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Daunomycin Inverts the Long-Range Chirality of DNA Condensed States†

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ABSTRACT: The effect of daunomycin upon DNA condensed states induced by poly(ethylene glycol) (PEG) was studied by circular dichroism (CD) and circular intensity differential scattering (CIDS). The CD spectra of these aggregates showed psi-type anomalies and intensities 10–100 times greater than those obtained with the dispersed DNA solutions in the absence of PEG. Increasing concentrations of daunomycin, added to the DNA solution prior to its aggregation, led, in the presence of PEG, to CD and CIDS signals which gradually decreased in magnitude and eventually inverted sign. The coincidence of the transition point of both signals and a careful characterization of the CD spectrum at the transition point clearly indicated that the inversion observed corresponds to an inversion of the handedness of the aggregates. The latter result suggests that the structure of the aggregates at the inversion point should resemble that of a nematic liquid-crystalline structure. The characteristic B-DNA spectrum obtained in this case further suggests that the packing process does not affect the secondary structure of the DNA molecules and that small changes in their local structure can induce dramatic changes in their long-range tertiary packing. The results obtained in this study represent a confirmation of a recent theory of psi-type CD in which the anomalous signals are interpreted as a manifestation of the long-range chirality of the aggregates.

In appropriate condensing agents, DNA molecules can condense and aggregate to form highly ordered structures which resemble in many ways those observed in vivo (Bouligand et al., 1968; Gourret, 1978; Brugerolles & Mignot, 1979; Livolant, 1984) and can be used as simplified models of the complex organization of DNA in eukaryotic chromosomes. These agents can be alcohols, such as ethanol (Reich et al., 1980; Huey & Mohr, 1981) and PEG (Lerman, 1971; Evdokimov et al., 1983; Evdokimov, 1988; Livolant & Maestre, 1988); H1 or H5 histones (Adler & Fasman, 1971; Sponar & Fric, 1972); polypeptides such as polylysine (Carrol, 1972; Shapiro et al., 1969; Haynes et al., 1970) and polyhistidine (Burchardt et al., 1973); divalent metallic ions (Simpson & Sober, 1970; Shin & Eichhorn, 1977); or lithium (Wolf et al., 1977).

The most obvious property displayed by these systems is the presence of anomalous circular dichroism (CD)[1] signals which are 10–1000 times larger than those of dispersed DNA. The shape of the spectrum is also greatly deformed by the presence of long tails extending toward the red wavelengths, outside the absorption bands of DNA. The anomalous spectra observed inside the absorption regions have been termed psi-type CD by Lerman et al. (1974), where "psi" stands for "polymer-and-salt-induced".

The liquid-crystal circular dichroism (LCCD) approach carried out by Saeva (1973, 1979) has been used to relate the anomalous signals to the presence of a long-range chiral organization in the DNA aggregates (Livolant & Maestre, 1988; Spada et al., 1988). However, this theory can describe only the optical activity of oriented cholesteric mesophases, and its extension to the treatment of rotationally averaged systems is by no means justified.

Recently, a general theory of CD of oriented and rotationally averaged systems has appeared in the literature (Keller & Bustamante, 1986a,b; Kim et al., 1986; Bustamante et al., 1988). This theory is valid for all dimensions of chiral order relative to the wavelength of light, and it reduces to the excitonic theories of Tinoco (1962) and De Voe (1965) in the limit of small chiral dimensions. This theory has shown that the anomalous psi-type signals are a manifestation of the long-range chiral structure in the DNA aggregates.

Evdokimov et al. (1983) have shown that complexes of DNA and certain drugs such as daunomycin, obtained in the presence of PEG, can give rise to psi-type signals of opposite sign to those obtained in the absence of the drug. The question then arises as to whether or not this sign inversion reflects an inversion in the handedness of the DNA aggregates induced by these drugs. In this paper, we present several lines of evidence, including CD, CIDS, and transmission electron microscopy (TEM), that the inversion in the sign of these

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[1] Abbreviations: PEG, poly(ethylene glycol); CD, circular dichroism; CIDS, circular intensity differential scattering; LCCD, liquid-crystal circular dichroism; TEM, transmission electron microscopy; $I_{L,R}(\vartheta)$, intensities scattered at an angle ϑ for incident L and R circularly polarized light; r, moles of drug bound per mole of phosphate.

