233. Temperature and Concentration Dependent Circular Dichroism of Mono- and Di-*cis* Isomers of (3*S*,3′*S*)-Astaxanthin Diacetate

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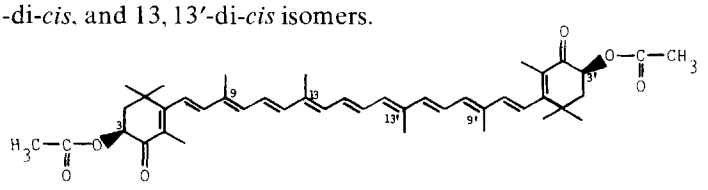
Summary

The circular dichroism (CD.) spectra of all-*trans*-(3*S*,3′*S*) astaxanthin diacetate and its 9-*cis*, 13-*cis*, 9,9′-di-*cis*, 9,13′-di-*cis*, and 9,13-di-*cis* isomers conform to the rules previously formulated for optically active carotenoids with a 4-oxo-β-end ring containing an asymmetric C-atom [1]. Thus the CD. bands of the all-*trans* and the di-*cis* isomers show the same signs whereas those of the mono-*cis* isomers have opposite signs. The CD. spectra of all the astaxanthin diacetate isomers invert sign upon cooling to −180°. The CD. spectra of the 9-mono-*cis* and 9,9′-di-*cis* isomers and to a lesser extent also those of the 9,13′-di-*cis* and 9,13-di-*cis* isomers are concentration dependent at −180°, with the longest wavelength band giving at the higher concentration a bisignate CD. curve under the main absorption characteristic of aggregation. This phenomenon has been observed only in isomers with a 9-*cis* linkage. It is suggested that steric hindrance prevents such aggregation taking place in the other isomers.

Introduction. – The stereoisomers of carotenoids which are optically active by virtue of a twist of the formally conjugated end-ring against the plane of the chain give CD. spectra which are related to one another in a characteristic manner [1–4]. The spectra of the mono-*cis* isomers are approximate mirror images of those of their all-*trans* counterparts whereas the di-*cis* isomers have spectra similar to those of the all-*trans* form. Furthermore in compounds with the 4-oxo-β-end group such as astaxanthin there is an inversion of sign of all the CD. bands upon cooling to −180°. This has been ascribed to temperature-dependent equilibria [1].

We have extended this study to include the temperature and concentration dependence of the CD. and absorption spectra of a series of mono-*cis* and di-*cis*-(3*S*,3′*S*)-astaxanthin diacetates, namely the all-*trans*, 9-*cis*, 13-*cis*, 9,9′-di-*cis*, 9,13′-di-*cis*, 9,13-di-*cis*, and 13,13′-di-*cis* isomers.



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Results. – The CD. and absorption spectra at $+20°$ and $-180°$ are shown in *Figures 1–7*. As in the case of all-*trans*-astaxanthin and other carotenoids whose optical activity is due to an asymmetric C(3) [1], there is a strong temperature-dependence of the CD. spectra of the astaxanthin diacetates. They invert sign upon cooling, as do the spectra of other 3-hydroxy-4-oxo-carotenoids. It has been shown [1], our rule 1, that the maxima in the CD. spectra of all-*trans* and di-*cis* isomers are opposite in sign to those of the mono-*cis* isomers of the same carotenoid. This rule is obeyed almost perfectly by the compounds in this study. This rule did not, however, specify the relative intensities of peaks within a spectrum, and the present spectra show considerable differences between compounds which will be discussed later.

A striking new observation is the strong concentration-dependence of the CD. of some of the compounds, especially in the region of the lowest-energy band *(Fig. 8* and *9)*. At the concentrations normally employed (0.05 to 0.09 mg/ml, 73 to 132 μM) all 9-*cis* compounds show at $-180°$ strong CD. maxima with pronounced structure and a change in sign of the signal across the region of the lowest energy absorption band ("bisignate CD. curve" [5]). (Note the $\Delta\varepsilon$ scale in *Fig. 8* and *9*). The positive and negative CD. maxima do not coincide with the maxima of the vibrational components in the absorption spectra at the same temperature. Upon dilution, the intensity in the CD. decreases sharply under the lowest-energy band, although the effect is much less in the other parts of the spectra. In very dilute solution (3 to 7 μM) the intensities of the lowest-energy band are weak and the frequencies of the fine-structure components are coincident with the absorption frequencies. This concentration dependence seems to be restricted to 9-*cis* isomers and is only observed at low temperature (between -150 and $-180°$). The 13-mono-*cis* and 13,13′-di-*cis* isomers do not show these effects. There is no concentration dependence of the $\Delta\varepsilon$ values between 90 μM and 10 μM and good coincidence of absorption and CD. maxima at $-180°$. At RT. the $\Delta\varepsilon$ values of all compounds are concentration-

