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Comparative studies of the high molecular weight amyloid fibril proteins and similar components from normal tissues

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SUMMARY

Analysis of purified amyloid fibrils by gel filtration, polyacrylamide gel electrophoresis in SDS and 8 M urea, and immunodiffusion and immunoelectrophoresis showed that, in addition to the specific amyloid proteins AA and AL, the amyloid preparations all contain a high molecular weight complex. The latter protein complex contains fibronectin, a component which reacts with a non-AA specificity of an antiserum to degraded AA amyloid fibrils (termed the 'B' specificity), and a high molecular weight component excluded by a Sepharose 2BCL column. Similar components were found in aqueous extracts of normal tissues prepared by an identical procedure, and these form aggregates of different size in non-dissociating conditions. It is suggested that amyloid fibrils are complexes of a variety of macromolecules in addition to the specific proteins AA and AL.

INTRODUCTION

Amyloid fibrils can be extracted from normal tissues with distilled water after more soluble materials have been removed by repeated saline washings (Pras *et al.*, 1968, 1969). This gives a relatively pure preparation of amyloid fibrils. When these are reduced and alkylated and chromatographed in dissociating conditions the individual amyloid proteins AA and AL can be isolated (Harada *et al.*, 1971; Glenner, Harada & Isersky, 1972; Husby *et al.*, 1972). However, under these conditions a large proportion of the material appears in the void volume peak of the columns used, thus having very high molecular weight (mol. wt) (Benditt & Erikson, 1972; Husby & Natvig, 1972; Levin *et al.*, 1972). The low mol. wt components of amyloid are well known and their characteristics have made it possible to establish a chemical classification of amyloid (Husby, 1980). The composition of the high mol. wt material has not, however, been completely characterized.

When a similar extraction procedure is applied to normal tissues a protein preparation is obtained. This has been termed non-collagenous reticulin component (NCRC) and when it is used as an antigen in rabbits it gives an antiserum that reacts with reticulin fibres in human tissues (Pras & Glynn, 1973; Pras *et al.*, 1974). Serological and immunohistological studies have shown that NCRC codistributes with fibronectin and collagen type III in reticulin fibres, although these antigens are immunologically distinguishable (Unsworth *et al.*, 1982). NCRC has a similar amino acid composition and some immunological cross-reactivity with the high mol. wt component of amyloid fibrils (Husby & Sletten, 1977).

We have investigated the biochemical and immunological properties of the high mol. wt components of aqueous extracts of normal and amyloid tissues to determine their characteristics and their relationship to other components of the connective tissue matrix.

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MATERIALS AND METHODS

Tissue specimens. Normal (three patients) and amyloid (six patients) spleens and livers were obtained and stored at −20°C until analysed. The amyloid tissues had been previously characterized: five cases were AA amyloid (BL, JL, AM, BOL, HAN) and one AL amyloid (λ MOL). Normal livers were also obtained from three Sprague–Dawley rats.

Preparation of aqueous extracts. These were prepared by the method of Pras *et al.* (1968, 1969). Briefly, 20–30 g (wet weight) tissue was homogenized in 300 ml 0·15M NaCl in ice for 5 min and the homogenate centrifuged at 20,000 ***g*** for 15 min at 4°C. The supernatant was removed and the sediment rehomogenized in further saline. This was repeated 7–12 times until no or negligible amounts of protein were detected in the supernatant by absorption at 280 nm. The last sediment was rehomogenized in distilled water (300 ml) and centrifuged at 48,000 ***g*** for 90 min at 4°C. This was repeated four times (once with 200 ml and three times with 150 ml). Extracts 2–5 with the water were pooled and lyophylized; these formed the 'crude' aqueous extracts. In two cases (normal and AA amyloid livers) each of the saline washes (third to final) were dialysed extensively against distilled water and lyophylized; distilled water extracts were also lyophylized separately; and with the amyloid material a final 2M urea extract was made, dialysed and lyophylized.

