its characteristic

staining properties with iodine, Congo red, and several metachromatic dyes (2). It can be associated with a variety of diseases such as chronic suppurative conditions, rheumatoid arthritis, and systemic lupus erythematosus, a variety of tumors, and all of the known plasma cell dyscrasias. Several genetically determined forms of amyloidosis are known. When no other cause is found, the disorder is classified as primary amyloidosis. Even here, however, several types have been described on the basis of differences in tissue distribution. In a significant per cent of elderly adults and in a number of species of animals, deposits having many of the properties of amyloid have been located in the brain, pancreas, heart, aorta, and certain other tissues (25). While there appear to be differences in the tissue distribution and staining properties in the deposits associated with the different clinical forms of amyloidosis, there are certain characteristics that suggest that all forms of amyloid share many common properties (2): among these are the typical fibrils seen on electron microscopy (2, 5, 9, 21, 26–28); the characteristic, albeit variable metachromasia; and the ability to bind Congo red.

A definitive decision as to the identity or nonidentity of different types of amyloid can be reached only chemical, physical, and immunological characterization of purified amyloid. The availability of a method to extract a relatively pure water-soluble protein from different types of amyloid deposits (5) has permitted a more direct approach to the study of amyloid fibrils and their subunits. It has also, for the first time, allowed comparative studies of the major constituent of amyloid derived from different sources. Before discussing the results of these studies, four major reservations must be made. Firstly, the material studied here constitutes only the major fibrillar part of amyloid and excludes all soluble constituents such as "doughnuts" and other serum components (9–11). Secondly, the number of samples studied is too small to permit clear-cut conclusions as to the existence of classes and subclasses of amyloid. However, differences in the ultrastructural appearance and sedimentation properties, coupled with the differences in Congo red binding, suggest the possible existence of several types of amyloid. Thirdly, the number of tissues studied from each individual precludes any conclusions regarding the relation of amyloid in different tissues from the same individual. Lastly, some of the variations noted in the chemical analyses are impossible to interpret because of uncertainties as to the purity of the samples studied.

In spite of these limitations, it would appear that amyloids from different individuals resemble each other but may not be identical. Differences in the chemical composition, in the ultrastructure of isolated fibrils, as well as in the antigenic properties, reported in the accompanying article, suggest that amyloid preparations may differ in primary structure. It is more difficult to interpret variations in the ultracentrifugal patterns, in the morphologic organization of amyloid in tissue sections, and in the staining characteristics. This heterogeneity

may be due either to extraneous substances which could react with the fibrils or to physical factors which may affect their organization in tissues. There was no obvious relationship between the physical, chemical, and ultrastructural properties that held for all of the preparations. However, we should mention that in amyloid 10, prepared from the spleen, the smaller fibrils corresponded to the 9S component, whereas larger fibrils were derived from the more rapidly sedimenting 95S fraction. It did not prove feasible, however, to convert the 95S component into the 9S component by any of the chemical procedures studied; and sufficient amounts of the 9S component were not available for detailed chemical or immunological analyses. While it seems possible that the 9S components are subunits of the larger fibrils and are joined together to form them, we could not test this directly.

To obtain smaller subunits of the large amyloid fibrils, we tried a number of procedures. The material proved to be remarkably resistant to a variety of proteolytic enzymes, a feature which may be significant in explaining the persistence of amyloid in tissues once it is deposited; only pronase, a potent and nonspecific enzyme, resulted in significant degradation. Neither urea, nor guanidine, nor reducing agents produced soluble subunits in large amounts in all instances, although it would appear that some of these reagents may sometimes prove of use (29, 30). Cyanogen-bromide treatment yielded several fragments that could be separated on Sephadex gel filtration, but they have not yet been fully characterized (unpublished observation).

The most informative approach proved to be the use of strong alkali. There is ample precedent for the use of alkaline extraction in studies of amyloid. Krawkow in 1898 extracted a chondroitin sulfate–like material from amyloid with $Ba(OH)_2$ (31). Hass and Schulz developed an extraction method which they thought yielded undegraded amyloid (32). While cutting fresh tissue into sections with a freezing microtome, they were able to extract with pH 11 sodium hydroxide a protein that remained in solution on neutralization of the solvent, but most of which was precipitated by acetic acid at pH 4–5.4. It seems possible that this corresponds to the DAM obtained in this study. Recently, Shirahama and Cohen (33) showed that the solubility of amyloid increased at pH greater than 9.5 and that solubilized alkaline extracts could regain their fibrillar structures after neutralization.

