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Congo Red Dichroism with Dispersed Amyloid Fibrils, an Extrinsic Cotton Effect*

E. P. Benditt,† N. Eriksen, and C. Berglund‡

DEPARTMENT OF PATHOLOGY, UNIVERSITY OF WASHINGTON, SEATTLE

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Abstract. The spectral absorption, optical rotatory dispersion, and circular dichroism associated with interaction of Congo red dye with partly purified suspensions of amyloid fibril fragments were examined. A set of phenomena consistent with a Cotton effect was found. A nearly identical set of phenomena was obtained with poly-L-lysine in its α-helical form. The dichroism seen when Congo red binds to amyloid substance in tissue sections can also be interpreted as a Cotton effect. This suggests that some special conformation, presumably in protein, is present in a major constituent of amyloid. This conformation is not present in gamma globulin, Bence-Jones protein, albumin, fibrinogen, or other proteins tested so far. These and other optical properties of amyloid substance can be used to compare amyloid deposits in different human cases and in different species. Extension of the use of polarization microscopy with other dyes that bind to other substances in tissue sections should permit more exquisite probing of the conformation of important macromolecules *in situ* in cells and tissues than has hitherto been possible.

Introduction. The strong affinity of amyloid substance for Congo red dye *in vivo* and *in vitro*[1] and the characteristic dichroism seen in polarized light[2] are generally accepted as the most specific properties of the substance. There exists no detailed description and, hence, no clear understanding of the optical phenomena seen when Congo red interacts with amyloid substance. Spectroscopic analysis of the interaction of partially purified preparations of the fibrillar component of amyloid substance with crystal violet has been made,[3,4] and binding of Congo red by similar preparations without detailed spectral analysis has been studied.[5,6] Since some fibril-fragment suspensions in aqueous media are sufficiently clear for optical examination by other means, we examined the interaction of Congo red with partially purified fibrils in aqueous suspension by optical rotatory dispersion and circular dichroism, as well as by optical absorption. When Congo red interacts with fibril-rich extracts of tissues there is seen a consistent set of phenomena which have the properties of an extrinsic "Cotton effect." This provides a basis for an explanation of the anomalous colors seen with the polarizing microscope in tissue sections stained with Congo red and is helpful in identifying some molecular features of amyloid substance responsible for the Congo red phenomenon.

Materials and Methods. The amyloid substance which forms the main material of this study was derived from liver and kidney tissues of a man 55 years of age who

died with massive amyloidosis of liver, kidney, and other organs secondary to tuberculosis. The case chosen represents a characteristic form of the disease. The amyloid substance exhibited fully the distinctive staining properties with Congo red and crystal violet and electron microscopically showed the characteristic fibrils.

Pieces of tissue, frozen within a few hours of autopsy, were stored at −20°C until the time of extraction. The amyloid fibrils were extracted from the tissue with water according to the method of Pras *et al.*[6] As a final step to remove some contaminating proteins, the suspension was sedimented for 1 hr at 100,000 *g*. The pellets thus obtained were resuspended in water, dialyzed exhaustively against water at 4°C, and centrifuged again for 1 hr at 100,000 *g*. The final pellets were suspended in a small amount of water and either lyophilized or stored frozen. The presence of native-type fibrils in the preparations was verified by electron microscopy of specimens negatively stained with phosphotungstic acid.

Congo red was obtained from National Aniline Co. A single batch (95% dye content, color index no. 22120) was used in all of the experiments recorded. Human albumin (crystalline) and human γ-globulin were Nutritional Biochemicals Corp. and Pentex, Inc., products, respectively. Human fibrinogen from human plasma was purified by the method of Laki.[7] Two Bence-Jones proteins were prepared from urines of patients with multiple myeloma. Protein content was estimated from the nitrogen found by micro-Kjeldahl analysis. Poly-L-lysine hydrobromide, mol wt 75,000 (Miles Laboratories, Inc.), was dialyzed against water and lyophilized, or converted to the hydrochloride by successive dialyses against dilute NaOH solution (pH 11), water and dilute HCl solution (pH 2) before lyophilization. Polymer content of the lyophilized material was calculated from nitrogen content.

Spectral absorption was measured in a Cary model 15 spectrophotometer; optical rotatory dispersion and circular dichroism, in appropriately equipped Cary model 60 spectropolarimeters (kindly put at our disposal by Drs. H. Neurath of the Department of Biochemistry and W. S. Chilton of the Department of Chemistry).