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signals reflects a change in handedness of the aggregates. These results also represent a confirmation of the predictions of the theory by Keller and Bustamante (1986a,b; Kim et al., 1986; Bustamante et al., 1988) on the origin of the psi-type spectra.

THEORY

The CD spectrum of an optically active molecule changes, sometimes drastically, when the molecule becomes part of a larger aggregate. If these aggregates have dimensions similar to the wavelength of light (>100 nm for light in the UV and visible), two kinds of anomalies occur in the CD spectra of suspensions of these aggregates.

First, long tails often appear in the CD at wavelengths outside the absorption bands of the constituent molecules. These tails are a manifestation of the ability of the particles to scatter more one circular polarization than the other (Bustamante et al., 1980a,b, 1981). They can be eliminated by methods designed to correct the scattering of the solution (Dorman & Maestre, 1973; Reich et al., 1980; Maestre & Reich, 1980).

Second, the magnitude of the CD signals inside the absorption band can be up to 1000 times larger than those observed for small systems. It was found that these anomalously large signals could not be eliminated by scattering correction methods, a fact that reveals the distinct physical origins of these two types of anomalies (Keller & Bustamante, 1986b).

Psi-Type Circular Dichroism Spectra. The theory of psi-type CD developed by Keller and Bustamante (Keller & Bustamante, 1986a,b; Kim et al., 1986) recognizes that in nonaggregated, small systems, the incident light induces oscillating dipole moments independent of each other. The spectroscopy of these systems (absorption and scattering) can be described in terms of a collection of independent, localized excitations (Figure 1a). To account for circularly dichroic phenomena, the excitation created at a given group must be coupled with that of an adjacent group (exciton interaction, Figure 1b). Traditional CD theory (Tinoco, 1962) has very successfully accounted for the usual CD spectra of nucleic acids and polypeptides within the frame of nearest-neighbor exciton interactions. If the system is large, but its chromophore density is low or inhomogeneous, again only local excitations can take place (Figure 1c).

On the other hand, when the particle is dense and extends to dimensions comparable to the wavelength of light in all directions in space, the induced oscillating dipole moments can couple to each other over the whole extent of the particle (Figure 1d). These couplings can take place by short-range (dipole–dipole) as well as long-range (scattering) interactions. As a result, the excitation created at a given group delocalizes throughout the whole aggregate, giving rise to *collective excitation modes* of the groups. These collective modes are the particle's eigenmodes, and are somewhat analogous to the effect observed in resonant cavities. The particle is said to *sustain a series of eigenmodes of excitation.*

Now we can no longer talk of the excitation of individual dipoles, but of a collective response of all dipoles in the aggregate. The shape of these eigenmodes depends on the long-range internal organization of the particle, i.e., in this case on how the DNA is packed in the condensate.

If the folding of the DNA molecule in the condensed particles is chiral, the eigenmodes of excitation can be preferentially excited by one circular polarization over the other. Furthermore, the long-range nature of the chirality in these condensates will permit a "*spatial-resonance*" between the circular polarization of the appropriate handedness and the

