

A HISTOCHEMICAL STUDY OF THE PROTEIN NATURE OF AMYLOID

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Amyloid is a hyalin-like, structureless, translucent substance which accumulates between parenchymatous cells and in connective tissues. Virchow¹ in 1854 proposed the name amyloid for this substance to denote its gross and microscopic appearance and its affinity for iodine (a characteristic of starch). It is believed to be a mixture of protein, glycoprotein, other polysaccharides and, occasionally, lipids.²⁻⁴

Giles and Calkins^{5,6} were able to study the composition of an almost pure amyloid deposit in the liver of a patient dying of tuberculosis and secondary amyloidosis. Their chemical analysis suggested that the substance was hydrophilic and chiefly protein in content, but also contained about 4 per cent carbohydrate, of which 1.5 per cent was aminosugar. By chromatography they demonstrated that both glucosamine and galactosamine were present in the ratio of 4 to 1, indicating that chondroitin sulfate was not a major carbohydrate component; this was contrary to the observations of other investigators.^{2-5,7-11}

Giles and Calkins^{5,6} were also able to demonstrate that almost 2 per cent of the carbohydrate content of amyloid occurred as bound neutral sugars (chiefly glucose and galactose in approximately equal amounts), as well as uronic acid (0.6 per cent). They observed also that the hexosamine concentrations found in several amyloid-containing livers from both horses and human beings was roughly proportional to the amyloid concentrations, as judged from histologic examination. The virtual absence of phosphorus, purines and pyrimidines, which they reported, would indicate that nucleic acids are not detectable in amyloid and that amyloid is not merely altered protoplasm.

Calkins and Cohen¹² published observations based on the analysis of amyloid recovered post mortem from the livers and spleens of 8 human subjects. Their findings were essentially similar to those of Giles and Calkins. Numerous investigators using electrophoretic and immunochemical methods have reported that amyloid contained appreciable

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quantities of serum globulins.^{5,7,9,13-16} Heparin has also been implicated as a possible component.¹⁷

Various theories of etiology have been advanced. Jones and Frazier¹⁸ believed that amyloidosis was an alteration of a mucopolysaccharide reticulum response. According to these authors, the proteins were precipitated by a reaction of a circulating antigen with antibody. Amyloid was especially abundant in the reticuloendothelial cells of affected animals.^{4,19} Several investigators have commented on the role of these cells in the pathogenesis of the deposit.^{4,19-23} Ehrlich²⁴ offered the theory that amyloid might be due to the production of a pathologic protein by normal or abnormal plasma cells and might be a precipitate of protein with mucopolysaccharide. Mallory²⁵ and Warren²⁶ suggested the possible role of perverted fibroblastic activity in the formation of the substance, while Geer, Strong, McGill and Muslow²⁷ suggested that it might arise as an alteration of the connective tissue ground substance. Teilum²⁸ noted that the development of deposits in the spleen was preceded by the accumulation of periodic acid-Schiff (PAS) positive and pyroninophylic substances within splenic reticuloendothelial cells. He regarded these substances as indicative of abnormal glycoprotein synthesis and as precursors of amyloid. In commenting on Teilum's findings, Cohen, Calkins and Levene²⁹ stated: ". . . the evidence that these histologic features *per se* denote abnormal glycoprotein synthesis would seem to be circumstantial. The conclusion that simply because the accumulation of PAS-positive and pyroninophilic material precedes the development of amyloidosis, the former substance is a precursor of the latter does not appear to be justified."

The view which has received considerable attention during the last decade, as summarized by Symmers,³ is that amyloidosis is a manifestation of immunologic disturbance. It was postulated that amyloid, which might be the insoluble glycoprotein product of a local antigen-antibody reaction, was deposited in prepared situations. The detection of globulin as a constituent of amyloid has given considerable impetus to the role of antigen-antibody reaction.^{5,7,9,13-16} Another factor in favor of this concept was the increase of the globulin and glycoprotein fractions in the serums of affected animals and man.³⁰⁻³³ Calkins, Cohen and Gitlin³⁴ showed, however, that human amyloid homogenates did not bind anti-gamma globulin as did precipitates of gamma globulin antibody. This would suggest that amyloid was not a simple antigen-antibody precipitate. These authors observed that globulins were present but pointed out that this alone did not constitute proof of an antigen-antibody reaction. Although numerous references have been made to the protein nature of amyloid, we were unable to find specific citations of its histochemical

demonstration. The investigation reported here was undertaken to demonstrate those protein-reactive groups characteristic of amyloid and also to determine the similarity or dissimilarity of amyloid found in various species.