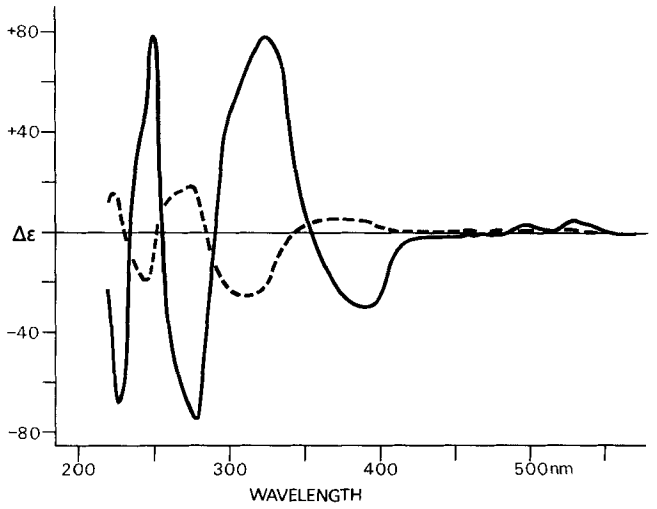

Fig. 1. *CD. of all*-trans-*(3S,3′S)-astaxanthin diacetate*, 0.095 mg/ml (140 μM); - - - - - + 20°, ——— – 180°

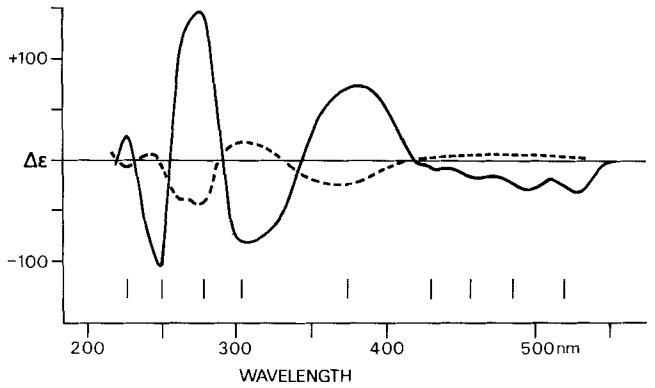

Fig. 2. *CD. of 9-cis-(3S,3'S)-astaxanthin diacetate,* 0.0049 mg/ml (7.2 μM); ----- +20°, ——— −180° (bars indicate positions of absorption maxima or shoulders at −180°)

independent (up to *ca.* 100 μM) and the fine-structure components of absorption and CD., where they are resolved, coincide. Higher concentrations could not be measured on account of the very strong absorption and the limitations in cell-length.

With the exception of 13,13'-di-*cis*-astaxanthin diacetate all isomers show a regular alternation of the sign of the CD. signals in progressing from low to high energy. A similar result has been observed for the carotenoids with an asymmetric center at C(3) [1]. The CD. of 13,13'-di-*cis*-astaxanthin diacetate under the low-energy absorption band has the opposite sign to that in the other di-*cis* compounds. The 9,9'-di-*cis* isomer shows a weak bisignate CD. under the low-energy band even at the extreme dilution of 2.5 μM (see *Fig. 4* and *9*).

