Liquid Crystals
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713926090

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To cite this Article Bonazzi, Stefania , De Morais, Monica Miranda , Garbesi, Anna , Gottarelli, Giovanni , Mariani, Paolo and Spada, Gian Piero(1991) 'Chromonic lyomesophases formed by the self-assembly of the cyclic dinucleotide d(cGpGp)', Liquid Crystals, 10: 4, 495 — 506
To link to this Article: DOI: 10.1080/02678299108036438
URL: http://dx.doi.org/10.1080/02678299108036438

LIQUID CRYSTALS, 1991, VOL. 10, NO. 4, 495–506

Chromonic lyomesophases formed by the self-assembly of the cyclic dinucleotide d(cGpGp)

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(*Received 11 February 1991; accepted 4 May 1991*)

The cyclic dinucleotide d(cGpGp) undergoes a self-association process in water to give, first, columnar aggregates similar to the four-stranded helix of poly(G). Successively, at higher concentration, these aggregates self-organize to give a cholesteric and a hexagonal mesophase, the former of which appears only in biphasic systems. The self-assembly process in isotropic solution has been studied by CD spectroscopy and the structure of the mesophases was investigated by optical microscopy and X-ray diffraction.

1. Introduction

We have recently reported that the deoxydinucleoside phosphate d(GpG) in water gives rise to cholesteric and hexagonal mesophases [1, 2]. This dimer in aqueous solution first undergoes a self-assembly process to give a chiral columnar aggregate similar to the four-stranded helix formed by polyguanylic acid. At higher concentration, the columnar aggregates self-organize to give a cholesteric phase; a subsequent increase of the concentration leads to a hexagonal mesophase. Similar behaviour has been reported also for other oligodeoxyguanylates as well as for the deoxynucleotide d(pG) [3].

We report here a study of the 3′-5′-cyclic deoxydinucleotide d(cGpGp). Formally, this cyclic dimer can be obtained from the linear precursor by adding a second phosphate to join the free 3′ and 5′ hydroxy groups; as a consequence, the conformational mobility of the molecule is restricted and the hydrophilic character enhanced. Besides the obvious curiosity to see the effect of these changes on the ability to give liquid-crystalline mesophases, cyclic dinucleotides have been the subject of recent attention [4]. In particular, this cyclic compound, as well as the ribo derivative cGpGp, were found to be regulators of the synthesis of cellulose in the bacterium *Acetobacter Xylinium* [4, 5]; the ribo derivative also behaves as a host molecule for planar intercalators [6]. The study of the liquid-crystalline phases can give information on the molecular structure in aqueous solution in conditions fairly similar to those found in biological systems.

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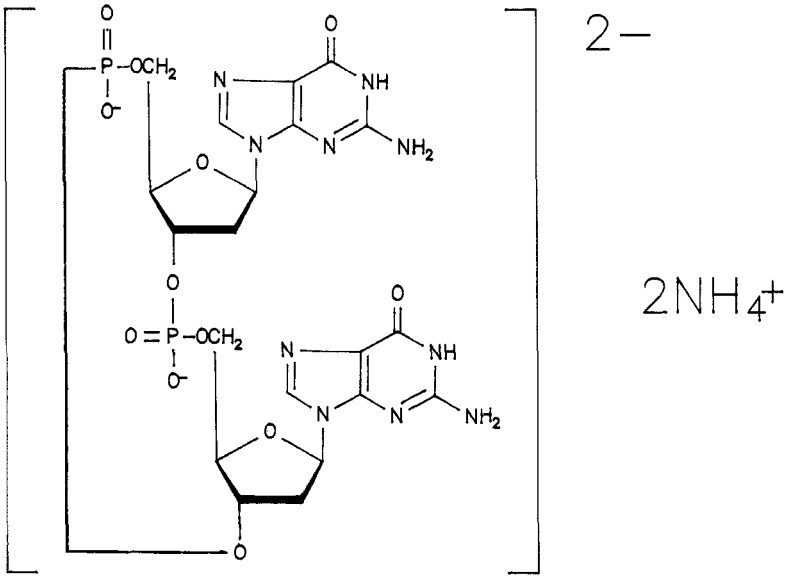


2. Results

2.1. *Optical microscopy*

Preliminary observations were carried out on samples with a concentration gradient obtained by peripheral evaporation or by allowing water to penetrate into the neat sample. Only in a few experiments do we have textural evidence for the cholesteric phase; generally only textures characteristic of the hexagonal phase (H) were observed (see figure 1), indicating that at room temperature only this phase is stable. The critical concentration for the hexagonal phase evaluated with samples of different concentration was *c.* 40 per cent at 22°C.

