

Circular Dichroism Measurements on Oriented Optically Active Species

BENGT NORDÉN

Division of Inorganic Chemistry 1, University of Lund, S-220 07 Lund, Sweden

The possibilities of measuring circular dichroism (CD) in oriented samples are discussed. A method is designed for obtaining CD of anisotropic optically active species oriented perpendicular to the direction of incidence of the circularly polarized light.

The quantitative CD is discussed in terms of orientation of the respective electric transition dipoles in the molecule, and of the orientation degree of the molecular species for two cases of uniaxial orientation, on the one hand the light is propagated parallel to the direction of orientation, on the other perpendicular to it.

The methods have been demonstrated by studies of CD in oriented solutions of a few steroids and poly- α -L-glutamic acid dyed with acridine orange.

The usefulness of circular dichroism (CD) spectra of *quasi*-oriented systems has been shown in previous investigations.^{1,2} It was then also concluded that measurement of CD, and especially optical rotation (OR), can be erratic in systems with birefringence. The present paper deals with methods of measuring CD in oriented systems and experimental criteria on the reliability of the resulting effects. As the methods are demonstrated on chemically interesting species, the results will be of value also from a chemical point of view.

METHODS

The following *semi*-quantitative discussion must not be confused with the general theories on optical activity of anisotropic solutions.* As the latter, however, are all very complex and require detailed knowledge about the electronic states of the molecule they are unsuitable for the purpose of a qualitative discussion on the CD of an oriented anisotropic chromophore. In our treatment we assume that the only detected effect is the molecular CD (secondary effects due to birefringence and linear dichroism could be neglected). Furthermore, we assume that the CD spectrum can be decomposed into CD-

* See, *e.g.*, Ref. 3 and references therein.

bands each of which corresponds to an electronic transition which could be represented by *one* transition dipole moment vector (more or less hypothetical). The direction of this vector should be defined by the molecular orientation, relative to the propagating light, at which optimum rotational strength should be observed for the considered transition *viz.* the orientation where the absorption intensity due to interaction with the transition dipole is optimum.

1. The sample is oriented perpendicularly to the direction of the luminous flux

Let us consider an ellipsoidal molecule with two energetically well separated transitions; one long-axis polarized and one polarized perpendicularly to the long-axis. If the transitions do not overlap, the recorded circular dichroisms for the two transitions ($\Delta\varepsilon_{\text{long}}$ and $\Delta\varepsilon_{\text{perp}}$, respectively) when the molecules are perfectly oriented with their long axes perpendicular to the direction of light (the "degree of orientation" $f=1$),³ is given by the relations

$$\Delta\varepsilon_{\text{long}}(f=1) = \frac{3}{2} \Delta\varepsilon_{\text{long}}(f=0), \quad (1)$$

$$\Delta\varepsilon_{\text{perp}}(f=1) = \frac{3}{4} \Delta\varepsilon_{\text{perp}}(f=0) \quad (2)$$

($f=0$ denotes the *random* case).³ This is true only if the interaction between the light and the sample is determined solely by the changed transition probabilities due to the different distributions of the potential electric transition dipoles.

In general, however, the oriented dipoles require "*the matter*" to be oriented thereby acquiring the doubly-refracting properties of a uniaxial crystal (*cf.* Maxwell effect, Kerr effect, Cotton-Mouton effect).⁴ The double-refraction (birefringence) will change the polarization of the light in a way which is schematically shown in Fig. 1. This figure illustrates in fact the principle of a

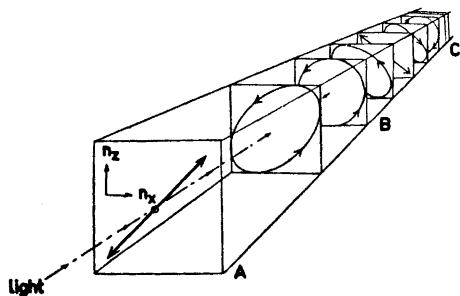


Fig. 1. Illustration of depolarization of circularly polarized light by a doubly refractive medium. A-B, the generation of circularly polarized light in the instrument. B-... the sample. Each section (AC/6) corresponds to a phase difference of $\pi/4$ between the two perpendicularly polarized component waves or a distance of $\lambda/8 |n_x - n_z|$.⁵

quarter-wave plate. If A-B is the optical unit in the instrument which transforms linearly polarized light into circularly polarized, B-... may represent the doubly refractive sample (we have then assumed the same refractive indices, n_x and n_z , and parallel optic axes, *e.g.* z , of the two systems). With 45° between the optic axis (or "principal plane")⁵ and the direction of polarization

of the incident light, the two components parallel to x and z , respectively, will have the same amplitude but different speeds due to the difference $n_x \neq n_z$.⁵ After traversing a certain distance, $A - B$, there will be a difference in phase of $\pi/2$, ($A - B = \lambda/4|n_x - n_z|$, the optical path difference being one quarter of the wavelength of the light, $AB = BC/2$, and at that point the light will be circularly polarized.

