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Differences in Ultrastructural Organization of Amyloid as Revealed by Sensitivity or Resistance to Induced Proteolysis

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Summary. Amyloid substance is known to be resistant to proteolytic enzymes. In the present investigations it was found that after mild preoxidation with potassium permanganate of tissue sections, the deposits of amyloid in 11 cases of secondary generalized amyloidosis were dissolved by trypsin. The amyloid deposits in three cases of primary generalized amyloidosis however, proved resistant to induced proteolysis. Furthermore, myeloma amyloid in a case of anuric nephrosis, local massive amyloid deposits in a case of medullary thyroid carcinoma, amyloid of an amyloid tumour of the skin, as well as all senile amyloid deposits investigated (in heart, aorta, meningocerebral vessels, senile plaques of cerebral cortex and in the pancreatic islets) were resistant to induced proteolysis.

These findings indicate essential differences in ultrastructural organization between the various amyloids; such differences are not detectable by any morphological method. Resistant and sensitive types of amyloid thus seem to be bound to clinical forms of amyloidosis and seem to correspond to types A and B amyloid as recently distinguished on a physico-chemical basis by Benditt and Eriksen.

The usefulness of testing sensitivity or resistance of amyloids to induced proteolysis in differentiating them according to their ultrastructural organization in pathohistology and the significance of this test as a new approach in amyloid research are discussed.

Zusammenfassung. Die Resistenz gegen proteolytische Enzyme ist eine charakteristische Eigenschaft der Amyloidsubstanzen.

In der vorliegenden Arbeit wird darüber berichtet, daß nach milder Voroxydation der Gewebschnitte mit Kaliumpermanganat die Amyloidablagerungen in allen Organen von Fällen sekundärer generalisierter Amyloidose durch Trypsin aufgelöst wurden, wogegen sich alle Amyloidablagerungen in drei typischen Fällen primärer generalisierter Amyloidose gegen induzierte Proteolyse als resistant erwiesen.

Weiterhin wurde in einem Fall von medullärem Schilddrüsen Carcinom mit massiven Amyloidablagerungen im Stroma, in den Amyloidablagerungen einer Myelom-Niere, und in einem Amyloidtumor der Haut sowie in allen untersuchten senilen Amyloidablagerungen (Herz, Aorta, cerebromeningeale Gefäße, senile Drusen in der Hirnsubstanz und Amyloidablagerungen in den Pankreasinseln) gegen induzierte Proteolyse resistentes Amyloid gefunden.

Diese Befunde weisen auf gewisse Unterschiede in der ultrastrukturellen Organisation der verschiedenen Amyloidsubstanzen hin, welche Unterschiede mit den üblichen morphologischen Methoden nicht erfaßbar sind.

Sensitives und resistentes Amyloid scheinen mit gewissen Erkrankungsformen der Amyloidose verbunden zu sein. Es scheint eine gute Übereinstimmung der Verteilung von sensitivem und resistentem Amyloid auf verschiedene Erkrankungsformen der Amyloidose im eigenen Material und der Verteilung von Typ A bzw. B Amyloid von Benditt und Eriksen, differenziert aufgrund physikochemischer Methoden, zu bestehen.

Es wird die Frage der Verwendung von histochemischer Prüfung der Sensitivität bzw. Resistenz gegen induzierte Proteolyse zur Differenzierung der ultrastrukturellen Organisation der Amyloidsubstanzen besprochen und auf die Bedeutung dieser Methode als eine neue Untersuchungsmöglichkeit in der Amyloidforschung hingewiesen.

The question whether all amyloid deposits in the different forms of amyloidosis (generalized secondary and primary, senile and local amyloids) are of the same chamical composition and structural organization and whether they are related in their pathomechanism, has been raised (Cohen, 1967; Pras *et al.*, 1969) but not definitely answered. That may be, in part, because the definition of amyloid is possible only on a morphological basis and not on a chemical molecular basis. However, the specific morphologic criteria of amyloid (such as its metachromasia with crystal violet, additive topo-optical staining reaction with Congo red, manifesting itself in increased positive birefringence, and its fibrillary ultrastructure in the electron microscope) do not provide evidence of the identity of the various amyloids in terms of chemical ultrastructure. Histochemical (Braunstein and Buerger, 1959; Diezel and Pfleiderer, 1959; Pearse, 1960, 1972; Pfeiffer, 1965; Ganter and de Saint-Maur, 1971) and chemical investigations (Goessner, 1961; Thompson *et al.*, 1961; Schmitz-Moorman, 1965; Cohen, 1966; Pras *et al.*, 1969) of the composition of amyloid have revealed some differences in the amyloids of different origin but do not provide a firm basis for differentiating between definite types of amyloid.

In more recent systematic studies on the chemical composition, physicochemical and optical characteristics of the amyloid substances Benditt and Eriksen (1971) have presented evidence for the existence of two chemically different types of amyloid substance. They distinguished a type A of amyloid in five samples, prepared from cases of generalized (secondary) amyloidosis associated with inflammatory diseases, and a type B amyloid prepared from 5 cases not associated with inflammatory diseases. The A and B types of amyloid differed greatly in amino acid composition, in the lack of a specific α-protein in type B amyloid and in the electrophoretic mobilities of their constituents as well as in pH stabilities of Congo red binding as revealed by the spectral shift of Congo red bound to amyloid.

These observations by Benditt and Eriksen point to definite differences in chemical-structural organization of the amyloids of different origin. From the morphological point of view, the different stabilities of Congo red binding of type A and type B amyloids appear of interest; it remains to be shown however, whether this criterion can be successfully used as an additional histomorphological test for differentiating between the type A and type B amyloid in situ.

A well-known further characteristic of amyloid is its resistance to proteolytic enzymes in spite of its proteinaceous or glycoproteinaceous nature (Cohen, 1966). The resistance of amyloid to proteolytic enzymes has been stated by many authors for amyloid in man and animals (Missmahl, 1950; Hüsselmann, 1955; Windrum and Kramer, 1957; Diezel and Pfleiderer, 1959; McAlpine and Radcliffe, 1963; Sorensen and Binington, 1964; Pfeiffer, 1965; Lehner *et al.*, 1966; Arvy and Sors, 1968; Stiller and Katenkamp, 1970) and it has also been confirmed for isolated amyloid fibrils in the electron microscope (Cohen and Calkins, 1964; Emerson *et al.*, 1966; Pras *et al.*, 1969; Kim *et al.*, 1969).

In view of the complete resistance of the fibrillary element—the essential component of amyloid—to proteolytic enzymes, we noted with some surprise the induced rapid proteolytic degradation of the amyloid substance in tissues during our studies on the topo-optical reactions of collagen (Romhányi *et al.*,

1970; Romhányi *et al.*, 1972). These investigations resulted in two observations relevant to the morphological study of the amyloid problem: a) the collagen-specific *inversive* topo-optical staining reaction with Congo red (Romhányi *et al.*, 1970), which, contrasting with the amyloid specific *additive* topo-optical staining reaction with Congo red, provided a new approach to selective differentiation between amyloid, hyaline and collageneous structures (Romhányi, 1971) and b) the selective proteolysis of acetyl collagen by trypsin (Romhányi, 1971b; Romhányi *et al.*, 1972), which opened up a new possibility to study the structural relation between amyloid and connective tissue structures.

During these investigations we carried out a large number of enzyme treatments on tissue sections pretreated in different ways and found that after pretreatment with potassium permanganate (as used in silver impregnation techniques) the amyloid substance revealed a rapid proteolytic dissolution in contrast to the resistance of reticulin and collagen fibers and several cell proteins.

These findings prompted us to start systematic studies of the sensitivity to induced proteolysis of amyloid deposits in different types of amyloidosis. In the course of these investigations we tested a great number of amyloid deposits localized in various organs in different forms of amyloidosis and found that on the basis of sensitivity to induced proteolysis two types of amyloid could be distinguished: 1 a sensitive type and 2 a resistant type. This difference in induced proteolytic sensitivity of amyloids appears to be of special interest in several respects, to be discussed later.

In the present paper we report on the differences in sensitivity to induced proteolysis, which we found between the amyloid deposits in different pathological entities of amyloidosis (as general secondary and primary amyloidosis, senile and local amyloids) and discuss the possible implication of these findings in the characterization and ultrastructural differentiation of amyloids in the tissues as a new morphological approach in amyloid research.

Material and Methods

Our material (Table 1) comprised a) 11 cases of generalized secondary amyloidosis, b) three cases of primary generalized amyloidosis with typical massive involvement of the heart, tongue, salivary glands, peripheral nerves, pulmonary alveolar septa and the vascular walls and capillaries in many organs; c) a case of medullary thyroid carcinoma with massive amyloid deposits in its stroma, d) a case of massive amyloid deposition in a myeloma kidney with renal failure, e) a case of amyloid tumour of the skin, f) and a large number of senile amyloid deposits (aorta, heart, congophil angiopathies of the meningocerebral vessels, senile plaques in the cerebral cortex and amyloid deposits in the pancreatic islets of diabetics and aged patients).

