Biophysical Chemistry 7 (1978) 339–346
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PHYSICAL PROPERTIES OF POLY (3,N^4-ETHENOCYTIDYLIC ACID)

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Received 15 July 1977

Homopolymers of etheno CMP have been prepared by the action of polyribonucleotide phosphorylase upon etheno CDP. At alkaline pH the optical properties are consistent with a structure consisting of partially helical single-stranded chains whose helical regions are stabilized by base stacking. At acid pH the degree of helicity increases markedly. The degree of cooperativity displayed by the helix → coil transitions induced by pH or temperature is less than for the case of polyribocytidylic acid. In the presence of acridine orange the alkaline form develops a strong extrinsic CD spectrum.

1. Introduction

The preparation of fluorescent etheno derivatives of adenosine, cytidine, and several of their nucleotide derivatives has stimulated studies upon the biological activity of these derivatives, as well as upon the physical properties of their polymers [1–5]. The etheno derivatives of AMP, ADP, and ATP have been shown to be capable of replacing their naturally occurring analogs as substrates or modifiers of several enzymes [2,5]. In particular, ϵADP and ϵCDP have been found to function as substrates of polyribonucleotide phosphorylase thereby making possible the biosynthesis of homopolymers of ϵAMP and ϵCMP [4]. This paper is concerned with the properties of homopolymers of the etheno derivative of CMP (poly ϵC).

Poly C itself has been found to exist in at least two distinct helical forms. At neutral pH it exists as a single-stranded structure containing alternating randomly coiled regions and helical regions stabilized by base stacking [6,7]. At acid pH it forms a double-stranded, parallel helical structure with a shared proton between the two N(3) nitrogens of the bases [6,7]. The base pair is further stabilized by two hydrogen bonds between the carbonyl and amino groups of the cytosine bases [6,7]. The acid form of poly C is ionic strength dependent and may be disrupted by either the binding or dissociation of a proton, depending upon solvent conditions [6,7].

Since formation of 3,N^4-ethenocytidine involves modification of the exocyclic amino group and the N(3) ring nitrogen, both of which are involved in the interstrand hydrogen bonding occurring in the two-stranded helical acid form of poly C, it is of particular interest to compare the properties of poly ϵC with those of poly C as a function of pH and temperature. The major issues include the extent to which poly ϵC can adopt helical forms and their relation to those formed by poly C. Previously we have described the structural effects of exocyclic modification of poly G [8].

2. Experimental

2.1. Materials

The etheno derivative of CDP was prepared by the reaction of chloroacetaldehyde with CDP, as described by Barrio et al. [2]. The product, etheno CDP, was pu-

rified on a DEAE cellulose column and used for the preparation of poly εC. Polymerization of εCDP to form poly εC was achieved by the action of polyribonucleotide phosphorylase (PL-Biochemicals, type 15). The polymerization, isolation and purification conditions were similar to those described for O^6-alkylguanylic acid homopolymers [9]. Polymerization was carried out in 0.15 M tris–K^+ cacodylate, 0.08 M $MgCl_2$, 30°C and followed by measuring viscosity as a function of time. The polymer was removed when viscosity attained a plateau in about 4 to 5 hours. The mixture was then deproteinized by $CHCl_3$ emulsification, fractionated on G-200 Sephadex, dialyzed, and lyophilized. The sedimentation coefficient of the preparation used here was 3.5 s at pH 8.5.

The sample of acridine orange used in these studies was purchased from Aldrich and recrystallized twice from ethanol prior to use. All other reagents were analytical grade. Glass redistilled water was used for the preparation of all solutions.

2.2. Methods

Determinations of absorption spectra were made using a Cary spectrophotometer. Measurements of absorbance at a single wavelength were made with a Gilford spectrophotometer.

Determinations of circular dichroism (CD) were made using the JASCO J-40 apparatus. This was equipped with a thermostatted cell holder permitting the circulation of water from a constant temperature bath. The molecular ellipticity at a particular wavelength, $[\theta]_\lambda$, was computed using the equation:

$$[\theta]_\lambda = 100\,\theta_\lambda / Cl\,, \qquad (1)$$

where θ_λ is the observed ellipticity in degrees at wavelength λ, C is the molar concentration of mononucleotide units, and l is the path length.

