

Molecular aspects on the interaction of sanguinarine with B-form, Z-form and H^L-form DNA structures

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The interaction of sanguinarine with right-handed (B-form), left-handed (Z-form) and left-handed (H^L-form) structures of poly(dG-dC).poly(dG-dC) has been investigated by measuring the circular dichroism (CD) and UV-absorption spectral analysis. Sanguinarine binds strongly to the B-form DNA and does not bind to Z-form or H^L-form, but it converts the Z-form and the H^L-form back to the bound right handed form as evidenced from CD spectroscopy. Sanguinarine inhibits the rate of B to Z transition under ionic conditions that otherwise favour the left-handed conformation of the polynucleotides. UV absorption kinetic studies show that the Z-form reverses back to B-form on binding to sanguinarine. Binding isotherms obtained from spectrophotometric data show that sanguinarine binds strongly to the B-form polymer in a non-cooperative manner, in sharp contrast to the highly cooperative interaction under Z-form and H^L-form polynucleotides. These studies reveal that the alternating GC sequence undergoes defined conformational changes and interacts with sanguinarine which may be an important aspect in understanding its extensive biological activities.

It is known that B-form DNA can adopt a variety of unusual conformations depending on the nucleotide sequence and environmental conditions¹. Of the unusual structures so far known, perhaps, the most striking example is the Z-DNA, which is a left-handed duplex conformation with Watson-Crick base pairs and can be generated in alternating purine-pyrimidine sequences under a variety of solution conditions²⁻⁵. X-ray crystal structure analysis of oligonucleotide crystals has further confirmed the left-handed Z-form structure⁶. Although, a precise biological role of Z-DNA has not yet been unambiguously identified⁷, evidence for the existence of Z-DNA *in vivo* has been provided⁸⁻¹¹. Both right-handed B-form and left-handed Z-form coexist in plasmids of *Escherichia coli* and, they have also been detected in chromosomes of *Drosophila* and in metabolically active mammalian cells¹⁰.

Protonation of DNA has been studied for several years¹²⁻¹⁶, but has gained considerable significance from the observation of Chen¹⁷ that a left-handed (Z-like) conformation could be generated in poly(dG-dC).poly(dG-dC) at acid pH. From extensive UV-spectroscopy and CD studies, the author's laboratory has advanced a model of left-handed structure¹⁸ with the formation of Hoogsteen base pairing at low pH. It is yet another polymorphic form of DNA and its name

has been coined as H^L-form¹⁹. This model of H^L-form DNA has recently been confirmed by the FTIR studies of Tajmir-Raiahi and co-workers²⁰ and by the Raman spectroscopic studies of Otto and co-workers²¹. The biological relevance of the protonation induced conformational study of nucleic acids with respect to biological systems has also been reported by several authors²²⁻²⁴.

The focus of the present study is the interaction of a biologically important benzophenanthridine plant alkaloid, sanguinarine (Fig. 1) with the three major polymorphic form of DNA, namely the right-handed B-form, the left-handed Z-form and the left handed H^L-form of poly(dG-dC).poly(dG-dC) polymer. Sanguinarine is a planar phenanthridine fused aromatic ring system which is extensively distributed in plant species and has displayed excellent antimicrobial, antitubulin, antiplaque and antitumour activities²⁵⁻²⁸. Using extensive UV-VIS spectroscopy, fluorescence spectroscopy, CD spectroscopy, NMR spectroscopy, thermal melting, viscometric and thermodynamic studies, the author's laboratory has

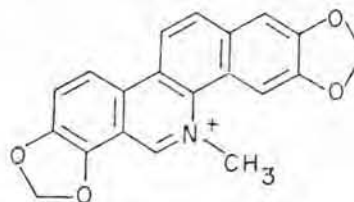


Fig. 1—Chemical structure of sanguinarine

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demonstrated for the first time that sanguinarine (iminium form) binds to DNA by a mechanism of intercalation with a high preference to GC base pairs²⁹⁻³⁸, while its alkanolamine form does not bind to B-form DNA³⁸. The present study provides information on the nature of the conformational transition induced by this alkaloid in the three polymorphic forms of DNA, B, Z and H^L-form of poly(dG-dC).poly(dG-dC) in solution.