2.2. *X-ray diffraction*

X-ray diffraction experiments were performed as a function of the water content of the samples. As usual for liquid crystal crystallography [10], the X-ray diffraction pattern was analysed by considering two different regions separately, the first at low angle ($S<(10\,\text{Å})^{-1}$, where $S=2\sin\Theta/\lambda$, 2Θ being the scattering angle and λ the X-ray wavelength) and the second at high angle ($S>(10\,\text{Å})^{-1}$), which corresponds to investigating the long range sample organization separately from the short range order. In particular, by considering the position of the low angle diffraction peaks (or better their spacing ratios), it is possible to recognize the presence of different phases and to identify their symmetries [10]. In the present case, the analysis of the low angle pattern clearly indicates the existence of a liquid-crystalline phase in the central part of the phase diagram. This phase appears to be bordered by a crystalline phase in the dry side of the phase diagram and by a more disordered organization (the cholesteric phase identified by optical microscopy) on the other side. Finally, an isotropic liquid phase follows the disordered organization when the water content increases further.

Before describing the diffraction data in more detail, some remarks should be made. First, considering our previous results on a series of linear derivatives of guanosine [2, 3], the extraordinary stability of the hexagonal phase must be underlined: this property results on the one hand from the difficulty in obtaining the crystal phase from

hydrated samples (two weeks under vacuum at 0·05 mmHg were generally insufficient to remove the water and to induce the phase transition) and on the other hand from the existence of a large biphasic region corresponding to the hexagonal–cholesteric transition. In fact the existence of the pure cholesteric phase could not be detected.

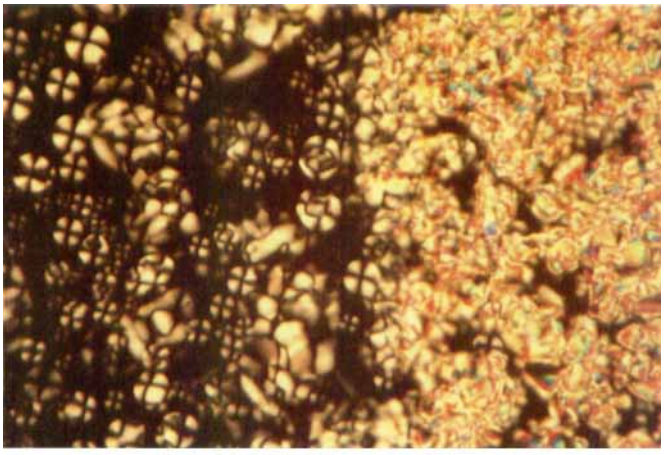
The X-ray diffraction profile of samples with a weight concentration of d(cGpGp) ranging from *c.* 40 per cent to *c.* 80 per cent, is characterized in the low angle region by the presence of a very strong peak associated with few (four in some samples) low intensity signals, the spacing ratios of which, in the order $1:3^{1/2}:4^{1/2}:7^{1/2}\ldots$, clearly demonstrate the hexagonal two dimensional symmetry [10, 11, 12]. Moreover, the position of these peaks appears to be strongly concentration dependent, indicating a continuous variation of the unit cell as a function of water content (see figure 2); a similar effect has already been described and discussed [2, 13]. Concerning the high angle diffraction region, a narrow peak centred at about $(3{\cdot}4\ \text{Å})^{-1}$ is observed: the spacing, the half-height width and the intensity of this peak appear to be insensitive to water concentration, even inside the large biphasic region. All of the structural results are summarized in the table.