The effect of double refraction on the apparent CD should thus be a decrease or even a change in sign (point C, Fig. 1). The effect will be wavelength dependent (*cf.* optical pathlength above) but should not produce any "CD bands" as long as no transitions are mixed up with it. However, if an anisotropic uniaxially oriented transition can interact, the sample may behave like a polaroid.⁷ In CD instruments with continuously modulated polarization (*e.g.* the Roussel-Jouan Dichrograph),⁶ the effect may be very erratic.¹ However, in the latter cases this effect can be detected by rotating the sample in a plane perpendicular to the direction of the incident light. The effect will change at the rotation and will be at a maximum with the direction of orientation in the sample parallel or perpendicular to the original direction of linear polarization of the light. Numerous experiments with anisotropic dyes (*e.g.* acridine orange)⁸ oriented in stretched polymer films (aqueous polyvinyl alcohol) in our laboratory have shown that the sum of apparent CD-deflections recorded at many different angles of orientation of the sample in a plane perpendicular to the light will equal zero, if the angles are equally distributed around the circle.^{1,8} For instance, $CD(0) + CD(90) + CD(180) + CD(270) = 0 \pm 2\%$ of the maximum deflection (the figures denote the angles in degrees between the direction of orientation and the original direction of polarization). With only four values, however, a comparatively high precision in the orientation is required (*e.g.* $0.0 \pm 0.5^\circ$) and it can be easier to record instead, *e.g.*, 20 values.

To avoid errors from a relatively low birefringence we would like to suggest the following method:

(a) Insert the oriented sample in the cell-compartment and a standard with known CD (in this order following the path of the light, see Fig. 2).

(b) If the minimum CD recorded is not much less than the CD of the standard (say 95% of it), measure the CD (without standard) at different orientations of the sample arranged symmetrically around the original direction of polarization and the direction perpendicular to it (can be determined by using a polarizer—disconnect the modulation of the electro-optic crystal in the instrument)⁶. The mean of the obtained values represents the true CD.

(c) If the birefringence is strong (the apparent CD of the standard is much lower than its true CD) we suggest that the orientation (starting from random state) is gradually increased and the apparent CD of the standard is simultaneously inspected. When the effect from birefringence is not too high, a qualitative result can perhaps be recorded. However, a better alternative should be that the orienting force (stretching) is turned 90° and when the CD of the standard shows that there is no effective birefringence, the value (without standard) is taken as the true CD. The result from stretching first in one direction and then in a direction perpendicular to the first can of course not be a uniaxial orientation. If the model representing the uniaxial stretch is the change of a sphere into an ellipsoid, the symmetric biaxial stretching

should be represented by a disc, which defines the *plane of orientation*. With no molecules oriented (with their long-axes) out of this plane the same relations will hold, as the above-mentioned for the case of a perfect uniaxial orientation.

We shall now expand these relations to include cases of partial orientation (defined by a "degree of orientation", f , which is the ratio of hypothetically perfectly oriented molecules, along a given direction, when the rest $1-f$, is perfectly random¹ and with transitions which are neither parallel nor perpendicular to the long-axis (but defined by the angle, α , they form to the long-axis). If $A(\parallel)$ is the absorption of linearly polarized light when the direction of polarization⁹ is parallel to the direction of orientation, and $A(\perp)$ is the corresponding perpendicular absorption, the following relations should give the absorption ($A(f)$) of unpolarized (or circularly polarized) light:¹⁰⁻¹²

$$A(f) = \frac{1}{2} (A(\parallel, f) + A(\perp, f)) \quad (3)$$

$$A(f=0) = \frac{1}{3} (A(\parallel, f) + 2A(\perp, f)) \quad (4)$$

The fact that the absorption is proportional to the square of the scalar product between the electric vector in light and the transition dipole in the molecule in combination with our definition of f gives (K proportionality constant):^{10,12-14}

$$\begin{aligned} A(\parallel, f) &= [f \cos^2 \alpha + (1-f)/3]K^* \\ A(\perp, f) &= [\frac{1}{2}f \sin^2 \alpha + (1-f)/3]K \end{aligned} \quad (5)$$