The material was fixed in formalin for 24 hrs. Paraffin sections were prepared. Deparaffinized sections (in some cases also unfixed frozen sections) were used for testing sensitivity to induced proteolysis of amyloid in the following way:

Deparaffinized sections were dehydrated and overlayered with a celloidin film: 3–4 drops of 0.3% celloidin were dropped on the section and when the celloidin had begun to dry, the slide with section was immersed into water. The celloidin film so formed protected the section from detaching during enzyme treatment but did not inhibit enzyme action on the tissue. The section was then surrounded with a paraffin ring of 1–2 mm height, 3–4 drops of the enzyme solution were placed on the tissue section and a coverslip applied. In this way it was possible to perform enzyme treatments with small amounts of enzymes under controlled standard conditions at 37° C for different periods of time.

Table 1. Survey of the material investigated

			Type of amyloidosis	Sensitivity of amyloid to induced proteolysis
1.	32 y., ♀, 881, 61	Pulmonary tuberculosis.	Second. gen. amyloidosis	Sensitive
2.	62 y., ♂, 701, 67	Bronchiektasis. Fibrotic. tuberculosis.	Second. gen. amyloidosis	Sensitive
3.	65 y., ♂, 494, 68	Tuberculosis. Bronchiekt.	Second. gen. amyloidosis	Sensitive
4.	54 y., ♂, 49, 69	Arthritis deformans. Steroid treatment.	Second. gen. amyloidosis	Sensitive
5.	69 y., ♂, 358, 69	Pulmonary tuberculosis. Amyloid nephrosis.	Second gen. amyloidosis	Sensitive
6.	65 y., ♀ 610, 69	Rheumatoid arthritis. 15 y. duration. Steroid therapy.	Second. gen. amyloidosis	Sensitive
7.	91 y., ♀, 71, 70	Rheumatoid arthritis. No medical treatment.	Second. gen. amyloidosis	Sensitive
8.	70 y., ♂, 1098, 71	Chronic pyelonephritis.	Second. gen. amyloidosis	Sensitive
9.	49 y., ♀, 1116, 71	Chronic pyelonephritis, anuria.	Second. gen. amyloidosis	Sensitive
10.	25 y., ♀, 71, 72	Lymphogranulomatosis. 4 years duration.	Second. gen. amyloidosis	Sensitive
11.	27 y., ♀, 267, 72	Lymphogranulomatosis. Cytostatic and steroid therapy.	Second. gen. amyloidosis	Sensitive
12.	59 y., ♂, 1036, 71	Tu. colonis ? Ileus, coecostomia.	Primary gen. amyloidosis	Resistant
13.	60 y., ♂, 673, 70	Kachexia. Tu. ventr. ? 5 y. duration. Klin. d. g.: Primary gen. amyl.	Primary gen. amyloidosis	Resistant
14.	71 y., ♂, 151, 69	Short clinical observ. Cardiac insufficientia.	Primary gen. amyloidosis	Resistant
15.	44 y., ♀, 350, 72	Myeloma nephrosis with renal failure.	Myeloma amyloid.	Resistant
16.	56 y., ♀, 6536, 69	Medullary thyroid cc. with amyloid masses in the stroma.	Local amyloid.	Resistant
17.	50 y., ♀,	Multiple nodules in the skin: Amyloid tumor.	Local amyloid.	Resistant
18.	Senile amyloid deposits in heart, aorta, meningocere bral vessels, senile plaques in the cerebral cortex and amyloid deposits in the pancreatic islets.		Senile amyloids	Resistant

For providing us with material and report of cases 14 and 17 we are indebted to Dr. St. Orbán (Hospital, Kaposvár) and Prof. L. Szodoray (Dermat. Klin. Debrecen).

1. Testing for Direct Proteolysis. The sections were treated with trypsin for 6–18 hrs or longer: with trypsin (Chem. Work G. Richter Ltd. Budapest, 2 mg/ml phosphate buffer 0.15 M pH 8.2).

2. Testing for Induced Proteolysis. Sections were pretreated with potassium permanganate (0.25% in 0.15% H_2SO_4, freshly prepared by mixing equal volumes of 0.5% potassium permanganate and 0.3% H_2SO_4) for 3 min (by dropping the solution onto the slide) followed by treatment with oxalic acid solution (5%) until the sections were decolorized. The time of treatment with potassium permanganate should not go beyond 3 min, for longer treatment results in a gradual decrease in both staining and birefringence of amyloid with Congo red. After thorough washing in distilled water the sections were incubated in the enzyme solution 6 and 18 hrs or longer. As controls, potassium permanganate-treated sections were treated with the buffer solution for the same length of time.

3. Testing of Amyloid for Sensitivity to Induced Proteolysis Combined with Selective Collagenolysis. Acetyl collagen is known to undergo selective proteolysis (Romhányi, 1971; Romhányi *et al.*, 1972). We tested whether, and how, the selective proteolysis of collagen, basement membranes and reticulin fibers, into and on which amyloid deposits are precipitated might influence the stability of amyloid deposits. Therefore a) acetylated sections were treated with trypsin for 18 hrs to induce selective collagenolysis and b) acetylated sections were treated with potassium permanganate and then trypsinized for 24 hrs.

Acetylation

A modification of the original method proposed by Lillie (1958, 1964) was used (Romhányi *et al.*, 1972). The material fixed in formol, not longer than 24 hrs, was washed in tap water for 12–24 hrs and then embedded in paraffin. The sections were not allowed to hydrate. After deparaffinization in xylol and washing in absolute ethanol for 5–10 min they were put in the following acetylation mixture for 48 hrs: 80 ml anhydrous acetic acid and 20 ml pyridine. The acetylation mixture was removed from the sections by washing them with absolute ethanol for 2–3 hrs.

Testing Sections for Amyloid-Lysis and Collagenolysis

The sections were stained with Congo red and mounted a) in gum arabic or b) in Canada balsam and examined in the polarisation microscope for the presence or absence of amyloid and collagen (Romhányi, 1971).

Optical demonstration of amyloid and its differentiation from collagen was achieved by mounting the Congo red stained sections in gum arabic. Due to their weak *inversive* topo-optical staining reaction the Congo red-stained collagen fibers are isotropic in this medium and therefore they are not apparent with polaroids crossed, however, amyloid with its *intensive additive* topo-optical staining reaction stands out most clearly, showing strong positive birefringence on an isotropic background (Romhányi, 1971).

Optical Demonstration of Collagen

a) In Canada balsam Congo red-stained collagen fibers, basement membrane and amyloid reveal a strong *additive* topo-optical staining reaction with greatly increased positive birefringence (Romhányi *et al.*, 1970). We used this method to demonstrate the birefringence of collagen fibers and basement membranes after the dissolution of amyloid.

b) Demonstration of Selective Collagenolysis in Acetylated Trypsinized Slices. Congo red-stained acetyl collagen fibers show strong negative birefringence in gum arabic (Romhányi *et al.*, 1972). In this way collagenolysis was easy to demonstrate optically when acetylated slices, treated with trypsin were stained with Congo red and mounted in gum arabic. Similarly the sensitivity or resistance of amyloid to induced proteolysis was established in this way against the background of collagenolysis in acetylated and potassium permanganate-treated slices incubated in trypsin.

When demonstration of cell nuclei in the enzyme treated sections was required the slices were stained with haematoxylin after they have been treated with permanganate and then they were incubated in the enzyme solution. Congo red staining was applied after enzym incubations.

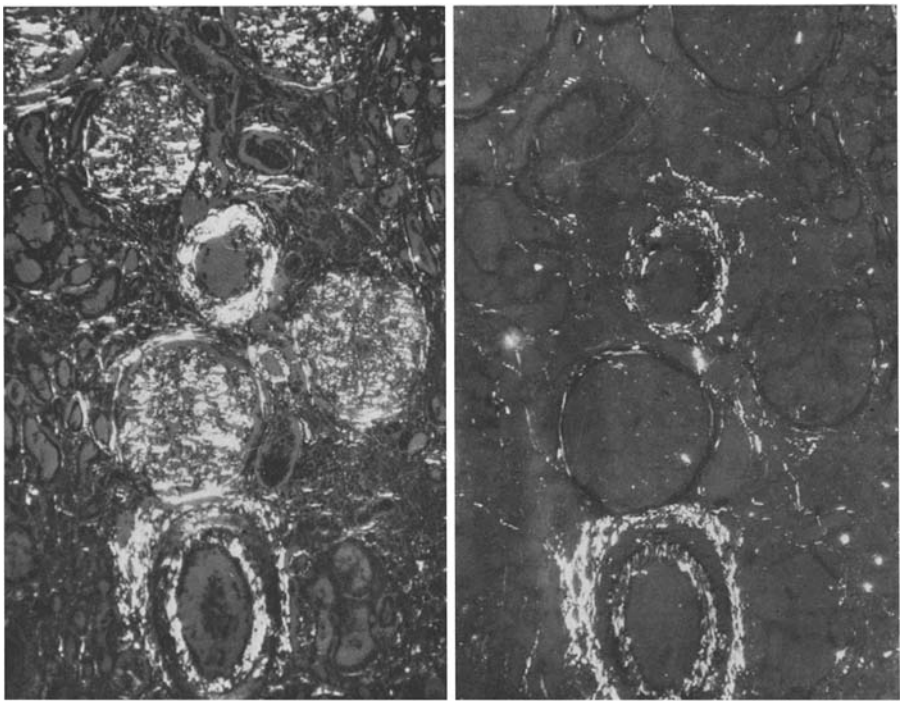

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Fig. 1a and b. Kidney; generalized secondary amyloidosis. a, Control, pretreated with potassium permanganate and incubated in buffer solution; b, the same visual field in the next section pretreated with $KMnO_4$ and incubated in trypsin for 6 hrs, haematoxylin-Congo red, mounted in Canada balsam; *X* polars. The birefringent masses of amyloid seen in (a) are absent in (b) in which only the birefringent connective tissue structures are apparent

Observations

1. Resistance of Amyloid Substances to Direct Proteolysis. In agreement with the data in the literature we have found all amyloid deposits in different types of amyloidosis (secondary, and primary generalized amyloidosis, myeloma, local and senile amyloids) resistant to trypsin even when treatment was prolonged for several days. We found that after trypsin treatment amyloid masses stained with a more reddish hue and more selectively with Congo red and produced a lighter green polarisation colour (Romhányi, 1971).