In all of the compounds some of the CD. bands which are rather broad at RT. show distinct fine structure at low temperature. This behaviour is especially marked for the lowest-energy band, and for the "*cis*"-band. The spacing of the components

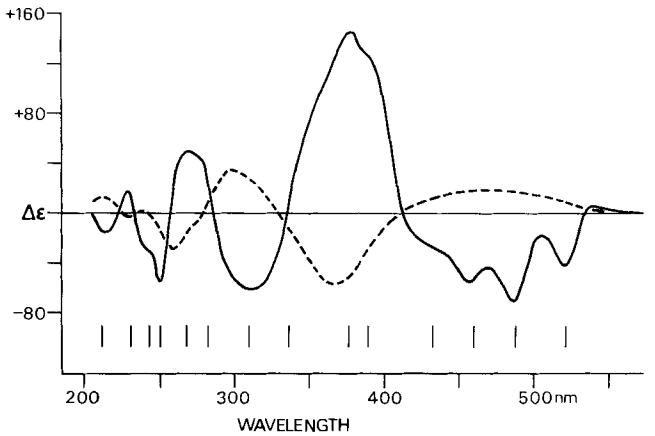

Fig. 3. *CD. of 13-cis-(3S,3'S)-astaxanthin diacetate,* 0.064 mg/ml (94 μM); -----+20°, ——— −180° (bars indicate positions of absorption maxima or shoulders at −180°)

corresponds roughly to a vibration of $\tilde{\nu} \sim 1300$ cm^{-1}. The determination of the spacings is not very accurate where the components are only resolved as shoulders.

The CD. spectrum of the 13-mono-*cis* isomer is especially striking *(Fig. 3)*. The $\Delta\varepsilon$ values between 400 and 500 nm (region of the lowest-energy absorption-band) and also at the wavelength of the so-called *cis*-band at 370 nm are considerably higher than in the other isomers. We checked that the $\Delta\varepsilon$ values are independent of concentration over the range from 16 to 94 μM. The vibrational structure of these two bands is also pronounced at low temperature. It may be that the 13-*cis* isomer forms a rather narrower range of conformers about the C(6),C(7)-single bond [1]. If one conformer predominates the CD. will be more intense and vibrational fine structure may become better resolved.

The CD. spectra of the 9-mono-*cis* and 9,9′-di-*cis* isomers and to a lesser extent also those of the 9,13′-di-*cis* and 9,13-di-*cis* isomers are concentration-dependent at $-180°$, with the longest-wavelength band giving at the higher concentration a bisignate CD. curve under the main absorption characteristic of aggregation. This phenomenon has been observed only in isomers with a 9-*cis* linkage. It is suggested that steric hindrance prevents such aggregation taking place in the other isomers.

Discussion. – The CD. spectra of the all-*trans*, mono-*cis* and di-*cis* isomers of (3*S*,3′*S*)-astaxanthin diacetate provide an excellent illustration of the operation of our rules [1], governing the CD. spectra of cartenoids with an asymmetric carbon atom in the ionone ring. The CD. spectra of the 13-*cis*, 9,13′-di-*cis*, and 9,13-di-*cis* conform well. However, the 13,13′-di-*cis* isomer shows a spectrum with a low-energy band of the same sign as that of the mono-*cis* compound. In addition the band at ~250 nm is weak. In other respects the spectra of this isomer conform to the rules. The 9-*cis* and 9,9′-di-*cis* isomers give the expected spectra both at RT. and at high dilution at $-180°$. However, at $-180°$ and concentrations of 0.1 to 0.03 mg/ml

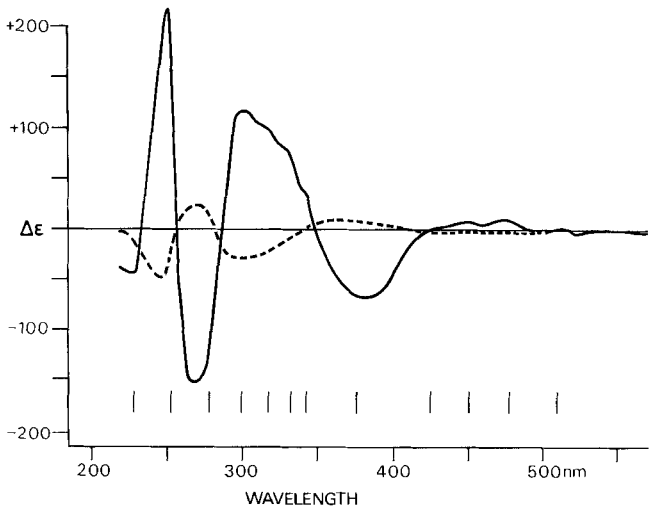

Fig. 4. *CD. of 9,9′-di-*cis*-(3S,3′S)-astaxanthin diacetate at high dilution,* 0.0016 mg/ml (2.4 μM); ----- +20°, ——— −180° (bars indicate positions of absorption maxima or shoulders at −180°)

which are normally employed, a different type of spectrum arises. We defer discussion of this until later in the account.

