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Poly(dG).poly(dC) at neutral and alkaline pH: the formation of triple stranded poly(dG).poly(dG).poly(dC)

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ABSTRACT

Alkaline titrations of different samples of poly(dG).poly(dC) and of the constituent homopolymers poly(dG) and poly(dC) have been performed in 0.15 M NaCl and their CD spectra followed. Sample I contained a slight excess of poly(dC) (52% C: 48% G) and showed a single reversible transition (pK=11.9) due to the dissociation of double stranded poly(dG).poly(dC). Sample II, containing an excess of poly(dG) (43% C: 57% G), showed two transitions (pK_1=11.4, pK_2=11.9) the first one being only partially reversible. Examination of the CD spectra along the alkaline titrations indicated the presence of another hydrogen-bonded complex of higher G content. Mixing curves performed at pH 8 have confirmed the presence of a 2G : 1C complex, besides the double stranded complex. It can be formed in amounts up to 30% by mixing the two homopolymers, alkali treatment and heating. The CD spectra of the two complexes have been computed from the CD data of the mixing curves. This permitted the determination of the concentrations of both complexes and homopolymers in all samples. The ratio of triple to double stranded complex is not only dependent on the G/C ratio of the sample, but also a function of the previous physico-chemical conditions. These results explain the variability of many properties of different poly(dG).poly(dC) samples observed by other workers.

INTRODUCTION

The data on poly(dG).poly(dC) in the literature point to the fact that this polynucleotide complex can exist in different forms under the same experimental conditions. Radding, Josse and Kornberg[1,2] have synthesized this complex for the first time in 1962 and studied it in some detail. The synthesis by DNA polymerase gave rise to a complex containing rarely a base ratio of 1/1, but often a large excess of poly(dG) (as much as 80%). The excess of G incorporated could be controlled by varying the pH conditions of the synthesis. According to these authors[1,2], the properties of such preparations were not only related to an excess of poly(dG). Green and Mahler[3] have published two spectra of poly(dG).poly(dC) samples containing 58 and 63 % G respectively; the excess of poly(dG) alone, however, does not explain the difference between their two CD spectra.

Poly(dG).poly(dC) samples containing an excess of poly(dC) are commerci-

ally available (Miles Laboratories, 50 to 65 % C). Such and similar preparations have been studied by Gray and Bollum[4] who showed that the variation of the CD spectra was due to the presence of different amounts of free poly(dC) in the samples.

The polymorphism of poly(dG).poly(dC) has also been observed by Wells and coworkers[5] by equilibrium centrifugation in cesium sulfate gradients, performed under the same experimental conditions on different samples containing strictly 1G/1C. These authors observed not less than six different values for the buoyant density of poly(dG).poly(dC) (between 1.473 and 1.552 g/ml). They explained their findings by the ability of the homopolymers (poly(dG) and poly(dC)) to give rise to various complexes between each other or themselves.

In order to check the hypothesis of the existence of another species in the samples containing an excess of poly(dG) and to understand the polymorphism of poly(dG).poly(dC), we have performed a detailed study of the interaction between poly(dG) and poly(dC) at pH 8. The next paper[6] will be devoted to the study of this system in acid medium.

MATERIAL AND METHODS

Alkaline titration: Two samples of different G/C ratio have been used : sample I was purchased from Miles Laboratories, Elkhart, IN, USA and contained 52% C and 48% G; sample II was from Boehringer, Mannheim, GFR and contained 57% G and 43% C. Samples were dissolved in 0.15 M NaCl, 0.001 M Tris, pH 8. pH was measured on a Tacussel Isis-4000 pH-meter with a high alkalinity electrode. Titrations were performed from pH 8 to pH 12.5 and back to pH 8, using 0.1 and 1.0 M NaOH and HCl, respectively and the CD spectra were recorded.