2. Sensitivity to Induced Proteolysis of Amyloid Deposits in Generalized Secondary Amyloidosis. In each of our eleven cases of generalized secondary amyloidosis all deposits of amyloid in various organs showed rapid proteolysis by trypsin in sections pretreated with potassium permanganate. Induced proteolysis of amyloid in a case of amyloid nephrosis is demonstrated in Fig. 1.

The birefringent masses of amyloid disappeared after enzyme treatment, only the birefringent connective tissue structures are seen. The details are shown in the light microscope

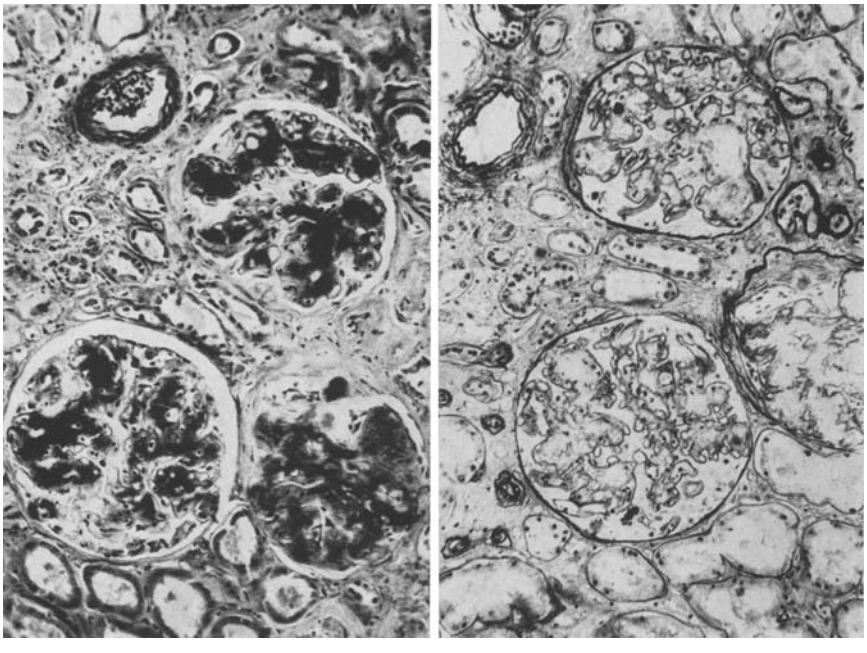

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Fig. 2a and b. Kidney; generalized secondary amyloidosis. In the light microscope (a) control, (b) $KMnO_4$ and trypsin, incubated for 6 hrs. Haematoxylin; Congo red. The amyloid masses, intensively stained with Congo red in (a) disappeared in (b). The basement membranes of the glomeruli stand out clearly after the dissolution of amyloid in (b)

in Fig. 2. The Congo red-stained amyloid masses have disappeared from the glomeruli in the enzyme treated section and the glomerular and peritubular basement membranes stand out clearly. Fig. 3 shows induced proteolytic dissolution of the amyloid masses in the splenic follicles in a case of generalized secondary amyloidosis. Again after dissolution of the amyloid masses (Fig. 3b) the cellular details, the birefringent reticulin fibers and elastic membranes of the arteries and the trabecular connective tissue, are well preserved.

Fig. 4 demonstrates enzymatic dissolution of diffuse amyloid deposits in the splenic pulpa and Fig. 5a, b show that the ultrastructural stability of amyloid is not influenced by selective proteolysis of acetyl collagen.

We can thus state that in all 11 cases of generalized secondary amyloidosis all deposits of amyloid in various organs showed the same sensitivity to induced proteolysis. They were rapidly dissolved by trypsin if the sections were mildly preoxidized with potassium permanganate.

3. Resistance to Induced Proteolysis of Deposits in Primary Generalized Amyloidosis. Each of our three cases was characterized by the typical massive and extensive amyloid deposits in the heart, tongue, striated muscles, by massive amyloid deposits in the peripheral nerves, in the alveolar pulmonal septa, and by widespread deposits in the vascular walls, as well as in the mucous membrane of the intestinal tract, and by varying involvement of spleen liver and kidney.

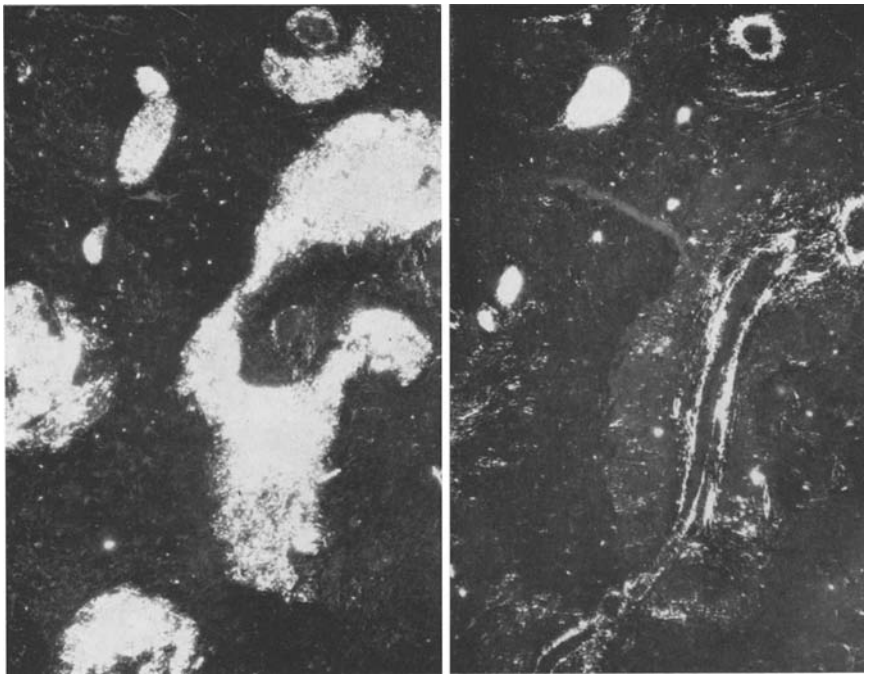

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Fig. 3a and b. Secondary follicular amyloidosis of the spleen. a, Control; b, $KMnO_4$-trypsin, incubated for 6 hrs. Haematoxylin-Congo red, Canada balsam, *X* polars. a and b, Represent the same areas of successive slices. The strongly birefringent amyloid masses seen in (a) are lacking in (b) as a result of amyloid lysis. Other structural details are well preserved. The birefringent collagen fibers of the trabecules and elastic lamellae of the follicular arteries can be recognized

In Fig. 6 massive amyloid deposits resistant to induced proteolysis are seen in the myocardium in a case with a generalized primary amyloidosis. Fig. 7 demonstrates masses of deposits of primary generalized amyloidosis resistant to induced proteolysis in the connective tissue of the mitral valve against the background of selective proteolysis of acetyl collagen.

The following illustrations display resistant amyloid deposits in the peripheral nerves and perineural vessels (Fig. 8), widespread enzyme-resistant pulmonary alveolar amyloidosis (Fig. 9) and enzyme-resistant massive amyloid deposits in the hepatic sinucoids and the periportal vessels [10].

Fig. 11 demonstrates enzyme resistant amyloid deposits in the splenic pulpa reticulum and in the collagen tissue of the trabeculae in a case of primary generalized amyloidosis and in Fig. 12 against the background of selective collagenolysis in acetylated section. Amyloid deposits, both in the pulpa reticulum and in the trabecular collagen and vascular wall, are equally resistant to enzymatic degradation.

In Fig. 13 the sensitivity and resistance to induced proteolysis of amyloid deposits in the adrenal cortex and capsular tissues in a case of secondary generalized amyloidosis (a, b) and in generalized primary amyloidosis (c) are compared. The amyloid deposits of the generalized secondary amyloidosis are dissolved, however deposits of the generalized primary amyloidosis (c) proved completely resistant.