Molecular weight determinations by light scattering were made using a Phoenix light scattering photometer. Calibration in terms of reduced intensities was made using a Ludox suspension whose turbidity was determined by transmission measurements with the Cary spectrophotometer, using 10 cm cells. Scattering measurements over a range of angles were made using a cylindrical cell, whose angular calibration was checked using an aqueous fluorescein solution.

Concentrations of polymer were computed from the absorbance at 272 nm, assuming a molar extinction coefficient of 1.00×10^4 at pH7, 25° [4].

The majority of measurements were made using a buffer consisting of 0.005 M Na^+ cacodylate (Na Cac), 0.005 M Na^+ acetate (Na oAc), 0.001 M EDTA. In this buffer the polymer was soluble over the entire pH range studied. At an ionic strength of 0.1 precipitation occurred upon standing at pH's below 5.

3. Results

3.1. Properties of εCMP

The CD spectrum of εCMP at pH 6.5 is shown in fig. 1. The spectrum in the region of the principal absorption bands between 330 and 240 nm consists of a weak positive band with little indication of structure. No major change in the spectrum occurred upon alteration of the pH to 4.5.

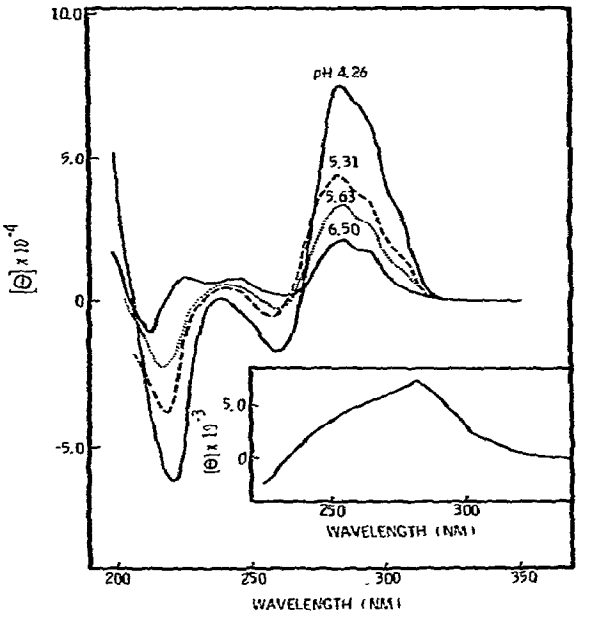

Fig. 1. CD spectra of poly εC (2.6×10^{-5} M) in 0.005 M Na Cac, 0.005 M NaoAc, 0.001 M EDTA, 25°, at a series of pH's within the region of the acid transition. Inset: CD spectrum of εCMP at pH 6.5. The other conditions are the same as above.

3.2. The alkaline form of poly εC

εCMP and poly εC both combine with one equivalent of hydrogen ion per nucleotide unit upon titration from alkaline pH to pH 3 [4]. The hydrogen ion titration curve of poly εC has been reported to have a midpoint close to 5.4 [4]. In the present study, the properties of poly εC were found to be independent of pH from pH 6.2 to pH 8.0, the highest pH to which measurements were extended. It is plausible therefore to consider the properties of the polymer in this pH range as corresponding to those of the alkaline, or unprotonated form of poly εC.

The CD spectrum of alkaline poly εC shows a considerable intensification over that of the monomer. The spectrum at long wavelengths (>260 nm) shows at least two positive bands: a main peak at 283 nm with a shoulder at 296 nm. At lower wavelengths there are weak positive bands at 240 and 223 nm and a negative trough at 212 nm (fig. 1).

