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Selective Differentiation between Amyloid and Connective Tissue Structures Based on the Collagen Specific Topo-optical Staining Reaction with Congo Red

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Summary. There are some difficulties inherent in the identification of amyloid by Congo red anisotropy in the polarisation microscope, mainly because of the possibility of a non-specific reaction of collageneous structures.

The newly recognized collagen-specific Congo red topooptical staining reaction provides a theoretically well-founded possibility for differentiating between amyloid and collagen. In Canada balsam, or other apolar mounting media the Congo red anisotropy of amyloid as well as that of collagen are of the same (additive) optical sign. In gum arabic however they are of opposite signs, additive for amyloid and inversive for collagen. That difference enables a definite differentiation between amyloid and collagen, since in gum arabic Congo red stained amyloid is positively birefringent, and collagen is isotropic or weakly negatively birefringent.

Pretreatment of the tissue sections with proteolytic enzymes resulted in a decrease in the general background staining of the tissues as well as in an increase of the inversive Congo red staining reaction of collagen and of the additive reaction of amyloid, providing thereby the possibility of differentiating sensitively and selectively between amyloid and collagen by Congo red anisotropy.

Enzymatic removal of the elastic fibers prior to Congo red staining facilitated the exact localisation of senile amyloid deposits in the aortic wall. The initial amyloid deposits were visualized in the smooth muscle cells of the aortic wall. This finding may lead to a new approach to the pathogenesis of senile aortic amyloid.

Zusammenfassung. Es bestehen Schwierigkeiten in der selektiven Differenzierung zwischen Amyloid und Bindegewebsstrukturen durch Kongorot-anisotropie, hauptsächlich wegen der Möglichkeit einer nicht spezifischen Mitreaktion von kollagenen Strukturen.

Die unlängst erkannte kollagenspezifische topooptische Kongorotreaktion ermöglicht eine theoretisch gut begründete Differenzierung von Amyloid und Kollagen.

In Canada Balsam, oder in anderen apolaren Einschlußmedien, ist die Kongorot-Anisotropie von Amyloid und Kollagen desselben optischen (additiven) Characters, wogegen in Gummi arabicum diese vom entgegengesetzten Charakter ist: *additiv* am Amyloid und *inversiv* am Kollagen. Dieses optische Verhalten ermöglicht eine klare Differenzierung zwischen Amyloid und Kollagen: da das kongorotgefärbte Amyloid in Gummi arabicum sich als positiv doppelbrechend erweist, kongorotgefärbtes Kollagen dagegen optisch isotrop oder schwach negativ doppelbrechend ist.

Nach Vorbehandlung der Schnitte mit proteolytischen Fermenten (Elastase, Trypsin) ist bei nachträglicher Kongorotfärbung, nebst Herabsetzung der diffusen Färbung des Gewebshintergrundes, sowohl die additive topooptische Reaktion des Amyloids, wie die inversive Kongorotreaktion des Kollagens erhöht. Dadurch ergibt sich ein hochempfindliches und selektives Darstellungsverfahren für Amyloid.

Die enzymatische Herauslösung der elastischen Fasern aus Schnitten der Aortenwand verbesserte bedeutend die Erkennung und Lokalization der Ablagerungen des senilen Amyloids

durch Kongorotanisotropie. Es konnte so erkannt werden, daß die initialen Ablagerungen des senilen Aortenamyloids intracytoplasmatisch in den glatten Muskelzellen der Gefäßwand auftreten. Diese Befunde können zur neuen Richtung in der Erforschung der Pathogenese des senilen Aortenamyloids führen.

The histopathological identification of amyloid, even in the case of micro-deposits, has become an essential requirement since clinical practice begann to rely-instead of on the in vivo dye-dilution tests (Bennhold, 1922; Jarnum, 1960) on the histological demonstration of amyloid deposits in biopsy materials from different organs for the diagnosis of general amyloidosis.

It is generally agreed that for the histological demonstration of amyloid, Congo red birefringence (Divry *et al.*, 1927; Romhányi, 1943, 1949; Ladewig, 1945) and thioflavine fluorescence (Vassar and Culling, 1959; Burns *et al.*, 1967; Schwartz *et al.*, 1964) are most reliable. Both methods are of great diagnostic significance, however, they should be used with criticism in view of the possibility of non-specific reactions of some tissue elements.