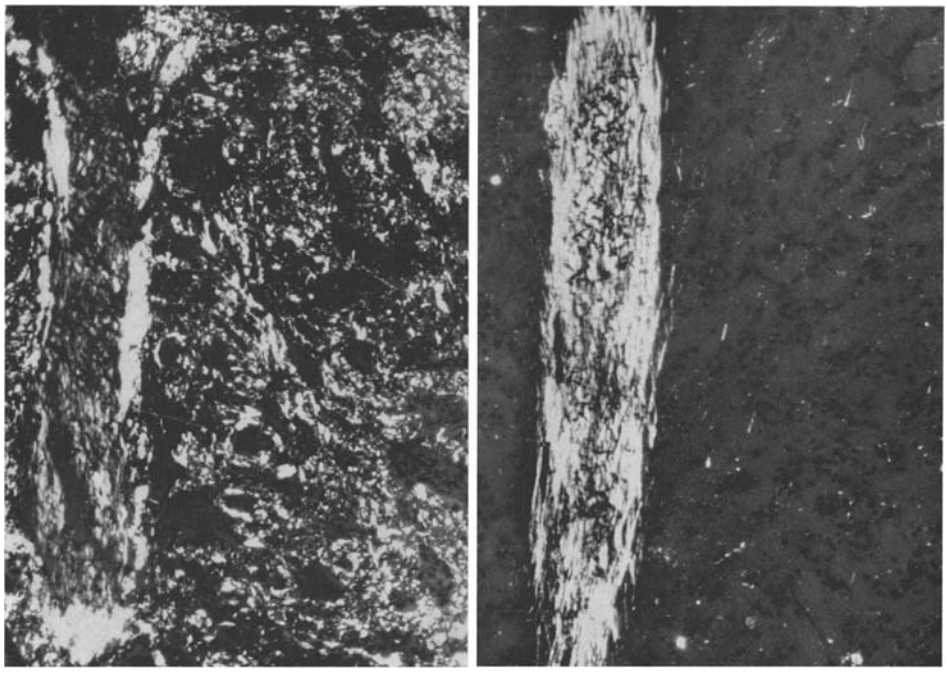

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Fig. 4a and b. Diffuse secondary amyloidosis of the splenic pulpa. a, Control; b, $KMnO_4$-trypsin, 6 hrs. Haematoxylin-Congo red, Canada balsam, *X* Polars. Complete dissolution of amyloid is seen in (b). Because of mounting in Canada balsam, the collagen fibers of the trabecule are strongly birefringent both in (a) and in (b). In (b) also the fine birefringent reticulin fibers can be recognized in the pulpa

4. Resistance of Induced Proteolysis of Myeloma Amyloid. As shown in Table 1, we had the possibility to investigate the sensitivity to induced proteolysis of myeloma amyloid in a case with massive intratubular amyloid deposits in the kidney, causing death of the patient by tubular obstruction and renal failure. As shown in Fig. 14, myeloma amyloid proved resistant to induced proteolysis.

5. Resistance to Induced Proteolysis of Local and Senile Amyloid Deposits. a) On testing the sensitivity to induced proteolysis of the massive amyloid deposits in the stroma of a case of medullary thyroid carcinoma and in one case of amyloid tumour of the skin, we found resistance of the amyloid masses in both cases (Fig. 15).

b) Deposits of senile amyloid in the aorta proved completely resistant to induced proteolytic degradation in all cases examined. The difference in sensitivity of deposits of generalized amyloidosis and of senile amyloid to induced proteolysis is demonstrated in a section of the aorta from a case with generalized secondary amyloidosis (Fig. 16). Senile amyloid deposits in other organs: as congophil angiopathies of cerebromeningeal vessels, senile cardiac amyloid, senile plaques in the cerebral cortex, and pancreatic islets of diabetics and aged persons, proved similarly resistant to induced proteolysis.

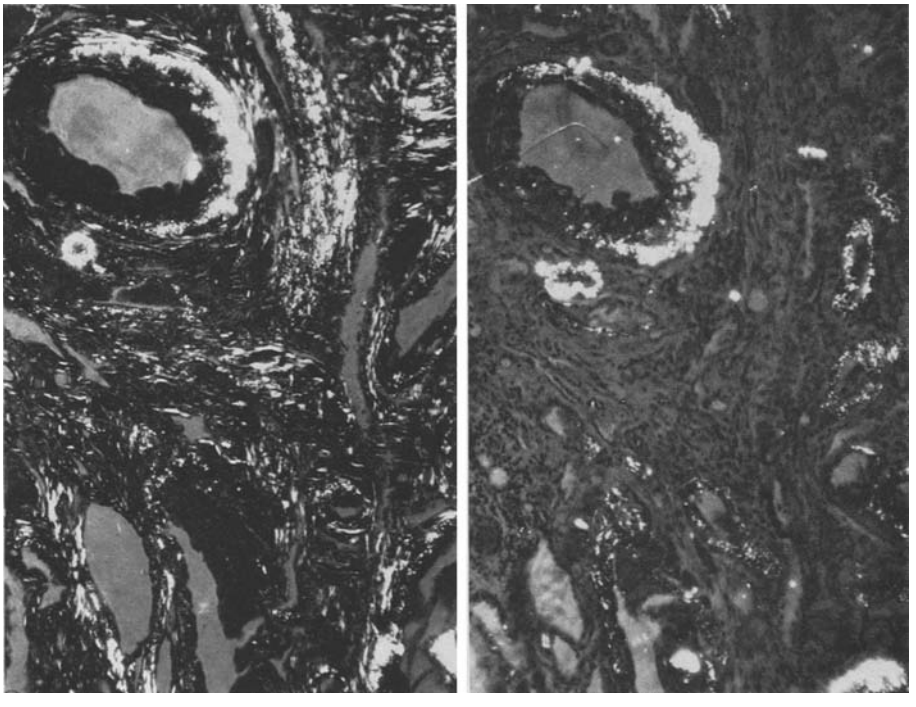

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Fig. 5a and b. Vascular and perivascular amyloid deposits in the periadrenal tissues from a case of generalized secondary amyloidosis. a, Acetylated, b, acetylated and incubated in trypsin for 20 hrs. Congo red, *X* polars, mounted in gum arabic. In (a) both the amyloid masses and the collagen fibers are strongly birefringent/Congo red-stained acetyl collagen fibers are known to show strong negative birefringence (Romhányi *et al.*, 1972), in (b) there is selective collagenolysis of acetyl collagen; however, the birefringent amyloid masses are well preserved

Discussion

Amyloid substance of different origin is known to be highly resistant to enzymatic degradation. As mentioned in the introduction, that resistance has been confirmed by many investigators using different enzymes (trypsin, pepsin, pronase, papain, collagenase) under different experimental conditions including prolonged autolysis (Dreher and Vandré, 1972) and the digestion process in the gastrointestinal tract (Missmahl, 1950). This resistance is due to the fibrillary element—the essential component of amyloid in the electron microscope. The fibril is also the substrate of Congo red birefringence (which is dependent upon the physical integrity of the fibril) (Glenner *et al.*, 1968); it represents the most reliable histomorphological criterion for amyloid (Cohen, 1967).

It is to be noted that, as yet, no satisfactory explanation of the resistance to proteolysis of the amyloid substance—as an essentially proteinaceous or glyco-

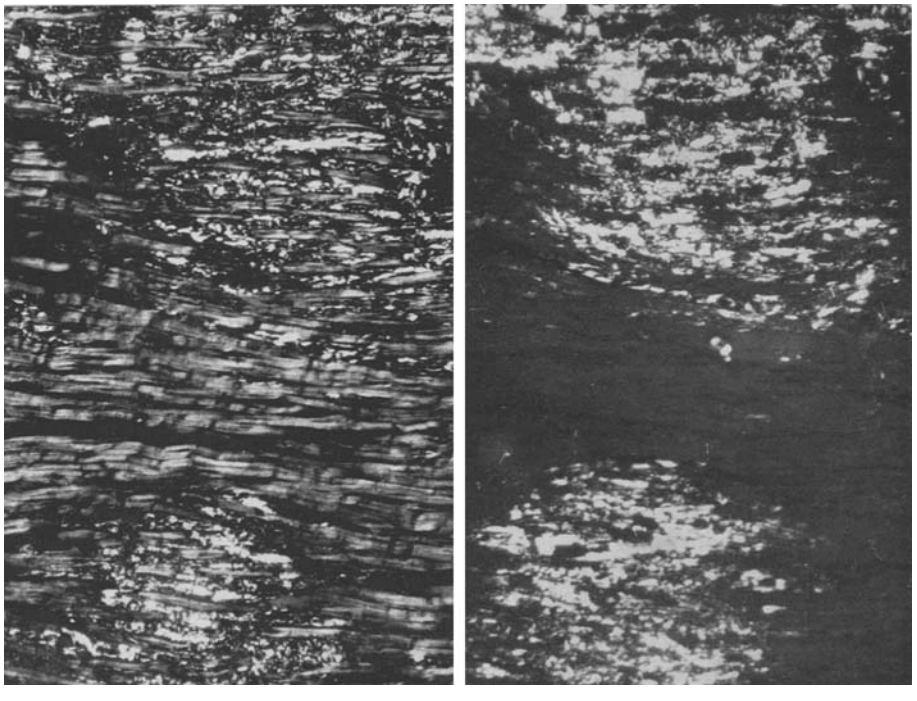

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Fig. 6a and b. Myocardium from a case (No. 14) of generalized primary amyloidosis (a) control, (b) after $KMnO_4$-trypsin, incubated 20 hrs. Congo red, gum arabic, *X* polars. In (a) amyloid masses are seen between the birefringent muscle fibers, in (b) the enzyme resistant amyloid masses are present and the muscle fibers are dissolved. Collagen fibers are nearly isotropic

proteinaceous substance—has been found. Recently Shirahama and Cohen (1971) have reported on the lysosomal dissolution of human amyloid fibrils by mouse peritoneal macrophages. They assumed that the intracellular degradation of amyloid fibrils resulted from the continuous effect of several enzymatic components. Such an intracellular enzymatic degradation of amyloid must however be considered very insufficient in the organism. The resistance of amyloid to enzymatic degradation has apparently not been used as a diagnostic tool in the histochemistry of amyloid (Pearse, 1960).