MATERIAL AND METHODS

Kidney, liver, spleen and heart were obtained at necropsy from examples of amyloidosis in rabbits, hamsters, horses, mice, chickens, ducks, dogs and human subjects (Table I). A total of 30 tissues from 23 cases were used. The tissues were

TABLE I
SOURCE OF SPECIMENS

Case no.	Tissue	Species	Remarks
1	Liver, kidney	Human	Secondary amyloidosis—primary disease unknown
2	Liver, kidney	Human	Secondary amyloidosis—primary disease unknown
3	Spleen, heart	Human	Secondary amyloidosis—primary disease unknown
4	Kidney	Human	Secondary amyloidosis—primary disease unknown
5	Spleen, kidney	Human	Secondary amyloidosis—primary disease unknown
6	Kidney	Human	Secondary amyloidosis—primary disease unknown
7	Spleen	Chicken	Amyloidosis secondary to tuberculosis
8	Spleen	Chicken	Amyloidosis secondary to tuberculosis
9	Spleen	Chicken	Amyloidosis secondary to tuberculosis
10	Spleen	Chicken	Amyloidosis secondary to tuberculosis
11	Spleen	Chicken	Amyloidosis secondary to tuberculosis
12	Spleen	Mouse	Secondary amyloidosis—primary disease unknown
13	Spleen	Mouse	Amyloidosis secondary to chronic nephritis
14	Spleen	Mouse	Amyloidosis secondary to tuberculosis
15	Spleen	Mouse	Amyloidosis secondary to tuberculosis
16	Spleen	Dog	Amyloidosis secondary to canine distemper
17	Kidney	Dog	Amyloidosis secondary to chronic nephritis
18	Kidney	Dog	Primary amyloid nephrosis
19	Liver, spleen, kidney	Duck	Experimental amyloidosis induced by methylcholanthrene
20	Liver	Duck	Experimental amyloidosis induced by methylcholanthrene
21	Spleen	Rabbit	Amyloidosis secondary to chronic pneumonitis and emphysema
22	Liver	Horse	Amyloidosis secondary to coccidioidomycosis
23	Spleen, liver	Hamster	Amyloidosis secondary to subcutaneous abscesses

fixed in neutral buffered 10 per cent formalin, and paraffin sections were cut at 6 to 8 μ . Deparaffined serial sections of each specimen were treated by a wide variety of techniques, as follows:

1. For demonstration of tissue structures: hematoxylin and eosin.²⁶
2. Localization and quantitation of amyloid: Congo red (Bernhold's),²⁶ crystal violet²⁶ and thioflavine T.²⁶ Sections stained by the latter method were examined by

fluorescent microscopy, using a Phillips CS 150 mercury vapor source and 6 mm. UGI transmission and 2.5 mm. Euphose absorption filters.

3. Demonstration of glycogen, mucopolysaccharides, polysaccharides and glycoprotein: periodic acid-Schiff (PAS),²⁸ acetylation-PAS,²⁹ acetylation-deacetylation-PAS,³⁰ bromination-PAS³¹ and performic acid-Schiff.³²

In order to reduce the number of tissue sections to be handled, 9 cases, all with significant deposits of amyloid, were selected for further study with protein stains. This group contained specimens representative of all species included in the study. The sections were celloidinized after being mounted on slides.

Danielli's dinitrofluorobenzene (DNFB) stains were used to demonstrate the presence of sulfhydryl, disulfide, tyrosyl and amino groups.³³ The staining procedures were performed according to schedules reported by Thompson and colleagues.³⁴ With the exception of the method for arginine, paired sections (experimental and control) from each case were stained by each of the following procedures:

1. The specific demonstration of sulfhydryl groups: Control sections were treated with benzoyl chloride in anhydrous pyridine (benzoylation) to block sulfhydryl groups as well as tyrosyl and amino groups. Both the experimental and control sections were then treated with sodium nitrite to block amino groups, diazoaniline to block tyrosyl groups, and then stained with DNFB coupled with H acid (the monosodium salt of 8-amino-1-naphthol-3, 6-disulfonic acid).