In inhibition tests we have demonstrated (1943, 1949) that unstained amyloid has positive form birefringence, and Congo red staining induces strong birefringence of amyloid with a positive optical character (with respect to the slow axis of retardation of unstained amyloid), which indicates that the linear dye molecules are arranged in parallel on amyloid and in turn suggests that amyloid, previously considered amorphous, has a submicroscopic micellar structure, which without detailed optical analysis was assumed also by Divry *et al.* (1927). This conclusion, however, was questioned by Missmahl and Hartwig (1953), and Missmahl (1955), who attributed the Congo red birefringence of amyloid to residual collagen and reticulin fibers included in it and not to the amyloid itself. However, using other topooptical staining reactions it could be definitely shown (Romhányi, 1956; Diezel and Pfleiderer, 1959) that it was not the masked collagen or reticular fibers but amyloid itself that was responsible for the Congo red birefringence and therefore it is not quite clear why Heller, Missmahl *et al.* (1964) referring to Missmahl and Hartwig (1953) claimed that they had predicted the micellar ultrastructure of amyloid on polarisation optical grounds. This was then established for human and experimental amyloid in the electron microscope by Cohen and Calkins in 1959, and Caesar in 1960, and soon confirmed by a number of investigators (Fruhling *et al.*, 1960; Battaglia, 1962; Gueft and Ghidoni, 1963; Manitz and Themann, 1963; Sorenson and Shimamura, 1964; Boeré *et al.*, 1965; Glenner *et al.*, 1968; Hirschl, 1969; Zucker-Franklin *et al.*, 1970).

Obviously it was the demonstration of the fibrillary ultrastructure of the amyloid substance in the electron microscope that had given universal credit to Congo red birefringence as optical evidence of the micellar ultrastructure of amyloid and as a most reliable method with great sensitivity for identifying it in histology (Cohen, 1967). However, only a clear understanding of the underlying molecular structural basis of the anisotropic staining reaction of amyloid and of other fibrillary textures can prevent a misinterpretation of the polarisation optical findings.

Thus the statement, often encountered in the literature, that green Congored birefringence is specific for amyloid seems to be an over-generalization, since this phenomenon is a function of both the thickness and the degree of orientation of the dye aggregates (Diezel and Pfleiderer, 1959; Wolman and Bubis, 1965) and furthermore other histological micellar components, specially dense hyalin collagen masses, can bind Congo red in an ordered pattern and appear in green polarization colour between crossed polaroids (DeLellis *et al.*, 1968; Klatskin,

1969; Reissenweber and Decavo, 1969; Cooper, 1969). Using the selective alkaline staining method of Puchtler *et al.* (1962) in studies of a great number of liver biopsies and of organs from autopsy material, Klatskin (1969) has more recently found that collagen shows green birefringence in a high percentage of specimens, in particular after Carnoy fixation. If such disturbing optical effects could not be eliminated or appropriately interpreted, serious limitation would be imposed on the usefulness of Congo red birefringence for the identification of amyloid in histopathology, specially in cases of microdeposits.

The present report deals with the selectivity problem of Congo red birefringence of amyloid and with the molecular structural basis of differentiating it from collagenous structures based on the newly recognized collagen-specific inversive topooptical Congo red staining reaction (Romhányi *et al.*, 1970). This provides a possibility of definite differentiation between amyloid and collagen even in the smallest deposits of amyloid, and is therefore of great value for histopathology being faced with such differential diagnostic tasks in everyday practice. In addition a useful way is described of a clear demonstration of senile aortic amyloid deposits as well as of microdeposits of general amyloid by proteolytic enzyme treatment prior to Congo red staining.

Materials and Methods

Our material comprised different organs from 15 autopsy cases with generalized secondary amyloidosis and 1 case of primary systemic amyloidosis, the deposits of senile aortic amyloid from a great number of cases and local amyloid deposits in pancreas islets and cerebromeningeal vessels. The organs were fixed in formol and, in several cases, for comparative purposes also in Carnoy's fluid for 24 hr.

1. The slices were stained with 1% Congo red for 10 minutes and, after rinsing in water, mounted in a) Canada balsam, b) in gum arabic, containing 1% fructose to inhibit cracking of gum arabic layer during drying. When gum arabic is acidic, it is rendered to about pH 7.0, adding 0.1 M sodium hydroxid. The gum arabic layer was allowed to dry on the slice since the optical effects to be described on the collagen, appeared only in the dry state of the mounting medium. If necessary, the dried layer was overlayered with Canada balsam and covered with cover glasses.

2. For a better differentiation between amyloid and elastic tissue in vascular walls, slices (coated with a 0.2% celloidinlayer) were pretreated with elastase for 2–4 hr (2 mg of Banga elastase 2 ml distilled water) and stained in the usual way and mounted in a) Canada balsam and b) in gum arabic. In addition: similar treatment was used with trypsin (Reanal, Bp.) (2 mg/ml M/15 phosphate buffer at pH 7.2).

