Film Dichroism. VI.[1] Linearly-polarized Absorption Spectra of Neutral and Divalent 3,6-Diaminoacridine Dyes in the Stretched Poly(vinyl alcohol) Film

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(Received April 16, 1982)

The linear dichroic spectra of the neutral (uncharged) and divalent (doubly charged) Acridine Orange, Acridine Yellow, and Proflavine were measured at 25 °C in the visible and UV wavelength regions by the film dichroism method. These 3,6-disubstituted symmetric acridine dyes were molecularly dispersed in the heat-treated poly(vinyl alcohol) film by the staining method. The neutral and divalent dye species were prepared by adding a sufficient amount of sodium hydroxide or sulfuric acid to the staining dye solutions. The wavelength dependence of the reduced dichroism, $\Delta A/A$, of the neutral species at a given stretch ratio was very similar to that of the monovalent species previously reported [*Bull. Chem. Soc. Jpn.*, **52**, 3163 (1979)]. Values of $\Delta A/A$ for the divalent species were nearly constant over the entire wavelength region 550—220 nm. The long-axis and short-axis polarized component (A_z and A_y) spectra were computed for the neutral dye species from the observed parallel- and perpendicular-polarized dichroic spectra by using a reduction procedure. The broad, principal band of each dye species in the visible region was deduced to be a composite of two orthogonal (a strong A_z and a weak A_y) electronic transitions.

In previous papers in this series,[2,3] we have shown that the monovalent, symmetrically-substituted acridine dyes can be oriented unidirectionally in the stretched poly(vinyl alcohol) (PVA) film and that the linearly polarized spectra of these dyes can be analyzed into the long-axis and short-axis (A_z and A_y) components whose transition moments are polarized in the molecular plane. For 3,6-disubstituted acridine dyes, such as Acridine Orange and Proflavine, the broad, long-wavelength absorption band in the visible region was resolved, without exception, into composite of a strong A_z-component and a very weak A_y-component.[2,3] Since these dyes are known to bind to various kinds of biopolymers, and since the binding has been studied mostly by the spectrophotometric methods, the optical properties of the individual dyes must be clarified thoroughly to facilitate the correct interpretation of the spectroscopic behavior of the dye–polymer complexes.

The monovalent 3,6-disubstituted acridine dyes are susceptible to deprotonation or further protonation, becoming neutral (uncharged) in alkaline solutions or divalent (doubly charged) in acid solutions. The visible absorption spectra of these neutral and divalent dye species are very different from those of the corresponding monovalent (singly charged) species,[4] but, interestingly, often resemble the metachromatic spectra of the monovalent species in the presence of various kinds of polyelectrolytes.[5] Therefore, the film dichroism study of the neutral and divalent acridine dyes is highly desirable for identifying the electronic transition moments responsible for the visible absorption bands, and, if possible, for confirming the systematic changes of these bands in terms of two overlapping (A_y and A_z) component spectra. For this purpose, painstaking efforts were made in the preparation of the neutral and divalent dye species molecularly dispersed in the PVA film, and in the measurements of the dichroic spectra of the stretched films.

Experimental

Materials. Monovalent dyes in the hydrochloride form have been described elsewhere.[2] Abbreviations used in this work are as follows: Acridine Orange, 3,6-bis(dimethylamino)acridinium chloride (AO); Acridine Yellow, 3,6-diamino-2,7-dimethylacridinium chloride (AY); Proflavine, 3,6-diaminoacridinium chloride (PF). Each of these dyes in the neutral form is denoted as AO°, AY°, and PF°, and the monovalent cations as AO^+, AY^+, and PF^+, whenever so needed. The divalent cations are denoted as AO^{2+}, AY^{2+}, and PF^{2+}. Poly(vinyl alcohol) with a nominal degree of polymerization of 2000 was purchased from Nakarai Chemicals, Ltd. (Kyoto). Other chemicals were of reagent grade.