Observations. Spectral phenomena resulting from interaction of Congo red with preparations rich in amyloid fibrils: Congo red alone above pH 6 has an absorption maximum at 497–500 nm, as illustrated in Figure 1. Absorption of the dye in the ultraviolet will not concern us here. From 10^{-7} M to 5×10^{-4} M

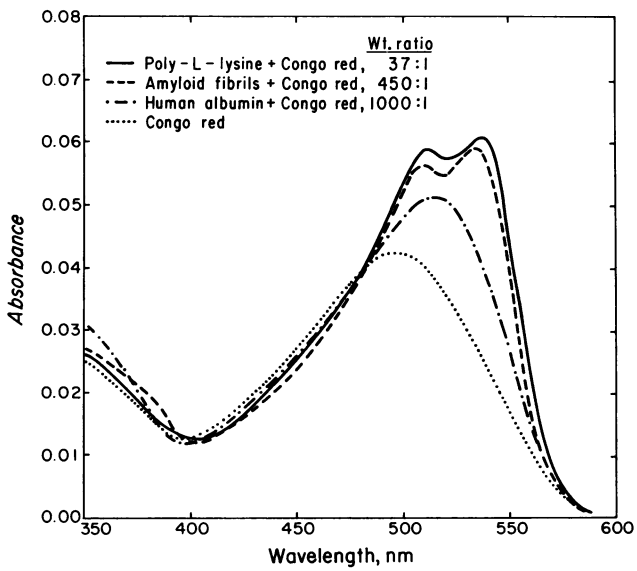

FIG 1.—Absorption spectra of Congo red in the presence of —— poly-L-lysine; - - -, amyloid fibrils; and ·-· human albumin. Substrate without added Congo red in the reference beam. Solvent, water at pH 11 (NaOH); 10^{-6} M Congo red; optical path length, 1 cm.

the dye obeys Beer's law; 10^{-6} M dye in the presence of 10^3 times its weight of albumin increases its absorbance from 4.3×10^{-2} to about 5.2×10^{-2} and the absorption maximum shifts to about 515 nm. A similar amount of γ-globulin produces little hyperchromicity and almost no shift in the wavelength of maximum absorbance. Bence-Jones proteins, fibrinogen, or random-coil forms of poly-L-glutamic acid or poly-L-lysine produce no significant spectral changes. In sharp contrast, addition of a suspension rich in amyloid fibril fragments to the dye solution produces marked increase in absorbance and two absorption maxima emerge, one at about 510 nm and the other at 538–540 nm. Dependence of the spectral shifts on concentration of the substrate is shown in Figure 2; 10^{-6} M

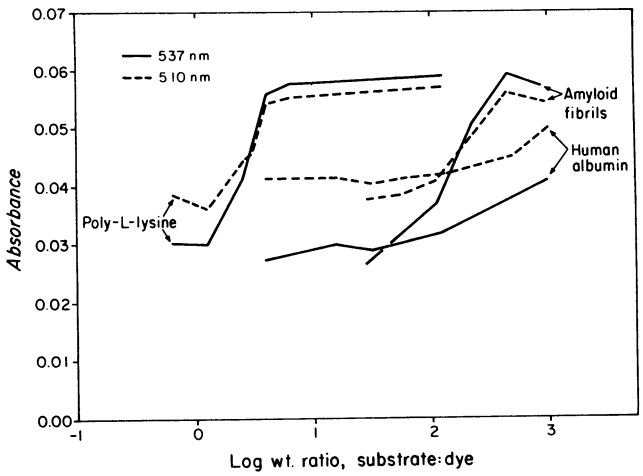

FIG. 2.—Effect of substrate concentration on optical absorption of Congo red at peak wavelengths. Congo red, 10^{-6} M; solvent, water at pH 11.

dye exhibits, with increasing protein concentration, a progressive rise in the intensity of absorption of the two spectral regions, 510 and 540 nm. The absorbance in the 540-nm region rises more rapidly than that in the 510 region and eventually exceeds that at 510 nm. The characteristic spectrum can be demonstrated spectrophotometrically in stained sections.