The polarisation optical investigations were made with a Leitz Ortholux polarisation microscope equipped with rotating compensators. Concerning the characteristics and types of the topooptical reactions the reader is refered to our earlier publications (Romhányi and Deák, 1967; Romhányi, 1968).

Observation

Differentiation between Amyloid and Collagen Based on the Inversive Topooptical Congo Red Staining of Collagen

Fig. 1 demonstrates how the optical behaviour of collagen in the adrenal capsule varies with the mounting media used. In Canada balsam both amyloid and collagen show increased positive birefringence, and even the polarisation optical colour of collagen and of amyloid were similar in the original so that it appeared quite impossible to differentiate between amyloid and collagen or to

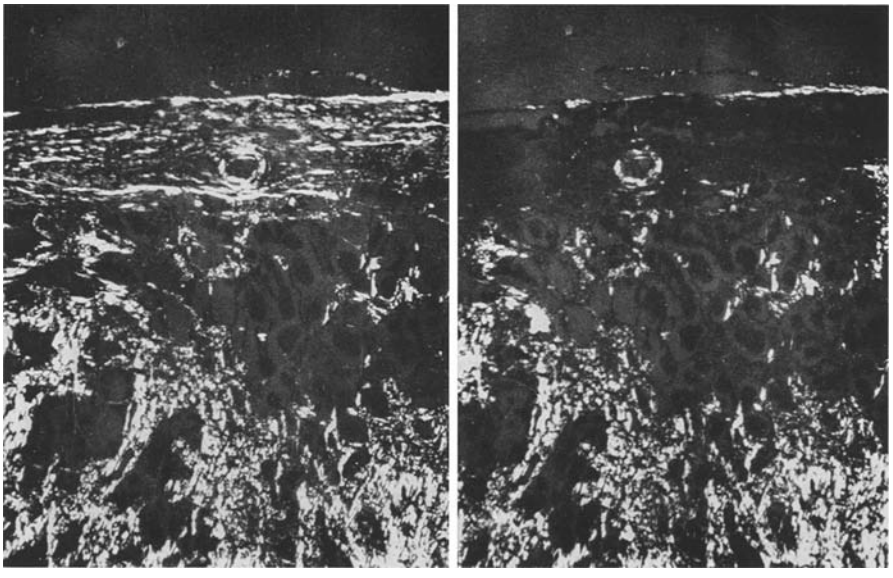

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Fig. 1a and b. Adrenal cortex with massive amyloid deposits. Congo red staining. Crossed polaroids. a) Mounted in Canada balsam, b) mounted in gum arabic. In a) there is a strong positive birefringence of collagen fibers of the capsule greatly interfering with a clear identification of amyloid deposits. In b) the collagen fibers appear isotropic because of the inversive nature of the Congo red staining reaction. Scattered amyloid deposits in the capsular connective tissue can be clearly made out

determine whether there were any amyloid deposits present in the adrenal capsule itself. However, by mounting the slice in gum arabic (Fig. 1b) the qualitative difference in optical behaviour between amyloid and collagen became obvious. The birefringence of the collagen fibers was depressed to isotropy as a result of their inversive anisotropic staining reaction with Congo red, amyloid retained unchanged its additive topootical Congo red staining reaction.

Fig. 2 shows the dependence on mounting media of the optical behaviour of collagen and amyloid in the tongue in a case of systemic primary amyloidosis. In Canada balsam (Fig. 2b) the intensive positive birefringence of the dense collagen fibers renders it difficult or even impossible to detect whether there are amyloid deposits present. However, when the slice was remounted in gum arabic (Fig. 2c) the collagen fibers appeared isotropic (black) because of the inversive character of the Congo red staining reaction, and thus even the smallest deposits of the positively birefringent amyloid, unrecognisable in Canada balsam, stood out clearly in the dense connective tissue and around the blood vessels.

The Effect of Fixation

After Carnoy-fixation, the Congo red binding of collagen fibers is increased, as compared with those fixed in formol, and accordingly their optical effects after Congo red staining are markedly increased in comparison to those fixed in formol