The thermal profile at pH 7 of molecular ellipticity at 283 nm showed only a gradual decrease over the entire temperature range observed, with no indication of

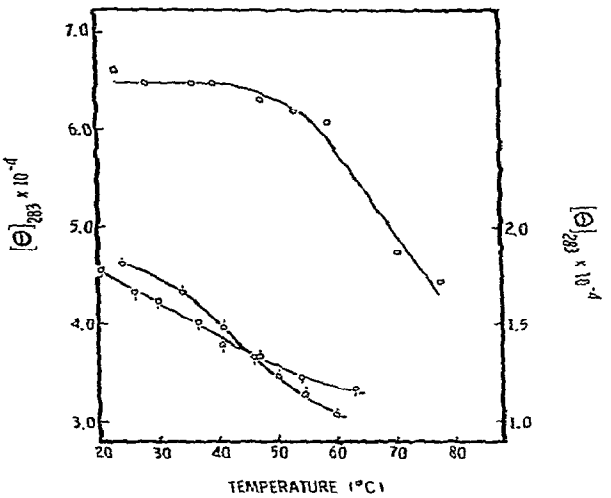

Fig. 2. Temperature dependence of molecular ellipticity for poly εC (3×10^{-5} M) in 0.005 M NaCac, 0.005 NaoAc, 0.001 M EDTA. ♀ pH 7.0 (right ordinate), ♁ pH 5.7 (left ordinate), ○ pH 4.9 (left ordinate).

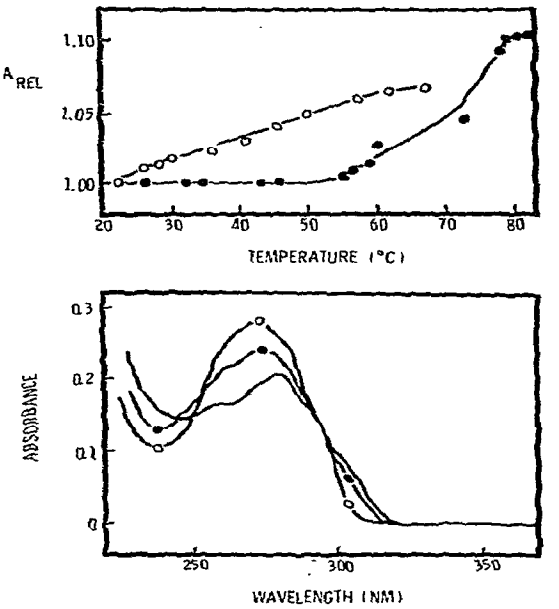

Fig. 3. Upper: Temperature dependence of relative absorbance at 272 nm for poly εC(2×10^{-5} M) in 0.005 M NaCac, 0.005 M NaoAc, 0.001 M EDTA. ○ pH 7.0, ● pH 5.3. Lower: Ultraviolet absorption spectra for poly εC in 0.005 M NaCac, 0.005 M NaoAc, 0.001 M EDTA, 23°, ○ pH 7.0, ● pH 5.5, — pH 4.5.

any hyper-sharpening suggestive of a cooperative transition (fig. 2). A tenfold increase in ionic strength, from 0.001 to 0.1 produced no significant change in either the magnitude of the molecular ellipticity at 23° or the shape of the thermal profile. The thermal behavior of the alkaline form of poly εC is thus qualitatively similar to those of poly C and polyriboadenylic acid [10].

The ultraviolet absorption spectrum at long wavelengths of alkaline poly εC consists of a principal band ($\lambda_{max} \approx 272$ nm) with shoulders at 280 nm and 295 nm (fig. 3). At least two of the corresponding electronic transitions appear to contribute to the CD spectrum (fig. 1). The temperature dependence of the absorbance at 272 nm, like the molecular ellipticity, shows only a gradual transition, reflecting a progressive loss of hypochromism with increasing temperature (fig. 3).

In summary, the optical properties of alkaline poly

εC, including the insensitivity to ionic strength and the noncooperative thermal transition, are generally typical of those of partially helical single-stranded polynucleotide coils, whose helical regions are stabilized by base stacking. The non-conservative nature of the CD spectrum at long wavelengths represents a difference in behavior from some other polynucleotides of this class. However, in the present case the observed spectrum reflects the summed contributions of several electronic transitions, which may mask a spectrum of the quasi-conservative type arising from a particular transition.