From eqns. (3-5) we obtain for the ratio of unpolarized (or circularly polarized) absorption

$$\frac{A(f)}{A(f=0)} = \frac{\frac{3}{2}f \cos^2 \alpha + \frac{3}{4}f \sin^2 \alpha + (1-f)}{f \cos^2 \alpha + f \sin^2 \alpha + (1-f)}$$

The circular dichroism of an oriented state at a function of the random state is thus given by

$$\Delta\varepsilon(\alpha, f) = \Delta\varepsilon(\alpha, f=0) \times \left\{ \frac{3}{2}f \cos^2 \alpha + \frac{3}{4}f \sin^2 \alpha + (1-f) \right\} \quad (6)$$

(For two different transitions with α equal to 0° and 90° , we obtain the relations 1 and 2, respectively.)

This method has been used in connection with the orientation of films of optically active cobalt(III) complexes.^{1,2} In the present investigation we have used stretched polyethylene films as an orienting base. The double refraction of the film has been depressed according to the above-mentioned procedure of biaxial stretching and the circular dichroism of some steroids has been measured using this method.

* From $[f \cos^2 \alpha + (\cos^2 \alpha + \frac{1}{2}\sin^2 \alpha + \frac{1}{2}\sin^2 \alpha)(1-f)/3]K$. The contribution from the "random part", $1-f$, is obtained by imagining the ratio $(1-f)/3$ of the total amount of molecules oriented along each of the axes in an orthogonal coordinate system.

2. The sample is oriented parallel to the direction of the luminous flux

The equations corresponding to (1) and (2) of the above treated method should in this case be

$$\Delta\varepsilon_{\text{long}}(f=1)=0 \quad (7)$$

$$\Delta\varepsilon_{\text{perp}}(f=1)=\frac{2}{3}\Delta\varepsilon_{\text{perp}}(f=0) \quad (8)$$

The general case of an electric transition dipole directed at an angle α from the long-axis of the ellipsoidal molecule ($0 < \alpha < 90^\circ$; cf. Ref.⁹) and with a degree of orientation, f (defined as before), with respect to the direction of the incident light is represented by the following expression

$$\Delta\varepsilon(\alpha, f) = \Delta\varepsilon(\alpha, f=0)[\frac{2}{3}f \sin^2\alpha + (1-f)] \quad (9)$$

(With α equal to 0° and 90° we obtain the relations 7 and 8, respectively.)*

This method has been applied in a CD study of streaming solutions of poly- α -L-glutamic acid (PGA) containing acridine orange (AO). As the optic axis, equal to the direction of orientation, should be expected to be parallel to the flow lines and thus parallel to the direction of the incident light, there should not be any error due to double refraction. However, experiments with different flow-cells followed by a CD standard (cf. Method 1) showed that in many cases serious double refraction was present, probably caused by traces of perpendicular flow at the out- and inlet of the cell. However, a cell where the fluid entered symmetrically from a core was considered satisfactory.

EXPERIMENTAL

Method 1. The experimental technique of preparing and stretching polyethylene films containing dissolved species has been described elsewhere.¹⁴ The biaxial stretching was performed in a stretching arrangement consisting of two orthogonally directed stretching devices for uniaxial stretching (a cross of four screws). The film was first stretched in one direction (two screws) with a stretch factor which did not make the apparent CD exceed the first minimum according to Fig. 2 (e.g. 1.1). Then a perpendicular stress was applied until the apparent CD had returned to the original deflection. Then the film could be stretched a little in the first direction again, and so on. By carefully checking that the birefringence did not pass the value with a phase difference of π one could be sure of no birefringence when the apparent CD of the standard was back at the origin (provided that this correction was performed in a wavelength region free of CD from the sample in the film). The best result, judging from shapes of CD standard spectra, was obtained if the biaxial stretching was performed by a high number of very small orthogonal stress contributions. (A device for *simultaneous* stretching along the two axes and measuring apparent CD is under construction).