Materials and Methods

Sanguinarine chloride was obtained from Aldrich (Milwaukee, WI, USA) and was used without further purification as no detectable impurities were observed by thin layer chromatography and proton-NMR spectroscopy. The solution of sanguinarine was freshly prepared each day and was always kept protected in dark to prevent any light induced changes. A molar extinction coefficient (ϵ) 30,700 $M^{-1} \text{ cm}^{-1}$ at 327 nm in 0.1 N HCl was used for determining its concentration³⁸. No deviation from Beer's law was seen in the concentration range employed in this study.

Poly(dG-dC).poly(dG-dC), hereafter poly[d(G-C)], was a product of Sigma Chemical Co. (MO, USA) and was used as such after checking its nativeness by UV and CD spectral measurements. The concentration of the polymer was estimated spectrophotometrically by using a molar extinction coefficient of 8400 $M^{-1} \text{ cm}^{-1}$ at 254 nm^{39,40}. Citrate-phosphate (CP) buffer containing 5 mM Na_2HPO_4 was adjusted to the specified pH using citric acid following the method of Gomori⁴¹. This buffer provided constant sodium molarity of 10 mM. CP buffer, pH 5.2, was used for studies with the B-form and Z-form DNA structures and its interaction with sanguinarine. This is because sanguinarine persists fully as charged iminium form^{36,37} at this pH at 20°C. Formation of H^L-form structure and its interaction with sanguinarine were performed at 10°C in CP buffer of pH 3.4. Analytical grade reagents and glass distilled deionized water were used throughout.

Spectrophotometric study

All UV-VIS absorbance studies were performed on a Shimadzu Model UV-260 automatic recording double-beam spectrophotometer (Shimadzu Corporation, Japan) equipped with a thermoelectrically controlled cell holder using a 1 cm path length in a matched quartz cuvette as described previously³⁹.

Circular dichroic (CD) study

CD measurements were carried out on a Jasco J-720 spectropolarimeter (JASCO International Co. Ltd., Japan) equipped with a Jasco temperature controller, Model PTC-343 as described^{39,40}.

Formation of Z-form and H^L-form

The B to Z and B to H^L transition were measured by absorption and CD spectroscopy. For B to Z transition potential, the poly[d(G-C)] polymer was treated with 40 μM hexamine cobalt chloride under continuous stirring of the solution as reported^{4-6, 19}. An equilibrium time of 20 min was generally allowed after initiating the conformational transition. The ratio of absorbance at 260 nm to that at 295 nm was used as a measure of the B to Z transition. The formation of H^L-form from B-form was shown to occur by slowly adding the polymer into 10 mM CP buffer, pH 3.4 at 10°C under constant stirring. Dry nitrogen gas was purged through the sample chamber to avoid moisture condensation on the optical windows. The transition from B to H^L form followed very fast kinetics and was found to be complete within a second. The hypochromicity change at 255 nm was generally about 23% during B to H^L transition resulting in a characteristic UV spectrum for the H^L-form of poly[d(G-C)]. This result is in conformity with our earlier observation^{18,19}. Duplex nature of the H^L structure was also confirmed by Tm measurement^{29,30} and the Tm value was found to be 40.5°C. The secondary structure of H^L-form was further generated on a Pentium II computer using Chem-Window programme.