We have argued [1] that the CD. spectrum is that of an inherently chiral chromophore and that this chirality is due to steric hindrance of the planar ring-chain configuration relieved by rotation around the C(6),C(7)-bond and thereby resulting in a conformation in which the end-rings are in a distorted *s-cis* relationship with the chain. Two such conformations of slightly different energy are possible with the ring "up" or "down". In carotenoids without asymmetric C-atoms one form is converted into its mirror image of identical energy by **simultaneous** rotation and ring inversion. In this case one has a (racemic) mixture of rapidly interconverting chiral molecules. When there is a substituent at either C(2) and/or C(3) the two ring conformations have different energies. The substituent is equatorial in one and axial in the other form. Therefore the two twist forms around the C(6),C(7)-bond become energetically different and hence in the equilibrium mixture one of the two forms predominates and a strongly temperature-dependent CD. is observed. This can apparently even lead to a change of sign upon cooling. It has been shown [1] [4] that the inversion of sign upon introduction of one *cis*-bond follows logically from the accompanying change of symmetry.

The phenomenon discovered in this work arises for the astaxanthin diacetates with 9-*cis* and 9,9'-di-*cis* configurations. At the normally employed concentrations of about 0.06 mg/ml (*ca.* 90 μM) the 9,9'-di-*cis* and 9-mono-*cis* isomers exhibit at low temperature (– 180°) a surprisingly strong CD. under the lowest-energy absorption-band with very pronounced splitting and change of sign of the components within the band (bisignate CD.). The CD. maxima and minima do not coincide with the peaks in the absorption at the same temperature (see *Fig. 8* and *9*). Upon dilution the $\Delta\varepsilon$ decreases and is then comparable with the values observed in the all-*trans* isomer. Although this phenomenon is very pronounced in the 9-mono-*cis* and 9,9'-di-*cis* isomers, a lesser concentration-dependence can also be detected in the 9,13-di-*cis* and 9,13'-di-*cis* isomers. No concentration dependence is observed in the all-*trans*, 13,13'-di-*cis* and 13-mono-*cis* isomers.

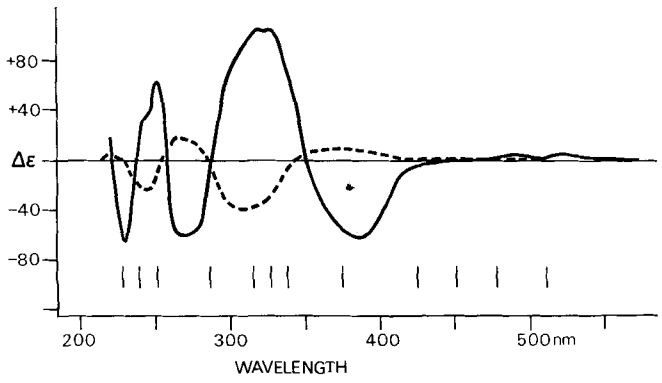

Fig. 5. *CD. of 9,13'-di-*cis*-(3S,3'S)-astaxanthin diacetate,* 0.0023 mg/ml (3.4 μM); – – – – – + 20°, ——— – 180° (bars indicate positions of absorption maxima or shoulders at – 180°)

The form of the CD. spectrum under the longest-wavelength absorption band is typical of that expected for an aggregated chromophore in which a resonance-excitation interaction (or exciton interaction) between identical monomers occurs [6]. The optical properties of the aggregate depend upon the optical properties of the monomer and the geometry of the aggregate. If the number of monomers in the aggregate is known it is in principle possible to deduce the geometry of the oligomer from the form of the absorption and CD. spectrum of the aggregate. However, in this case we have no evidence about the size or uniformity of size of the oligomers in the glassy solution. It may in future be possible to obtain an estimate of this by studying the intensity of the CD. spectrum as a function of monomer concentration.