Our findings indicate that the primary resistance of amyloid to enzymatic degradation by proteolytic enzymes can be abolished by mild preoxidation with potassium permanganate, inducing a rapid proteolytic dissolution by trypsin of certain types of the amyloid substance but not of others.

From the pathomorphological point of view it is of significance that the difference in sensitivity to induced proteolysis of the amyloid substances is not

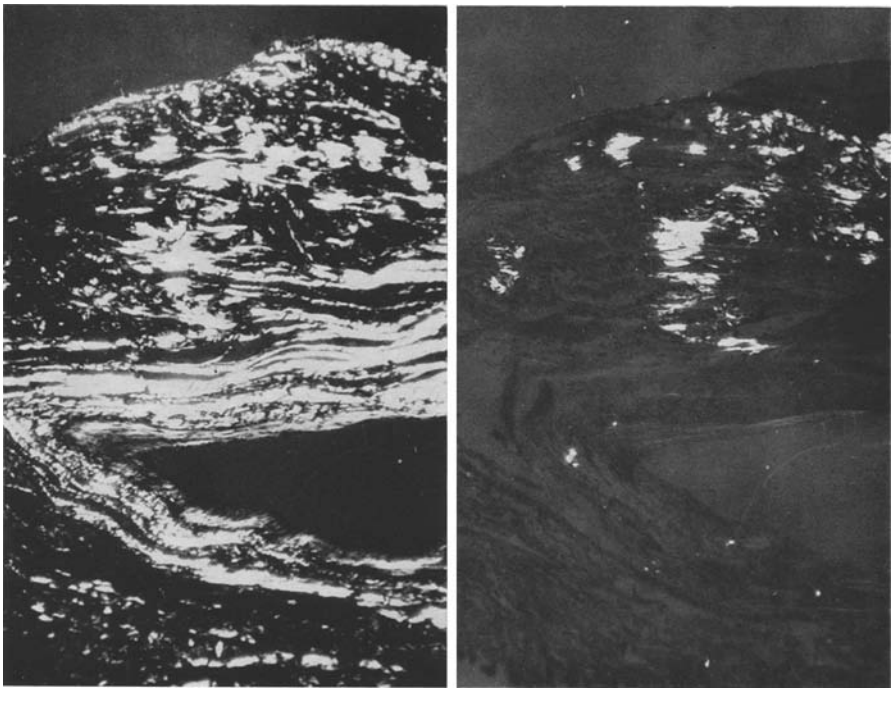

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Fig. 7a and b. Mitral valve from a case (No. 12) of primary generalized amyloidosis. a, Control, stained with Congo red and mounted in Canada balsam; b, the same field in the next section, which was acetylated an then treated with potassium permanganate and incubated in trypsin for 20 hrs. Congo red, Canada balsam. In (a) the birefringent amyloid masses are quite overshadowed by the strongly birefringent Congo red stained collagen fibers. In (b) there is selective proteolysis of acetyl collagen, only the birefringent amyloid masses, resistant to induced proteolysis, are seen

a chance phenomenon but is associated with well-defined forms of amyloidosis as well as with some types of local and senile amyloid deposits.

We found that amyloid deposits of *generalized secondary* amyloidosis, in all cases and in all organ localisations, irrespective of their location in collagen, basement membrane or on reticular fiber-systems, were invariably sensitive to induced proteolysis.

By contrast, all organ deposits of the three cases of *generalized primary* amyloidosis, independent of their localisation in collagen, basement membranes, or reticular fibers proved resistant to induced proteolysis. Similarly the ultrastructural stability of both types of amyloid was not disturbed by selective proteolysis in acetylated sections of the underlying connective tissue structures (such as collagen, reticular fibers and basement membranes) (Fig. 5). Thus in our material we did not find evidence for significant distinction between peri-

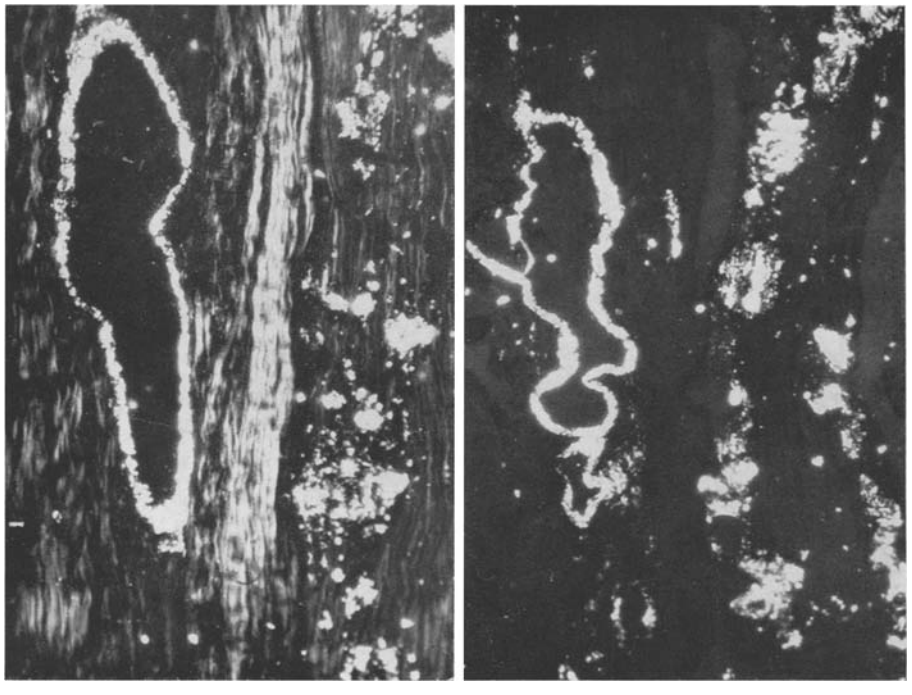

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Fig. 8a and b. Peripheral nerve from a case (No. 13) of primary generalized amyloidosis. Congo red, Canada balsam, *X* polars. a, Control; b, acetylated, then treated with $KMnO_4$ and incubated in trypsin for 20 hrs. In (a) birefringent amyloid masses are seen in the vascular wall and within the nerve fibers as spherites. The collagen structures are birefringent. In (b) there is selective collagenolysis, the amyloid masses, resistant to induced proteolysis, are well preserved in the vascular wall and within the nerve

collagen and perireticulin types or for localizations of amyloid as suggested by Heller *et al.* (1964).

Furthermore, we found resistant-type amyloid in a myeloma kidney with renal failure, in the stroma of a case of medullary carcinoma of the thyroid,—a recently recognized type of local amyloid (Hazard *et al.*, 1959; Müller, 1969; Lietz and Donath, 1970) and in one case of amyloid tumour of the skin, as well as in all types of senile amyloid deposits such as in the aorta, the heart, in the congophil angiopathies of the meningocerebral vessels, in the senile plaques the cerebral cortex and in the pancreatic islets of diabetics.