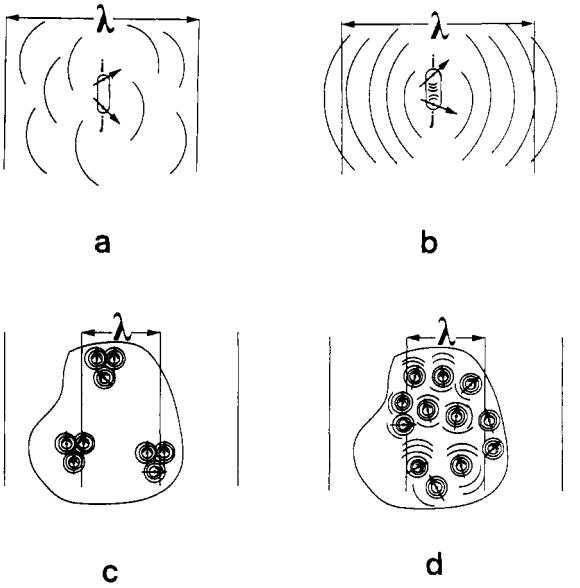

FIGURE 1: (a) In small particles, whose dimensions are much smaller than the wavelength of the light (λ), the electric field of the incident radiations, E_0, induces dipole moments $\mu_i = \alpha_i E_0$ on the particle anisotropically polarizable groups. (b) The classical CD theories account for circularly dichroic phenomena by coupling the oscillating point dipoles on the polarizable groups i and j within the particle. The induced dipole i therefore becomes $\mu_i = \alpha_i(E_0 + E_j)$, where E_j is the electric field scattered from group j on group i. (c) In large and nondense particles, only localized and short-range dipole–dipole interactions can take place. (d) In large and homogeneously dense particles, dipole–dipole coupling over their whole extent takes place, and collective excitation modes are sustained.

object, giving rise to the excitation of a pure or quasi-pure mode. In this case, the exchange of energy between the particle and that particular polarization is very efficient. On the other hand, the opposite circular polarization "misses", so to speak, the long-range chiral organization of the particle, and it cannot induce a single pure eigenmode, but a superposition of modes of different shape, sign, and phase which tend to cancel each other. The energy exchange in this case is therefore small. The difference in the interaction of these two incident polarizations with the sample can then be all vs none, and the circular dichroism signal can be very large.

Experimentally, the magnitudes are reduced by orientational averaging of the particles, by various degrees of inhomogeneity in the condensate's population, by imperfect "spatial-resonance", etc. The important point is that the CD is no longer the result of the nearest–neighbor interaction between adjacent induced dipoles, as in Tinoco's theory, but the result of the dipoles coupled by the light throughout the entire particle. A new "form-CD" due to the long-range chirality superimposes and dominates the regular short-range CD.

Circular Intensity Differential Scattering. Long-range chiral aggregates can scatter preferentially one circular polarization more than the other and give risk to anomalous CD signals also outside the absorption band (see above).

This property varies as a function of the scattering angle and is a sensitive probe of the long-range handedness of the scatterer. It is convenient to measure this property in the form of a normalized ratio called the circular intensity differential scattering (CIDS) ratio:

$$\mathrm{CIDS}(\vartheta) = \frac{I_L(\vartheta) - I_R(\vartheta)}{I_L(\vartheta) + I_R(\vartheta)}$$

where I_L and I_R are the intensities scattered at an angle ϑ for incident left and right circularly polarized light, respectively. A theory of the angular dependence of the CIDS ratio as a

function of the structural parameters of the scatters has been formulated (Bustamante et al., 1980a,b, 1981, 1985; Keller et al., 1985), and the differential scattering patterns of a variety of chiral systems have been measured (Wells et al., 1986; Garab et al., 1988). The most significant advantage of the CIDS ratio is that it can be positive or negative and the scattering patterns often show lobes of alternating sign as a function of the scattering angle. Since CIDS is an interference phenomenon, the sign of the CIDS ratio is controlled by the handedness of the scatterer and not by the details of the polarizability tensors of the groups in the scatter. Right- and left-handed structures otherwise identical will have identical CIDS but with opposite sign.

The number of alternating lobes in the CIDS pattern depends on the dimension of the chiral scatterer relative to the wavelength of the light. For chiral structures from one-fifth to one-tenth of the wavelength of light, the scattering patterns possess a single sign (Wells, 1987). In these cases, a change in sign necessarily implies an inversion of the chirality.