However, it is possible to make some qualitative observations about the nature of the effect. A degenerate exciton interaction between two or more identical chromophores results in both in a shift of the centre of gravity of the excited state and a splitting of the excited states of the aggregate. In the present case the low-temperature absorption spectra of the aggregated species are virtually indistinguishable from those of the monomer species. This is clear from a comparison of the low-temperature absorption spectra of the concentrated and dilute solution of the 9-*cis* and 9,9'-di-*cis* isomers (*Fig. 2* and *8*, and *Fig. 4* and *9*, respectively). Therefore we conclude that the exciton shift and splitting is less than the width of one vibronic component of the absorption envelope, *i.e.* $< 600\ cm^{-1}$. The splitting is, however, revealed in the CD. spectra (*Fig. 8* and *9*), because the components of an exciton couplet are oppositely circularly polarized. Hence, although the exciton splitting is not resolved in the absorption spectrum it is observed in the CD. spectrum. If the exciton splitting is less than the width of one vibrational component of the absorption envelope this corresponds to the so-called weak coupling limit [7]. In this case a bisignate CD. couplet should be observed under each vibronic peak. Convolution of a progression of couplets will lead to a curve of the type seen in *Figures 8* and *9*. Each couplet should cross the base-line at the same wavelength as the absorption maximum corresponding to it. This accounts for the displacement of the CD. maxima and minima to either side of the absorption maxima seen in *Figures 8* and

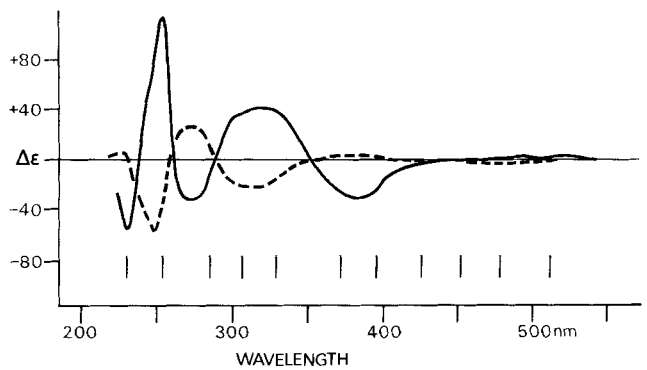

Fig. 6. *CD. of 9,13-di-*cis*-(3S, 3'S)-astaxanthin diacetate*, 0.026 mg/ml (38 μM); - - - - - + 20°, ——— – 180° (bars indicate positions of absorption maxima or shoulders at – 180°)

9. It will be of great interest to repeat these measurements at ultra-low temperatures, say at 4.2 K, in order to see whether individual exciton couplets can be completely resolved from one another. At this stage of the experiments we tentatively conclude that the exciton splitting is $< 600\ cm^{-1}$, smaller than the vibronic progression of the main absorption band. A curve fitting procedure is being undertaken to obtain a more accurate estimate.

Although the excitonic contribution to the CD. spectra of the 9-*cis* and 9,9′-di-*cis* isomers is very evident between 400 and 550 nm, the CD. bands at shorter wavelengths merely intensify somewhat as aggregation takes place and no bisignate CD. bands become apparent. There are likely two related reasons for this. The first is that the magnitude of the excitonic splitting is dependent upon the square of the transition dipole moment of the monomer absorption band. The shorter wavelength absorption bands of the carotenoid chain are considerably weaker than those of the longest-wavelength band. Therefore the excitonic splitting will be proportionately less and the degenerate-exciton contribution to the CD. spectrum rather weak. The second point is that the CD. signals of the monomer itself at wavelengths shorter than 400 nm are relatively intense. Therefore a weak excitonic contribution may go undetected. There may, in addition, be other reasons for the weakness of the degenerate excitonic contribution related to the polarisations of the transitions and perhaps to the nature of the vibronic envelope of the absorption bands.