The difference between the sensitive and the resistant type of amyloid proved to be definite, since under the experimental conditions used the deposits in generalized secondary amyloidosis were completely dissolved in about 4–6 hrs of digestion, whereas the amyloid substances of the second group firmly resisted enzymatic degradation even under prolonged enzyme treatments. Thus we may

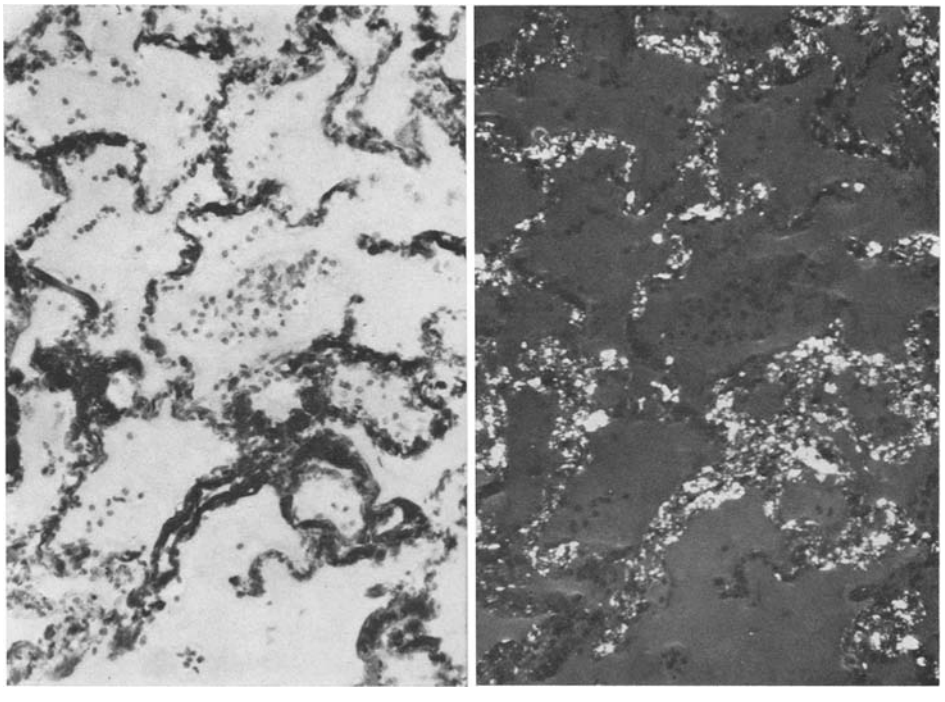

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Fig. 9a and b. Lung, primary generalized amyloidosis (No. 13). Pretreated with potassium permanganate and then incubated in trypsin for 20 hrs. Haematoxylin-Congo red-gum arabic. a, In the light microscope; b, the same between crossed polaroids. The strongly birefringent widespread alveolar septal amyloid deposits—characteristic of primary generalized amyloidosis (Rajan and Kikkawa, 1970)—are resistant to induced proteolysis

conclude that the sensitivity and resistance of different amyloids to induced proteolysis can be considered indicative of essential differences in their molecular ultrastructural organization. These differences are the more remarkable since they are not detectable by any available morphological method, including electron microscopy. Thus we can conclude that although the amyloid fibrils show the same appearance in the electron microscope in different forms of human cases and even in experimental and spontaneous amyloid of different species (Cohen and Calkins, 1959; Cohen, 1959, 1966, 1967; Caesar, 1961; Bladen *et al.*, 1966; Husband and Lannigen, 1968; Rajan and Kikkawa, 1970; Zschiesche and Fritsch, 1970) they have different ultrastructural organisation at the molecular level not apparent at the electron microscopic level, a possibility suggested by Cohen (1967).

So far we have found no evidence to suggest that with time the sensitive type of amyloid becomes transformed into the resistant type, in other words, no sensitive form precedes the resistant stage. Even the smallest deposits of primary

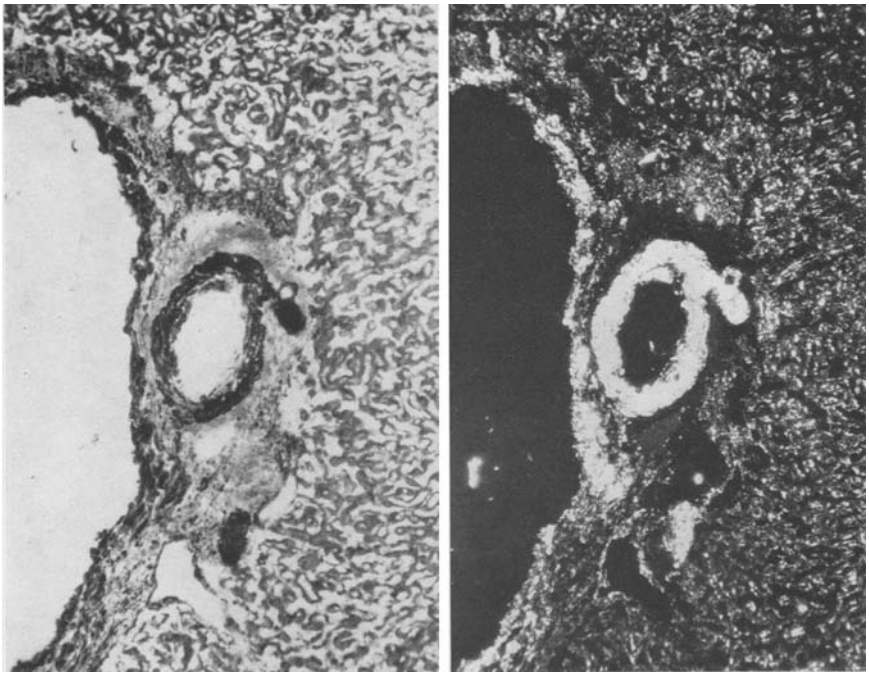

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Fig. 10. Liver, primary generalized amyloidosis (case 13). $KMnO_4$-trypsin incubation for 48 hrs. Congo red, gum arabic. There are heavy, widespread amyloid deposits along the sinusoidal walls and in the vascular walls of the periportal area. Amyloid deposits in the sinusoidal walls as well as in the vascular tissues are firmly resistant to induced proteolysis

generalized amyloidosis resisted induced proteolysis (Figs. 8–11, 13c). In fact, there is a possibility for the coincidence of senile amyloid deposits (relatively frequent in aged patients, Schwartz, 1967), with generalized secondary or primary amyloidosis in elderly patients (Fig. 15). However such findings are easily recognized and can not interpreted as transition between the two types of amyloid. When, on the basis of their resistance to induced proteolysis, we group together the amyloid substances of primary generalized amyloidosis, myeloma amyloid, and the local and senile amyloid deposits of our material, that should probably not imply any pathogenetic relation between them or an ultrastructural identity of them. Senile amyloids, grouped together by Symmers (1956) with primary generalized amyloidosis, may probably be regarded as a distinct heterogeneous group, although chemical and histochemical or ultrastructural criteria (Porta *et al.*, 1962; Schlote, 1965; Pauli *et al.*, 1971) are at present lacking to allow further definite differentiation between these groups of amyloid.

If the distribution of the amyloids, sensitive and resistant, to induced proteolysis is compared to that of the two types of amyloid as distinguished by Benditt

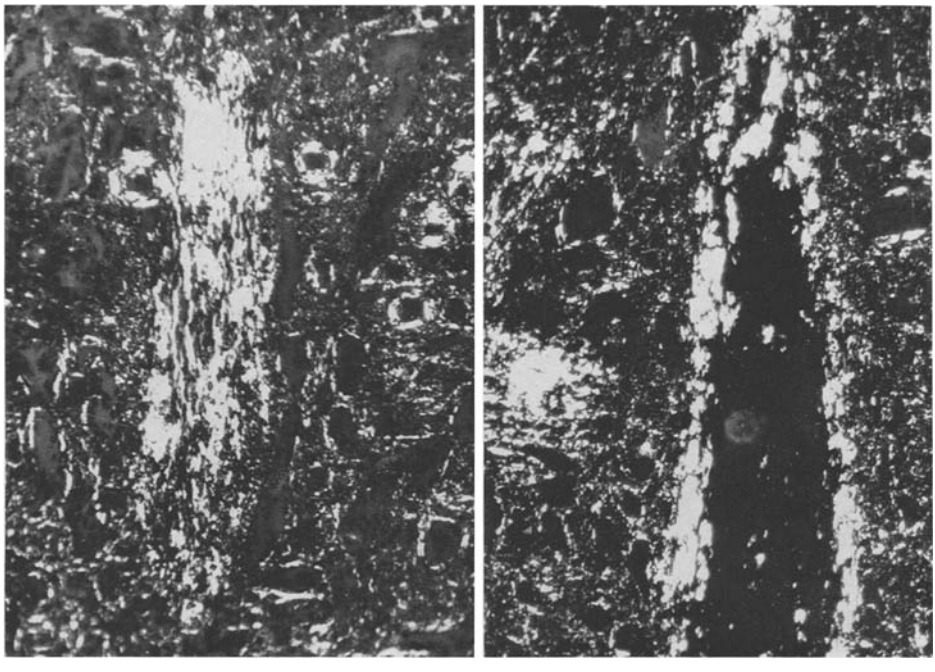

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Fig. 11 a and b. Diffuse amyloidosis of the splenic pulpa from a case (No. 13) of primary generalized amyloidosis. Both (a) and (b) pretreated with $KMnO_4$ and then incubated in trypsin for 20 hrs. Congo red, (a) mounted in Canada balsam, (b) mounted in gum arabic. The perireticular amyloid masses in the red pulpa proved completely resistant to induced proteolysis (see Fig. 4). In (a) the trabecular collagen is strongly positively birefringent, in (b) the trabecular collagen is isotropic due to its inversive topo-optical staining reaction with Congo red in gum arabic and therefore amyloid deposits at the periphery of trabecula are clearly visible

and Eriksen (1971) then a fairly good correspondance can be recognized between our sensitive type of amyloid and the type A amyloid of Benditt and Eriksen on the one hand, and the resistant amyloid in our experiments and type B amyloid of Benditt and Eriksen on the other. These authors have found type A amyloid in their 5 samples, obtained from secondary generalized amyloidosis. In our material all secondary amyloid proved sensitive to induced proteolysis. In the material of Benditt and Eriksen type B amyloid was established a) in a case of primary cardio-vascular amyloidosis, b) in a case of medullary carcinoma with massive amyloid deposits in the stroma, c) in a case of myeloma-amyloid and d) in two cases of amyloid with other malignancies.