Materials and Methods

CD, CIDS, and Absorbance Measurements. The CD spectra were recorded with a J-600 spectropolarimeter (Jasco Inc.). UV–visible spectra were run by a Shimadzu UV-260 spectrophotometer. The CIDS instrument was designed and built at UNM, Albuquerque (Katz et al., 1984). The detection level of the instrument was 1 part of differential scattering per 10 000 of total scattering. The preferential scattering of the samples was measured at 488 nm (argon ion laser). The laser's power was 100–200 mW; its attenuation by the optics was up to a maximum of 50%. The magnitude of CIDS signals is independent of the actual beam intensity because the differential signal is normalized by the total differential scattering. The differential scattering intensities are measured as a function of the angle from the direction of the incident beam. Angles <20° and >165° were not accessible (Wells et al., 1986). A filter was adapted between the sample and the detector to cut down the fluorescence of daunomycin. Prior to and after each CIDS measurement, the instrument calibration was checked by using a suspension of polystyrene beads (0.14-μm diameter) in water.

Transmissison Electron Microscopy. Best TEM results were obtained when the samples were deposited on formvar and glow-discharged carbon followed by uranil acetate treatment (Williams, 1977; Chattoraj et al., 1978). Generally, the reproducibility of the TEM images was poor.

DNA Preparation. Highly polymerized calf thymus DNA was purchased from Sigma Chemical Co. Stock solutions of 12 ODU were prepared in Tris buffer (10 mM high-purity Trizma base, pH 6.8, and 0.3 M NaCl) by stirring overnight at 4 °C. The Model W185D sonifier cell disruptor (Heating Systems-Ultrasonic Inc.), set in position 5, was used for the sonication as follows: 25 mL of the DNA solutions in a 40-mL glass centrifuge tube was sonicated with the microtip immersed 3 cm for 30 s at 4 °C. Samples were cooled down for 2 min for every 30 s of sonication. Polydispersed DNA solutions (12 ODU) were sonicated for 1, 2, 3, 4, and 5 min, and sizes were determined by agarose gel electrophoresis to be 4.2, 3.5, 3.0, 1.5, and 0.8 kbp, respectively (Maniatis et al., 1982).

Deproteinization of DNA was done repeatedly with phenol, phenol–chloroform, and chloroform (Maniatis et al., 1982) followed by a series of dialyses of 10 mM Tris-HCl and 1 mM EDTA, pH 7.0. The last two dialyses were done without EDTA. DNA solutions were frozen in small aliquots at −20 °C. As needed, DNA solutions were defrosted, and the concentration in nucleotides was determined spectrophotometrically by using an extinction coefficient $\epsilon_{260} = 6600\ M^{-1}\ cm^{-1}$ (Mahler et al., 1964). The purity of DNA was evaluated by the ratio A_{260}/A_{280} which ranged in our solutions between 1.80 and 1.88.

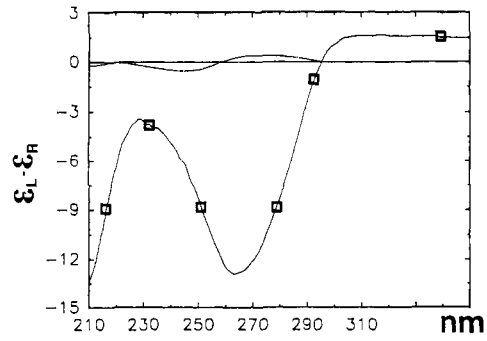

FIGURE 2: CD spectra of B-DNA (0.8 kbp) prior to (—) and after condensation (□) with PEG.

DNA–Daunomycin and Condensate Solutions. The DNA–daunomycin solutions were prepared by diluting and mixing stock solutions of sonicated DNA (3.67×10^{-4} M) and daunomycin (1.12×10^{-4} M in 0.3 M NaCl and 10 mM Tris buffer, pH 6.9). The final concentration of DNA was 3.67×10^{-5} M. The drug concentrations varied between zero and 3.0×10^{-5} M. Daunomycin solutions were stored in the dark and were freshly prepared every day. The values of the binding ratios, r (moles of drug bound per mole of phosphate), calculated spectrophotometrically (Chaires, 1985) ranged from 0.01 to 0.151.