Spectrophotometric titration study

The absorption spectra of the alkaloid mixed with or without the B, Z and H^L-form DNA structures were obtained using the same Shimadzu UV-260 spectrophotometer following the method described earlier¹⁹. The binding studies with the B- and Z-form DNAs were performed at 20°C, while that with the H^L-form was done at 10°C. Sufficient stock of the polymer was added to the buffer in a 1 cm path length cuvette to yield a concentration of 40 μM . Successive aliquots from a stock solution of sanguinarine were added to the cuvette at 20 min intervals. The amount of free (C_f) and bound sanguinarine (r_b) was determined following the method described by Chaires *et al.*⁴². The total alkaloid concentration CT and the amount of bound alkaloid (CB) were calculated using $\epsilon_{327}(\text{bound}) = 15,430 \text{ M}^{-1} \text{ cm}^{-1}$ and

$\epsilon_{353} = 14,520 \text{ M}^{-1} \text{ cm}^{-1}$, at the isosbestic point for sanguinarine as described earlier.

Results

Characteristic of B, Z and H^L -form

The CD spectrum of the B-form poly[d(G-C)] is characterized by a positive band in the 275 nm followed by a large negative band in the 250 nm region. This CD spectrum undergoes large changes as the polymer switches from the right-handed B to either left-handed Watson-Crick base paired Z-form or to the left-handed Hoogsteen base paired H^L -form. The Z-form CD spectrum generated presently under conditions of 40 μM hexamine cobalt chloride is in good agreement with that reported in the literature by previous workers⁴⁻⁶. In the B to H^L transition, a weak negative band appears around 300 nm, followed by a broad positive ellipticity in the 250-280 nm region. The formation of Z-form and H^L form is shown in Fig. 2. These three polymorphic forms exhibit a single isoelliptic point at 277 nm, indicating a complete transition equilibrium of each form of the polymer. The computer generated complementary hydrogen-bonded base pairs of B-form and H^L -form poly [d(G-C)] are depicted in Fig. 3A and B respectively. However, the hydrogen-bonded base pairs of Z-form

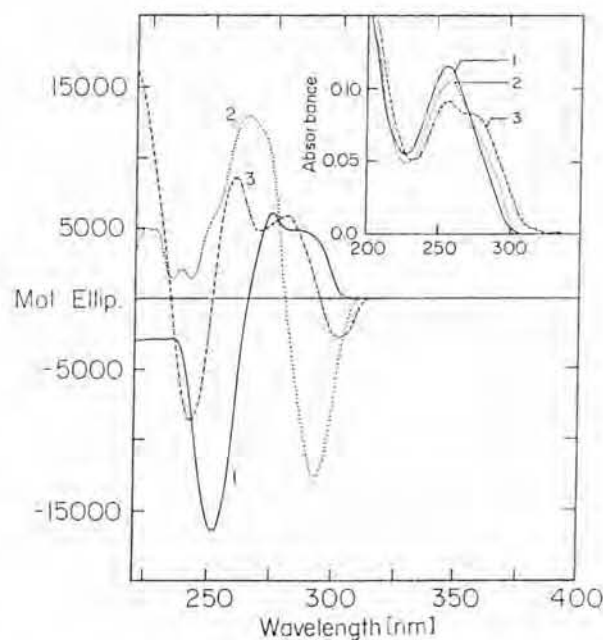


Fig. 2—CD spectra of 40.33 μM poly [d(G-C)] of B-form (curve 1) in 10 mM CP buffer, pH 5.2, 20°C; Z-form (curve 2) in 10 mM CP buffer containing 40 μM hexamine cobalt chloride, pH 5.2, 20°C and H^L -form (curve 3) in 10 mM CP buffer, pH 3.4, 10°C. Inset : UV spectra of B-, Z- and H^L - forms of 14.0 μM poly [d(G-C)] in identical buffer condition as above.