2. The specific demonstration of disulfide groups: The control and experimental sections were benzoylated to block any sulfhydryl, amino or tyrosyl groups present. The experimental sections were then treated with sodium cyanide to reduce non-reactive disulfide groups to reactive sulfhydryl groups. Both experimental and control sections were subsequently stained with DNFB coupled with H acid. Additional sections from each case were methylated in acidified methyl alcohol before benzoylation and subsequent DNFB-H acid staining. This was done to block carboxyl³⁵ groups which might stain with DNFB and lead to a false interpretation of the presence of disulfide groups.

3. The specific demonstration of amino groups: Control sections were benzoylated to block amino groups, together with sulfhydryl and tyrosyl groups. Both experimental and control sections were then treated with diazoaniline to block tyrosyl groups and hydrogen peroxide to oxidize sulfhydryls to nonreactive disulfides and stained with DNFB coupled with H acid.

4. The specific demonstration of tyrosyl groups: Control sections were benzoylated to block tyrosyl groups as well as sulfhydryl and amino groups. Both experimental and control sections were then treated with sodium nitrite to block amino groups, hydrogen peroxide to block sulfhydryl groups and stained with DNFB coupled with H acid.

5. The specific demonstration of tryptophan: Control sections were benzoylated to block tryptophan, then both the experimental and control sections were stained with the p-dimethylaminobenzaldehyde method of Adams.³⁶

6. The specific demonstration of arginine: Sections from each case were stained by the 8-hydroxyquinoline technique.³⁷ Sections of mature rat testes were used as positive controls for this stain.

RESULTS

The staining reactions of amyloid in the tissue sections from the various species were sufficiently uniform so that the results may be reported without reference to the affected tissue or species (Table II). The tissue sections stained for sulfhydryl, disulfide, tyrosyl and amino groups were evaluated by comparison with the specifically blocked control sections.

The characteristic structure, as well as the uniform positive reaction to accepted tests for amyloid (crystal violet, Congo red and thioflavine T) indicated that the tissues used in this study contained substances which would usually be interpreted as amyloid. The established fact that amyloid contains a carbohydrate component was confirmed by the presence of 1,2-glycol groups or their amino or alkylamino derivatives as

TABLE II
STAINING CHARACTERISTICS OF AMYLOID IN TISSUES FROM 8 SPECIES

Stain	Staining reaction to amyloid
Congo red	Positive
Crystal violet	Positive
Thioflavine T	Positive
Periodic acid-Schiff (PAS)	Positive
Acetylation-PAS	Negative
Acetylation-deacetylation-PAS	Positive
Bromination-PAS	Positive
Performic acid-Schiff	Positive
Sulphydryl DNFB	Negative to weakly positive
Disulfide DNFB	Strongly positive
Amino DNFB	Positive
Tyrosyl DNFB	Negative
Tryptophan	Positive
Arginine	Negative

denoted by the consistently positive PAS reaction.⁴⁰ The latter, observed subsequent to bromination, would suggest that glycogen was not a part of the carbohydrate component of amyloid or at least was not present in demonstrable quantities.³⁵ These procedures were performed to establish the fact that the amyloid in the various tissues employed in this study adequately conformed to a sufficient number of the histochemical characteristics of this substance.

That amyloid contains substances of a glycoprotein nature was suggested by the demonstration of hydroxyl or amino groups and by the negative PAS reaction following acetylation and the restoration of PAS positivity upon deacetylation of the acetylated sections.³⁵ The consistently positive performic acid-Schiff reaction indicated the presence of ethylenic linkages,³⁵ and possibly cystine.⁴⁰ The latter reaction could be interpreted as denoting the presence of carbohydrates, lipids or proteins, and is not particularly enlightening.

The specific protein stains employed were used to determine the nature of the protein components of amyloid as indicated by the presence or absence of certain reactive groups characteristic of proteins in general. The results obtained confirmed the previous reports attesting

to the protein nature of amyloid.^{2-7,9,12-16} However, the specific reactive groups of protein present in amyloid have not, to our knowledge, been previously reported. Variable quantities of these specific groups were thought to be in an available reactive form prior to staining⁴¹ and not appreciably altered by the fixative which was used.^{40,42}

The protein stains used in this study demonstrated that amyloid in rabbits, hamsters, horses, mice, chickens, ducks, dogs and human beings was characterized by the consistent presence of disulfide linkages and amino, tryptophan and sulfhydryl groups. This was only a qualitative demonstration and did not necessarily reflect their relative abundance. However, the extremely intense staining reaction obtained with the stain for disulfide linkages, and the weak reaction with the sulfhydryl stain suggested that disulfides were present in greater abundance than sulfhydryls. If one accepts Pearse's view,⁴⁰ the presence of disulfide groups in amyloid was also confirmed by the positive performic acid-Schiff reaction.