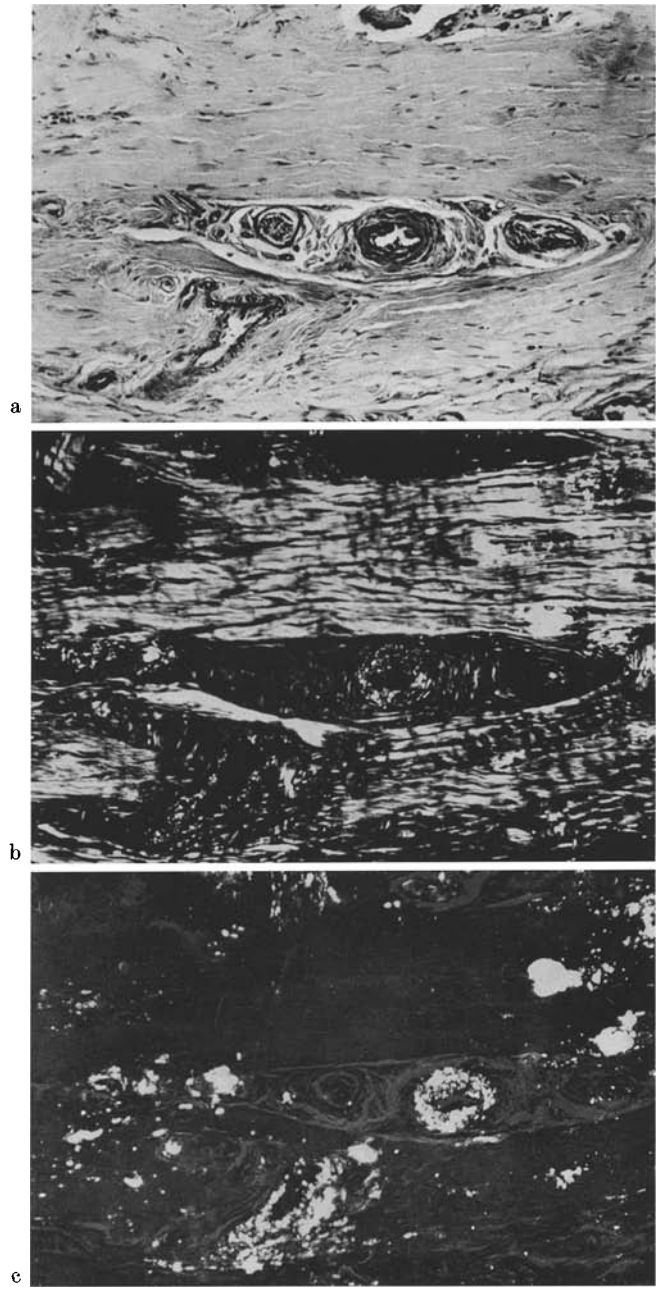

Fig. 2a–c. Slice from the tongue in a case of generalized primary amyloidosis. Congo red staining. a) In the light microscope; b) and c) the same field with crossed polaroids. b) Mounted in Canada balsam; c) mounted in gum arabic. In b) the strong positive birefringence of Congo red stained collagen fibers greatly inhibits the recognition of amyloid deposits. In c) collagen fibers are isotropic therefore the positively birefringent amyloid deposits stand out clearly

a b

Fig. 3a and b. Slice of human aorta with senile amyloid deposits. Congo red staining. Crossed polaroids. a) Mounted in Canada balsam, b) the same field in gum arabic. In a) the strong positive birefringence of the collagen fibers greatly interferes with the recognition of amyloid deposits. In b) the birefringence of collagen fibers in depressed to isotropy, and therefore the amyloid deposits are clearly recognized. Elastic fibers appear positively birefringent

(Romhányi *et al.*, 1970). Therefore in Canada balsam they showed, in addition to an intensive green polarisation colour, about twice as intensive positive birefringence as those fixed in formol and similarly intensive negative birefringence in gum arabic.

Effect of Pretreatment with Proteolytic Enzymes (Elastase, Trypsin)

Elastic fibers are known to give intensive staining with Congo red and to show moderate positive birefringence in red colour but not in green. Notwithstanding they may greatly interfere with the detection of amyloid deposits in vessel walls of the elastic type both in the light and in the polarisation microscope. Although the detection of senile aortic amyloid deposits is greatly facilitated by mounting of Congo red stained slices in gum arabic which depresses the birefringence of collagen fibers to isotropy, yet the birefringence of the Congo red stained elastic fibers continues to remain a disturbing factor in the identification and localisation of amyloid deposits in the aortic wall (Fig. 3).