Preparations of Dye–PVA Films. A stock PVA solution was prepared by dissolving the powdered PVA sample (10 g) in redistilled water (100 ml) under gentle stirring at 80—90 °C. The clear solution was filtered through a G-1 sintered glass filter, cooled to ambient temperature, and then spread onto a glass plate to prepare a clear film (10 cm × 10 cm).[2,6] The well-dried PVA film was cut to rectangular pieces; each piece was subjected to heat-treatment at 80 °C for four hours. The preparation of the dye-containing PVA film from a dye–PVA solution by the standard method[2,6] was unsuccessful because of the following reasons. To retain the respective dye species in the purely neutral or divalent form without being contaminated by the stable monovalent form, a sufficient amount of sodium hydroxide or sulfuric acid must be added to the aqueous dye–PVA solution. Attempts to cast a good-quality film from this mixture failed; the mixture either remained translucent for the neutral dye–PVA system or jellified for the divalent dye–PVA system.

After many trials-and-errors, film-staining was found to be the best method. Two heat-treated PVA films (one for the sample, the other for the reference) were immersed into the aqueous sulfuric acid solution (*ca.* 1.8 M, 1 M = 1 mol dm^{-3}). (If the concentration of the acid is too high, the film tends to dissolve. If it is too low, the film contains both the mono- and divalent dye species.) After sufficient time for swelling, the sample film was stained by the acid dye solution. The reference film was kept immersed in the dye-free acid solution for the same period of time. Both films were then dried on a glass plate. (When hydrochloric acid was used for sulfuric acid, the divalent dye species in the sample film was partly converted back to the monovalent form, probably because of the evaporation of hydrogen chloride from the film.)

The sample and the reference films for the neutral dye species were similarly prepared by immersing two heat-treated PVA films in the aqueous sodium hydroxide solution (*ca.* 2.5 M). The swollen sample film was transferred to the alkaline dye solution which contains ethanol enough for the uncharged dye not to precipitate. On being dried on a glass plate, both sample and reference films formed the white powder of carbonate on the surface. The carbonate was washed off with distilled water and then with ethanol. The transparent films were kept in a desiccator.

Measurements. A large number of dye–PVA films were prepared and the spectra of the nonstretched sample films were carefully compared with the solution spectra of the respective dye species, in order to select the films which were not contaminated by the monovalent species and were suitable for the dichroism measurement. The linear dichroic spectra, which are polarized parallel ($A_\parallel$) and perpendicular ($A_\perp$) to the direction of the film stretch, and the isotropic nonstretched spectrum (A) of the dye–PVA film[6] were measured, together with the visible spectra of dye solutions, at 25 °C as described previously.[2,3] Because of the difficulty in the multiple stretching of a given film, each film was stretched only once to a predetermined macroscopic stretch ratio S, as defined previously.[2,3,6]

Analytical Procedure. The dichroic data were expressed with the reduced dichroism $\Delta A/A$:

$$\frac{\Delta A}{A} = \frac{(A_\parallel - A_\perp)}{1/3(A_\parallel + 2A_\perp)}. \tag{1}$$

For symmetric 3,6-diaminoacridine dyes which belong to the C_{2v} point symmetry group, the reduction procedure[7,8] may be utilized for evaluating the A_y- and A_z-spectra (the short-axis and the long-axis polarized component spectra, respectively) from the observed dichroic $A_\parallel$- and $A_\perp$-spectra as before:[2]

$$\left.\begin{aligned} A_\perp - d_\perp A_\parallel &= \frac{K_z - K_y}{2K_z} A_y \\ A_\parallel - d_\parallel A_\perp &= \frac{K_z - K_y}{1 - k_y} A_z, \end{aligned}\right\} \tag{2}$$

where $d_\parallel$ and $d_\perp$ are the reduction factors, and K_y and K_z are the orientation factors.