Gel filtration. The following columns, gels and eluents were used: a 2·4 × 100 cm Sephadex G-100 column with 5M guanidine/0·1M acetic acid; two 1·5 × 90 cm Sephacryl S-300 columns with 0·1M $NaHC0_3$ and 6M guanidine/0·5M Tris/0·001M EDTA, pH 8·5, respectively; two 1·5 × 90 cm Sepharose 4B columns with 0·3M NaCl and 0·3M NaCl/1% SDS, respectively; a 2·4 × 100 cm Sepharose 2BCL column with 6M guanidine/0·5M Tris/0·001M EDTA, pH 8·5 and finally a 1·5 × 90 cm Ultrogel A2 column with 0·3M NaCl/2M urea. Calibration was done with dextran 200, thyroglobulin, ferritin, catalase, immunoglobulin G, albumin, trypsin inhibitor and insulin and Kav values were calculated (Laurent & Killander, 1964). Prior to application to the Sephadex G-100 column tissue preparations were treated with 6M guanidine/0·5M Tris/ pH 8·5 containing 0·5M dithiothreitol and 0·001M EDTA and in some cases were alkylated with 0·3M iodoacetamide (Husby *et al.*, 1972). Prior to application to the Sepharose 4B column in experiments using SDS, samples were suspended in 0·3M NaCl containing 10% SDS, incubated at 90°C for 3 h and centrifuged at 15,000 ***g*** for 15 min at 22°C. Columns were loaded with 10–15 mg protein in 1 ml or 40–50 mg in 5 ml. After fractionation the main peaks were extensively dialysed against distilled water and lyophylized.

Polyacrylamide gel electrophoresis (PAGE). This was performed in slab gels of 5–10% acrylamide in 0·1M phosphate buffer, pH 7·1, containing 0·1% SDS (Weber & Osborne, 1969) with the addition of 8M urea. The sample buffer of 0·01M phosphate, pH 7·1 contained 2·5% SDS and 8M urea.

Immunological studies. Immunodiffusion and immunoelectrophoresis were performed in 1% agarose in barbital buffer, pH 8·6, ionic strength 0·025. The following monospecific antisera to human proteins were used: rabbit antisera to protein AA, protein AL (λ MOL), SAA and fibronectin, produced as previously described (Marhaug & Husby, 1981; Scott, Bedford & Walton, 1981); rabbit antisera to pre-albumin, albumin, and β_2-microglobulin (DAKO immunoglobulins); rabbit antisera to IgG (γ-chains), IgA (α-chains) (Behring); goat antiserum to P component (Atlantic Antibodies). Rabbit anti-whole human serum was from DAKO immunoglobulins; rabbit antiserum to NCRC was kindly provided by Professor E. J. Holborow; an antiserum to alkali degraded AA amyloid fibrils (DAM) was prepared as previously described. It had two main specificities: (i) protein AA and (ii) part of fractionated amyloid fibrils eluted in the void volume of a Sephadex G-100 column (Husby *et al.*, 1972). The latter has been previously termed the 'Vo material' but in the present communication will be termed the 'B specificity'. The anti-DAM was used with and without immunoabsorption with normal human serum. DAM, purified AA, SAA and fibronectin were prepared as previously described (Husby *et al.*, 1972; Marhaug & Husby, 1981; Scott *et al.*, 1981).

Recombination experiments. The high mol. wt void volume component of aqueous extracts of normal human spleen, and purified protein AA and SAA, suspended in 0·1M $NaHCO_3$ (2·5 mg/ml)

were chromatographed separately and together (after incubation for 2 h at 37°C) on Sephacryl S-300 eluting with the same buffer. The protein peaks were dialysed against distilled water, lyophylized and analysed for AA and SAA by immunodiffusion. Similar experiments were not performed with amyloid void volume material due to its insolubility in non-dissociating conditions.

RESULTS

Aqueous extracts 2–5 from amyloid tissues stained avidly with Congo red exhibiting green birefringence when microscoped in polarised light; corresponding extracts from normal tissues showed only slight Congo red staining without birefringence.

Gel filtration

In non-dissociating conditions (0·3M NaCl or 0·1M $NaHCO_3$) aqueous extracts of normal tissues showed a reproducible and similar chromatographic pattern with Sepharose 4B and Sephacryl S-300. An example is shown in Fig 1a. There was a large peak of high mol. wt eluting in the void volume (50–60% material), a long tail of material of decreasing mol. wt (30–40% material), and a small retarded peak (5% material). To study further the high mol. wt material aqueous extracts of normal human spleen were also chromatographed on Ultrogel A2 (exclusion limit 50×10^6 daltons). This latter experiment showed that material excluded by Sepharose 4B and Sephacryl S-300