Sulfhydryl groups are found in the amino acid cysteine, which is not usually encountered in tissues as such, but is present in the form of glutathione.⁴² This polypeptide is widely distributed throughout the animal body, and is certainly not foreign to the tissues in which amyloid was observed in this study. As for amino groups, these are found as components of a wide variety of polypeptides, and their presence would be expected to be demonstrable in nearly all the sites of protein in the animal body. Of particular interest was the consistently positive reaction obtained with the stain employed for the demonstration of tryptophan. This amino acid is present in many animal proteins, among which are fibrinogen, gamma globulin, and casein.⁴³ From a review of the literature it is evident that tryptophan-containing proteins have frequently been reported to be incorporated in amyloid or related to it etiologically. On the other hand, tryptophan is noticeably absent in animal proteins such as collagen and elastin,⁴³ which have been frequently considered as possible precursors of amyloid.

The absence of arginine, or at least our inability to demonstrate it, is also of interest. Arginine is present in most proteins, particularly the histones,⁴³ and has been shown to be a component of Mallory's "alcoholic" hyalin.⁴⁴ The methods available for the demonstration of arginine are not extremely sensitive. However, we are of the opinion that the absence of this amino acid in amyloid was not related to any fault of the method since we obtained consistently positive results with control tissue (mature rat testes). Similarly, the consistently negative reaction in sections stained for the demonstration of tyrosyl groups is significant since this amino acid is widely distributed throughout the animal body and is a component of many proteins.⁴²

Our most striking observation was the consistent demonstration of large quantities of cystine, as denoted by the intense positive staining reaction for disulfide linkages (Figs. 1 to 6). Disulfides may occur in tissues as a result of oxidation of free cysteine with formation of cystine. As mentioned previously, free cysteine as such is generally not found in animal tissues, but large quantities are present in the form of polypeptide glutathione. Therefore, the disulfides (cystine) demonstrated were more likely a result of the oxidation of glutathione.⁴⁵ The abundance of stainable sulfhydryl groups (cysteine in glutathione) in the cellular components of all tissues and the weak staining reaction obtained for these groups in amyloid itself suggests that glutathione was present in large quantities in the former and in minimal quantities in the latter sites. Conversely, the absence of stainable disulfides in all tissues and their abundance in amyloid suggests that glutathione was present in the former sites in an unoxidized form and for the most part in the latter site in an oxidized form.

It can be postulated that a significant portion of the protein component of amyloid apparently represents a protein degradation product (oxidized glutathione) which is not usually found in parenchymatous tissues of the animal body. One might wonder if amyloid was not therefore related to some form of abnormal protein metabolism, influenced by factors as yet unknown. It is also evident that the presence of cystine would readily account for reactive sulfur groups. The availability of such sulfur groups could account for the earlier beliefs that chondroitin sulfates or heparin were components of amyloid.^{2-5,7-11,17}

SUMMARY

Amyloid deposits in tissues from chickens, dogs, hamsters, horses, mice, rabbits, and human subjects were subjected to a battery of staining procedures. Specific stains for disulfide linkages, sulfhydryl, tyrosyl and amino groups, tryptophan and arginine were employed. Amino acids and polypeptides containing disulfide linkages were found to be a constant and significant component of amyloid, regardless of the tissue or species in which it occurred.

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[Illustrations follow]

LEGENDS FOR FIGURES

All photomicrographs were made from tissue sections stained specifically to demonstrate disulfide linkages.

FIG. 1. Hepatic amyloidosis secondary to multiple subcutaneous abscesses in a hamster. $\times 220$.

FIG. 2. Amyloidosis in the spleen of a chicken, secondary to tuberculosis. $\times 220$.