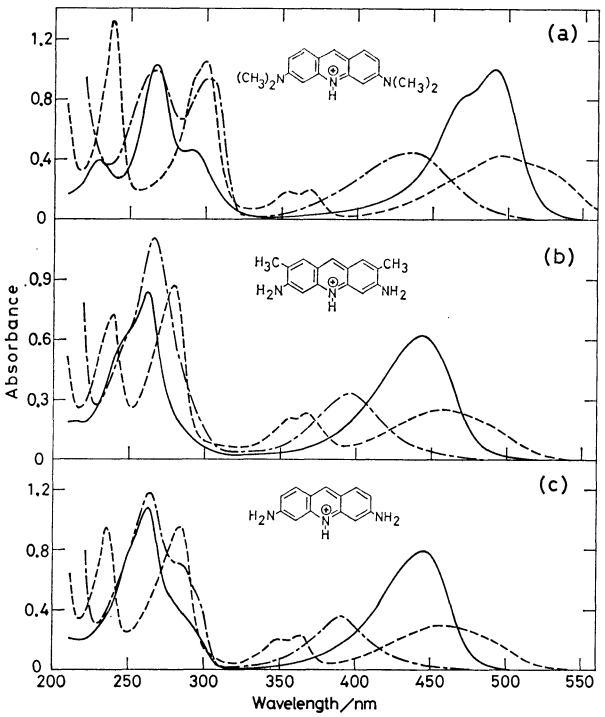

Fig. 1. Absorption spectra of three diaminoacridine dyes in solution at three representative pH regions. The neutral (uncharged) forms in alkaline solution (—·—), the divalent (doubly charged) forms in acid solution (----), and the monovalent (singly charged) forms in aqueous solution (——) for which the structural formulas are given. Conditions are: (a) Acridine Orange, AO° (21 μM) in 0.17 M NaOH solution, AO^+ (25 μM) at a neutral pH, and AO^{2+} (16 μM) in 2.3 M HCl; (b) Acridine Yellow, AY° (16 μM) in 0.17 M NaOH, AY^+ (19 μM) at a neutral pH, and AY^{2+} (14 μM) in 1.7 M HCl; (c) Proflavine, PF° (19 μM) in 0.17 M NaOH, PF^+ (22 μM) at a neutral pH, and PF^{2+} (14 μM) in 2.3 M HCl. The absorption of each solution was measured in a 1-cm cuvette cell.

Results and Discussion

Solution Spectra. Figure 1 shows the solution spectra of three diaminoacridine dyes in the acid, neutral, and alkaline pH regions. In all cases, the neutral (uncharged) dye species give rise to the visible spectra which are hypochromic and hypsochromic relative to those of the monovalent dye cations. The visible spectra of divalent dye cations reveal some remarkable features; the principal band shifts toward the long wavelength and a new twin-peaked band appears in the wavelength region 370—340 nm, where the monovalent and neutral species show only monotonically-decreasing absorption profile. It should be noted that this twin-peaked band, which was earlier observed for AO^{2+} by Zanker,[4] is in fact a feature common to the divalent 3,6-diaminoacridine cations. The purely divalent Trypaflavine, the quaternized homolog of PF^{2+}, was found to show the twin-peaked band at 365—350 nm.[9] It was also confirmed in this work that the divalent cations of the 10-methyl derivatives of AO and AY[3] give rise to the absorption spectra with the twin peak very much similar to those in Fig. 1. Therefore, no further details will be given on the dichroic behavior of the divalent species of those quaternary 3,6-diaminoacridine dyes.

Dichroic Spectra and Reduced Dichroism. Figures 2—4 show the dichroic spectra, *i.e.*, the $A_\parallel$- and $A_\perp$-spectra, and the wavelength dependence of the reduced dichroism $\Delta A/A$ (hereafter denoted as the RD-spectrum) for the neutral species (upper half) and for the divalent species (lower half). The results may be summarized as follows. (1) Regardless of the dye species examined, the $A_\parallel$-spectra are more intense than the $A_\perp$-spectra at a given stretch ratio; hence, the sign of the RD-spectra (filled circles) remains positive throughout the wavelength region. (2) The RD-spectra of the neutral species (AO°, AY°, and PF°) are not constant but decrease on the long and short wavelength sides of the principal band (the error is large for PF° due to the background scattering). This behavior is very similar to that of the corresponding monovalent dye species,[2] immediately indicating that the broad and featureless band of each