* The crudeness of our approximation should be stressed once again. Eqns. (6) and (9) imply that the CD of a random solution should equal the arithmetic mean of the CD viewed down the long-axis ($\Delta\varepsilon_{11}$) and those for the light propagating perpendicular to it ($\Delta\varepsilon_{22}$, $\Delta\varepsilon_{33}$): $\Delta\varepsilon_{\text{rand}} = \frac{1}{3}(\Delta\varepsilon_{11} + \Delta\varepsilon_{22} + \Delta\varepsilon_{33})$. Though *this* should be always true the inefficiency of (6) and (9) is obvious e.g. from the experimentally and theoretically proven fact that $\Delta\varepsilon_{22} = \Delta\varepsilon_{33} = 0$ for DNA but $\Delta\varepsilon_{11} \neq 0$.⁴⁷ According to our approximation, this should require two CD components with opposite signs being perpendicular to each other, $\Delta\varepsilon_{22} = \Delta\varepsilon_{33} = \Delta\varepsilon_{\text{long}}(f=1) + \Delta\varepsilon_{\text{perp}}(f=1) = 0$ (eqns. (1), (2)) and $\Delta\varepsilon_{11} = \Delta\varepsilon_{\text{perp}}(f=1) \neq 0$, (eqns. (7), (8)). This seems incompatible with the LD studies⁴⁷ but might function as a working model.

To obtain more precise measurements it was found necessary to perform the stretch adjustments with CD standards at different wavelengths ((+)Co(en)₃(ClO₄)₃ at 490 nm, *epi*-androsteron at 304 nm). By this means wrong adjustments due to tilted baseline were avoided (*cf.* Ref. 3).

The steroids used were of *pro analysi* purity (Merck Biochemica or Koch Light). They were immersed into polyethylene films *via* concentrated chloroform solutions at 62°C (24 h). The CD standards are described elsewhere ((+)Co(en)₃(ClO₄)₃²³ *epi*-androsteron = 3β-hydroxy-5α-androstan-17-one⁹).

Experiments with 2,2',4,4'-tetrabromo-3,3'-bithienyl did not reveal any orientation effect, which was not either expected,⁹ but an interesting effect was observed, *viz.* a reversal of sign of the long wavelength CD band upon aging the film. This effect was observed with both enantiomers and must be explained by a conformational change^{9,29} at the transition from the solution state in the film to a crystal-like state on the surface of the film (actually a powder could finally be observed on the surface).

Method 2. The flow experiment was performed by pumping the solution from an open deposition flask through the flow cell and back to the flask. The pump was a cog-wheel pump made of stainless steel. By modulating its speed, flow rates, 0–15 ml sec⁻¹, could be produced. The optical cell consisted of a 4 cm long tube of perspex with quartz windows at the ends. The outlet and inlet were both circular cores feeding the cell through a small circular slit between the perspex wall and the quartz window. In this way the fluid entered symmetrically (checked by using unmixed dyed solutions) from the core and was therefore considered not to give rise to birefringence. The total volume of the flow system (pump, tubings and cell) was only 9 ml.

The extraordinary effect of gelatine on the orientation effect was discovered by accident. Earlier attempts with more diluted solutions had been unsuccessful due to precipitation. The solution was prepared from solid gelatine (Merck, *p.a.*) which was heated together with an equal amount of water, diluted to a concentration of 20 % with cold water. 3–5 ml of this solution was added to 30 ml of 1 % PGA (aged 2 days at +4°C), *x* M AO solution (pH = 4.5) during vigorous stirring.

The PGA solution was prepared as follows. 0.30 g PGA sodium salt (Pilot Chemicals) was dissolved in water (10 ml) during vigorous stirring. 0.10 M HCl was added (very slowly) until pH = 4.5 (about 14 ml and in one hour). 0.5 ml 2.00 mM pure AOCl¹⁵ was the added.

The most critical step appears to be the mixing with gelatine. Due to lack of PGA it has not been possible systematically to investigate optimal conditions. We can only state that the result was capricious and the solution sometimes destroyed by precipitation.

EXPERIMENTAL RESULTS

Fig. 2 depicts a typical result from measurements of the apparent CD of a (+)Co(en)₃³⁺ solution when the light has first passed a polyethylene film. In the unstretched state the birefringence of the film is obviously of practically no importance. That this was not due to a chance phase lag of 2π (*cf.* Fig. 1) was checked by using other thicknesses and films. The fact was also in accordance with the observation that the function $R_D = A(//)/A(\perp)$ could be extrapolated to 1.0 at a stretch factor (Fig. 2) of 1.00 at linear dichroism measurements on biaryls in polyethylene films.⁹

The decrease in apparent CD and the change in sign was expected from the discussion of Method 1 (Fig. 1). The peak at stretch factor 1.2 obviously corresponds to a birefringence causing a phase difference of π (when right circularly polarized light enters the film, left circularly polarized light will exit, *etc.*). This fact, and the high precision of the measurement, might suggest a method for studying polymer species in stress.