The low angle diffraction profile of the samples in the cholesteric phase is expected to be quite different; it should be characterized typically by the presence of a broad band [2]. However, as noticed before, we never observed the pure cholesteric phase: the diffraction pattern of samples containing *c.* 30–40 per cent of d(cGpGp) shows in fact a diffuse broad band, centred at about $(50\ \text{Å})^{-1}$, superimposed on a Bragg peak, the intensity of which appears to decrease as the isotropic phase is approached. Even if the presence of a different phase cannot be excluded, as other reflections are not detectable and the samples are reproducible with difficulty in this region, by argument of continuity (in particular the position of the Bragg peak ranges from $S=0{\cdot}035$ to $0{\cdot}028\ \text{Å}^{-1}$), we assume that the cholesteric and hexagonal phases coexist in this part of the phase diagram.

Concerning the high angle diffraction profile, the peak at $S=(3{\cdot}4\ \text{Å})^{-1}$ still remains without variation in position and shape with respect to the hexagonal phase. However, it disappears when the isotropic solution is reached.

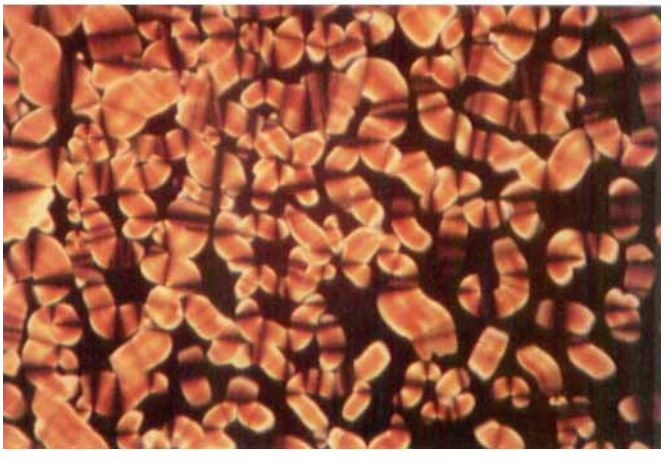
All of the data are consistent with the molecular aggregation model proposed for the linear guanosine derivatives previously investigated [2, 3]: the liquid-crystalline phases consist of rod-shaped aggregates, each rod being composed of a stacked array of planar tetramers, equally spaced, formed by Hoogsteen-bonded guanosine moieties. Also in this case the rod-shaped aggregates appear to be the structural elements which interact to form, depending on the sample concentration, either hexagonal or cholesteric mesophases. The presence of the rod is confirmed by the observation that the peak at 3·4 Å, which corresponds to the distance between the stacked planar

Figure 1. Textures of the phases obtained from d(cGpGp) in water. (*a*) Cholesteric droplets obtained from rehydration of a hexagonal phase. (*b*)–(*f*) Different textures originating from different local alignment of the hexagonal phase; (*b*) star-domain and (*c*) ribbon-like planar textures growing in the homeotropic (or isotropic) solution; (*d*) herring-bone texture; (*e*) undulating-pattern with twisted domain; (*f*) broken fan-shaped texture in which the director orientation is underlined by fine striations running normal to the radial sectors' boundary. All pictures were taken with crossed polars in samples with coverslips (original magnification ×400). Textures (*b*)–(*f*) are reminiscent of those exhibited by hexagonal phases formed by chromonics [7] and DNA [8]. For a topological interpretation see [9].



(*a*)

(*b*)



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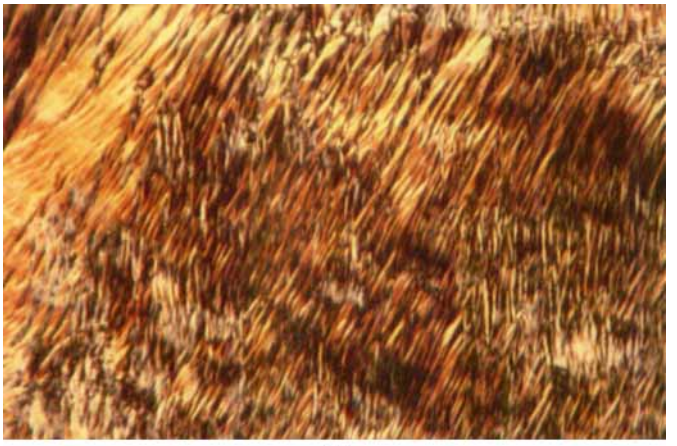