We therefore, pretreated the slices of human aorta with elastase and, after staining with Congo red and mounting in gum arabic, investigated them for

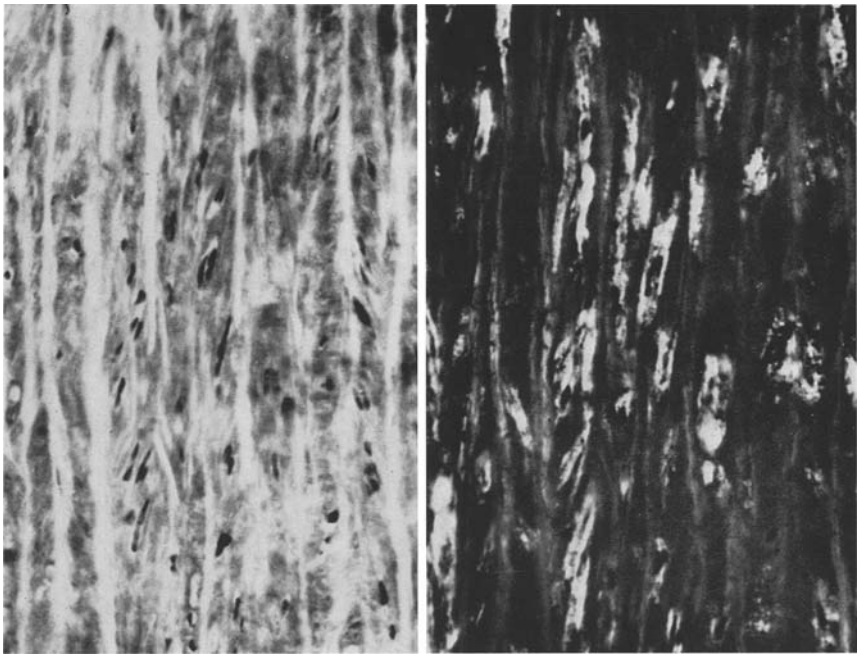

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Fig. 4a and b. Human aorta. Haematoxylin-Congo red staining, following pretreatment of the slice with elastase to remove elastic fibers. a) In the bright field microscope, b) the same field with the crossed polaroids, mounted in gum arabic. The selective localisation of amyloid deposits in the smooth muscle cells can be recognized

(senile) amyloid deposits in the polarisation microscope. Fig. 4 demonstrates the effect of such an approach: the amyloid deposits can be recognized more definitely and a clear picture emerges also of the intracellular localization of amyloid in the smooth muscle cells. Sections pretreated with proteolytic enzymes (elastase and trypsin) showed, in addition to a decrease in the background staining of the tissues an increase in the inversive anisotropic staining reaction of collagen and in the additive staining reaction of amyloid, which resulted in most clear cut optical differences between amyloid and collagen and basement membranes (Figs. 5, 6).

Discussion

Because of the possibility of a non-specific reaction of some birefringent histological elements, particularly of collageneous structures, there are some difficulties inherent in the specific identification of amyloid by Congo red birefringence. The practical interest in this question stems from the everyday task of identifying amyloid deposits in biopsy material (Beneke *et al.*, 1970) and is evidenced by a number of recent papers dealing with the evaluation of Congo red birefringence and attempting to establish a more definite basis for the recognition of amyloid deposits in tissues (Kelényi, 1967; DeLellis *et al.*, 1968; Mowry and Rodeubonh,