Studies are currently in progress to study the mechanism of alkaline degradation. While it is not yet possible to clearly delineate the nature of the bonds holding the subunits together, it is possible, on the basis of available data, to hazard some speculation regarding the approximate size of the basic subunit. The almost complete recovery of DAM from amyloid and the striking similarity in the peptide maps of the native and degraded preparations make it reasonable to estimate the size of the subunit from the number of peptides and the content

of arginine and lysine. However, possible inaccuracies in the amino acid analyses should be allowed for. On the basis of approximately 110–127 arginine and lysine residues per 100,000 g in the different samples (unpublished observation) and the presence of about 40–45 peptides in some of the peptide maps, one can estimate a molecular weight of about 35,000–40,000 for the basic subunit. A comparable value is obtained from the content of an average of seven methionine residues and the production of four peaks by cyanogen bromide cleavage. Whether these subunits correspond to some of the protofibrils described by Shirahama and Cohen (21) remains to be determined.

One other noteworthy feature is the unusual insolubility of this material and its resistance to proteolytic attack in its native state. Both of these properties may be significant in the localization, deposition, and persistence of amyloid in a variety of tissues.

SUMMARY

Amyloid fibrils were isolated from the tissues of nine patients with amyloidosis in a state of high purity by homogenization of the tissue followed by extraction with distilled water. Physical, chemical, and ultrastructural studies suggest that amyloid fibrils from different individuals resemble each other, but are not identical. In tissue sections as well as by negative staining of isolated fibrils, morphologic variations were observed. Among the isolated fibrils at least three types were noted. The majority resembled those described previously. However, one subject had two types of fibrils which differed in size and appearance.

Most of the preparations sedimented as a single component with a sedimentation coefficient of 45–50S or as a larger polymer. However, two of the preparations had sedimentation coefficients of 8–9S, and a third one had a major 95S component and a minor 9S fraction.

While the preparations of amyloid were not sufficiently pure for amino acid analyses, peptide maps demonstrated differences among amyloid preparations from different subjects.

The amyloid fibrils in their native state proved to be remarkably resistant to digestion by a number of proteolytic enzymes. Several chemical methods were tried to produce smaller subunits. Of these, the most successful one was the use of 0.1 M NaOH which yielded a smaller, soluble fraction with sedimentation coefficients ranging from 1.1 to 2.8S. Accompanying this degradation, there was little loss of peptides or carbohydrates. Based on the results of the chemical analyses, it is estimated that the subunit produced by sodium hydroxide had a molecular weight of approximately 35,000–40,000.

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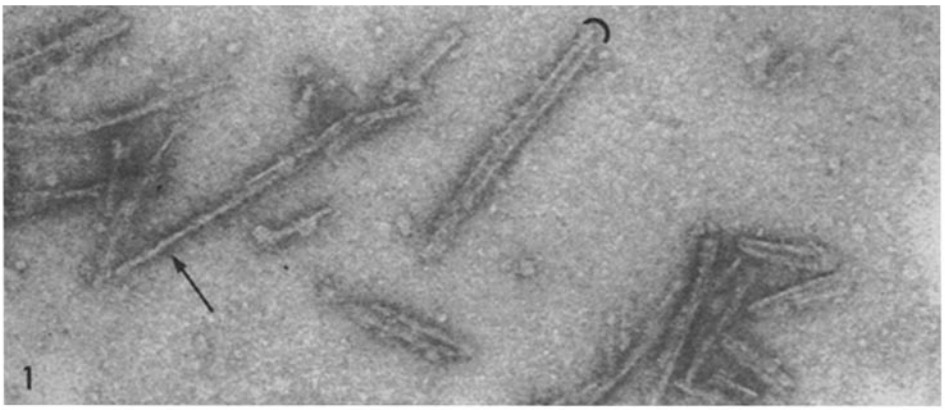
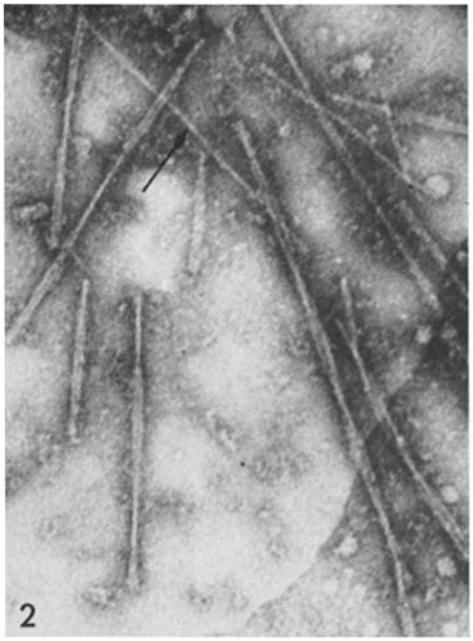
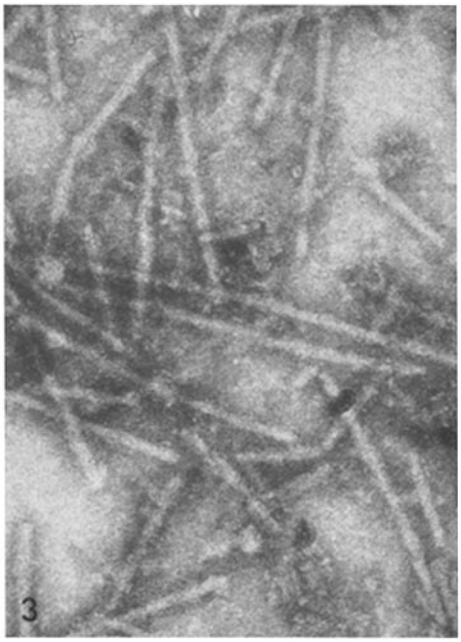