The occurrence of aggregation appears to be critically controlled by the stereoisomeric form of the carotenoid chain. The 9-*cis* and 9,9′-di-*cis* configurations seem to be a necessary condition. The reasons for this rather striking selectivity are not clear since the geometry and size of the aggregates have not been determined. However, the forces which hold the aggregates together must be rather weak because the phenomenon is observed only at low temperature. At RT. no concentra-

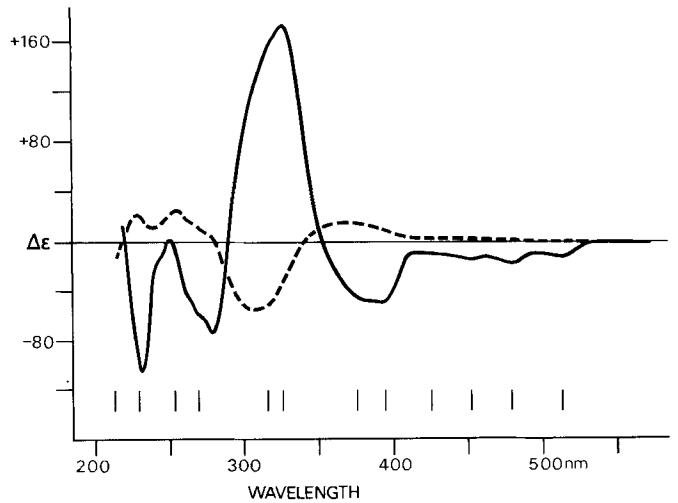

Fig. 7. *CD. of 13,13′-di*-cis-*(3S,3′S)-astaxanthin diacetate*, 0.062 mg/ml (91 μM); – – – – – + 20°, ——— – 180° (bars indicate positions of absorption maxima or shoulders at – 180°)

tion dependence of the $\Delta\varepsilon$ values has been detected in any of the carotenoids examined. There is a dependence of aggregation upon the nature of the end-group. For example, in the spectra of 9,9′-di-*cis*-zeaxanthin at $c = 100$ μM there is good coincidence of absorption and CD. peaks at low temperature and no bisignate bands appear [8]. Thus the phenomenon appears to be unique to astaxanthin diacetates although we have been unable, because of the unavailability of materials, to investigate whether free 9-*cis*, 9,9′-di-*cis*-astaxanthin or 9,9′-di-*cis*-zeaxanthin diacetate shows the same effect.

It is difficult to see how interaction between end-groups on different molecules could be responsible for aggregation. Hydrogen-bonding is not possible between ester acetate groups nor is the chain highly polar. We favour the idea that aggregation is a consequence of interaction between the π-electrons of the polyene chains. It is invariably observed that, in the crystalline state, carotenoid molecules pack with parallel π-chains separated only by *van der Waals* contact between the polyene-chain methyl groups [9]. However, in order to obtain oligomerization in solution it will be necessary for as long a portion as possible of the π-chains to be in contact between interacting molecules. The ionone ring contains the rather bulky geminal dimethyl groups which protrude on either side of the plane of the polyene chain. Hence the end-rings may provide a steric hindrance to close approach and perfect overlap of the π-clouds of two chains placed adjacent. The 9-*cis* configurations place the ionone rings out of the line of the polyene chain. It may be that the 9-*cis* and 9,9′-di-*cis* conformations allow close approach of the π-clouds of the polyene chain without bringing the ionone rings into close contact with one another and with the chain of an adjacent molecule. Molecular models lend some support to this idea. However, it is not possible to make it more formal without knowledge of the oligomer size and geometry.

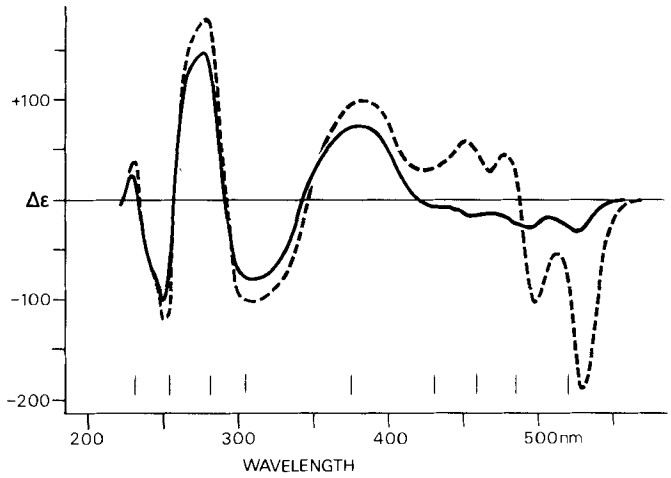

Fig. 8. *CD. of* 9-cis-*(3S, 3′S)-astaxanthin diacetate* at −180°, ——— 0.0049 mg/ml (7.2 μM); - - - - - 0.097 mg/ml (142 μM) (bars indicate positions of absorption maxima or shoulders)