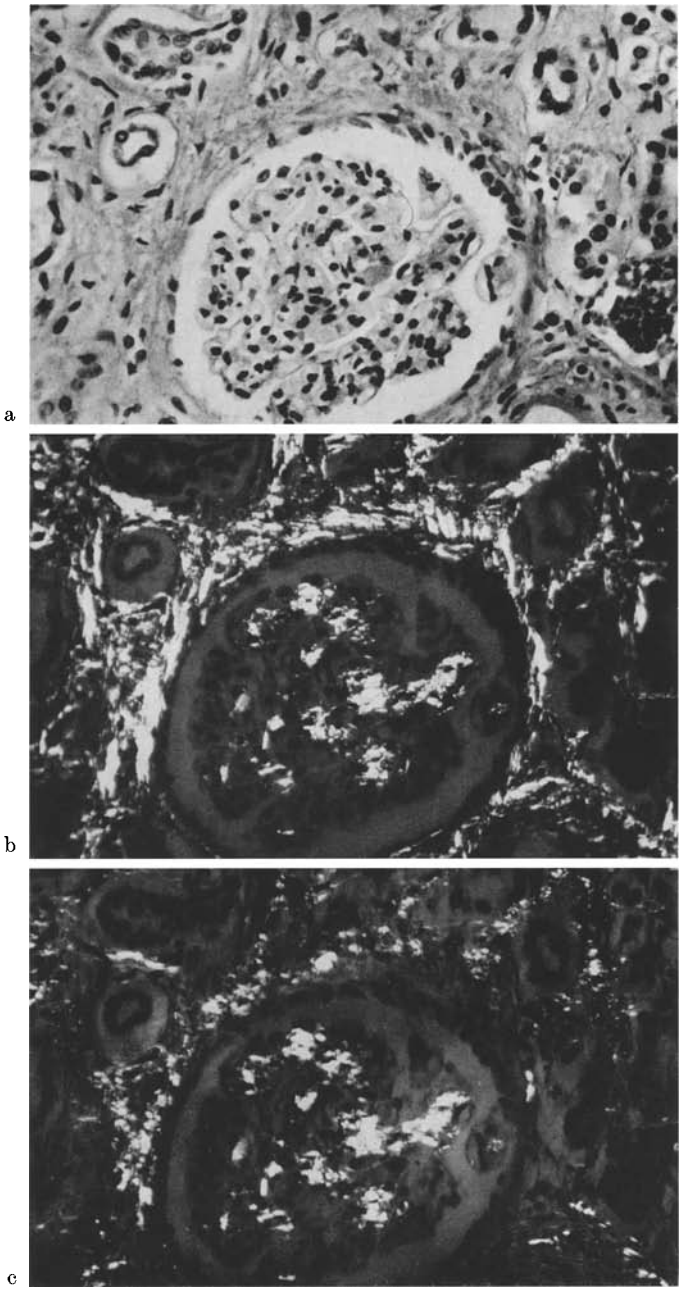

Fig. 5a–c. Amyloid deposits in the kidney. Haematoxylin-Congo red staining after pretreatment of the slice with trypsin, a) in the light microscope, b) mounted in Canada balsam, c) mounted in gum arabic. In b) the basement membranes as well as amyloid deposits are positively birefringent and therefore no differentiation between them is possible. In c) the Congo red stained basement membranes are isotropic because of their inversive Congo red reaction. All birefringent structures represent amyloid deposits

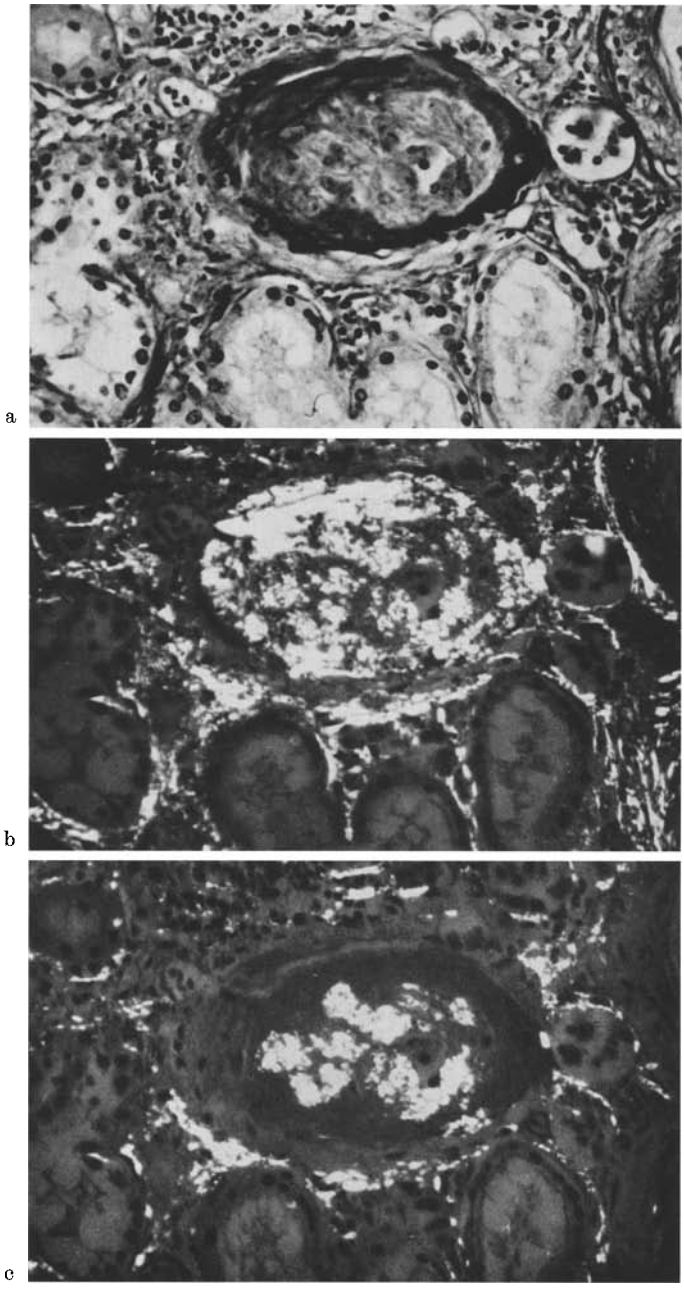

Fig. 6a–c. Amyloid deposits in a hyalinized glomerulus. Haematoxylin-Congo red staining after pretreatment of the slice with trypsin. a) In the light microscope, b) the same field mounted in Canada balsam c) mounted in gum arabic. In b) basement membranes, hyalin masses and amyloid deposits are strongly positively birefringent. In c) basement membranes and hyaline masses are isotropic. In this way the most selective differentiation and identification of the smallest amyloid deposits becomes possible