The CD spectra of poly C [11] and poly εC differ substantially. The positive band displayed by the former at 275 nm is symmetrical, in contrast to that shown by poly εC, while the positive bands observed at 240 and 223 nm for poly εC are absent for poly C.

3.3. The acid form of poly εC

A reduction in pH produces major changes in the CD spectrum of poly εC (figs. 1 and 4). The positive bands at 295 and 283 nm are greatly intensified and an additional positive band appears at 305 nm. The positive bands at 240 and 223 nm are lost, while negative bands appear at 260 and 218 nm (fig. 1).

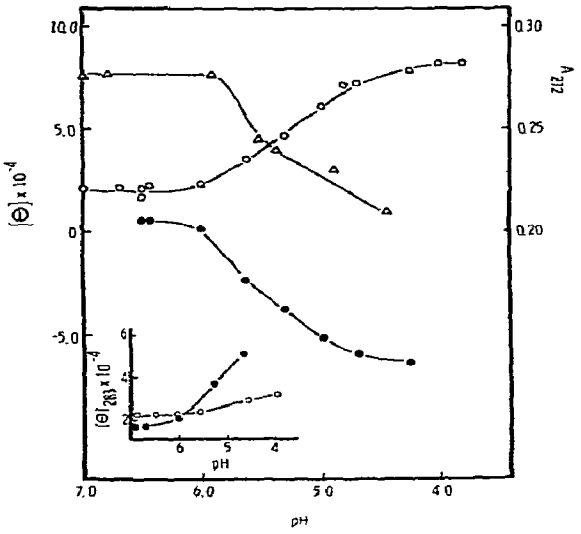

Fig. 4. pH dependence of molecular ellipticity ($[\theta]$) at 283 nm (○) and at 219 nm (●) and of absorbance at 272 nm (△) for poly εC in 0.005 M NaCac, 0.005 NaoAc, 00.01 M EDTA, 23°. Inset: pH dependence of $[\theta]_{283}$ in buffer (No EDTA) plus 0.01 M Mg^{2+} (○) and buffer plus 5.9 M urea (●).

The pH profiles of molecular ellipticity at 283 nm and at 218 nm do not show the pronounced hypersharpening characteristic of a *highly* cooperative coil → helix transition, such as that occurring in the case of polyriboadenylic acid [10], but rather a progressive change below pH 6 (fig. 4). However there is indication of some degree of cooperativity. A logarithmic ("Hill-type") plot of $x/(1-x)$ versus pH, where x, the apparent fractional conversion to the acid form, is equal to $([\theta]_{283,\text{pH}\,3.8} - [\theta]_{283})/([\theta_{283,\text{pH}\,3.8} - [\theta]_{283,\text{pH}\,6.5})$, showed significant curvature, the slope or Hill coefficient, decreasing from a value of 2 to 3 at high pH to a value of 1.2 below pH 5.6.

However a simplified interpretation of the pH profile in terms of a transition between only two species is probably unjustified. A comparison of the CD spectra at a series of pH's within the transition region reveals no well-defined isoelliptic point, as would be expected for a transition between only two distinct species (fig. 1).

Parallel measurements of the ultraviolet absorption spectra at a series of pH's likewise revealed a considerable dependence upon pH. Between pH 7 and pH 6 the spectrum at long wavelengths (>250 nm) consists of a principal band ($\lambda_{max} \approx 272$ nm) with shoulders at 280 nm and 295 nm (fig. 3). At lower pH's a significant additional hypochromism is developed at the wavelength of maximum absorbance, whose position shifts to 280 nm (fig. 3), while a new band arises at 306 nm. The pH profile of absorbance at 272 nm shows some indication of sharpening in the initial region of the transition between pH 6 and pH 5.6, followed by a more gradual decrease at lower pH's (fig. 4).

The presence of 5.9 M urea did not appear to alter qualitatively the shape of the pH profile of molecular ellipticity (fig. 4), although the increase in ellipticity between pH 6 and pH 4.7 became somewhat more gradual. The presence of 0.01 M Mg^{2+} greatly reduced the magnitude of the increase in molecular ellipticity at 283 nm between pH 6 and pH 5 (fig. 4), the region of steepest change being displaced to lower pH values. The binding of Mg^{2+} thus appears to interfere with the acid transition.