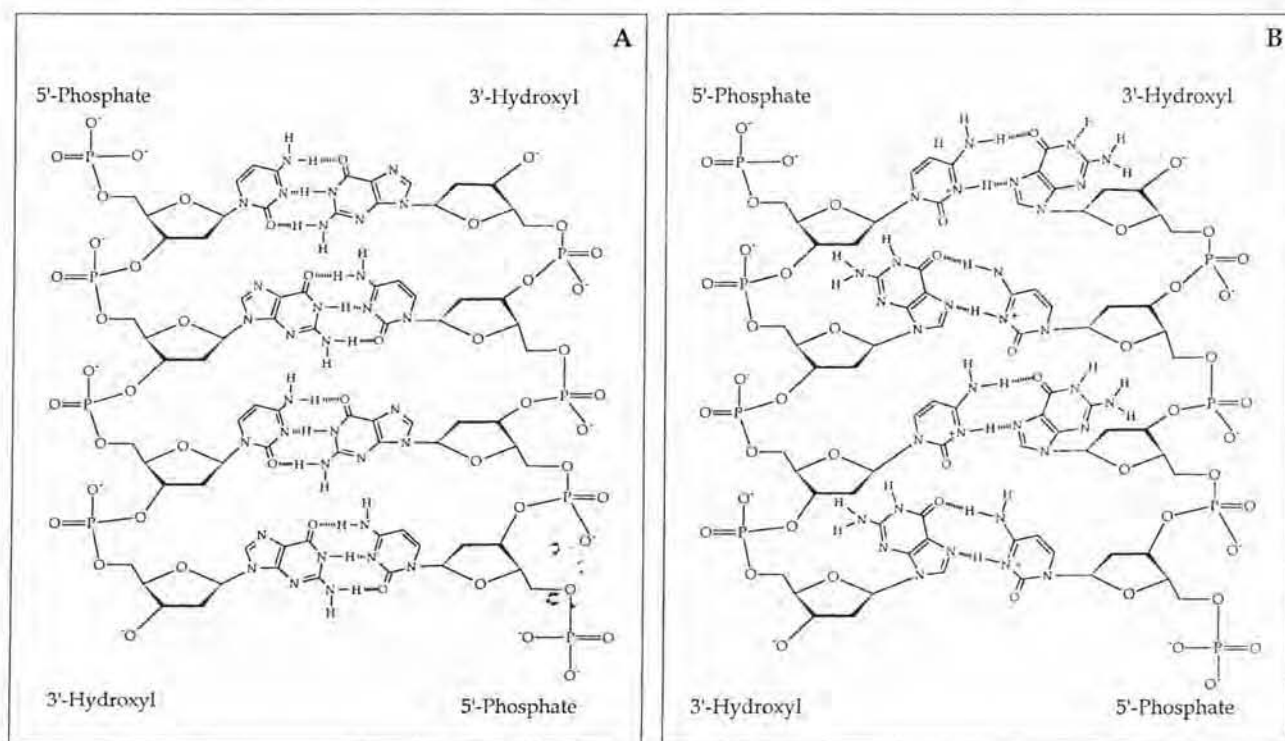


Fig. 3—A computer generated complementary hydrogen-bonded base pairs of the secondary structure of B-form (A) and H^L -form (B) of the polymer poly [d(G-C)].

poly [d(G-C)] could not be generated two-dimensionally as it follows the zig-zag path of its phosphate backbone¹. Fig. 3B clearly demonstrates the formation of secondary structure of H^L-form with Hoogsteen base pairs at acidic pH which results from the protonation of the N7-position of guanine bases and subsequent swing to *syn*-conformation from *anti*-conformation (B-form) to share the proton with the N3 of cytosine in alternating GC polymer.

CD spectral changes of B-form, Z-form and H^L-form DNA interaction with sanguinarine