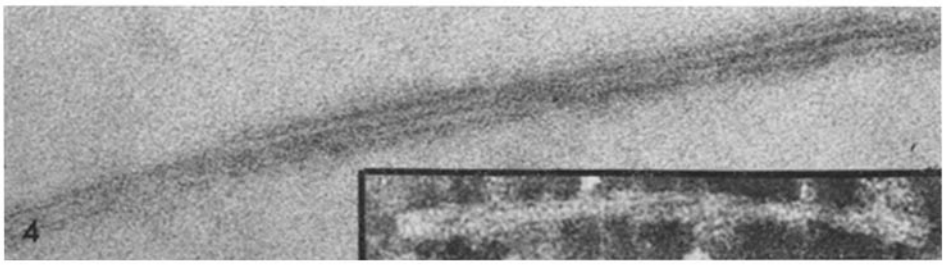
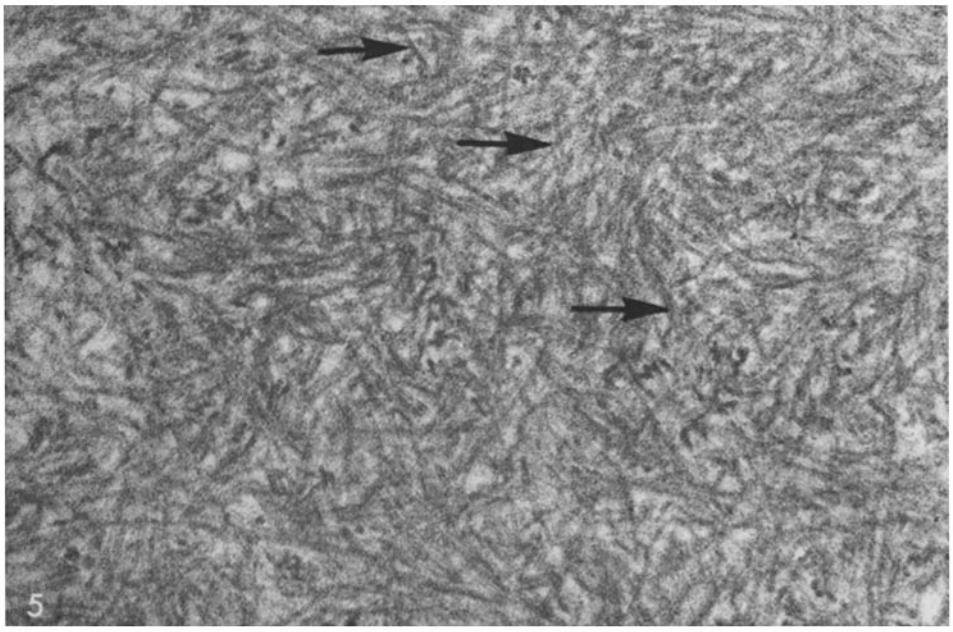


FIG. 1. Amyloid 1 contrasted with 2% PTA. Filaments often run in parallel pairs with intervening electron-dense space. (See half circle.) Arrow indicates single filament. × 160,000.

FIG. 2. "Small" filaments obtained from spleen of case 10. Arrow indicates single filament. Majority of filaments are loosely twisted around each other. × 160,000.

FIG. 3. "Large" filaments obtained from same specimen as filaments shown in Fig. 2. These filaments are almost twice as large as those in Fig. 2. Twisting is rare and lateral aggregation is not often seen. × 160,000.