The CD spectrum of poly εC at pH's below the transition region can probably be attributed to an ordered, presumably helical, structure. Its quasi-conservative character at long wavelengths is reminiscent of the behavior of other highly helical polynucleotides [11], although the spectrum is, in the present case, complicated by the presence of multiple electronic transitions. The alteration in CD spectrum probably reflects the summed effects of the structural transition and the spectral changes accompanying protonation of the base.

Qualitatively, many features of the pH dependence of absorbancy and molecular ellipticity could be accounted for in terms of an initial significantly cooperative structural transition of the partially protonated polynucleotide, which is subsequently protonated further at lower pH's. The existence of the ordered form in a range of ionization states would account for the absence of an isoelliptic point.

The thermal profile at acid pH of molecular ellipticity at 283 nm (fig. 2) showed a relatively broad transition, whose region of steepest change is displaced to higher temperatures with decreasing pH. The thermal profile of absorbance showed qualitatively similar behavior (fig. 3) in agreement with an earlier report [4].

The properties of the acid form of poly εC differ from those of the doubly stranded acid form of poly C in several significant respects. Both the pH-induced and the thermal transitions have a lower degree of cooperative character [6,7,10]. The ordered form of poly εC is not disrupted by complete protonation at pH 4.

An attempt was made to compare the molecular weights of the alkaline and acid forms of poly εC by light scattering measurements. While an apparent increase in molecular weight by a factor of three occurred at pH 4.5 as compared with pH 7, the apparent molecular weights at the former pH were time-dependent and suggestive of a slow aggregation with time. It was thus not possible to dissociate the effects of a possible formation of a multistranded structure from those of non-specific aggregation.

3.4. Interaction of poly εCMP with acridine orange

The alkaline form of poly εCMP, in the presence of the metachromatic dye acridine orange, in a 1 : 0.5 mole ratio, develops a strong extrinsic CD spectrum at visible wavelengths, with a positive band centered close to 444 nm and negative troughs at 480 and 420 nm (fig. 5). The dependence of molecular ellipticity at 444 nm upon the dye: polynucleotide (D/P) mole ratio (fig. 5) is sigmoidal for values of $D/P < 1$. This is the case whether the molecular ellipticity is computed per nucleotide unit or per dye molecule. In the former case the variation of $[\theta]_{444}$ with $(D/P)^2$ is linear.

The dependence upon D/P of $[\theta]_{444}$ computed per dye molecule (fig. 5) indicates that the induced CD spectrum cannot arise from isolated and independently bound dye molecules. If this were the case, the molecular ellipticity would be constant, or decrease slowly with increasing D/P.

An increase in molecular ellipticity with increasing values of D/P has been reported for the interaction of several dyes with DNA [12–15]. This has been interpreted as indicating that dye-dye interactions are responsible for the induced dichroism. However, for the DNA-dye systems a maximum value of induced circular dichroism is attained at much lower degrees of saturation than in the present case where it occurs for values of D/P close to unity [12–15].

As discussed earlier, the most plausible model for the alkaline form of poly εCMP at 23° is that of a partially helical single strand, whose limited helical regions are stabilized by base stacking interactions. In all probability the observed dependence of ellipticity upon D/P reflects both the increasing importance of pair interactions of bound dye with increasing D/P and also the cooperative induction of increased helicity of the polynucleotide upon increasing saturation with bound dye.

At D/P values close to unity, where the induced ellipticity attains its maximum value, the visible absorption spectrum is characteristic of that expected for externally stacked dye [16], with λ_{max} at about 460 nm (fig. 5). From a comparison of the value of absorbance at 492 nm with the value for free dye under these conditions it is estimated that the dye is over 90% bound for a D/P of 0.91.