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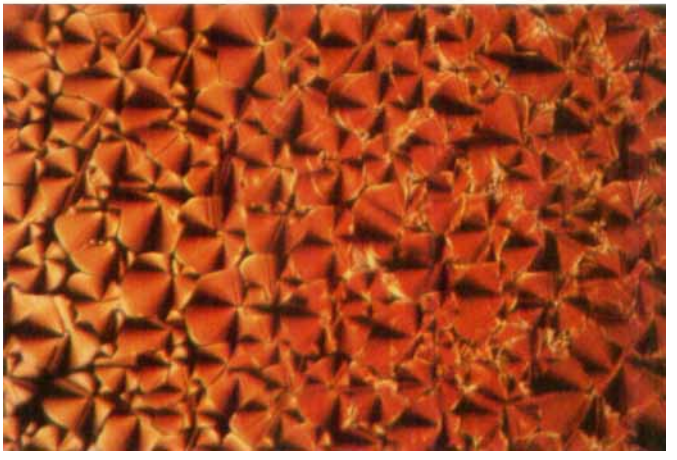

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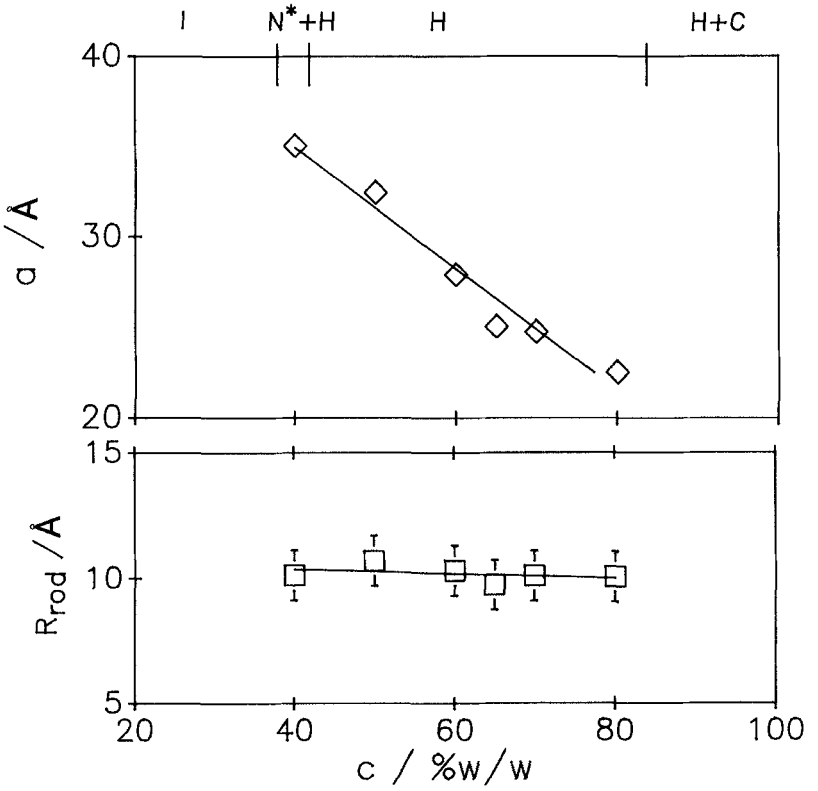

Figure 2. Variation of the dimension of the unit cell, *a*, (upper part) and of the radius of the rod-shaped aggregate, R_{rod}, (lower part) as a function of d(cGpGp) concentration at 25°C. At the top of the figure the approximate phase sequence is reported (I, isotropic; N*, cholesteric; H, hexagonal; C, crystal).