With higher stretch factors, the orientation further increases (at the value

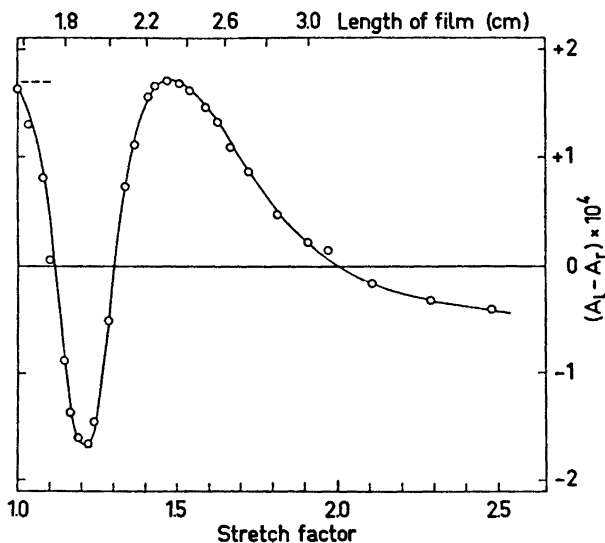


Fig. 2. Apparent CD of a (+)Co(en)₃(ClO₄)₃ solution preceded in the light path by a polyethylene film which was uniaxially stretched. The *stretch factor* is the ratio between the length of the stretched film and its length in the unstretched state. (--- denotes the CD without film.) $\lambda = 490$ nm.

1.5, the phase difference is 2π), but finally a maximum orientation (and birefringence) is reached (at about 2). The decay at higher stretch factor values is not only caused by this but also by the successively decreasing thickness of the film (*cf.* Fig. 1). (The film to which Fig. 2 refers was at stretch factor 1, 0.030 mm thick, and at 2.5, 0.021 mm.)

Table 1. CD changes upon biaxial stretching of films containing steroids. Stretch factors:^a 1.2–1.3. Molar absorption coefficients (ϵ) and molar circular dichroisms ($\Delta\epsilon$) of the solutions (cyclohexane). Corresponding wavelengths (nm) in parenthesis.

Sample	ϵ_{\max} , solution (M ⁻¹ cm ⁻¹)	$\Delta\epsilon_{\max}$, solution (M ⁻¹ cm ⁻¹)	CD(stretched film) CD(unstretched film)
Ergosterol	1.1×10^4 (282)	-17.3 (270) + 9.6 (220)	0.5 ± 0.5 10 ± 5
5- α -Cholestan-3-one	20 (289)	+ 0.94 (296)	1.0
5- α -Pregnan-3,20-dione	48 (290)	- 0.01 (340) + 3.72 (287)	- 0.8
5- β -Pregnan-3,20-dione	50 (288)	- 0.02 (340) + 2.80 (297)	- 0.7
Pregn-4-ene-3,20-dione	39 (288)	- 1.44 (338) + 3.12 (296)	0.5 0.9
Androst-4-ene-3,17-dione	39 (288)	- 1.32 (338) + 2.77 (297)	0 0.9
Androsta-1,4-diene-3,17-dione	38 (288)	- 0.32 (354) + 4.01 (306)	- 0.7

In Table 1 some results from the application of Method 1 are collected, which are of interest in connection with the rotational strengths of the $n \rightarrow \pi^*$ carbonyl transitions or $\pi \rightarrow \pi^*$ transitions in some steroids. The most striking feature of Table 1 is the generally small change in CD when stretching a polyethylene film containing the steroid (except in the cases of ergosterol and pregn-4-ene-3,20-dione). This fact indicates, if we assume that orientation takes place, that the apparent CD bands in general fairly well represent the CD of the respective component transitions¹ (*cf.* Discussion). This means that the dissymmetry factor, $^{16}g \equiv \Delta\epsilon/\epsilon$, reflects the relative proportion between the rotational strength and the dipole strength and can thus, *e.g.*, indicate if the transition in question is electrically or magnetically forbidden.¹⁷

The result should be treated with some care, however, as the CD changes are related to the CD of the unstretched film and when possible to another CD band (with opposite effect). Due to low molar absorption (except for ergosterol) the absorption spectra could not be used for determination of concentration. With 5- α -pregnan-3,20-dione, *e.g.*, the apparent decrease of the positive 287 nm band thus may be due to a compensating increase of the adjacent negative band.