Similarly, in our material we found amyloid resistant to induced proteolysis a) in 3 cases of primary generalized cardiovascular amyloidosis, b) in one case of medullary-thyroid carcinoma with massive amyloid deposits, c) in one case of

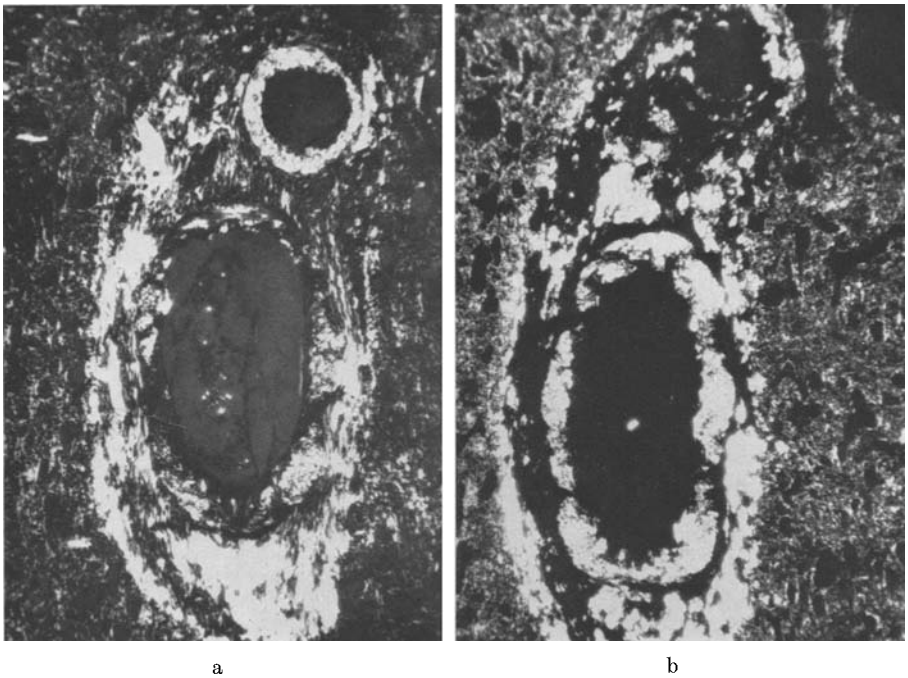

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Fig. 12a and b. Splenic trabecular area from the same case as Fig. 11. a, Control: acetylated and treated with $KMnO_4$; b, the same pretreatment as in (a) and incubated in trypsin 20 hrs, both stained with Congo red and mounted in gum arabic, in which acetyl collagen fibers are negative birefringent. In (a) the strongly birefringent collagen fibres and amyloid masses are not clearly delineated. In (b) there is selective collagenolysis and birefringent amyloid masses, resistant to induced proteolysis, are seen in the trabecular tissue, in the vascular wall and in the pulpa reticulum

myeloma amyloid, d) in one case of local amyloid tumor of the skin, and d) in all senile amyloid-deposits. As may be noted, a fairly good agreement exists between the distribution of the amyloid types A and B of Benditt and Eriksen and of our sensitive and resistant types of amyloid. However the final identification of sensitive and resistant amyloids with the types A and B of Benditt and Eriksen must await integrated morphological and histochemical investigations on the amyloid substance in the same case. Therefore we use the term: sensitive and resistant amyloid by which sensitivity or resistance to induced proteolysis as a histochemical characteristic is meant.

The question now arises how the difference in sensitivity to induced proteolysis of amyloid substances can be explained in molecular structural terms, and whether that difference can be correlated with the chemical structural data as reported for types A and B amyloid by Benditt and Eriksen. In view of the resistance of primary amyloids to induced proteolysis an earlier statement of Benditt and Eriksen (1964) may be of interest viz. that primary amyloid proved

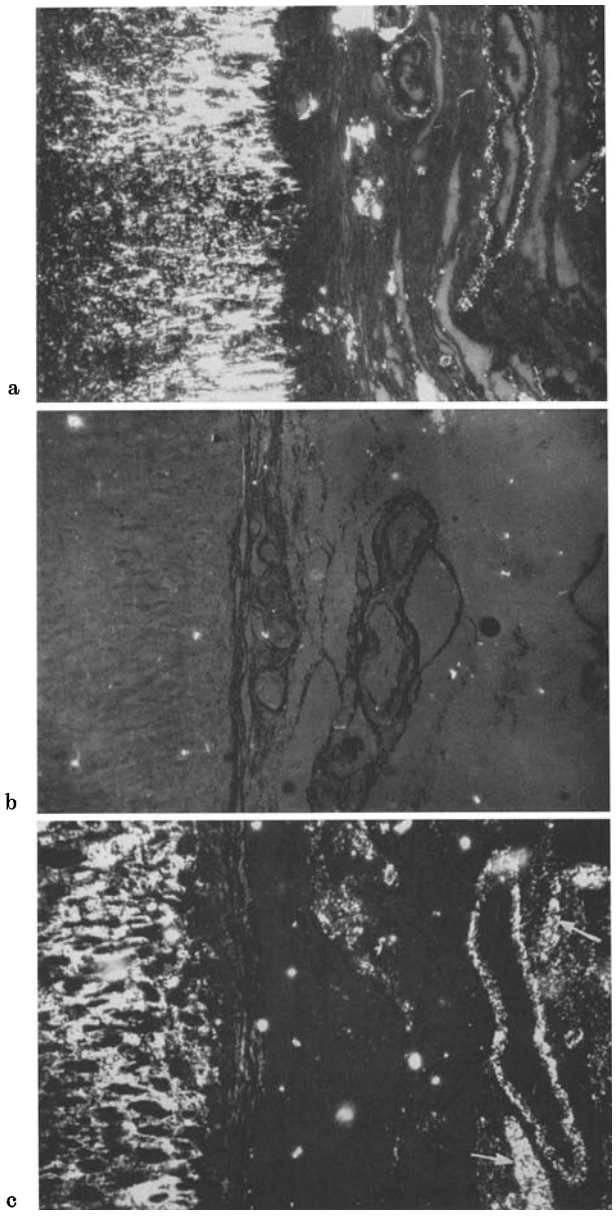

Fig. 13a—c. Adrenal cortex, a and b, From a case of generalized secondary amyloidosis. c, From a case (No. 12) of generalized primary amyloidosis. Congo red, gum arabic, *X* polars. a, Massive amyloid deposits are seen in the adrenal cortical substance as well as in the vascular walls in the capsule. b, $KMnO_4$-trypsin treatment for 6 hrs. Complete dissolution of amyloid in the cortical capillaries and the capsular vessels is seen. c, Adrenal cortex from a case (No. 12) of primary generalized amyloidosis, $KMnO_4$-pretreatment followed by trypsin incubation for 20 hrs. Amyloid deposits in this case proved completely resistant to induced proteolysis. Note the birefringent areas at the arrows which correspond at higher magnification to ringlike amyloid deposits around fat cell membranes, a characteristic feature of primary amyloidosis (Symmers, 1956)

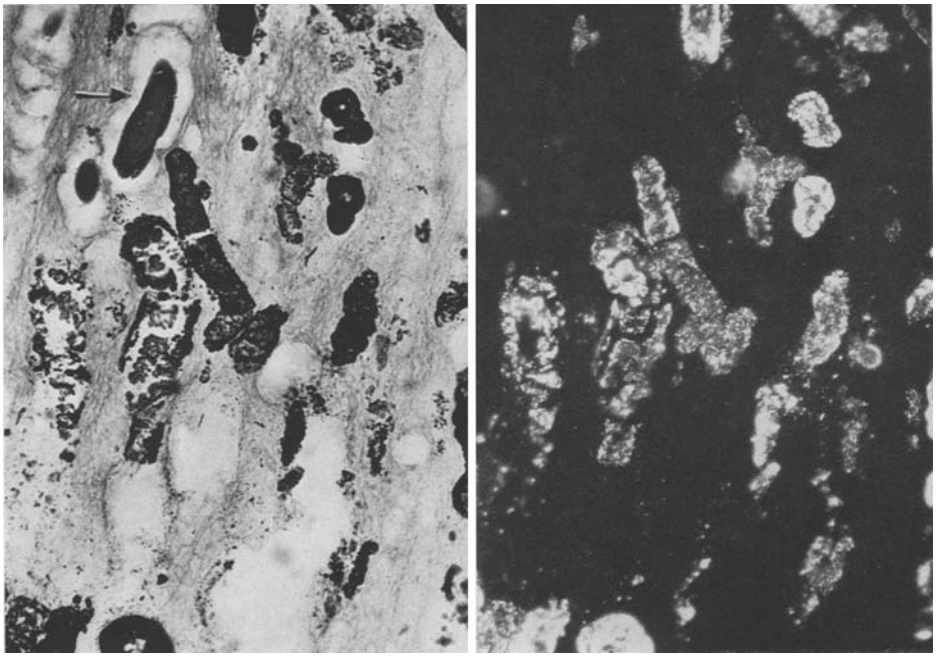

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Fig. 14a and b. Myeloma kidney. $KMnO_4$-trypsin incubation 18 hrs. Congo red, gum arabic; a, in the light microscope; b, with polaroids crossed. Numerous cylindrical birefringent amyloid masses, displaying characteristic spheritical structures on cross sections (Schubert *et al.*, 1972) replacing and obstructing renal tubuli and resistant to induced proteolysis are seen. The arrow points to an amorphous congophil cast

much more difficult to dissolve in urea than secondary amyloid. We may assume that the sensitive amyloid undergoes changes at some strategic points of its structure as a result of potassium permanganate pretreatment, which renders it accessible to the attack by the proteolytic enzyme, whereas the resistant type of amyloid remains unaffected by the same pretreatment.