Optical rotatory dispersion (ORD) and circular dichroism (CD): As shown in Figure 3*A*, Congo red in aqueous solution has no optical rotatory effects at a concentration of 10^{-6} M. However, in the presence of the material rich in amyloid fibrils there is dramatic induction of a complex, anomalous ORD effect (Fig. 3*B*). Furthermore, it can be seen (Fig. 4*B*) that this effect in the region between 475 nm and about 600 nm appears to be composed of two negative Cotton effects with troughs at 520 and 565 nm and corresponding crossover points at about 500 and 540 nm, respectively. This is consistent with what is known about the relationship of absorption maxima and the anomalous optical rotatory phenomena which may occur with these.[8] A related circular dichroism with negative ellipticity and two minima is shown in Figure 4*A*.

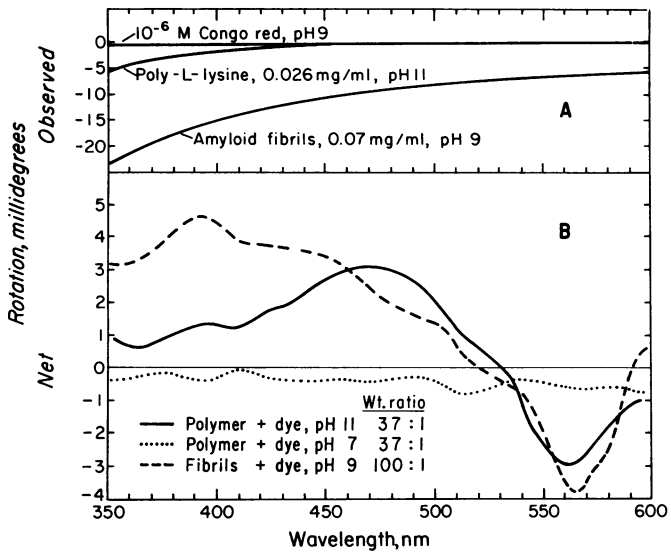

FIG. 3.—(*A*) Optical rotatory dispersion of poly-L-lysine in water at pH 11, and of amyloid fibrils and Congo red in 0.001 M $Na_2B_4O_7$ at pH 9. Optical path length, 10 cm. (*B*) Optical rotatory dispersion of 10^{-6} M Congo red in the presence of poly-L-lysine in water at pH 7 and 11, and in the presence of amyloid fibrils in 0.001 M $Na_2B_4O_7$ at pH 9. The positive and negative deviations were measured from an appropriate reference curve such as shown in (*A*). The rotation of 10^{-6} M Congo red alone was negligible except at pH 7, where it was taken into account.

Interaction of albumin with Congo red shows spectral evidence of binding and also a simple *positive* Cotton effect with a crossover at 520 nm corresponding to the shifted absorption maximum induced by the albumin substrate. The CD curve has a single positive ellipticity peak at 515–520 nm.

The interaction of Congo red with poly-L-lysine in its several forms: Stryer and Blout[9] showed that extrinsic as well as intrinsic chromophores exhibit Cotton effects. In an examination of three different cationic dyes interacting with polyglutamic acid, they observed that a helical conformation of the polymer was required for the production of Cotton effects in the bound dye and that the screw sense of the helix determined the sign of the Cotton effect. Because poly-L-lysine is currently one of the best-characterized polymers, from the conformational standpoint,[10,11] and because Congo red is used histologically at a high pH (about 11), where poly-L-lysine is in an ordered state, we chose to study its interaction with Congo red. Figure 1 shows the effect of interaction of Congo red with poly-L-lysine on the spectrum of the dye. At pH 11 in aqueous solution and room temperature (24 ± 1°C), where poly-L-lysine is in the α-helical form, the spectral configuration mimics almost exactly that of Congo red treated with the fibril-rich amyloid preparation. Figure 2 shows the dependence of the spectral shifts on concentration of poly-L-lysine. On a weight basis, the synthetic polymer is much more potent than the fibril preparation in inducing the spectral changes.

The poly-L-lysine-dye complex has a nearly identical ORD curve in the 475–600 nm region of the spectrum with that of the fibril-dye complex (Fig. 3*B*).