The condensates were prepared by adding a 360 mg/cm^3 buffered solution of poly(ethylene glycol) (PEG) 4000 GC grade (EM Science) to the DNA–drug solutions. The final concentration of PEG was 150 mg/cm^3, unless otherwise specified.

The PEG solution was added slowly and steady as to form initially a phase separation. The samples were slowly agitated and then allowed to equilibrate for 15 min. CD, CIDS, and TEM measurements were carried out within 2 h from the sample preparation.

Results

Condensates were prepared from variously sonicated DNA solutions to which gradually increasing amounts of the intercalating daunomycin were added before the PEG condensing agent. The resulting suspensions of these aggregates were investigated by CD, CIDS, and TEM.

Psi-Type Circular Dichroism. In the presence of PEG, the CD spectrum of B-DNA is drastically altered. The new, psi-type spectrum shows a very strong negative band centered at about 270 nm and a long scattering tail extending toward the visible spectral region (Figure 2).

The effect on the psi-type spectrum of DNA of increasing additions of daunomycin (prior to aggregation) is shown in Figure 3. As the drug binding ratio increases, the magnitude of the negative CD band decreases and nearly disappears at a value of $r = 0.108$. Beyond this point, a positive psi-type signal appears. Its magnitude increases with the binding ratios and reaches saturation at $r = 0.151$.

Figure 4 shows that, at the binding ratio at which sign inversion occurs, the CD spectrum of the condensates displays no psi-type anomalies. Instead, a spectrum very similar to that of the DNA–drug complex in the absence of PEG is obtained, which is also shown in Figure 4 for comparison. Both spectra just show the contributions of the intrinsic and induced CD signals of B-DNA and the drug. A comparison of these spectra

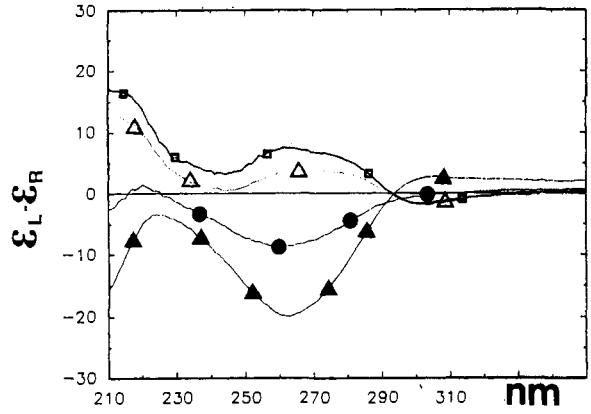

FIGURE 3: Psi-type CD spectra of condensed DNA (3.0 kbp)–daunomycin complex at different binding ratios: $r = 0$ (▲), 0.095 (●), 0.123 (△), and 0.143 (□).

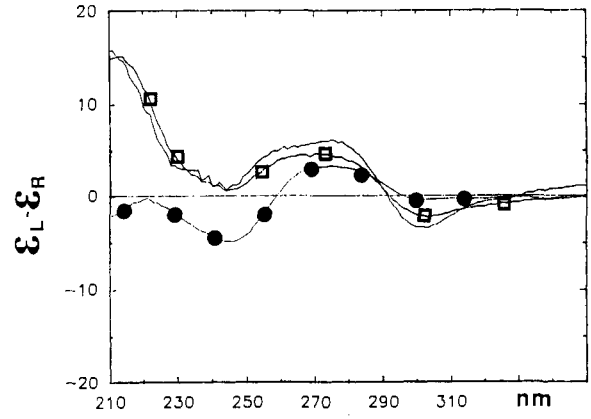

FIGURE 4: CD spectra of B-DNA (●) and of DNA–daunomycin complex (at $r = 0.108$) prior to (□) and after (—) condensation at the nematic-like inversion point.

with that of B-DNA shows that the signals at about 240 and 300 nm correspond to the most prominent bands in the CD spectrum of daunomycin (Samori et al., 1987).