In addition to the visible CD band at 400–500 nm, a set of strong bands also arises in the ultraviolet CD spectrum (fig. 6) with a positive peak at 257 nm and negative peaks at 330, 295, and 275 nm. The values of molecular ellipticity attained at values of D/P close to 1.0 are remarkably high, $[\theta]_{275}$ being close to

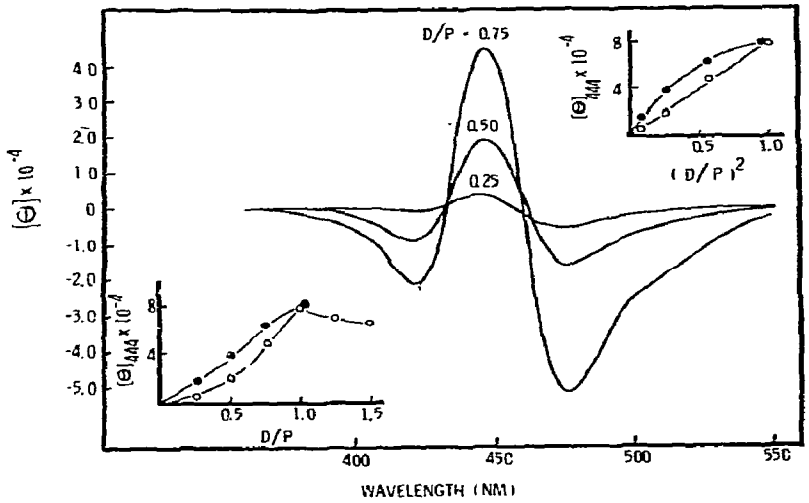

Fig. 5. Visible CD spectra shown by complexes of poly εC (3.0×10^{-5} M) with acridine orange. The buffer is 0.005 M NaCac, 0.001 M EDTA, pH 7.0, 23°. Left inset: Dependence of molecular ellipticity upon dye/polymer (D/P) mole ratio. Molecular ellipticities computed with respect to nucleotide concentration: —○—. Molecular ellipticities computed with respect to dye concentration: —●—. Right inset: Variation of molecular ellipticity with $(D/P)^2$. Molecualr ellipticities computed with respect to nucleotide concentration: —○—. Molecular ellipticities computed with respect to dye concentration: —●—.

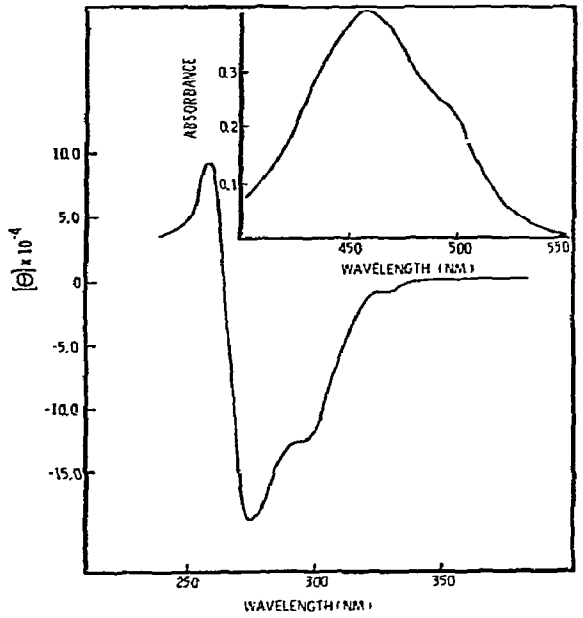

Fig. 6. Ultraviolet CD spectrum under above conditions for $D/P = 1.5$. Inset: Visible absorption spectrum under above conditions for $D/P = 0.91$.

-18×10^4.

Since the conformation and CD spectrum of the polynucleotide alone are unknown for these conditions, it is not possible to isolate the CD spectrum arising solely from bound dye. However, from the magnitude of the observed ellipticities it is likely that base-dye interactions are making a significant contribution. Since poly εC and acridine orange show extensive overlap of their ultraviolet absorption spectra, it is plausible that interactions of this kind could be of importance in determining the CD spectrum [17,18].