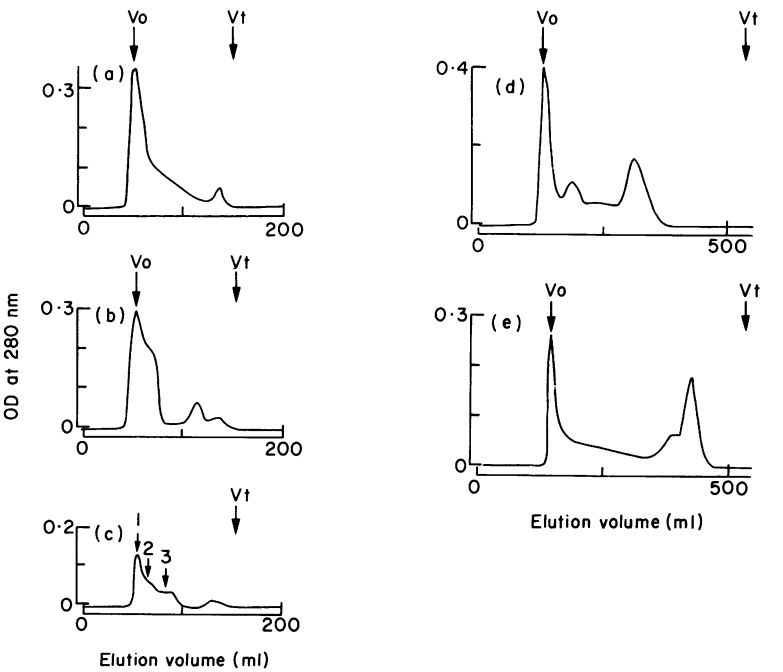

Fig. 1. (a) Sepharose 4B column showing chromatography of crude aqueous extract of normal human spleen eluted with 0·3 M NaCl. (b) Sepharose 4B column loaded with crude aqueous extract of normal human spleen treated with SDS and eluted with 0·3 M NaCl/1% SDS. Note the presence of three peaks; the void volume, as intermediate peak and a retarded peak. (c) Ultrogel A2 column loaded with crude aqueous extract of normal human spleen eluted with 0·3 M NaCl/2M urea. The high mol. wt material forms three components (1, 2 & 3). (d) Sephadex G-100 column loaded with aqueous extract of AA amyloid liver treated with 6 M guanidine and reduced and alkylated; elution was with 5 M guanidine/0·1 M acetic acid. The retarded peaks have been shown to be protein AA. (e) Sepharose 2BCL column loaded with void volume material from Sephadex G-100 after chromatography of AA amyloid fibrils; elution was with 6 M guanidine/0·5 M Tris/0·001 M EDTA. Note the large void volume peak, an intermediate peak and a retarded peak. Vt = total bed volume.

consisted of several components (Fig. 1c). Similar experiments were not performed with amyloid tissue extract due to its insolubility in these conditions.

Reduced and alkylated crude aqueous extract of normal and amyloid tissues were chromatographed on Sephadex G-100 eluting in dissociating conditions. In all cases there was a major symmetrical peak in the void volume. Extracts of normal tissue had small second peak (5% material; mol. wt 10,000). Extracts of amyloid material had a number of peaks; an example for AA amyloid is shown in Fig. 1d. These retarded peaks of amyloid have been previously shown to be composed of either protein AA or AL and have been fully characterized (Husby, 1980).

The high mol. wt void volume material from the Sephadex G-100 column (Fig. 1d) was rechromatographed in dissociating conditions on Sephacryl S-300 and Sepharose 2BCL. Similar results were obtained with both gels. Amyloid material (Fig. 1e) had a large void volume peak of high mol. wt (>40% material), an intermediate peak of varying size (mol. wt 50–160,000) and a retarded peak (mol. wt 10,000). Material from normal tissues also gave a large void volume peak, in most cases an intermediate peak and invariably a retarded peak (mol. wt 10–15,000). Experiments using the crude initial aqueous extracts of normal and amyloid tissues gave similar results, although the retarded peak was larger especially with AA amyloid.

Crude aqueous extracts of normal tissues and void volume materials from the Sephadex G-100 column (normal and amyloid) were treated with SDS and chromatographed on Sepharose 4B eluting with 0·3M NaCl/1% SDS. There was a similar pattern of a large void volume peak of high mol. wt material, a variable intermediate peak, and a small retarded peak of low mol. wt. An example for normal material is shown in Fig. 1e.