X-ray diffraction data.†

c/%w/w	40		50		60		65		70		80	
a/Å	35·0		32·5		27·9		25·1		24·8		22·5	
	Low angle $S/10^{-3}$ Å^{-1}											
Ratio	Cal.	Obs.	Cal.	Obs.	Cal.	Obs.	Cal.	Obs.	Cal.	Obs.	Cal.	Obs
1	33·0	33·0	35·6	35·6	41·4	41·3	46·1	45·9	46·6	46·7	51·3	51·3
$3^{1/2}$	57·1	—	61·7	61·5	71·7	71·9	80·0	80·6	80·8	80·6	88·8	88·4
$4^{1/2}$	65·9	66·1	71·2	71·7	82·8	82·8	92·2	92·6	93·3	93·4	102·6	103·1
$7^{1/2}$	87·2	87·2	94·2	94·7	109·5	—	122·0	—	123·4	—	135·8	—
$9^{1/2}$	98·9	98·5	106·8	—	124·1	125·3	138·3	—	139·9	—	154·0	—
$12^{1/2}$	114·2	—	123·3	122·2	143·3	143·0	159·7	159·3	161·6	—	177·8	—
$13^{1/2}$	118·8	—	128·4	—	149·2	147·8	166·2	165·4	168·2	—	185·0	—
	High angle S/Å^{-1}											
	$(3{\cdot}47)^{-1}$		$(3{\cdot}44)^{-1}$		$(3{\cdot}46)^{-1}$		$(3{\cdot}45)^{-1}$		$(3{\cdot}41)^{-1}$		$(3{\cdot}43)^{-1}$	

† *c* is the concentration; *a* is the dimension of the two dimensional hexagonal unit cell; low and high angles refer to the two distinct regions of the diffraction pattern (see text); *S* is the reciprocal spacing of the reflection, Obs. refers to the observed spacings while Cal. refers to the ones calculated for two dimensional hexagonal structures with the reported lattice parameter.

tetramers, is insensitive to the sample concentration. As the intensity, shape and position of this high angle diffraction peak do not change through the biphasic hexagonal–cholesteric region, whereas the low angle signals relative to the hexagonal organization decrease in intensity, we can infer that also in the cholesteric phase the structural element remains the same, the differences observed in the low angle diffractions being due only to a continuous variation of the long range order. Moreover we can assume that water does not penetrate the rods [2]: for the hexagonal phase it is then possible to calculate the radius of the cylindrical aggregates by using [2, 10]

$$R_{\text{rod}} = (\sigma c_v/\pi)^{1/2},$$

where σ is the unit cell surface and c_v the volume concentration of d(cGpGp) in the sample. The values calculated by using the specific volume of 0·651 $\text{cm}^3\,\text{g}^{-1}$ [14] are plotted in figure 2: it appears that the value of the rod radii of all the hexagonal samples investigated is approximately constant (10 Å), indicating that the rod-shaped aggregates are almost rigid, that their distance increases as a function of the water content and confirming that their inner structure is insensitive to concentration.

The analysis of the diffracted intensity confirms this view. A classical approach [15] was used to solve the crystallographic phase problem, namely the swelling experiment. Diffraction experiments give a set of integral intensities from which the modulii of the structure factors can be obtained. In order to calculate the electron density maps (which give direct information about the structure), the signs of these factors must be determined. In our case it is possible to assume, to a first approximation, that at all concentrations the electron density distribution inside the cylinders is a radial function of the ratio R/R_{rod} and that the electron density is fairly uniform outside the rods. With this condition, at all concentrations, the observed structure factors will sample a continuous function of the product $R_{\text{rod}}S$. By analysing this curve and determining the position of zeros (inversion points), the reflections can be phased [15]. To reconstruct the continuous structure factor function i.e. the form factor of the rod-shaped aggregates) we have measured the diffraction peak intensities in samples with different concentrations and plotted the properly normalized structure factors [15–17] as a function of the product $R_{\text{rod}}S$. This plot is shown in figure 3: the positions of the sign inversions are easily identified.

Figure 4 shows the structure of the hexagonal phase of the sample containing 50 per cent of d(cGpGp) calculated by using the correct assignment of the signs and after data normalization which leads to dimensionless expressions of both the structure factors and the electron density distribution [18]. The map clearly shows the electron dense rod section, which after our normalization appears to be positive, while the outside rod region appears smoothly negative owing to the lower value of the water electron density with respect to the d(cGpGp) molecule. Two interesting features which strongly support our proposed model must be underlined: first, the rod dimensions, which can be directly measured in the electron density maps, are in excellent agreement with the experimental data (see figure 2); secondly, in analogy to what was reported for the linear guanosine derivative [3], the central region of the rods appears to be characterized by a gradual decrease of the electron density, which can be explained by considering the presence of a hole in the middle of the guanosine tetramer [19].