Recently somewhat similar effects have been observed in the CD. spectrum of lutein [10]. When water is added to alcoholic solutions of lutein a drastic change of the CD. (**and** absorption) is observed when more than about 50% water is present. There are strong positive and negative CD. maxima in the visible region and no coincidence with absorption maxima. In this case, however, the CD. in the near UV. is also strongly affected. The authors state [11]: "The addition of water, from 0 to 100% *(v/v)* to ethanolic solutions of lutein (1–10 μM) gave rise to **homogeneous,** fluid and **clear** liquid mixtures" (our emphasis). They explain the spectral changes by an aggregation of lutein molecules. Since this could be related to our observations on the astaxanthin diacetate isomers, we repeated part of this work on lutein. It is especially important to establish whether the samples are true solutions. Therefore we prepared a solution of lutein in water/ethanol 3:2 *(v/v)*. This gave a CD. very similar to the one published [10]. This "solution" was filtered through a milliporefilter. A 1 μM pore size (PTEE, type FALPO 1300)-filter retained the "solute" completely, a colourless liquid passing through the filter. A 5 μM filter (PTFE, type LSNPO 1300) retained part of the suspended particles, the filtrate which was still coloured gave a CD. spectrum identical in form though weaker than the original mixture. Aggregates with a diameter of 1 μM contain about 10^9 molecules (assuming a density of 1 and a molecular weight of 600). Therefore we conclude that the results observed for lutein arise from the presence of microcrystals of carotenoid suspended in solution.

We consider it unlikely that the cooling of our astaxanthin diacetate solutions has, by reducing the solubility at low temperature, induced a similar aggregation or microcrystallization. It is known that *cis*-isomers invariably have a higher solubility than the *trans*-form [11] and the concentration-dependent CD. is observed in the *cis*-isomers, and not in the *trans*-astaxanthin diacetate. Furthermore, in the case of lutein, the UV. region of the CD. and the absorption spectrum are also affected. For the 9-*cis*-astaxanthin diacetates, a specific di- or oligomerization appears to be occurring.

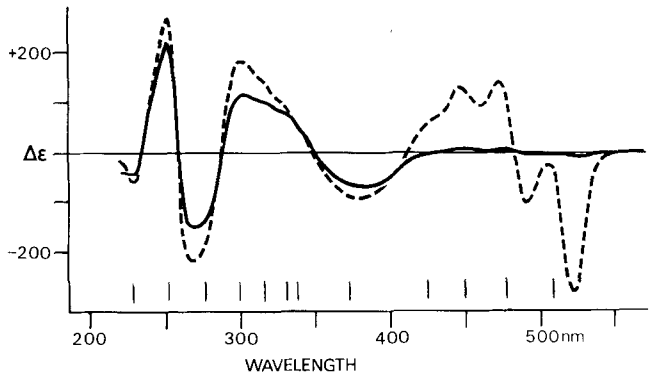

Fig. 9. *CD. of 9,9'-di-*cis*-(3S, 3'S)-astaxanthin diacetate* at −180°, ——— 0.0019 mg/ml (2.8 μM); - - - - - 0.059 mg/ml (87 μM) (bars indicate positions of absorption maxima or shoulders)

Experimental Part

The stereoisomers were separated by high pressure liquid chromatography from a mixture obtained by isomerization of (3*S*,3'*S*)-astaxanthin diacetate with I_2. The procedure was identical to that of [12] with the exception that optically active starting material was used instead of the racemate[1]). The structures were assigned by ^{1}H-NMR. spectroscopy[2]). Only in the case of the all-*trans* compound was the amount of sample sufficient for a quantitative absorption and CD. spectrum (on a solution of known concentration). All other isomers were assumed to have the same extinction coefficient ($\varepsilon \sim 163000$ M^{-1} cm^{-1}) as the all-*trans* form and the concentrations (and $\Delta\varepsilon$ values) calculated accordingly. The solvent was EPA (ether/isopentane/ethanol 5:5:2). The details for the measurements of the CD. spectra at different temperatures were as previously given [1].

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[2]) We thank Dr. *Vecchi* for the separation of the isomers, and Dr. *Englert* for the identification of the isomers.