Ergosterol takes up a special position. Firstly, the hydroxy group makes the solubility in polyethylene rather low (10^{-2} M compared with 10^{-1} M for the

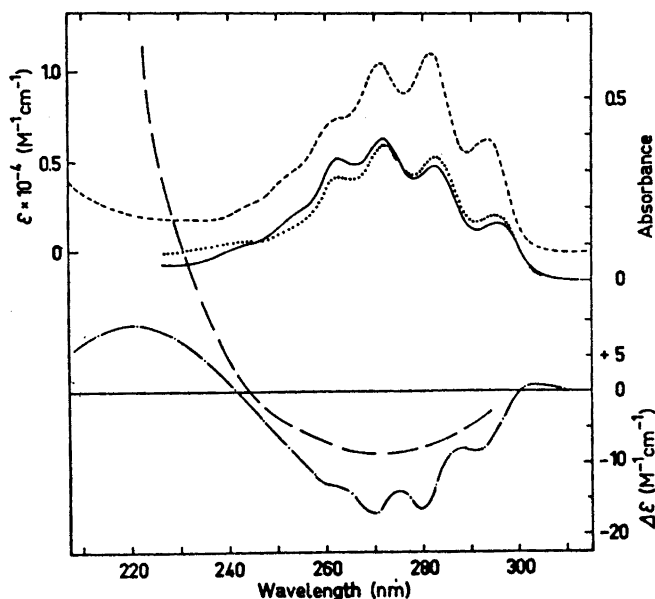


Fig. 3. Ergosterol. Above: Absorption spectrum of solution, left scale (cyclohexane, ethanol, or isooctane). Middle: Linearly polarized spectra in stretched polyethylene film (stretch factor $R=1.50$) (\dots , \parallel , *i.e.* electric vector of light parallel to the direction of orientation; — , \perp , *i.e.* perpendicular). Below: Circular dichroism of solution ($-\cdot-$), (cyclohexane, ethanol or isooctanol) and in oriented film ($-\text{—}$), biaxial stretching, stretch factors 1.2–1.3, see Table 1.

other steroids, judging from the absorbances or the circular dichroisms of the films). Secondly, ergosterol exhibits a high molar absorption (due to $\pi \rightarrow \pi^*$ transitions) which made it possible despite the low solubility even to register a distinct *linearly polarized* absorption spectrum (Fig. 3). The effect, however, of low values of $\Delta\epsilon/\epsilon$ was to increase the uncertainty of the polarized CD determination (Table 1, Fig. 3). Also with a very careful adjustment to zero birefringence in the film, a certain degree of linear dichroism remained, causing the characteristic "oriented dye artefact".¹ However, relying upon the rule of a zero sum artefact (see the introduction) the CD has been obtained as the mean of the CD's registered at different, equally distributed positions of the film around a circle in the plane perpendicular to the incident ray of light.

Fig. 3 indicates that the CD band at 220 nm is the residue of two transitions, to a high extent cancelling each others CD, one polarized parallel to the molecular long axis,* with positive rotational strength, and one perpendicularly polarized, with negative rotational strength. A comparison between the polarized absorption spectra and the solution CD spectrum reveals two transitions at about 295 nm, one intensive with negative CD and parallel polarization, and one smaller with positive CD and perpendicular polarization. Then one band (282 nm) follows which has parallel polarization. The low accuracy in polarized CD in this region (Table 1) and the fact that the strong CD band at short wavelengths may interfere make us neglect the apparent CD polarization in the 250–300 nm range. At 253, 262, and 272 nm three perpendicularly polarized bands appear. They have probably all negative rotational strengths.

The change in intensities between the two absorption bands at 272 and 282 nm when passing from ordinary solution (ethanol, cyclohexane or isooctane) to the random state in a polyethylene film ($[A(\parallel) + 2A(\perp)]/3$ or absorbance of an unstretched film) indicates some sort of "solvent" effect in polyethylene.

The induced CD of the $\pi \rightarrow \pi^*$ transitions in acridine orange^{20,21} (AO) in the presence of α -helical L-glutamic acid (PGA) was studied under flow conditions. The result is represented in Fig. 4. The random spectrum exhibits three clear Cotton effects, the rotational strengths of which, however, are a complicated function of the concentrations of PGA and AO and of the ionic strength.⁴⁶ Anyhow, the effects of flow on the different CD bands seem at least from a qualitative point of view to be independent of variations in concentrations of PGA and AO. (However, the magnitudes of the flow effects are higher in aged solutions.) Thus the effect of flow parallel to the incident light causes an increase of the first, an increase of the second, and a decrease of the third CD band, respectively, the bands in order of increasing energy. The fact that the first two bands are those dominating at low AO concentrations and the fact that the $\pi \rightarrow \pi^*$ transitions of AO are polarized parallel to the long axis⁸ and in the molecular plane of the dye can help us to make conclusions about the structure of the complex between PGA and AO (the induced optical activity in the dye proves the existence of such a complex). As the poly-peptide will be oriented with its long axis (helix-axis) along the flow lines and the CD bands, for which the $\pi \rightarrow \pi^*$ transitions were responsible, increased, the dye molecules