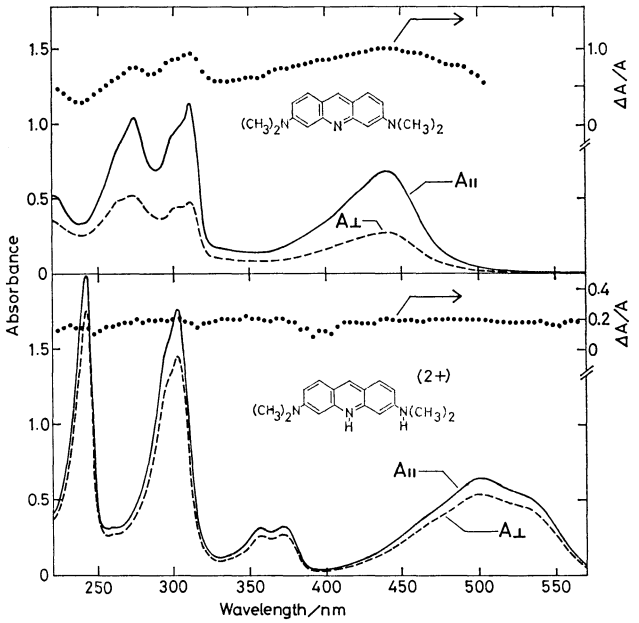

Fig. 2. The dichroic spectra polarized parallel ($A_\parallel$) and perpendicular ($A_\perp$) to the direction of stretch and the reduced dichroism ($\Delta A/A$) of Acridine Orange in the stretched PVA film. Upper: the neutral form. Lower; the divalent ionic form. The nominal stretch ratio S of the PVA film is 3.8,[2,6] which corresponds to the rotation of the mechanical stretcher of 15 turns.[6] This, in turn, corresponds to the threefold elongation of the PVA sample film along the stretching direction.

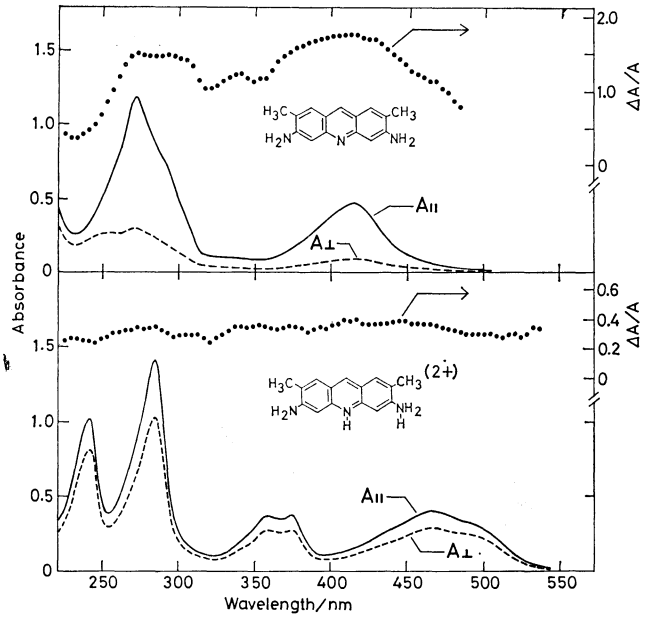

Fig. 3. The dichroic spectra ($A_\parallel$ and $A_\perp$) and the reduced dichroism ($\Delta A/A$) of Acridine Yellow in the stretched PVA film. The symbols and conditions are the same as in Fig. 2.

neutral species in the visible region (Fig. 1) is only apparent and contains at least two electronic transition moments.[2,3] The close similarity between the monovalent and neutral species may be accounted

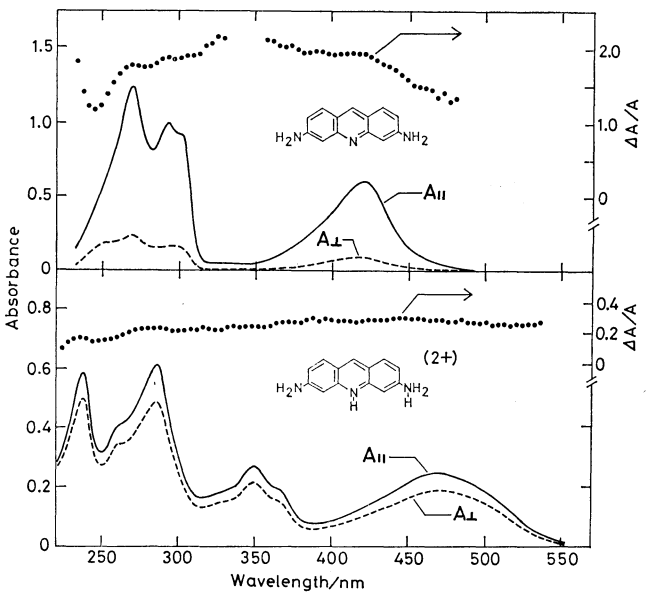

Fig. 4. The dichroic spectra ($A_\parallel$ and $A_\perp$) and the reduced dichroism ($\Delta A/A$) of Proflavine in the stretched PVA film. The symbols and conditions are the same as in Fig. 2.