Potassium permanganate is not infrequently applied in histotechnique as silver impregnation and other staining techniques and recently it has been introduced also as a stain and fixative for electron microscopy (Luft, 1965). Data in the literature indicate (Hake, 1965; Hopwood, 1969), that oxydation of reactive side-groups, cleavage of disulfide bridges, even of peptide bonds, may result from oxydation with potassium permanganate. Furthermore, carbohydrate units possibly built into the glycoprotein framework of amyloid may also be considered as the a possible target of the potassium permanganate effect.

Regardless of the unclarified molecular mechanism of the different sensitivity to induced proteolysis of amyloids, the clear-cut histochemical difference between the sensitive and resistant amyloids provides a new line of approach to characterize and differentiate amyloids in histopathology.

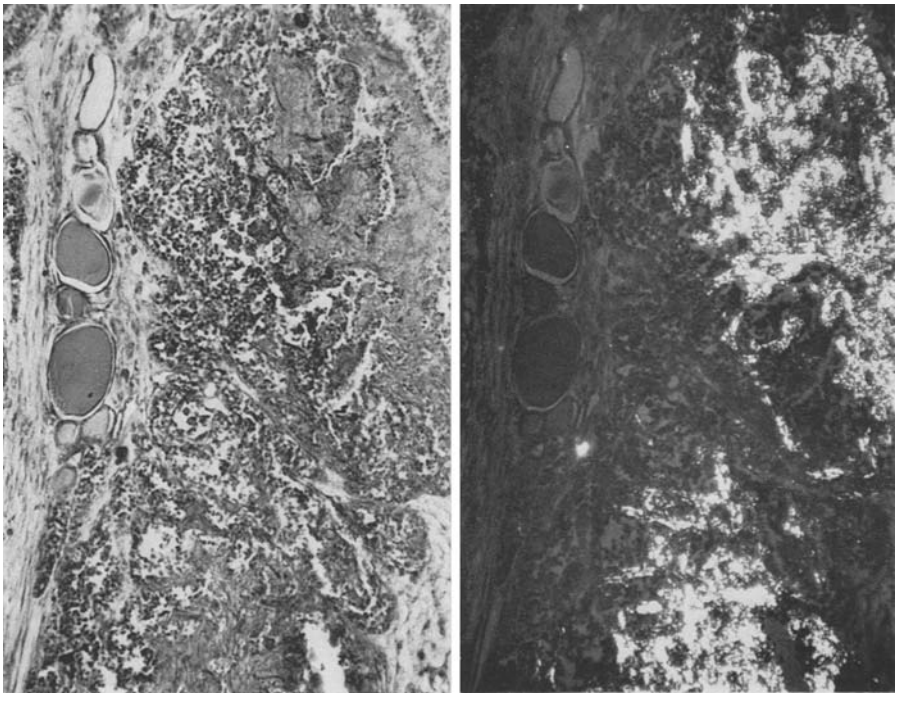

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Fig. 15a and b. Medullary carcinoma of the thyroid. $KMnO_4$-pretreatment followed by trypsin incubation 18 hrs. Haematoxylin. Congo red, gum arabic; a, in the light microscope; b, the same field with polaroids crossed, in (b) the birefringent amyloid masses in the stroma, resistant to induced proteolysis, are seen

Our material is relatively limited, especially as regards the three cases of primary generalized amyloidosis (relatively infrequent forms of amyloidosis, Symmers, 1956) as well as the local forms of amyloid. Therefore further investigations are needed along these lines on more material and also on special forms of primary and local amyloidosis. However, the present findings support the assumption that the resistance of amyloid to induced proteolysis can be taken as a distinctive histochemical characteristic of primary amyloid (and of senile amyloids); all secondary amyloids being of the sensitive type. That can be considered as important since until now no histomorphological distinctive features of primary generalized amyloidosis of local and senile amyloids were known.

We suggest that the testing of sensitivity to induced proteolysis may be of value in establishing the secondary or primary nature of generalized amyloidosis even in cases where apparently no associated disease was found and/or no classical organ distribution of amyloid deposits could be established. Also in biopsy material, the testing of sensitivity to induced proteolysis can be useful in clarifying the primary or secondary nature of the disease as in one of our cases (No. 13) which was then confirmed at post mortem.

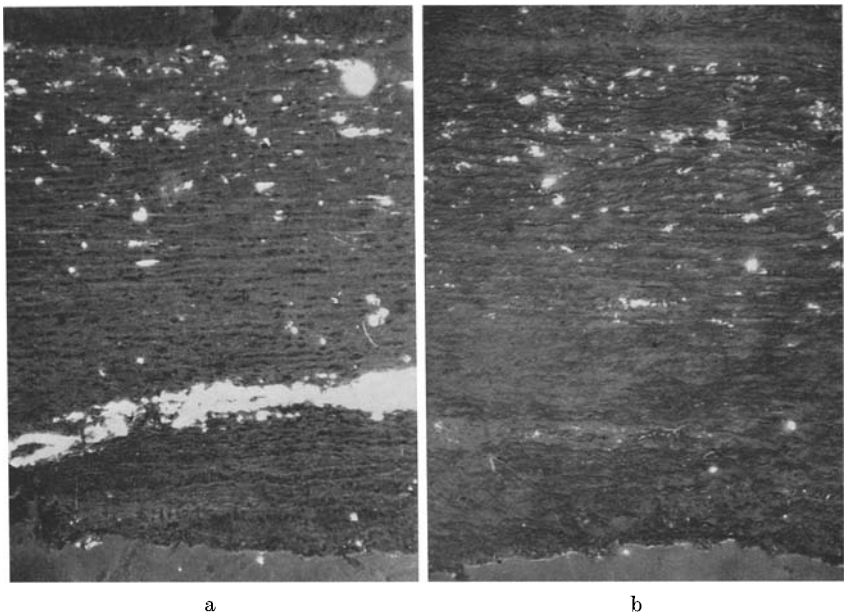

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Fig. 16a and b. Aorta from a case of generalized secondary amyloidosis (case No. 5), showing coexistence of deposits of secondary amyloidosis and of senile amyloid. a, Control; b, incubated in trypsin following a pretreatment with potassium permanganate. Congo red-gum arabic, *X* polars. In (a) the perivascular amyloid deposits of generalized secondary amyloidosis are seen in the outer zone of the aortic media, in the mid- and inner zone of the media the small birefringent spots correspond to senile amyloid. In (b) deposits of generalized amyloid are dissolved, however, the senile amyloid deposits are resistant to induced proteolysis

A new possibility arising from the proteolytic degradation of amyloid deposits for histomorphology (and electron microscopy) is worth mentioning. After the proteolytic removal of massive amyloid deposits from tissues, a new insight is possible into the morphology of the tissues into which amyloid masses have been precipitated, such as basement membranes, reticulin and collagen fibers. An example of that was shown in Fig. 2.

In connection with the induced proteolytic sensitivity or resistance of different amyloids in human pathology, the question of such sensitivity of different experimental or spontaneous animal amyloids appears to be of interest. According to the findings in our laboratory by Horváth [22] casein-induced murine amyloid deposits proved completely resistant to induced proteolysis.

Note Added in Proof. After submitting the manuscript for publication we observed at autopsy (435/72) another case of primary cardiovascular amyloidosis in a 73-year old man who had complained of general weakness, faintings and loss of weight for seven months. On admission, two weeks before death, his blood pressure was between 100/50 and 40/0, which could not be restored by noradrenalin infusions. At autopsy the heart weighed 600 gr, the myocardium was highly transparent and turned black in Lugol solution. Light microscopic study revelaed masses of amyloid deposits replacing the myocardial fibers, which could be recognized only sparsely in the amyloid masses.

Amyloid deposits were present in many organs, which proved resistant to induced proteolysis in all organ localisations.

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