* For description of structures of steroidal skeletons, see Refs. 13, 18 and 19

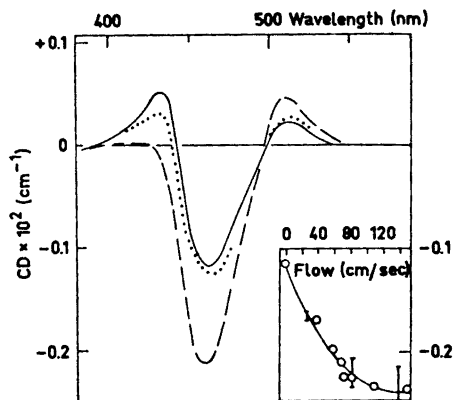


Fig. 4. The flow experiment with polyglutamic acid (PGA) and acridine orange (AO). Solution (1 % PGA, 4×10^{-5} M AO) at rest (*i.e.* random —) and in flow parallel to the incident light (70 cm sec⁻¹, . . .). With 2.7 % gelatin the effect of the flow was strongly increased (70 cm sec⁻¹, - -). The dependence of CD (468 nm) on the flow rate is also illustrated.

must be bonded preferentially with their planes perpendicular to the helix axis. The third positive (long-axis polarized) CD band almost vanishes in the oriented condition indicating a very high degree of orientation (*eqn.* (7)) and the increments of the two perpendicularly polarized bands are obviously larger than to 3/2 of the random values. This may seem dubious but can be explained by the fact that the CD bands overlap. Thus, an apparent increase in CD can partly be referred to a corresponding decrease in intensity of a band with oppositely directed rotational strength. Fig. 4 also depicts a typical dependence of CD on flow rate. It seems interesting that the effect still increases at rather high rates (80 cm sec⁻¹) because flow-pressure studies⁸ indicate turbulent flow and the orientation effect might be expected to decrease.

DISCUSSION

The interest in polarized CD spectra of steroids is closely related to questions about the characters of the studied transitions.¹⁷ Thus, the dissymmetry factors for the *resolved* bands can show if the transitions are, *e.g.*, magnetic dipole allowed.^{17,22} Knowledge about the transitions can then be used to explain or predict the influence of certain substituents on the spectral features of the molecule.²³

The original interest, however, was concentrated to the question if dihedral ketones might exhibit superposition effects similar to those observed in transition complexes with D_3 symmetry²⁴ (*i.e.*, of two orthogonally polarized transitions with opposite rotational strengths and with very low energy separation between). In such cases departures from current rules relating apparent rotatory power to chirality might be expected.²⁵ Actually, strongly solvent- and temperature-dependent CD bands of certain cyclohexanone derivatives have been explained by the superposition of two large CD bands at approximately the same wavelength but with different signs.⁴⁴ However, the steroids available for the present study all have very low symmetry. This is, *e.g.*,

reflected in the fact that in almost all cases the CD band and the corresponding absorption band have their maxima at about the same wavelength (*i.e.* do not originate from split bands). It is therefore not surprising that no "superposition effects" have been detected for such bands.

A more precise analysis of the result from the flow experiments with PGA : AO is impossible as the solutions can contain many species with different ratios of dye/peptide. Furthermore the dye is probably present partly in dimer form, the distribution between monomer and dimer being determined (at least to some extent) by the total dye concentration and the electrolyte content in the solution.^{15,26,27} (The interaction between pure acridine orange and chloride has been studied in this laboratory. It was shown that the chloride does not participate in the dimerization.¹⁵) It is rather clear, however, that the absorption bands at 490 and 460 nm can be associated with the monomer and dimer, respectively. The CD bands at 510 and 465 nm (which dominate at lower concentration ratios AO : PGA^{20,21}) may thus originate from complexes with those two species. We have observed that the "dimer" band is considerably increased when adding PGA, thus suggesting a preferential stabilization of the dimer in the PGA : AO complex.⁸ Stryer and Blout²¹ considered three possible models to explain their results with inducing optical activity into dyes. Two models assumed helical arrangements of the dye molecules, one with a super helix around the peptide helix (Model II) and one in which the dye molecules formed a "tangential" helix (Model III).²¹ One model (Model I) had the monomeric dye bound near the asymmetric α -carbon atom.