It is of interest to compare the extrinsic CD spectra developed by acridine orange with poly εC and with polyribouridylic acid. The latter spectrum shows qualitative differences from the former (fig. 7). A positive band appears at 425 nm and a negative trough occurs at 460 nm; the second negative trough found in the poly εC spectrum is absent. The signoidal variation of molecular ellipticity with D/P is present, as in the case of poly εC.

One can only speculate as to the nature of the helical structure formed by poly εC saturated with bound

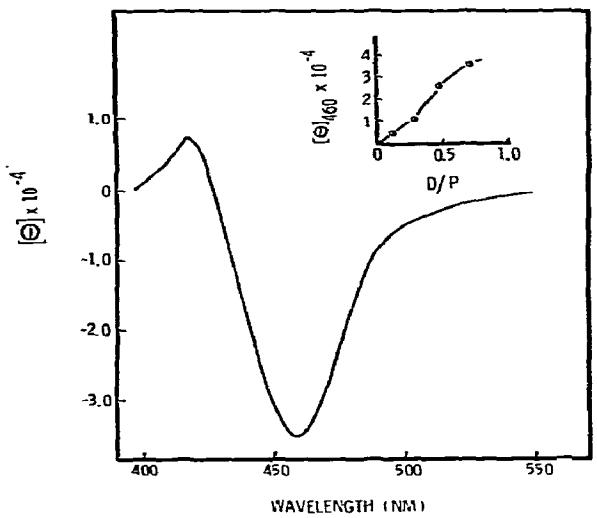

Fig. 7. Visible CD spectrum for a complex of acridine orange ($D/P = 0.71$) with poly U (6.22×10^{-4} M) in 0.005 MNaCac, 0.005 M NaoAc, 0.001 M EDTA, pH 7.0, 23°. Inset: Variation of the molecular ellipticity with D/P for the above conditions.

acridine orange. An obvious possibility is a single stranded helix stabilized by both dye–dye and base–base stacking interactions, possibly with significant overlap of the planar bases and bound dye.

3.5. *Interaction of poly ϵC with mercuric ion*

Fig. 8 shows the effects of the addition of Hg^{2+} to alkaline poly ϵC. The structure of the positive CD band at long wavelengths is progressively lost, while the maximum is shifted to longer wavelengths. The negative trough at 212 nm is intensified and shifted to longer wavelengths. The formation of the etheno bridge does not appear to block the interaction of poly ϵC with mercuric ion.

4. Discussion

The optical properties of the alkaline form of poly ϵC which prevails at pH's above 6, are somewhat similar to those of several well-characterized single-strand

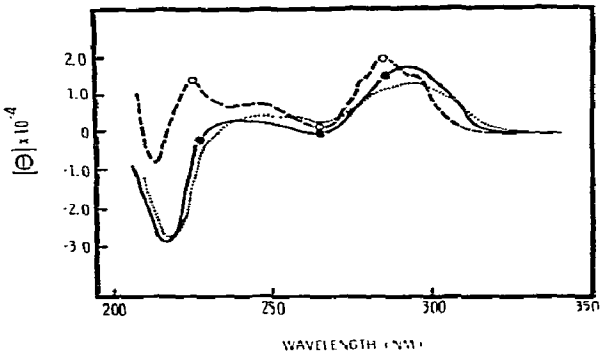

Fig. 8. CD spectra for poly ϵC (2.74×10^{-5} M) in the presence of varying levels of $HgCl_2$. The buffer is 0.005 M NaCac 0.005 NaoAc, pH 7.0, 23°. –o– 0 Hg^{2+}, –●– 2.4×10^{-5} M Hg^{2+}, ●●● 9.5×10^{-5} M Hg^{2+}.

polyribonucleotide systems possessing a partially helical structure stabilized by base stacking, such as poly C and polyriboadenylic acid [10]. The points of resemblance include the presence of significant temperature-dependent hypochromism, the enhanced magnitude of molecular ellipticity relative to that of the monomer, the noncooperative thermal transition, and the insensitivity to ionic strength. It appears reasonable to assign provisionally to the alkaline form of poly ϵC a structure of this kind. This model corresponds to a single stranded polymer in which randomly coiled regions alternate with helical segments of limited extent. The latter are progressively melted out with increasing temperature by a noncooperative mechanism.