2.3. *Circular dichroism*

The CD spectra of the cyclic dimer (10^{-4} $\text{mol}\,\text{l}^{-1}$ of guanine residue) in a neutral phosphate buffer (isotropic solution) as a function of temperature is reported in figure 5.

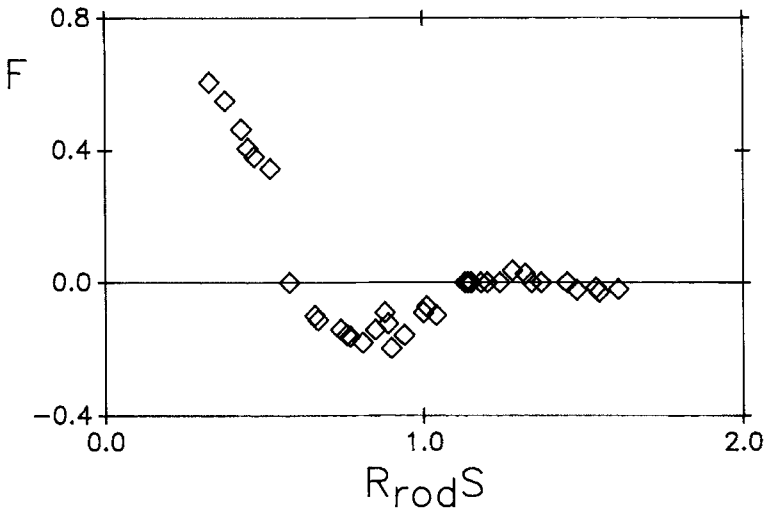

Figure 3. Determination of the signs of the structure factors in the hexagonal phase. The experiments were performed at different water content at 25°C: the normalized structure factors are plotted as a function of the product $R_{rod}S$.

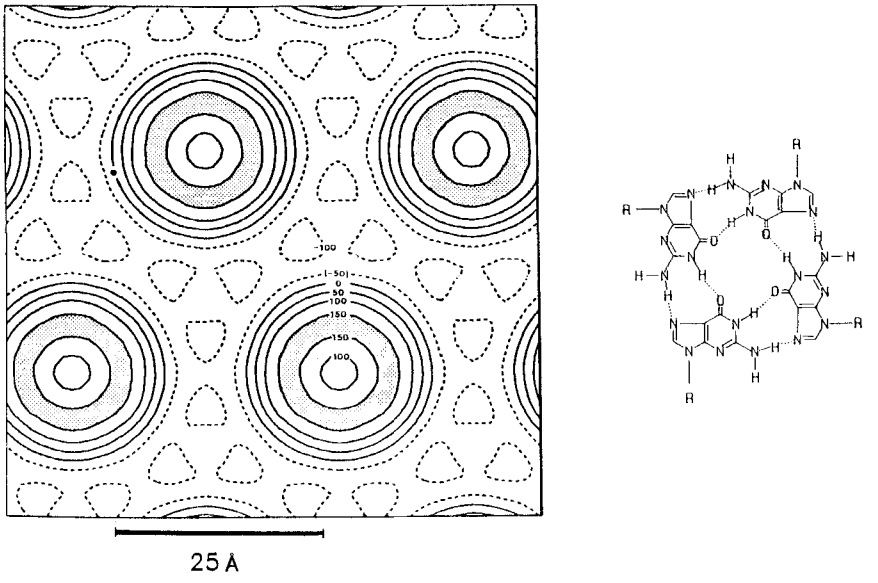

Figure 4. Electron density distribution of the hexagonal phase in the 50 per cent sample at 25°C. The relative value (in arbitrary units) of a few equidensity lines is reported and the highest density area is shaded. On the right hand side, the tetrameric arrangement of guanine bases bonded in a Hoggsteen mode is sketched.