Because of the total lack of examples of induced CD according to Model I for monomeric systems (in our laboratory we have, *e.g.*, investigated solutions of AO and *mono-L*-glutamate⁻) we feel inclined to rule it out. Model III is improbable, as the only polymer form so far known of acridine orange in solution is the dimer.* The result from our flow experiment also makes this model impossible as the planes of the dye molecules could not then have a common orientation. This leaves the model with a super helix.

It is difficult to interpret the signs of the CD bands in terms of absolute configuration of the complex(es). Current theories, however, of the optical activity of helical polymers distinguish two types of interaction between the transition dipoles of monomeric chromophores in a helical array:²⁸ one associated only with resultant electronic transitions polarized perpendicular to the helix axis ("pitch-dependent") and one with parallel and perpendicular polarizations with opposite rotational strengths, respectively ("radius-dependent"). The two CD bands with lowest energy (465 and 510 nm) which were perpendicularly polarized can belong to both categories but the third (430 nm) parallel polarized must be of the "radius-dependent" kind. The signs of parallel and perpendicular components are obtained for this case by combining transition dipoles (on two adjacent chromophores) which are polarized tangentially and parallel to the helix axis.²⁸ It has been pointed out that the rotational strength of the parallel component will be positive if the array of

* With another dye, *viz.* 1,1'-diethyl-2,2'-cyanine, which we have studied in streaming solutions (CD) of tartrate,¹⁰ however, CD is produced by a helical arrangement²⁹ in a separate dye polymer molecule.³⁰

transition dipoles in the bound dye molecules forms a segment of a right-handed helix (and *vice versa*).^{31,32} Obviously such an arrangement is the case with the transition responsible for the third positive CD band (430 nm). As polypeptides derived from L-amino-acids form right-handed helices, this indicates an arrangement in the actual PGA : AO species (obtained with high AO concentration) with a right-handed super helix of dye molecules (the long-axes which contain the transition dipole⁸ should form a right-handed helix).⁴⁵

These questions have been discussed in detail by Ballard *et al.*⁴⁰ who have also studied CD under streaming condition of PGA : AO. Their result, however, does not at all agree with ours, a fact which with respect to the complicated nature of the solutions⁴¹⁻⁴³ and the possibilities of serious experimental error (due to birefringence) cannot be discussed here. (The reported CD changes had the same signs for all bands: positive for the case of PGA : AO⁴⁰ and in the corresponding case with DNA : AO, negative. Though this behaviour could be the result of a linear dichroism³⁰ we are not inclined to call it an artifact: In very diluted PGA : AO, 0.1 % PGA, we have in fact recorded traces of a reversed effect, of that in Fig. 4, without gelatine.) Our result also disagrees with that reported by Powers³³ from measurements of Kerr constants on solutions of PGA : AO (*cf.* Ref. 34).

There are several features of Method 2 that make it seem more favourable than Method 1. One is the fact that there will be a constant number of molecules in the light path independently of whether the system is oriented or not. The stretching procedure of a film should always decrease this number. The natural absence of double refraction is also very favourable. It might be suggested that the numerous hydrodynamic theories and orientation models that have been made for explaining streaming birefringence (for a review, see Ref. 37) could be important in this connection. As Peterlin^{35,36} points out, however, higher concentrations make the molecules intertwine and they will lose their individuality. It should therefore be necessary to perform measurements in a very wide range of concentration, at different flow gradients and using molecules with different sizes and to extrapolate to conditions where the proposed theories hold (infinite dilution).^{35,36} As the increased effect when adding gelatin probably is due just to this interaction between PGA molecules and gelatin molecules, the state of our system is indeed very unfavourable from a theoretical hydrodynamic point of view.

The possibilities of Method 2 are strongly limited in general application by the requirement of long macro-molecules. The effect of gelatine, however, suggests a method using an orienting macromolecular medium. Attempts with the strong³⁸ complex (+)Co(en)₃EDTA⁺ in a concentrated gelatine solution have not revealed any orientation of the outer sphere complex,⁸ but we hope to be able to perform experiments with other (longer) counter-ions. In this way it could be possible to obtain information about the steric arrangement in the outer sphere complex in water solution which may be compared with the results from outer sphere studies in less polar media.^{1,2}

Of the two "sub-methods" of Method 1 the one using biaxial stretching appears very favourable. The uniaxial orientation has one disadvantage: As the standard CD inspection must be performed at a wavelength far from the CD bands of interest of the sample in film, there may be con-

siderable birefringence at the wavelengths of these bands. With the method of biaxial stretching, however, the birefringence should be zero at all wavelengths.

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