for by considering their molecular symmetry and chemical structure: one class is deprotonated and the other is protonated at the ring nitrogen. (3) The RD-spectra of the divalent dye cations (AO^{2+}, AY^{2+}, and PF^{2+}) are nearly constant throughout the visible and UV regions; both the principal band near 450—500 nm and the new twin peak behave similarly on stretching. This is a remarkable contrast with the cases of the neutral and monovalent dye species. (4) The divalent dye cations are less orientable than the uncharged counterparts, *i.e.*, values of $\Delta A/A$ are smaller by a factor of 10—5, at a given stretch ratio; a feasible explanation is that the H_2SO_4-containing PVA film matrix is less rigid than the NaOH-containing one.

Reduction Procedure. Because of the additives (H_2SO_4 and NaOH) in the dye–PVA films, the dichroic measurements for each film could be carried out only at a single stretch. It was not possible to determine the dependence of $\Delta A/A$ on stretch ratio at some selected wavelengths as in previous studies;[2,6,10] hence, the intrinsic value of the dichroism at infinitely high stretch ratio could not be estimated. Nor could the orientational behavior inside the "orientation triangle"[2,10] be established. Since the reduction procedure does not require the exact degree of orientation of the guest molecules,[7,8] this technique will be employed to estimate the short-axis polarized (A_y) and the long-axis polarized (A_z) component spectra. Figure 5 shows an example of the reduction procedure applied to AO°. Both ($A_\parallel - d_\parallel A_\perp$) and ($A_\perp - d_\perp A_\parallel$) were searched for A_y and A_z with a HITAC M-180 computer. While the factor $d_\perp$ (hence, the value K_z and an unnormalized A_y-spectrum) could be determined (Fig. 5b), the other factor $d_\parallel$ (hence, the value K_y) could not, because none of the trial spectra ($A_\parallel - d_\parallel A_\perp$) falls on (or crosses over) the zero absorbance line. Thus, the normalized A_y- and A_z-spectra are undeterminable. In order to circumvent this prob-

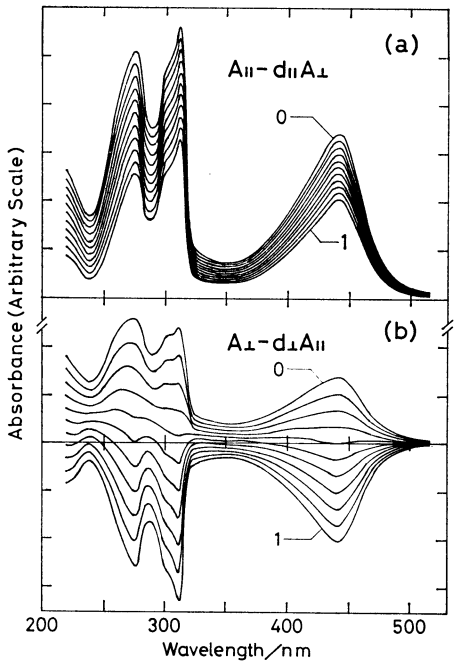

Fig. 5. The reduction procedure applied to the dichroic spectra of Acridine Orange in the neutral form. The $A_{\parallel}$- and $A_{\perp}$-spectra were measured at a nominal stretch ratio of 3.8. The reduction factors $d_{\parallel}$ and $d_{\perp}$ were varied from 0 to 1 at intervals of 0.1.

lem, a plausible assumption is made: the orientation behavior of the neutral form (AO°) is practically the same as that of the monovalent form (AO^+), so that the orientation triangle for AO^+ (Fig. 7 of Ref. 2 or Fig. 4 of Ref. 10) can be utilized to evaluate the orientation factor K_y by using the above K_z. Then, both A_y and A_z may be computed from Eq. 2. The same general techniques apply to other neutral dyes (AY° and PF°). The values of the reduction factors and the orientation factors are given in Table 1.