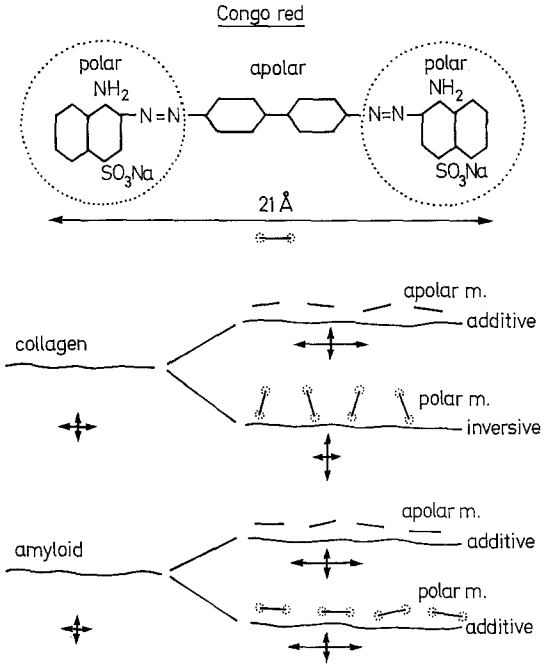

Fig. 7. Shematic drawing of the different topooptical staining reactions of collagen and amyloid depending on the mounting media. In Canada balsam Congo red molecules align on collagen in axiparallel orientation causing increased positive birefringence and green polarisation colour. In gum arabic the dye molecules are arranged transversally, as indicated by the inversive topooptical staining reaction. Amyloid binds Congo red molecules in axiparallel orientation in both polar and apolar mounting media

1964; Reissenweber *et al.*, 1969; Klatskin, 1969; Cooper, 1969; Beneke *et al.*, 1970).

DeLellis *et al.* (1968) pointed out that even when using the selective alkaline Congo red staining method of Puchtler *et al.*, (1962) for amyloid, dense collagen fibers may retain Congo red and appear in green polarisation colour and can therefore hardly be distinguished from amyloid. Attention has been called by Reissenweber and Decavo (1969) to the frequent appearance of green birefringence in Congo red stained collageneous structures, and we have already referred to similar observations by Klatskin (1969). Apparently these investigations could not throw new light upon this controversial issue.

In the present paper we have shown that the collagen specific, inversive topooptical staining reaction with Congo red, described in our previous paper (Romhányi *et al.*, 1970) provides a theoretically well founded new basis for definite differentiation between collagen and amyloid. At the same time it offers a possibility of explaining, on the molecular structural level, the non-specific green Congo red polarisation colour of collagen fibers, often stressed in the literature as the major disturbing factor in the polarisation microscopy of amyloid.

The relevant optical findings can shortly be explained by using the shematic drawing in Fig. 7. The Congo red molecule has a linear conformation. It is 21 Å in lenght with both ends negatively charged (in hydrophilic media) (Wälchli, 1945). A) In apolar hydrophobic mounting media (as Canada balsam) in which neither dye molecules nor collagen possess electric charges, Congo red molecules are bound by intermolecular forces and hydrogen bonding in nearly axiparallel order to the surface of collagen (as on amyloid) as indicated by the strong (about threefold) increase in positive birefringence with a greenish polarisation colour. B) In apolar media, as gum arabic or fructose, however, the electronegatively charged Congo red molecules are attached to collagen — as evidenced by the optically inversive character of the staining reaction — in a more or less axiperpendicular orientation. This can be explained as resulting from a low surface charge density in the positively charged side groups of collagen (Romhányi *et al.*, 1970). For this reason the linear Congo red molecules can be bound only unipolarly, by one of their electronegatively charged endgroups, their other ends being repelled by the interposed negative side groups of the collagen surface. The dye molecules are then further transoriented (to different degrees) by the various hydrophilic mounting media. This results in depression to isotropy or inversion to negative of the positive birefringence of the collagen fibers.

The inversive topooptical staining reaction of collagen with Congo red in gum arabic and fructose is highly specific for collagen (Romhányi *et al.*, 1970) which can be explained in terms of the specific surface charge distribution of collagen and of the molecular conformation of Congo red. In contrast to collagen, amyloid binds Congo red molecules in a highly ordered surface parallel alignment in both apolar and polar mounting media, as evidenced by the strongly increased positive birefringence with green polarisation colour at appropriate thickness of the slides.