FIG. 4. Higher magnifications of a pair of "small" filaments obtained from specimen 10. Filaments appear relatively smooth. Inset shows a higher magnification of the type of filament surveyed in Fig. 3. A twisted substructure is evident. At one end periodicity of individual strands is suggested. Both types of filaments × 300,000.



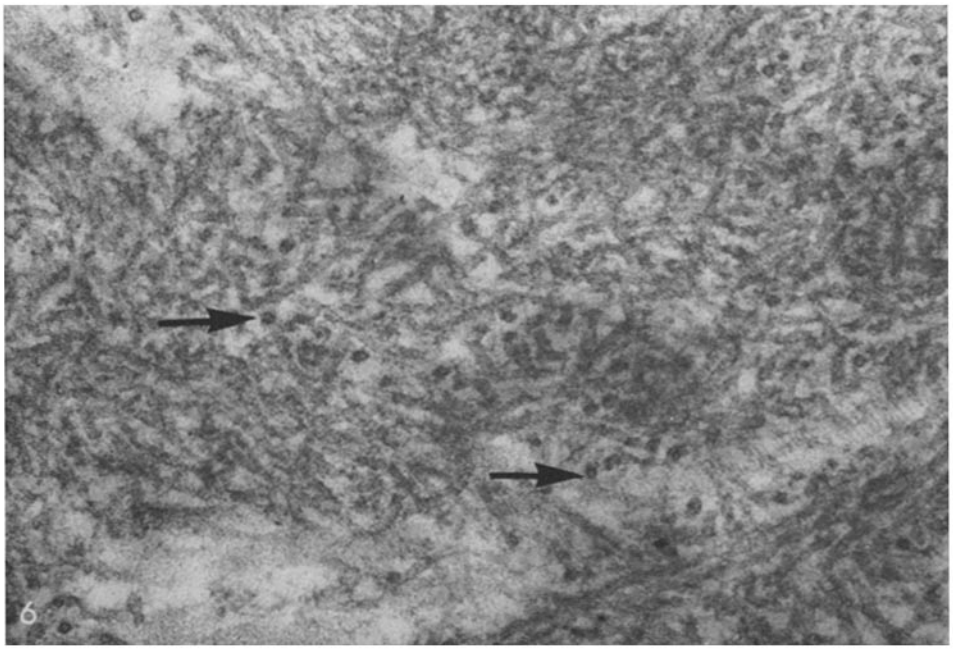


FIG. 5. Thin section of amyloid-laden lymph node of patient 7. Fibrils often show parallel filaments which could represent longitudinal sections through a tubular structure (arrows). Cross- and tangential sections suggest this possibility. Fibrils are randomly oriented. × 100,000.

FIG. 6. Section of amyloid-laden spleen obtained from case 8 at same magnification as section shown in Fig. 5. These tubular structures are more easily appreciated in cross sections which appear as circles (arrows). Fibrils have a less rigid appearance. × 100,000.

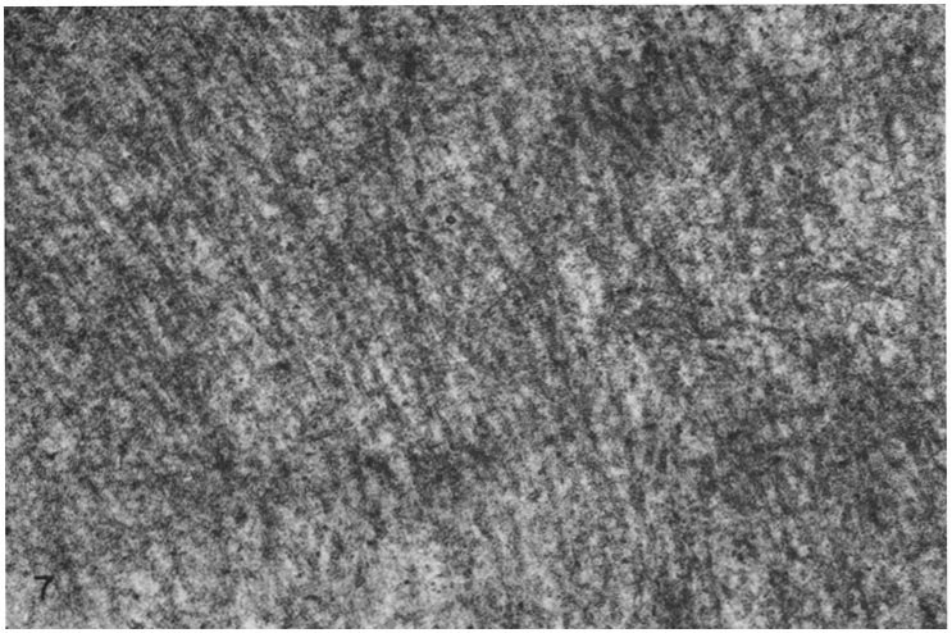


FIG. 7. Section of amyloid-laden spleen obtained from case 10, showing parallel orientation of fibrils probably responsible for the birefringence often displayed by this protein. × 100,000.