Electrophoresis

PAGE of crude aqueous extracts showed all preparations had some high mol. wt material which did not enter the gel. In addition aqueous extracts of normal tissues had two components (mol. wt 18,000 and 14,000) with additional minor ones in some tissues. The main components of aqueous extracts of amyloid tissues were: in AA amyloid a protein band of mol. wt 8,800 (protein AA); and in AL amyloid a band of mol. wt 37,000 reacting with anti-AL (λ). PAGE of the Sephadex G-100 void volume materials from amyloid fibrils showed a number of additional components in several

Table 1. Summary of the chromatographic and electrophoretic results

Tissue	Chromatography			Polyacrylamide gel electrophoresis
	Sephadex G-100	Sephacryl S-300*	Sepharose 4B/2BCL†	
Normal	Vo material mol. wt 10,000	Vo material mol. wt 10–14,000‡	Vo material mol. wt 50–70,000‡ mol. wt 10–15,000	High mol. wt material mol. wt 46,000 (liver only) mol. wt 18,000 mol. wt 14,000 mol. wt 12,000 (spleen only)
Amyloid	Vo material various amyloid subunits of AA and AL types§	Vo material mol. wt 50–160,000‡ mol. wt 10,000	Vo material mol. wt 50–70,000‡ mol. wt 10,000	High mol. wt material mol. wt 220,000 mol. wt 60,000 void volume material mol. wt 44,000 from Sephadex G-100 column mol. wt 30,000 mol. wt 37,000 (AL amyloid only) mol. wt 8,800 (AA amyloid only)

* Rechromatography of high mol. wt material from Sephadex G-100.

† Composite data from chromatography under various dissociating conditions.

‡ Variable Kav values from different tissues and different preparations.

§ Mol. wt data for these have been previously published in detail (Husby, 1980).

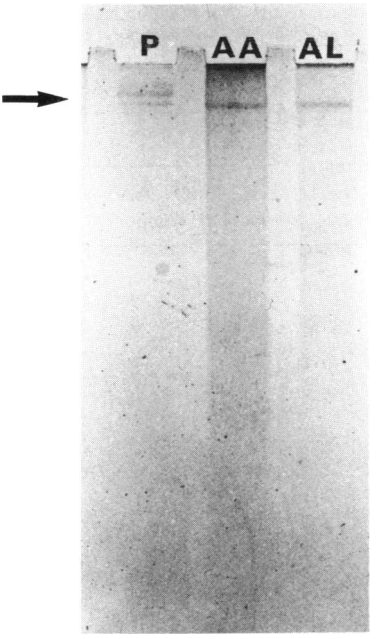

Fig. 2. Polyacrylamide gel electrophoresis (5% gels). The tracks contain purified plasma fibronectin (P), and void volume materials from the Sephadex G-100 column for AA amyloid (AA) and AL amyloid (AL). Note the double band given by plasma fibronectin and the single band by amyloid tissue extracts (arrows shows mol. wt 220,000 mark).

amyloids (Table 1). A notable feature of the amyloid preparations was that in addition to giving definite bands they gave diffuse protein staining throughout the gel. The component of mol. wt 220,000 had a similar mobility to plasma fibronectin, although if migrated as a single band and not a dimer; (Fig. 2); it was present in both AA and AL amyloids.

The relationship of chromatographic to PAGE results is shown in Table 1. Crude aqueous extracts of normal tissue and Sephadex G-100 void volume materials (normal and amyloid) were all rechromatographed on Sepharose 2BCL and then studied by PAGE. There were three peaks eluted from the Sepharose 2BCL column: the material eluted in the void volume was of high mol. wt and did not enter the polyacrylamide gel; with normal tissues the intermediate and retarded peaks contained material of mol. wt 18,000 and 14,000 with the former predominating in the intermediate peak; with amyloid preparations the intermediate peak contained material of mol. wt 60,000, 44,000, 30,000 and 8,800 and the retarded peak only mol. wt 8,800 material (protein AA).

Immunological studies

A variety of antigens were present in the tissue extracts (Table 2). Several antigens reacting with anti-whole human serum were detected on immunoelectrophoresis although their mobility and appearance did not always correspond exactly to the precipitin arcs given by normal human serum. The tissue preparations were also tested against antisera to prealbumin, IgG (γ-chains), IgA (α-chains), β_2-microglobulin, and NCRC with negative results in all cases.