The CD spectral changes associated with the interaction of sanguinarine with the B, Z and H^L-form of poly[d(G-C)] are shown in Fig. 4. Fig. 4A shows that on binding to sanguinarine, the CD spectrum of B-form structure remarkably perturbs producing pronounced enhancement of the 275 nm long wavelength band ellipticity. The changes in the 250 nm negative CD band were considerably less. An isoelliptic point was observed at 255 nm indicating an equilibrium in the binding process. Although sanguinarine is an optically inactive alkaloid, it acquires optical activity on binding to DNA. This is manifested by the CD spectral band arising out of the 320-370 nm region where sanguinarine has an absorbance band at around 327 nm. As the binding progresses, the ellipticity of this band also increases and later, attains a saturation at D/P of 0.38. The generation of this extrinsic band is due to asymmetric arrangement of sanguinarine molecules on binding to DNA structure. The CD spectral change on binding of sanguinarine to Z-form poly[d(G-C)] structure is depicted in Fig. 4B. The ellipticity of the 295 nm negative band decreases and crosses over to the positive side and increases in intensity as the concentration of the alkaloid increases. A saturation in the CD spectral changes was attained at a D/P of 0.38 and the generation of extrinsic CD band was also observed in the region 320-370 nm. At this point, the CD spectrum has features identical to the sanguinarine bound B-form of the polymer (curve No. 5 of Fig. 4A) indicating clearly that sanguinarine could convert the Z-form structure of poly[d(G-C)] to bound B-form structure. The effect of sanguinarine with H^L-form of poly[d(G-C)] is depicted in Fig. 4C. The spectral changes, essentially show similar features as the Z-form of the polymer. At saturation (D/P of 0.35), the CD spectral features of the sanguinarine bound polymer were similar to the bound B-form (curve No. 5 of Fig. 4A) but the ellipticity nature was a little lower.

Effect of sanguinarine on the kinetics of B to Z transition

The kinetic experiments were performed spectrophotometrically monitoring the change in absorbance at 295 nm, which is generally considered to be a diagnostic feature of the B to Z transition. The variation of absorbance with time is shown in Fig. 5. The reaction was initiated by adding poly[d(G-C)] to a buffer solution containing 40 μ M hexamine cobalt chloride in presence and absence of sanguinarine. At D/P of 0.28, complete inhibition of B to Z transition

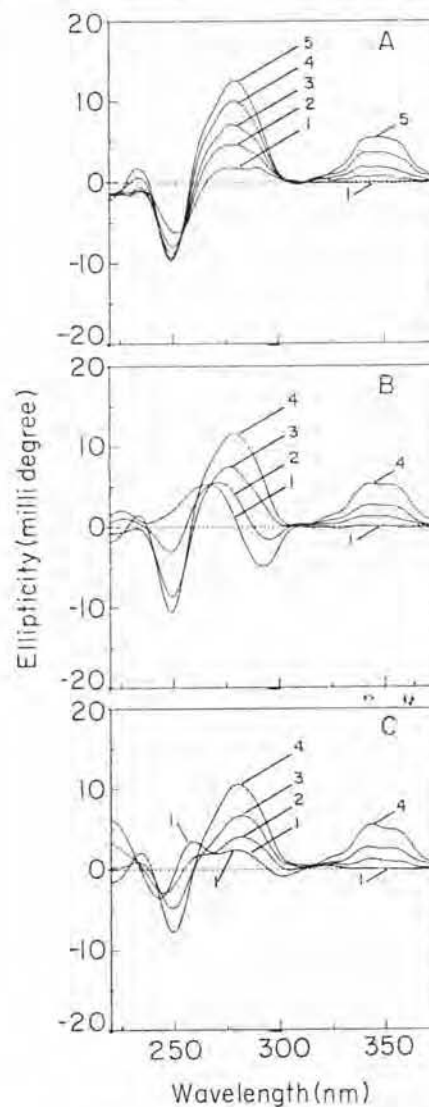


Fig. 4—CD spectra resulting from the interaction of 40.33 μ M poly [d(G-C)] in B-, Z- and H^L-forms with sanguinarine; (A) curves (1 to 5) denote the B-form polymer treated with 0, 4.63, 7.71, 10.77 and 15.34 μ M of sanguinarine; (B) curves (1 to 4) denote the Z-form polymer treated with 0, 3.92, 7.83 and 15.60 μ M of sanguinarine; (C) curves (1 to 4) denote the H^L-form polymer treated with 0, 4.77, 9.52 and 14.25 μ M of sanguinarine.