The psi CD intensities are drastically affected by the details of the preparation. Figure 5a shows the effect of PEG concentration and Figure 5b the effect of sonication of the DNA prior to the addition of PEG. Increasing additions of PEG and longer sonication times lead to higher psi-type CD intensities.

Circular Intensity Differential Scattering. CIDS patterns of aggregates of sonicated DNA (0.8 kbp), with increasing amounts of intercalated daunomycin, can be seen in Figure 6. The CIDS pattern corresponding to the condensates of DNA in the absence of daunomycin is positive and makes a maximum at 90°. The magnitude of this lobe decreases gradually with increasing values of drug saturation and disappears completely at a value of $r = 0.108$. Higher intercalating ratios induce a negative CIDS lobe whose absolute magnitude increases until it reaches saturation at a value of $r = 0.151$. Thus, the sign inversion and the saturation observed in the CIDS measurements occur at the same binding ratios as those observed in the CD inversion experiments (Figure 3).

Transmission Electron Microscopy. The TEM picture shown in Figure 7 is representative of many obtained using different sample preparations and staining techniques (Williams, 1977; Chattoraj et al., 1978). Almost always, the condensates are built up of beads "glued" to each other, forming various branched structures. Similar condensates are also obtained with increasing amounts of intercalated daunomycin. Rodlike condensates were seldom observed, and their

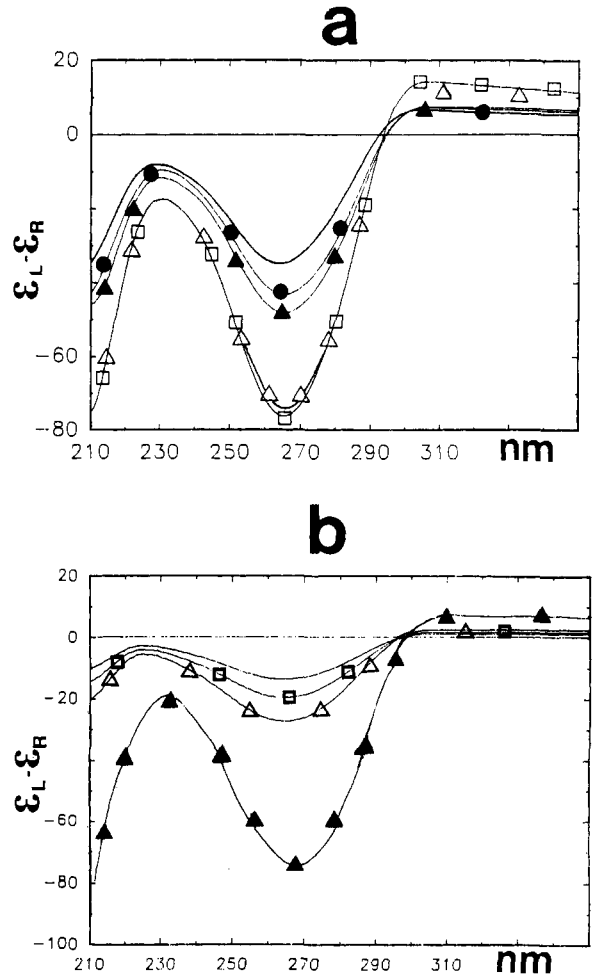

FIGURE 5: (a) Psi-type CD spectra of DNA (0.8 kbp) condensed by 112.0 (—), 125.0 (●), 135.0 (▲), 150 (△), and 170 (□) mg/mL PEG. (b) Psi-type CD spectra of DNA of various lengths: 4.2 (—), 3.5 (□), 3.0 (△), and 0.8 (▲) kbp.