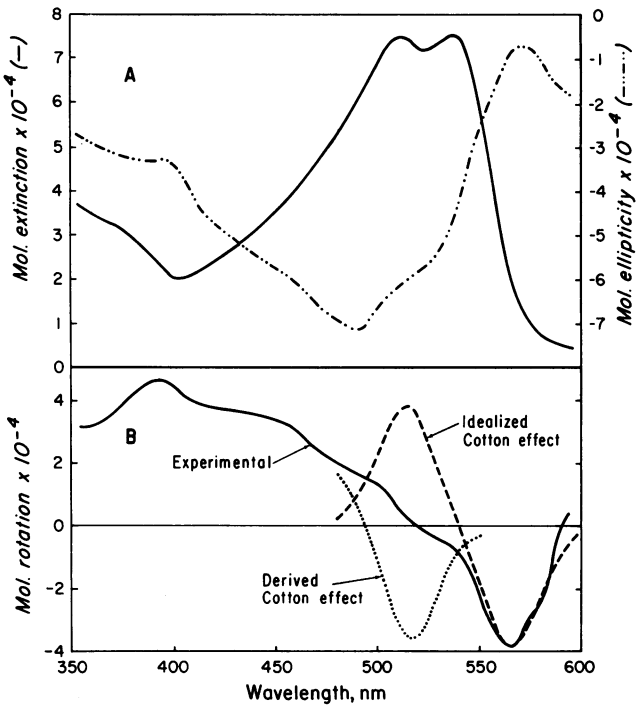

FIG. 4.—(*A*) Optical absorption and circular dichroism and (*B*) graphically resolved rotatory dispersion of Congo red in the presence of amyloid fibrils, in 0.001 M $Na_2B_4O_7$ (pH 9) at a protein:dye weight ratio of 100:1. The extinction is expressed in absorption units (log I_0/I)/cm path length per mole/liter; the ellipticity, in degrees/cm path length per decimole/ml; the rotation, in degrees per decimeter path length per mole/-deciliter.

At pH 7, where poly-L-lysine is in a random-coil form, Congo red exhibits neither a spectral shift nor anomalous rotatory dispersion (Fig. 3*B*). Finally, at pH 11 a CD curve with negative ellipticity and two peaks corresponding closely to the 510 and 538 nm absorption maxima is obtained.

Gentle heating of α-helical poly-L-lysine in aqueous solution at pH 11+ converts the polymer to the β-conformation, a structure which it retains for some hours even after being cooled to room temperature.[10,11] We compared the optical absorption spectra and ORD curves of Congo red in the presence of the polymer in each of the two conformations. A water solution (pH 11) 2×10^{-4} M in lysine monomer was heated for 30 min at 50°C, cooled to room temperature, treated with 0.01 vol of 10^{-4} M Congo red, and immediately examined for ORD. The ORD of an unheated but otherwise identical polymer solution with added Congo red was also obtained. The ORD curves are shown in Figure 5, together with that of the same concentration of dye interacting with the amyloid fibrils. As shown, the α-helical form of the poly-L-lysine induces in the dye an ORD effect more nearly resembling that of the amyloid fibrils than does the β form of the polymer.

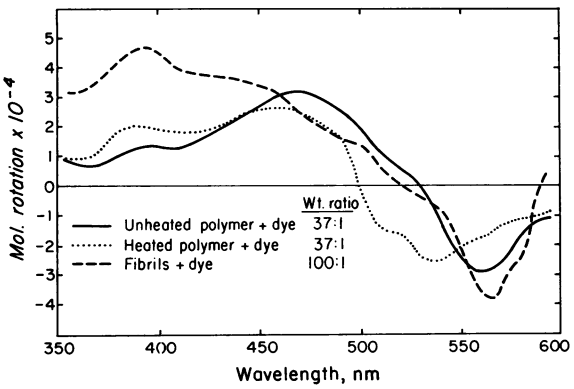

FIG. 5.—Optical rotatory dispersion of Congo red in the presence of — poly-L-lysine; ····, poly-L-lysine heated at 50°C for 30 min before addition of Congo red; and - - -, amyloid fibrils. The positive and negative deviations were measured from an appropriate reference curve for the substrate in the absence of Congo red. Solvent for poly-L-lysine, water at pH 11.6; for amyloid fibrils, water at pH 11.2. Congo red, 10^{-6} M; optical path length 10 cm.