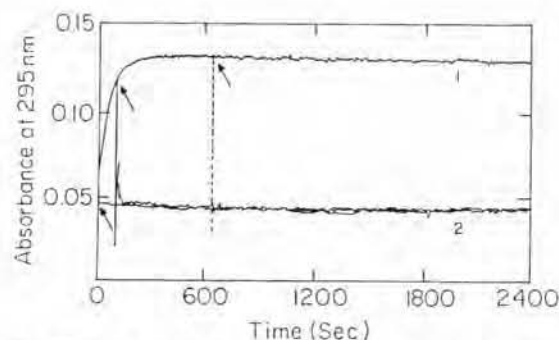


Fig. 5—Effect of sanguinarine on the kinetics of B to Z transition of 50.0 μM poly [d(G-C)] polymer in absence (curve 1) and in presence (curve 2) of 14.2 μM sanguinarine. The reaction was initiated by adding the polymer to the buffer containing with and without sanguinarine. The transition was interrupted by addition of 14.2 μM sanguinarine at the points indicated by the arrows.

was observed as indicated in Fig. 5 (curve No. 2). The reversal of Z to B transition was interrupted by the addition of the same amount of alkaloid at time intervals as indicated by arrows (Fig. 5). The kinetics of B to H^{L} transition followed a very fast kinetics which could not be monitored in the time scale of UV-VIS spectrophotometer. However, in a separate CD experiment, a complex of sanguinarine-B form poly[d(G-C)] was prepared and this was then transferred to CP buffer at pH 3.4. Under these experimental conditions, formation of H^{L} -form structure was not observed indicating that the alkaloid binds strongly to B-form structure and prevents its conversion to the H^{L} -form structure.

Binding studies of sanguinarine with B-, Z- and H^{L} -forms

To understand the nature of the conformational transitions, spectrophotometric binding studies of the alkaloid with the three forms was performed. The binding isotherms of sanguinarine on B-form, Z-form and H^{L} -form of poly[d(G-C)] are shown in Fig. 6. Significant differences are observed between the two left-handed forms (Z- and H^{L} -forms) in contrast to the results for B-form. The nature of binding isotherm for B-form DNA is indicative of a non-cooperative type of binding, while that of Z-form and H^{L} -form DNA is cooperative. The results show that sanguinarine interacts with Z-form and H^{L} -form DNA in a cooperative manner to convert both the polymorphic structures to bound B-form structures.

Discussion

The results presented here demonstrate that sanguinarine converts the left-handed Z-form and left-handed H^{L} -form of poly[d(G-C)] to the right-handed

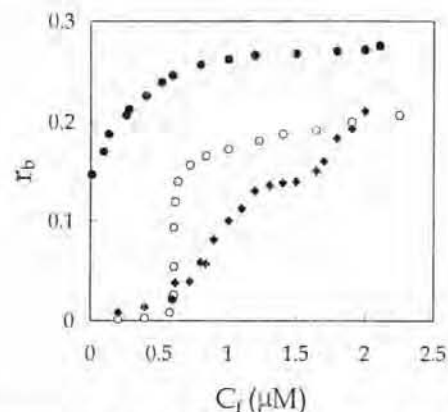


Fig. 6—Binding isotherms for sanguinarine on the interaction with B-form (●-●), Z-form (O-O) and H^{L} -form (◆-◆) of poly [d(G-C)].

bound B-form, in conditions otherwise favouring the existence of Z- and H^{L} -forms. The present data is restricted only to the Z-form in 40 μM hexamine cobalt chloride. In fact, attempts have been made to perform similar kinetic and CD studies under conditions of 4.5 M NaCl and 1.0 M MgCl_2 , the other two ideal conditions favouring Z-form^{4,6}. Since sanguinarine itself gets aggregated at those high salt conditions, such studies could not be performed unambiguously. Nevertheless, the studies with the Z-form of poly[d(G-C)] generated with 40 μM hexamine cobalt chloride is sufficient to demonstrate and generalize the ability of sanguinarine to induce Z to B transitions as $[\text{CO}(\text{NH}_3)_6]^{3+}$ ion has shown to be the best stabilization of Z-form, five order of more effective than $[\text{Na}^+]$ ions and four order of magnitude more effective than $[\text{Mg}^{2+}]$ ion respectively⁴³.