Thus the Congo red anisotropy of amyloid and that of collagen are of the same (additive) optical character with increased (positive) birefringence and green polarisation colour in Canada balsam, however, in gum arabic they are opposite in sign: additive for amyloid and inversive for collagen. This difference in optical chracter provides a firm basis for definite differentiation even of the smallest amyloid deposits from collagen.

Thus it is obvious that mouting of Congo red stained slices in gum arabic is the method of choice for the identification of amyloid by polarisation microscopy. Besides the high specificity and sensitivity of this method a point worth stressing is that no procedure of differentiation is included in it. In this way not only a constant optimal staining intensivity of amyloid can be secured, but all variations and uncertaities in staining effect possibly resulting from over-differentiation or suppression of Congo red staining of amyloid by alkaline media used in different staining modifications are completely abolished. The alkali stability of Congo red binding of amyloid is relative, variable and at about pH 11.0 (Benditt and Eriksen, 1970). Congo red binding, stable to pH 11.0 and above is a characteristic of primary amyloid, and instability to pH 11.0 is the property of secondary amyloid. These various dye affinity-constants and even differences in size and thickness of amyloid deposits invariably must result in various degrees of differentiation of amyloid staining with alkali. In this way it is understandable why a highly sensitive and selective Congo red staining of amyloid for polarisation microscopy could not

be attained by using differentiation procedures, and by mounting the slices in Canada balsam.

We have schown that mounting of Congo red stained slices in Canada balsam or other apolar media, as generally practiced, is not adventageous for the identification of small amyloid deposits by polarisation microscopy. In spite of the many recommended refinements of the original Bennhold (1922) staining method (Highman, 1946; Pearse, 1960; Puchtler *et al.*, 1962) the selective differentiation of small amyloid deposits from connective tissue structures may be troublesome or even impossible because of the additive topooptical staining reaction of collagen fibers, which can manifest itself even when the collagen fibers appear unstained under the light microscope. This is especially the case in Carnoy fixed material in which collagen fibers show a more intensive anisotropic Congo red staining reaction with strongly increased positive birefringence and a green polarisation colour in Canada balsam (Romhányi *et al.*, 1970), because the NH_2 side groups of collagen, responsible for binding Congo red, are not blocked by this fixative, as they are partly blocked by formalin (Romhányi and Deák, 1967). This explains why Klatsky has so often observed a green polarisation colour of collagen fibers and basement membranes in his material mainly fixed in Carnoy's fluid. Similarly the interpretation as amyloid microdeposits of spot-like Congo red anisotropies with a green polarisation colour of collagen fibers and basement membranes by Ravid *et al.* (1967) in a high percentage of organs from routine autopsy cases, clinically unrelated to amyloidotic diseases, cannot be considered as conclusive.

We have also demonstrated that the identification of senile amyloid deposits in the aortic wall is facilitated by the enzymatic removal of the elastic fibers prior to staining. In this way it became possible to recognize the intracellular localisation of the initial senile amyloid deposits in the smooth muscle cells of the vascular wall. This finding may open a new line of research about the pathogenesis of this type of "amyloid". In this connection it is of interest to refer to the observation of Lietz and Donath (1970) that in a case of medullary thyroid cracinoma with amyloid stroma fibrillary amyloid masses were present in smooth muscle cells, and electron microscopic findings suggested that amyloid had been secreted by or liberated from these cells into the stroma.

The finding that after pretreatment of the slices with proteolytic enzymes (elastase and trypsin) in addition to the decrease in intensity of background staining both the inversive Congo red anisotropy of collagen and the additive Congo red anisotropy of amyloid were increased, appears to be of practical interest, since this phenomenon can be used with great adventage to achieve the most sensitive differentiation between amyloid and collagen especially in basement membranes. The increase of inversive topooptical Congo red reaction of collagen after pretreatment with proteolytic enzymes had been explained in our previous paper as caused by the removal of some structural components from collagen interfering with the orientation of the Congo red molecules on the ultrastructural framework of collagen. Similarly it can reasonably be assumed that the increased anisotropic Congo red staining reaction of amyloid after proteolytic enzyme treatment is the result of the enzymatic elimination of an amorphous component from the micellar texture of amyloid interfering with the dye molecule

orientation on the paracrystalline component of amyloid. The question whether this is an enzyme soluble component of amyloid itself (Wössner, 1961), or contaminating plasmaproteins (Horowitz *et al.*, 1965) cannot be decided as yet.

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