The presence of fibronectin suggested by PAGE was confirmed immunologically (Fig. 3). It was detected in all AA and AL amyloid extracts by immunodiffusion, although it was also present in apparently minute amounts in some normal tissue preparations (Table 2). Immunodiffusion also showed that anti-fibronectin reacted with DAM and that fibronectin reacted with unabsorbed anti-DAM, the latter being abolished by immunoabsorption with normal human serum. The precipitin line given by anti-DAM with fibronectin showed a reaction of non-identity with both the AA and B specificities of the anti-serum and a series of immunoabsorption experiments confirmed these specificities were all different.

Table 2. Summary of the immunological results (Vo is Sephadex G-100 void volume material)

Antigens	Antisera							
	Whole human serum (No. components	DAM						
		B component	AA component	AA	AL(λ)	P component	Fibronectin	Albumin
Normal tissue								
Aqueous extract spleen	1	+	0	0	0	0	0	0
Aqueous extract liver (a)	3	+	0	0	0	+	+	+
Aqueous extract liver (b)	2	+	0	0	0	0	0	0
Vo spleen	2	0	0	0	0	0	0	0
Vo liver	2	+	0	0	0	0	0	0
AA amyloid								
Aqueous extract (JL)	3	+	+	+	0	+	+	0
Vo (JL)	1	+	+	+	0	0	+	0
Vo (BK)	3	+	+	+	0	0	+	0
Vo (AM)	2	+	+	+	0	0	+	0
Vo (BOL)	3	+	+	+	0	0	+	0
Vo (HAN)	2	+	+	+	0	0	+	0
AL amyloid								
Aqueous extract (MOL)	3	+	0	0	+	0	+	0
Vo (MOL)	3	+	0	0	+	0	+	0

Crude aqueous extracts of normal tissue and Sephadex G-100 void volume materials (normal and amyloid) were all rechromatographed on Sepharose 2BCL and were then studied by immunodiffusion. There were three peaks eluted from the Sepharose 2BCL column: none of the high mol. wt void volume material was immunoreactive the intermediate peaks contained the DAM B antigen and, in AA amyloid preparations protein AA and fibronectin; protein AA was also present in the retarded peak of the amyloid preparations.

Studies of saline extracts

Small amounts (less than 10%) of high mol. wt void volume material were present in the saline extracts of normal and amyloid tissue chromatographed on Sepharose 2BCL in dissociating

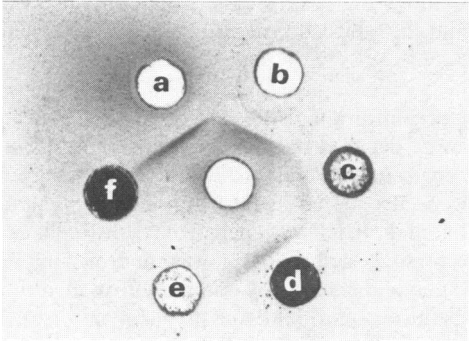

Fig. 3. Immunodiffusion in 1% agarose after staining with Coomassie brilliant blue R250. Anti-human fibronectin was in the central well. The outer wells contained: (a) normal human serum; (b) purified fibronectin; (c–f) void volume material from Sephadex G-100 column; (c) AA amyloid, (d) AL amyloid, (e) normal spleen, (f) normal liver. The serum and purified fibronectin and amyloid preparations give reactions of identity while the normal tissue preparations do not react.

conditions. The saline extracts showed immunoreactivity to the DAM B specificity, and in the case of the early saline extracts, with fibronectin. AA was also present in the saline extracts of amyloid tissue. However, analysis by PAGE showed a number of components were present in the saline extracts that were not in the aqueous extracts. The final urea extracts of AA amyloid tissue did not contain AA but gave a sharply defined, rapidly developing precipitin line on immunodiffusion with anti-fibronectin.