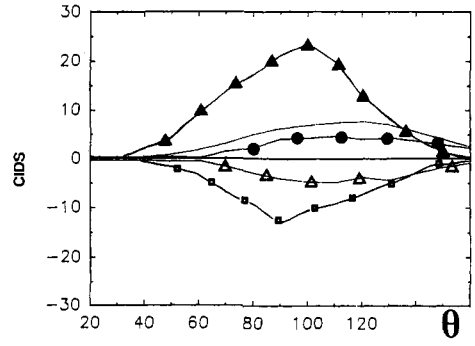

FIGURE 6: CIDS curves of condensed DNA (3.0 kbp)–daunomycin complex at different binding ratios: $r = 0$ (▲), 0.059 (—), 0.095 (●), 0.123 (△), and 0.143 (□).

presence was not related to the amount of intercalated drug, suggesting that they were artifacts of the staining or the dehydration step in the sample preparation (Chiu, 1986). The average diameter of the beads was 500 Å.

Discussion

The TEM pictures of the condensates show multiformed objects made up of spherical beads attached to each other (Figure 7). The beads are likely to be molecules of DNA collapsed in a spool or toroidal fashion. PEG is a dehydrating agent that can induce a closer contact between DNA segments in the aggregate. The tendency of the DNA to collapse in toroidal or spoollike structures has been reported with several

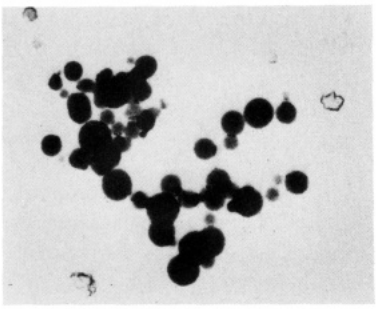

FIGURE 7: TEM picture of DNA condensates.

other condensing agents (Gosule & Schellman, 1976; Wilson & Bloomfield, 1979; Chattoraj et al., 1978), and it is likely to correspond to a thermodynamically stable form. These forms have been observed in a large range of DNA concentrations and therefore can result from intramolecular collapse as well as from intermolecular aggregation. At the concentrations utilized in these experiments, the beads observed are most likely the result of intermolecular aggregation.

The packing density and the size of the aggregates are drastically affected by the details of the preparation and are monitored by the psi-type CD signals. The psi-type CD theory predicts that the magnitude of the psi-type CD signal is controlled by the chromophore density, the volume, and the pitch of the aggregates. In particular, it has been shown (Keller & Bustamante, 1986a,b) that the magnitude of the CD signals per chromophore and its deformation away from the conservative, short-range excitonic contributions increase with the overall volume of the aggregate. The increase in the psi-type CD signals observed with increasing additions of PEG and with smaller DNA dimensions (Figure 5a,b) is thus interpreted in terms of an increase in the packing density of the aggregates and the formation of larger aggregates. In fact, as the amount of added PEG increases, closer contacts between the condensed DNA segment chains and larger aggregates must occur. Also, longer sonication times increase the packing density: reducing the average length of the DNA molecules can make the chain folding and packing meet less restrictions.

It has been shown that if the aggregates are concentrated either by centrifugation (Evdokimov et al., 1988) or by inducing phase separation (Livolant & Maestre, 1988), cholesteric textures can be observed under the optical microscope. This observations indicate that the close molecular packing leads to the formation of superhelical structures with higher order chirality.

Daunomycin Inverts the Long-Range Chirality of the Aggregates without Altering Their Overall Shapes. The CIDS theory (Bustamante et al., 1981, 1985; Wells, 1987) predicts that for objects whose dimensions are smaller than one-tenth of the wavelength of light, the CIDS patterns consist of a single positive or negative scattering lobe. This is consistent with the dimensions of the aggregates of about 500 Å observed under the electron microscope. Although the morphology of these aggregates (Figure 7) is not altered by daunomycin in the range of binding ratios studied, the changes in the magnitude and eventual sign inversion of the CD and the CIDS signals indicate that this intercalating drug can affect substantially the internal chiral structure of the aggregates. For CIDS patters consisting of a single lobe, as those obtained with suspensions of the DNA aggregates (Figure 6), sign inversion implies necessarily the inversion of the chirality of the scattering objects (Bustamante et al., 1981, 1985). Thus, *the CIDS patterns provide the first experimental evidence that intercalation of daunomycin can invert the handedness of the long-range Chiral organizations of DNA obtained in the presence of PEG.*