The Long-axis and Short-axis Polarized Spectra. Figure 6 shows both the long-axis polarized (A_z) and the short-axis polarized (A_y) spectra for three neutral dye species. It is clear that, for each species, the isotropic absorption spectrum in the visible region (Fig. 1) belongs predominantly to the A_z-spectrum, but that the contribution from the A_y-spectrum should not be ignored (dashed lines). The featureless principal band is now concluded to be a composite of two mutually perpendicular transitions (the y- and z-polarizations or the ^1L_a and ^1L_b bands[2-4]). The A_z-spectrum is also predominant in the UV region, but the A_y-spectrum substantially contributes to the observed isotropic band in each case.

The C_{2v} symmetry should be assumed if the reduction procedure is to be applied to the observed $A_{\parallel}$- and $A_{\perp}$-spectra of the divalent dye species on a reliable basis. The C_{2v} symmetry could be preserved for a given divalent species only if an additional proton is attached to the 10-position of the corresponding monovalent acridinium ion. The divalent species probably belong to a class of lower symmetry,[11] since the second protonation is believed to occur at the 3- or 6-position.[4,12] As a consequence, the directions

TABLE 1. THE REDUCTION FACTORS ($d_{\parallel}$ AND $d_{\perp}$) AND THE ORIENTATION FACTORS (K_y AND K_z) AT A CONSTANT STRETCH RATIO S FOR NEUTRAL ACRIDINE DYES

Dyes	$d_{\parallel}$	$d_{\perp}$	K_y	K_z
AO°	0.62	0.38	0.237	0.568
AY°	0.28	0.16	0.123	0.758
PF°	0.37	0.09	0.156	0.847

The nominal value of S is 3.8.[2]

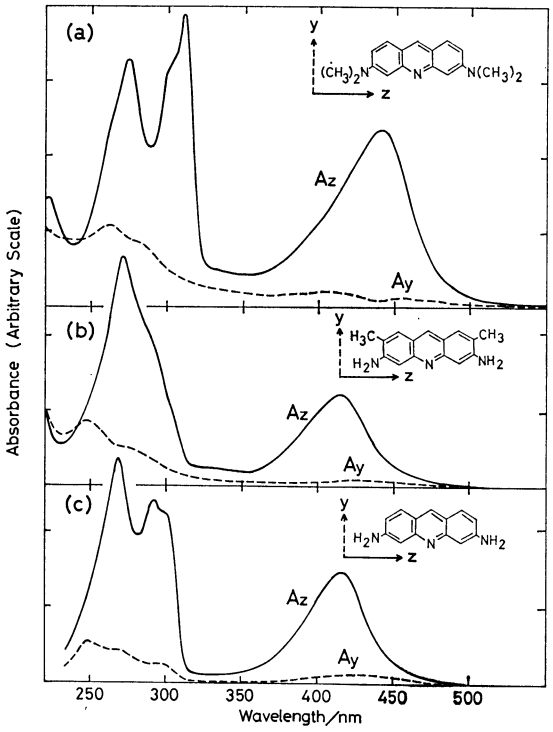

Fig. 6. The short-axis (y) and long-axis (z) polarized component spectra (A_y and A_z) of three 3,6-diaminoacridine dyes in the neutral forms. (a) Acridine Orange, (b) Acridine Yellow, and (c) Proflavine.

of the in-plane transition moments may no longer be mutually perpendicular. We are therefore obliged to postpone the analysis of the dichroic data of the divalent dye species, until an analytical method suitable for the film dichroism of guest molecules of low symmetry is developed.

Closing Remarks. The film dichroism studies on the symmetric 3,6-diaminoacridine dyes in the neutral, monovalent, and divalent forms may be summarized as follows. All dye species in each form clearly show some regular spectral features in the visible absorption spectra. The twin-peaked band appears only in the spectrum of the divalent species. For the neutral and monovalent forms examined so far,[2] the long-axis and the short-axis polarized component spectra overlap each other; the A_z-spectrum is always located on the wavelength side slightly shorter than the A_y-spectrum. We may now conclude that the broad, visible absorption band of the symmetric 3,6-diaminoacridine dyes is a composite of two mutually-polarized, overlapping component spectra regardless of the substituents at the 3,6-nitrogen, at the 2,7-carbon, and at

the 10-nitrogen of the acridine nucleus. These findings should facilitate a new interpretation of the spectral changes of these dyes when bound to various synthetic polyelectrolytes and biopolymers.

We acknowledge with deep gratitude some initial contributions to the present work by Dr. Yukio Matsuoka, who formerly belonged to our research group and was actively engaged in developing film dichroism techniques.

References

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