Discussion. Aside from adventitious serum constituents, three characteristic entities have now been isolated from amyloidotic tissues: fragments of the electron microscopically demonstrable fibrils,[5,6] a protein electron microscopically seen as a double pentagonal structure,[12] and a low molecular weight protein of unusual composition.[13] The exact relationship of these three entities to each other and to the amyloid substance complex is not clear. Examination of the optical properties of the three entities in the presence of Congo red by the methods described here thus far shows that only fibril-rich preparations exhibit the several phenomena. Other evidence indicating the conformation dependence of the extrinsic Cotton effect with Congo red is that treatment of the fibril-rich material with 8 M urea and removal of the urea by dialysis abolishes the capacity to induce the spectral changes. We conclude, therefore, that the fibrils are the sites of dye binding and the repository of the molecular constitution and conformation inducing in the dye the spectral, the ORD, and the CD effects observed.

Recently there have appeared x-ray diffraction studies from three laboratories on amyloid fibril preparations similar to those used here.[14-16] Two of these[14,15] have specially emphasized the presence of a 4.67 Å spacing considered to be due to the cross-β structure. We cannot yet say what relationship, if any, the conformation-dependent extrinsic Cotton effect may bear to this cross-β structure. The x-ray diffraction studies require drying in contrast to the optical properties measured here. Hence, one cannot be certain that the cross-β conformation is a property of the hydrated state of the macromolecular complex.

It is assumed currently that all amyloid fibrils have the same composition. However, it is clear from examining the reported data[5,13,17,18] that either this is not so or that the "purified" fibril preparations have varying degrees of contamination with other substances, both protein and nonprotein. The main material for this study was derived from a case of amyloidosis selected for reasons given above. In addition, two other cases were examined in somewhat less detail, one associated with multiple myeloma and the other with a carcinoma of the mouth. While the polarizing microscope showed the typical dichroism, fibril dispersates from these latter cases treated with Congo red and examined by spectroscopy and optical rotatory dispersion exhibited some differences. Similar

spectra with hyperchromism and two maxima but less pronounced separation of the two peaks were seen. ORD effects were not nearly as striking as in the case presented in detail. We suggest that material from these other cases may contain contaminants or constituents which alter the optical phenomena and possibly other properties of the fibrils. Independent evidence of the presence of contaminating material comes from the fact that it takes larger amounts of protein from these latter two cases than from the case detailed here to induce the maximum degree of hyperchromicity at 538 nm.

Currently it appears that the phenomena exhibited when Congo red interacts with amyloid have a high order of specificity. Recent evidence which we have obtained indicates binding constants of about 10^7 M^{-1}. The distinctive character of the combined optical phenomena (absorption, rotation, and circular dichroism) should be useful both in identifying and in probing the structure of a characteristic part of amyloid substance. In addition to this extrinsic Cotton effect, the ORD and CD in the ultraviolet should be of considerable value. We have already observed in this region of the spectrum some similarities and some differences among amyloid preparations derived from different cases.

The demonstration of the Cotton effect exhibited by the product of the interaction of amyloid fibrils and Congo red provides the basis for a reasonable explanation of the "green" birefringence of amyloid substance seen in tissue sections stained with Congo red. It can easily be shown that the color is a yellow-green only when the polarizer and analyzer are accurately crossed. Slight uncrossing (1–2°) of the polarizer in one direction produces a yellow to yellow-orange color in the stained material, and uncrossing a similar amount in the opposite direction produces a blue-green color. The direction of rotation required to observe a particular color depends upon the orientation to the polarizer of the fibril mass being viewed. The rotation of the yellow-orange and blue-green light in opposite directions is obviously consistent with a Cotton effect. Furthermore the spectral region in which it occurs is the same as that shown here for the dispersed fibrils. One cannot measure this with the usual visual compensators because of the complex mixture of birefringence, optical rotation, and circular dichroism not only of the amyloid but of the interwoven tissue elements such as collagen. The eye, however, easily sorts out the anomalous color shifts from the plain dispersion and birefringence. The high intensity of the Cotton effect as seen in the microscope probably depends upon the fact that the fibrils in the tissue are in an ordered arrangement as opposed to the random distribution of the fragments in suspension. These considerations suggest that with appropriate apparatus (e.g., of Allen *et al.*[19]) one may be able to use dyes as probes of molecular conformation in tissue sections.

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† Requests for reprints may be addressed to Dr. E. P. Benditt, Department of Pathology, University of Washington School of Medicine, Seattle, Wash. 98105.

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