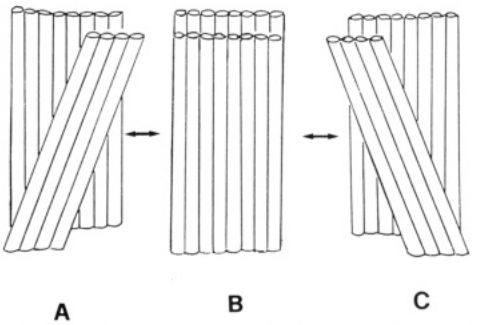

FIGURE 8: Schematic picture of handedness inversions in cholesteric-like macroorganizations (A and C). Single rodlike segments represent single B-DNA double chains. The inversion of this long-range chirality takes place at a transition point (B) in which a nematic-like stacking of the DNA segment chains takes place.

The inversion of the psi-type CD signal at the same binding ratio at which the CIDS patterns invert demonstrates that the sign and magnitude of the psi-type spectrum are controlled by the handedness and structure of the long-range chirality of the sample. The coincident behavior of the CD and the CIDS results obtained in this work provides, in this way, a strong confirmation of the predictions of the psi-type CD theory.

Aggregates Have Nematic-Like Liquid-Crystalline Properties at the Chirality Inversion Point. The similarity between the psi-type CD spectrum of the aggregates *at the point of inversion* and that of the DNA–drug complexes prior to PEG addition reveals *a loss of the long-range chirality*. This is not due to a gradual decondensation of the DNA induced by the successive additions of daunomycin, since the transmission electron micrographs shows the presence of substantial amounts of condensates at similar concentrations of the drug. Rather, the inversion of the long-range chirality must involve a transition from a cholesteric-like to a nematic-like phase within each aggregate (Figure 8). In these conditions, the CD spectrum of the aggregates is determined by the short-range chiral structure of the individual DNA chains, and it is no longer dominated by psi-type anomalies. This interpretation is further supported by the nematic-like textures observed through the polarizing microscope by Evdokimov (1988), in the same range of drug concentrations.

Furthermore, the complete absence of a psi-type signal at the inversion point and the small amounts of drug required to induce the inversion beyond the transition point have two additional implications: (1) The packing of the molecules does not affect the secondary structure of the DNA molecules. This fact is consistent with X-ray diffraction studies showing that the DNA molecules in the aggregates remain in the B conformation (Haynes et al., 1970; Liquier et al., 1975; Herbeck et al., 1976). (2) Small changes in the local structure of the DNA molecules, possibly involving subtle modifications in their hydration and surface charge distribution upon binding of daunomycin, can induce dramatic changes in the long-range tertiary packing of the molecules.

Conclusions

Binding of daunomycin to DNA prior to its aggregation by PEG can induce a decrease of the psi-type CD signals of the resulting aggregates and eventually induce an inversion of sign. Parallel CD and CIDS measurements on samples prepared at various binding ratios of daunomycin to DNA have shown that these two signatures invert sign at the same critical binding ratio. This coincidence has been interpreted in terms

of the CIDS theory, and it strongly suggests that the sign inversion responds to a change in handedness of the long-range chiral packing of the aggregates. A careful analysis of the CD spectrum of the aggregates at the inversion point shows that the aggregates at the midpoint of the transition have a nematic-like structure. The effect of less or more drug at this point is to induce the packing of the molecules into opposite senses of coiling.

The inversion appears to be related to the ability of daunomycin to alter the intermolecular contacts between the DNA molecules by changing their hydration and surface charge distribution. Preliminary results showing that minor changes in the daunomycin molecular structure markedly affect its ability to induce this inversion (Samori et al., 1991) appear to confirm this interpretation.

The results presented here represent the first experimental confirmation of the psi-type CD theory of Keller and Bustamante (1986a,b) which interprets the psi-type CD signals as a manifestation of the long-range chirality of large three-dimensional aggregates.

Acknowledgments

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