The spectra change dramatically on increasing the temperature indicating the occurrence at low temperature of a self assembly process [20]. In particular, the spectrum at the highest temperature, which should correspond to the unassociated molecules, is very similar to that of the unassociated linear dimer d(GpG) [3] and consists of a weak broad negative band in the region of the first two guanine absorptions. When the sample is cooled to room temperature, this spectrum still

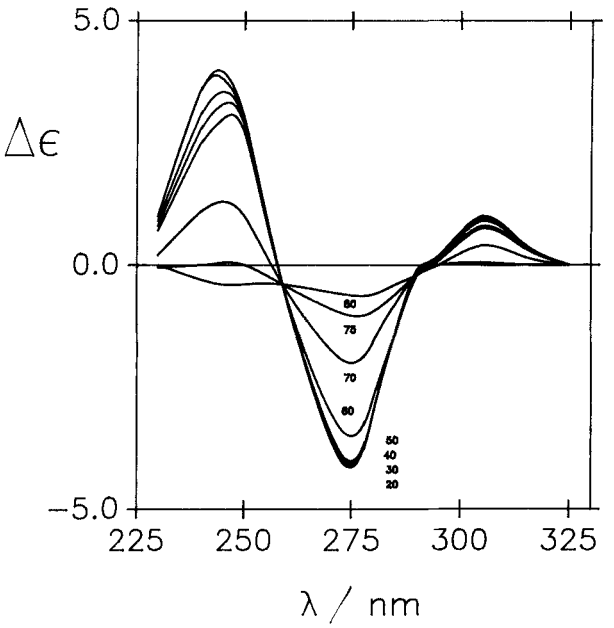

Figure 5. CD spectra of a buffered solution (pH = 7·0) of d(cGpGp) (10^{-4} mol l^{-1} of guanine residue) recorded at different temperatures in a heating experiment: From top to bottom in the positive branch of the exciton couplet centred at *c.* 260 nm: 20, 30, 40, 50, 60, 70, 75, 80°C. The sample suffers a high hysteresis and the solution cooled back to 20°C exhibits the same spectrum as that recorded at 80°C for several hours.

appears for several hours then the spectrum corresponding to the associated species slowly appears. This unassociated spectrum is very different from that of the analogous adenine [21] and thymidine [22] derivatives. For both A and T linear dimers, the CD spectrum is in fact similar to that of DNA with positive exciton couplet (positive band at *c.* 270 nm and negative band at *c.* 245 nm). Cyclization in both A and T derivatives causes a dramatic sign inversion of both bands. This particular behaviour of the guanine derivatives demonstrates that their conformation is much more mobile than in the A and T derivatives. On the other hand, it is well known that in guanine derivatives the rotation process around the glicosyl bond is different from other bases and, in particular, the *syn* conformation is highly populated [23]. CD spectra further show that during the self-assembly process exciton components appear and that there is a considerable increase in their intensity. All of this indicates that the conformation changes and becomes gradually more rigid during the aggregation process. From the sign of the exciton couplet at *c.* 260 nm we can deduce a left handed chirality of the columnar aggregate [24].

3. Discussion

From the data presented here, it seems evident that, despite the steric constraint imposed by the second phosphate bridge, the cyclic dimer also gives rise to a liquid-crystalline hexagonal phase with a mechanism similar to its linear counterpart: a chiral rod-shaped aggregate is formed which is composed of a stacked array of Hoogsteen-bonded guanine tetramers. This aggregate resembles the four-stranded helix formed by polyguanylic acid. However, CD data indicate that in the present case we have a left

handed structure, while for poly(G) [24] (and also for d(GpG) [3]) a right-handed four-stranded helix is observed. The stereochemical constraint imposed by the bridge has, therefore, little effect on the ability to form chiral columnar aggregates, but a remarkable effect on its stereochemistry. At concentrations below 35 per cent these aggregates are still in isotropic solution, but, at the critical concentration (*c.* 40 per cent), a hexagonal mesophase is obtained.