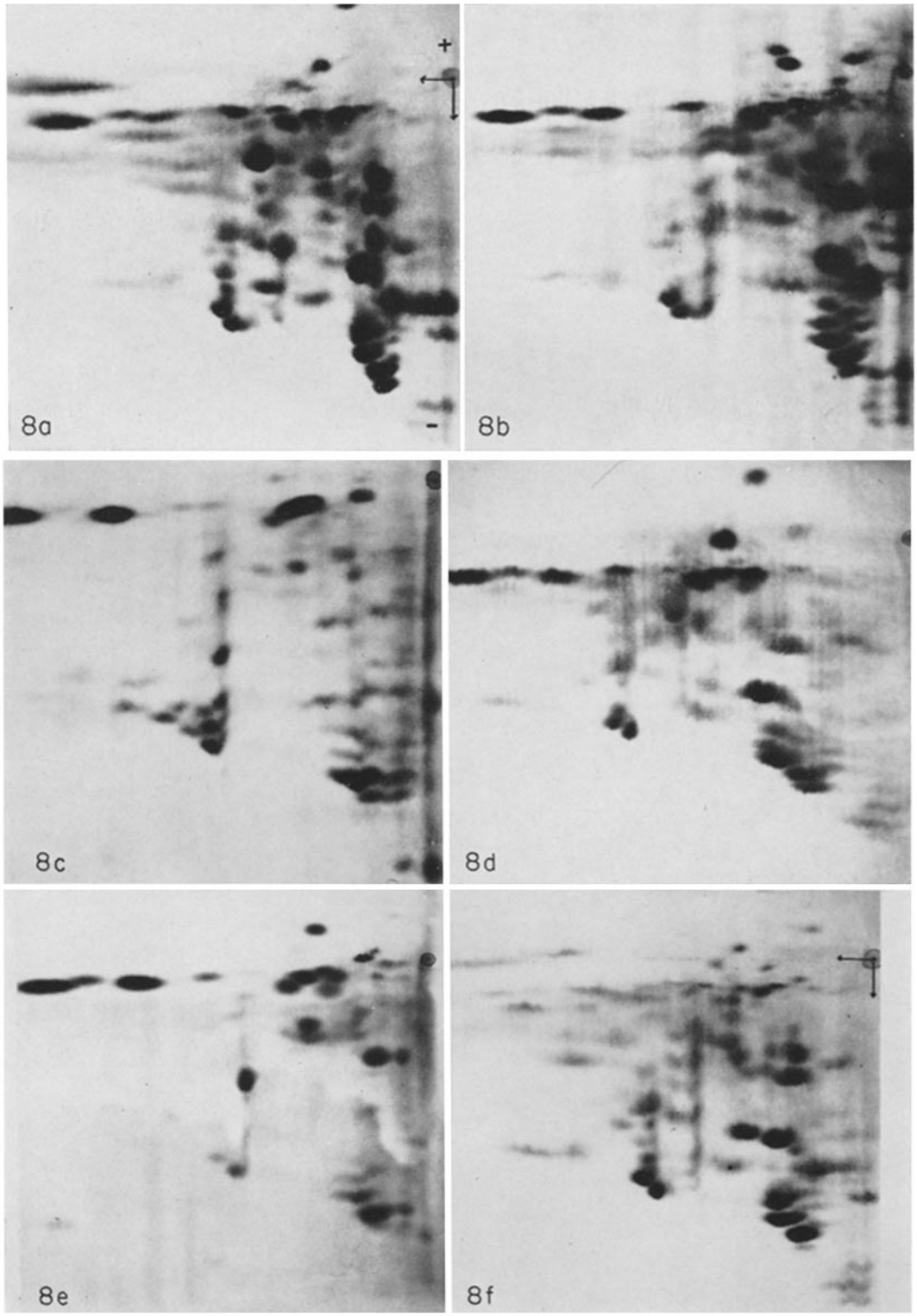


FIG. 8. Peptide maps of: *a*, amyloid 1; *b*, amyloid 2; *c*, amyloid 3; *d*, amyloid amyloid 5; *f*, DAM 1.