The structure of the helical species formed at acid pH remains uncertain. The $3,N^4$ ethenocytosine groups cannot participate in the normal base pairing interactions of cytosine and stabilization of the helical structure must occur by some other mechanism. The blocking of the acid transition by Mg^{2+} suggests that electrostatic interactions may make a significant contribution to the free energy of stabilization.

The noncooperative nature of the thermal melting curve makes it unlikely that the acid form of poly ϵC consists of doubly stranded helices of considerable extent. The possibilities include a bihelical form with numerous interruptions, so that a series of short bihelical regions are present, as well as single stranded helices, which undergo lateral aggregation.

The strong negative trough at 218 nm appears to be in juxtaposition to a strong positive band at lower wavelengths (fig. 1), which was not completely resolvable with the available instrumentation. A conservative spectrum in this wavelength region has been observed for several helical polynucleotides [9] and has been attributed to $\pi \rightarrow \pi^*$ transitions corresponding to absorption bands related to the E_{1u} band of benzene [19]. An alternative explanation [9] is in terms of an $\eta \rightarrow \pi^*$ transition involving the nonbonding electron pairs of the N and O atoms.

The strong extrinsic CD spectrum developed by the alkaline form of poly ϵC in the presence of acridine orange is clearly suggestive of an induced helical conformation. The stabilizing factors include the charge neutralization of the polynucleotide strand resulting from the binding of the cationic dye, the base stacking interactions of the ethenocytosine groups, and perhaps the stacking interactions of externally bound dye.

Acknowledgement

This research was supported by Grant Numbers CA 20129 and CA 20292 from the National Cancer Institute, DHEW, and by Grant Number 74 B from the American Cancer Society.

References

[1] J.R. Barrio, J.A. Secrist and N.J. Leonard, Biochem. Biophys. Res. Commun. 46 (1972) 597.
[2] J.A. Secrist, J.R. Barrio and N.J. Leonard, Science 175 (1972) 646.
[3] J.A. Secrist, J.R. Barrio, N.J. Leonard and G. Weber, Biochemistry 11 (1972) 3499.
[4] B. Janik, R.G. Sommer, M.P. Kotick, D.P. Wilson and R.J. Erickson, Physiol. Chem. and Physics 5 (1973) 27.
[5] R.F. Steiner, FEBS Letters 23 (1972) 139.
[6] W. Guschlbauer, Proc. Natl. Acad. Sci. V.S. 57 (1967) 1441.
[7] W. Guschlbauer, Nucleic Acids Res. 2 (1975) 353.
[8] J.R. Mehta, D.B. Ludlum and R.F. Steiner, Biochim. Biophys. Acta, in press.
[9] J.R. Mehta and D.B. Ludlum, Biochemistry 15 (1976) 4329.
[10] A.M. Michelson, J. Massoulie and W. Guschlbauer, Prog Nucleic. Acid Research, eds. J. Davidson and W. Cohn, Vol. 6 (Academic Press, New York, 1967) p. 83.
[11] F.H. Wolfe, K. Oikawa and C.M. Kay, Canadian J. Biochem. 47 (1969) 637.
[12] K.R. Bhat, Doctoral Thesis, Physiochemical investigations of the magnesium – DNA – proflavine system, Rutgers University (1974).
[13] H.J. Li and D.M. Crothers, Biopolymers 8 (1969) 217.
[14] D.G. Dalgleish, H. Fujita and A.R. Peacocke, Biopolymers 8 (1969) 633.
[15] A. Blake and A.R. Peacocke, Biopolymers 5 (1967) 38.
[16] D.F. Bradley and M.K. Wolfe, Prod. Natl. Acad. Sci. U.S. 45 (1959) 944.
[17] I. Tinoco Jr., J. Chim. Phys. 65 (1968) 91.
[18] I. Tinoco Jr., J. Am. Chem. Soc. 86 (1964) 297.
[19] J. Brahms, J.B. Pilet, H. Damany and V. Chansekharan, Proc. Natl. Acad. Sci. U.S. 60 (1968) 1130.