For the linear dimer instead, a cholesteric phase is formed first at very low concentrations (*c.* 2·5 per cent) and then the hexagonal phase is formed at *c.* 18 per cent. The cyclic dimer, therefore, requires a much higher concentration and at room temperature forms only a stable hexagonal phase. The presence of the second phosphate hydrophilic group is certainly connected to the higher concentration required and at this concentration the chiral columnar aggregates have not enough free volume to form the cholesteric structure. On the other hand, in the various examples of chromonic phases reported in the literature [7, 25], at concentrations higher than 40 per cent, only hexagonal phases have been observed at room temperature.

With regard to the conformation of cyclic dimers, X-ray diffraction studies on d(cApAp) and cGpGp in the crystalline phase are reported in [4] and [6]. In [4] an analysis of the molecular conformation in solution by means of NMR and molecular mechanics is also presented which indicates that the 12-membered circular sugar-phosphate backbone provides a rigid framework to hold the two adenines 6·8 Å apart in parallel planes. The same molecular conformation is assigned also to cGpGp in solution [6] in which the two bases are still 6·8 Å apart in parallel planes. Our X-ray data on the hexagonal phase (obtained obviously in very concentrated solutions) display only a 3·4 Å reflection which corresponds to the stacking of the aromatic guanine tetramers; no peaks corresponding to a 6·8 Å periodicity could be detected. On the other hand, CPK models show that, starting from the structure given in [4] for the adenine dimer, the aromatic bases can easily be placed at nearly contact distance without severe distortion of the 12-membered ring. The fact that the mobility of the guanine containing dimers is different to that of the adenine derivative is also illustrated by the difference of CD spectra discussed here. The diameter of the columnar aggregates obtained from X-ray diffraction seems to be slightly smaller than that observed for the linear derivative. Also the 3·4 Å vertical spacing is slightly larger than those observed for linear oligomers [3]. This could be due to the steric constraint exerted by cyclization.

4. Experimental

4.1. *Synthesis*

The cyclic dimer was prepared according to a recently developed procedure [26] and purified by anion-exchange chromatography with DEAE-Sephacell (eluent: linear gradient of TEAB from 0·05 to 1·0 mol l^{-1}). The fractions with HPLC titre $\geqslant 95$ per cent were pooled, coevaporated several times with water to remove the buffer and converted into the ammonium salt by several coevaporations with concentrated ammonia. The final product was lyophilized and kept under vacuum for 24 h in the presence of phosphoric anhydride.

4.2. *Optical microscopy*

Microscopic observations of samples between slide and coverslip were performed with a Zeiss polarizing microscope equipped with a photocamera. Samples with a

concentration gradient were obtained by peripheral evaporation of a water solution or by allowing water to penetrate the neat compound.

4.3. *X-ray diffraction*

X-ray diffraction experiments were made using a rotating anode generator Rigaku Denki RV 300 equipped with a powder diffractometer: Ni-filtered Cu–K_α radiation (average wavelength 1·54 Å) was used. The samples were prepared at the required concentration and left for at least two days at room temperature to avoid inhomogeneity: sometimes, but in many cases unsuccessfully (see Results), a longer time was used to reach equilibrium. The samples were held between two mica windows in a vacuum tight cylindrical cell. To measure the diffraction intensities, X-ray measurements were also performed using a conventional X-ray generator equipped with a camera operating in vacuum: a bent quartz crystal monochromator was used to select the Cu–$K_{\alpha 1}$ radiation. In this case, in order to reduce the spottiness arising from eventual macroscopic monodomains, the sample cell was rotated continuously during the exposure. The diffraction patterns were recorded on a stack of four Kodak DEF-392 films: densitometric traces were obtained by using a Joyce–Loebl microdensitometer and the relative intensities of the reflections were calculated as reported in [11]. The sample cell temperature was controlled by using a circulating thermostat with an accuracy of 0·5°C.

4.4. *Circular dichroism*

CD spectra were recorded with a JASCO J500A spectropolarimeter equipped with a DP100 data processor and with a thermostated water jacket cell holder (thermal stability 0·5°C). The solution was prepared by dissolving the compound (concentration of guanine residue: 10^{-4} mol l^{-1}) in a phosphate buffer (pH = 7·00, Merck) and allowing it to stand refrigerated for a week before recording the spectra.

We thank MURST and CNR for financial support.

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