CD spectra presented in Fig. 4 show the nature of the conformational transition associated with the conversion of the Z- and H^{L} -forms to the bound B-form in presence of the alkaloid. The spectral changes from Z- and H^{L} -forms directly towards a characteristic right-handed B-form DNA-sanguinarine complex, is without any indication of intermediate structures. Similar kind of CD spectral changes have been reported in the literature with other DNA intercalating ligands^{44,45}. Therefore, it is likely that the binding site of sanguinarine is a right-handed intercalation site. Formation of alternate binding sites such as left-handed intercalation site is not required to explain the binding under the Z-forms structure. Although the complex may form *via* intercalation into left-handed DNA with subsequent change in handedness of the intercalation site. Intercalators like ethidium⁴⁶, actinomycin D⁴⁵ and dannomycin⁴⁴ have

been shown to induce cooperative binding effects with left handed Z-form of poly[d(G-C)]. In case of the recently studied antibiotic, elsamicin, similar effects have been reported⁴⁷. Sanguinarine inhibits the rate of B to Z transition. Similar kinetic data for several intercalators has been reported by Mirau and Kearn⁴⁸ who have suggested that the effectiveness of inhibition of B to Z transition of an intercalator is related to its binding kinetics rather than the binding constant. Earlier studies from the author's laboratory have revealed that sanguinarine intercalates to B-form DNA and exhibits a remarkably high GC specificity in the binding²⁹⁻³⁸. This affinity for GC specific B-conformation is practically responsible for effectively inhibiting the B to Z transition in poly[d(G-C)] and inducing Z to B transition in this polymer.

It has been clearly demonstrated that poly[d(G-C)] structure is also the ideal model for B to H^L equilibrium transformation (Fig. 2). In solution at pH 3.4 at 10°C, the formation of a characteristic negative CD band around 300 nm in this polymer is considered to be the diagnostic feature for the isomerization of the polymer to the left-handed H^L-conformation. These data are in conformity with the earlier observations¹⁹. This conformational isomerization under the influence of low pH exhibits an isoelliptic point with the B-form that indicates that the two conformations of the polymers coexist under these experimental conditions. Further support to the left-handed type helical conformation under the influence of low pH comes from the absorbance data, where hypochromic effect of the band around 280 nm and in isosbestic point similar to the B to Z isomerization was observed. Again, computer generated secondary structure of H^L-form of poly [d(G-C)] clearly shows that it forms guanine-cytosine base pairing with double hydrogen bonds, while triple hydrogen bonds occurs in B-form structure. It has been found that in some biological systems, certain biological functions are relatively favoured at low pH. A pH dependent enhancement of DNA binding by the ultra biothorax homeo domain has been studied by Li *et al.*²². This report highlights the relevance of the protonation induced conformational study of nucleic acids with respect to biological systems. Robert-Nicoud *et al.*⁴⁹ had found that the anti-Z-DNA antibody binding to the polytene chromosome isolated from the salivary gland of *Chironomus thummi* larvae is enhanced when DNA is exposed to low pH as revealed from the dramatic increase in the immunofluorescence intensities of the left-handed DNA immunoglobulin.

A recent Raman spectroscopic study of low pH induced changes in DNA structure of polytene chromosomes has revealed that left handed DNA structure⁵⁰ is present at low pH. There are also many other reports that describe various cell processes at low pH conditions in several biological systems that draws considerable attention to pH regulated gene expression and related environmental influences over DNA topology and subsequent effect on selected gene expression^{24,50,51}. Thus, as DNA undergoes defined conformational changes under the influence of low pH, the ability of these conformational status to interact with sanguinarine may convey some specified meaning for its regulatory role leading to its usefulness as an anti-tumour agent.

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