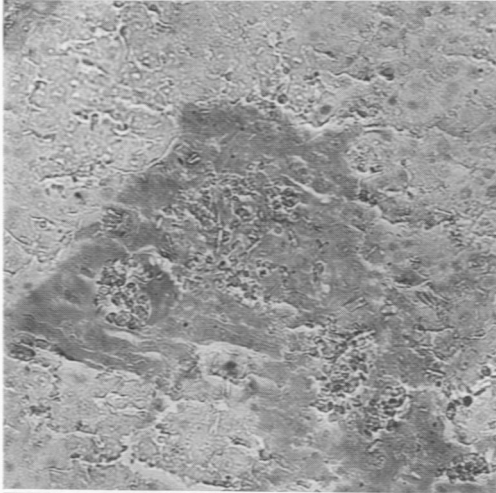
FIG. 3. Secondary amyloidosis in a human kidney; the primary disease is unknown in this case. $\times 220$.

FIG. 4. Splenic amyloidosis secondary to distemper in a dog. $\times 220$.

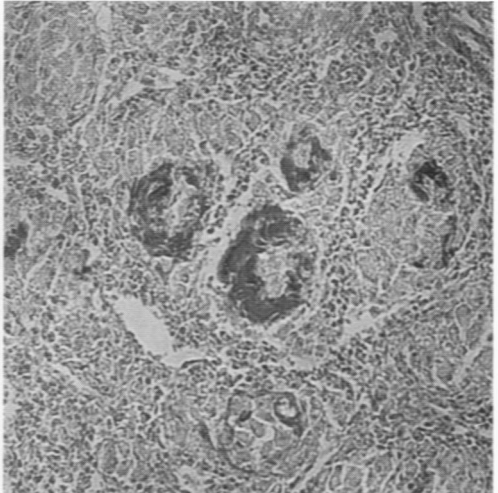
FIG. 5. Hepatic amyloidosis secondary to coccidioidomycosis in a horse. $\times 550$.

FIG. 6. Duck. Hepatic amyloidosis experimentally induced by the administration of methylcholanthrene. $\times 880$.

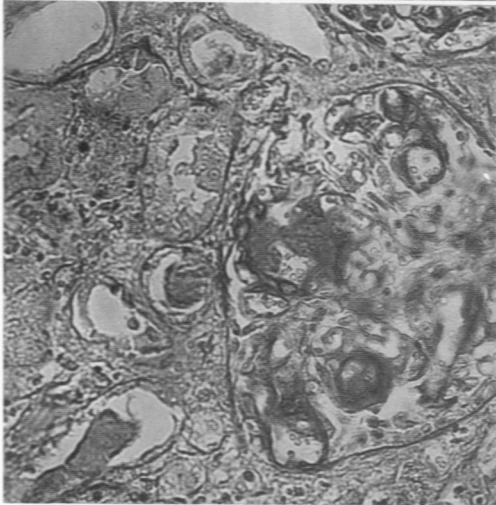
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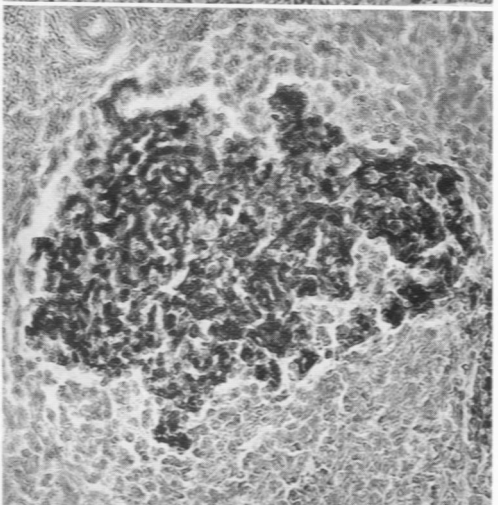
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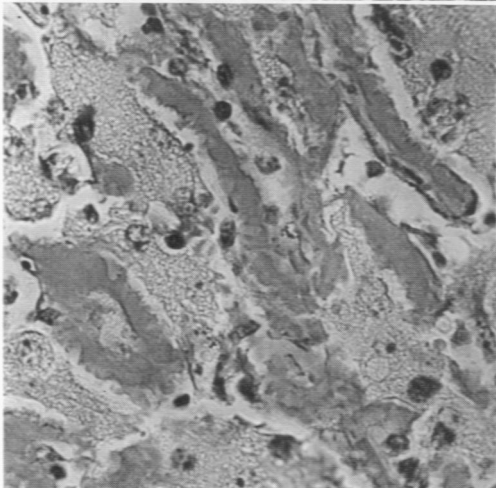
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