Recombination experiments

The separate and combined gel filtration studies of purified protein AA and SAA with the high mol. wt component of normal tissue aqueous extracts showed no evidence that there was any association between the high mol. wt material and the other proteins

DISCUSSION

The major amyloid proteins are AA and AL representing primary/myeloma associated and secondary amyloidosis; they are respectively related to immunoglobulin light chains and SAA (Husby, 1980). Other amyloid proteins include AFp, related to prealbumin (Costa, Figueira & Bravo, 1978), and AEt, related to calcitonin (Sletten, Westermark & Natvig, 1976). P component is also present in amyloid deposits. However, our results showed that amyloid fibrils contain a number of other components, including a high mol. wt complex, the DAM B antigen and fibronectin. While it may be argued that these components are not an integrated part of the amyloid fibril but merely contaminants which are extracted at the same time, this is unlikely to be the case. The proteins were extracted from all the amyloid tissues we examined, and previous studies have shown that high mol. wt material is present in apparently pure preparations of amyloid fibrils (Benditt & Eriksen, 1972; Husby & Natvig, 1972; Levin *et al.*, 1972; Husby, Natvig & Sletten, 1974).

Previous studies of amyloid have used gel filtration in dissociating conditions with gels that have low exclusion limits; for example Sephadex G-100, which under these conditions only separates proteins with mol. wt less than 60,000 daltons (for review see Glenner, 1980). But when a range of gels is used with different exclusion limits the heterogeneity of the void volume material from the Sephadex G-100 column is demonstrated. Some of the material is undoubtedly aggregated protein AA or AL, and these were present in both the intermediate and retarded peaks when the void volume material from the G-100 column obtained respectively from AA and AL amyloid preparations was refractionated on Sepharose 2BCL, but they were not the major components.

The high mol. wt void volume material from the Sepharose 2BCL column is probable the same in preparations of amyloid and normal tissue, although further studies will be needed to confirm this. We suggest that the antigen which forms a component of reticulin, NCRC, is part of this complex and it will not react in immunodiffusion due to its size (Unsworth *et al.*, 1982). We have been unable to further degrade this complex using reducing and dissociating conditions, or in additional experiments with 0·1M NaOH. However, the present evidence suggests that it is not a single component but a complex of various size molecules. On the other hand the DAM B antigen which fractionates in the intermediate peak from the 2BCL column is of smaller molecular size. It is difficult to immediately correlate the immunological, chromatographic and electrophoretic findings between normal and amyloid preparations unless the DAM B antigen has a different apparent mol. wt in these preparations. Given the tendancy for amyloid proteins to aggregate this is very likely.

Fibronectin, which is present in amyloid fibril preparations, is a normal component of connective tissue, plasma and tissue fluids in different biochemical forms which cross-react immunologically (Yamada & Olden, 1978; Yamada & Kennedy, 1979). It is a structural component of connective tissue which co-distributes with reticulin fibres (Stenman & Vaheri, 1978). Its presence in the early saline washes of amyloid and normal tissues is compatible with the removal of a normal plasma protein, while its presence in the final urea extracts of amyloid tissue, without amyloid proteins such as AA, suggests the specific solubilisation of a connective tissue protein, indeed, urea specifically solubilises fibronectin from cell cultures (Yamada & Olden, 1978). Fibronectin has specific binding sites for macromolecules such as collagen and heparin (Ruoslahti *et al.*, 1981); its

consistent presence in preparations of amyloid fibrils may be of significance in their deposition. Serum amyloid P component immobilised on Sepharose beads specifically binds fibronectin; this interaction is calcium-dependent and related to the conformation of the P component (De Beer *et al.*, 1981). P component is an important constituent of amyloid fibrils (Holck *et al.*, 1979) and this interaction may explain the presence of fibronectin in amyloid preparations. Fibronectin is susceptible to proteolysis and denaturation, and our results must be interpreted with this in mind.

The components of normal and amyloid tissues we have studied have a tendency to aggregate. Chromatography of aqueous extracts of normal tissues under non-dissociating conditions suggests in these circumstances they form a heterogenous group of aggregates of different sizes. Additional experiments with agarose gel electrophoresis (results not shown) revealed these complexes were electrophoretically heterogenous. When the ionic strength is changed aqueous extracts of normal tissue and amyloid tissue form large visible particulate aggregates. The physical properties of self aggregation may be important in developing structural elements of connective tissue such as reticulin fibres. It is tempting to suggest amyloid is deposited in tissues by an abnormal protein such as AA attaching to a normal connective tissue component. Our recombination experiments did not provide evidence for this. Nevertheless, it may occur when certain critical conditions are met; and there must be a basis for the non-random distribution of amyloid in tissues.

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