Preparation of samples for mixing curves: The homopolymers poly(dG) and poly(dC) were obtained by strand separation in an alkaline (pH 12) cesium sulfate gradient by equilibrium centrifugation[7]. Pure poly(dG) and poly(dC) were isolated and extensively dialyzed against 0.15 M NaCl, 0.001 M Tris, pH 8. Samples for continuous variation experiments were mixed in the usual manner. In order to obtain maximal pairing, all samples were brought to pH 12.5 after mixing, neutralized to pH 8.6, heated to 80 C for one hour and then allowed to return slowly to room temperature; the CD spectra were then recorded (mixing curve I) . The samples were kept at 4 C for 48 hrs. and the CD spectra recorded again (mixing curve II)

Recording of CD spectra: The CD spectra were recorded on a Roussel-Jouan Dichrographe II. recording specifications were: sensitivity $10^{-4} \Delta \epsilon$ /cm, scanning speed 0.125 nm/sec, time constant 2 sec., path length 1 cm, temperature

22 C. Spectra are digitalized during recording with a remote PDP-12 computer The CD signal value is obtained by the analog to digital conversion of a signal read on the Philips recorder of the Dichrographe. Eight equidistant readings are made in a 0.5 nm interval and then averaged. The 0.5 nm intervals are given by an angular optical recorder fitted on the wavelength axis of the Dichrographe. Since the wavelength range used was 210.5 to 330.0 nm, each spectrum consists of 240 values. All further computations with the experimental spectra were always performed with this 0.5 nm resolution. In order to improve the signal to noise ratio of the base lines (recorded for every cuvette before the titration of each sample), four scans were performed. Base line correction and normalization to molar dichroism were performed on line during the recording of the spectra. All computations were carried out on the same PDP-12 computer used for CD data acquisition. The computation of the number of independent molecular species has been described elsewhere[8].

RESULTS

Alkaline titrations: The alkali induced helix-coil transition of poly(dG).poly(dC) has first been studied by Radding et al.[1]. These authors had used two different techniques for alkaline titrations: spectrophotometric titrations in 0.2 M Na^+ led to a sharp reversible hyperchromic transition with a pK=11.4; the study of viscosity changes showed a sharp transition with a pK=11.1, the poor reversibility of the viscosity data indicated, however, a failure to obtain the original structure. Inman and Baldwin[7] found a helix-coil transition with a pK=11.8 by spectrophotometric titration in 1.8 M Cs_2SO_4; in 0.4 M KCl the pK was 11.9. Our results for the two different poly(dG).poly(dC) samples and for the homopolymers poly(dG) and poly(dC) are shown in figs. 1 and 2. Curve b, fig. 1 shows the alkaline titration of sample I (48% G: 52% C). A single reversible transition is observed with a pK=11.9, as previously found. Curve c, fig. 1 is the alkaline titration of sample II (57% G: 43% C). In this case two transitions are observed, the first one is only partially reversible and has a pK=11.35, the second one is fully reversible and shows a pK= 11.95. Both samples show transitions over a very narrow pH range, suggesting that the first transition of sample II reflects also the strand separation of a highly ordered structure.

Alkaline titration of poly(dC) shows only a very small change on the CD spectrum, compared to the other transitions, and the pK=11.9 coincides with the pK of sample I and the second pK of sample II. In this case the transition corresponds to the deprotonation of the amino group of the poorly stacked

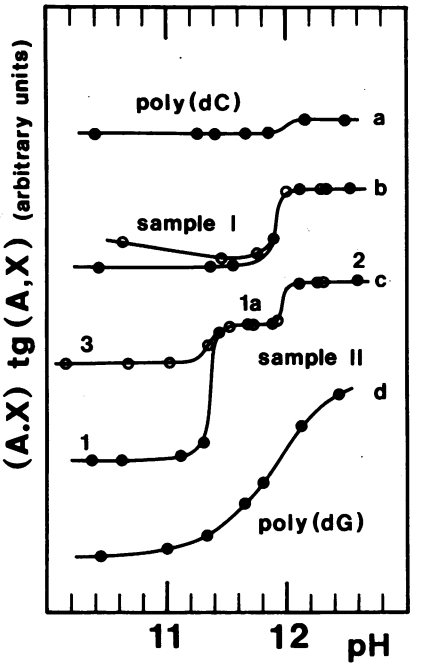

Fig. 1. Alkaline titrations of
a) poly(dC)
b) poly(dG).poly(dC) sample I
c) poly(dG).poly(dC) sample II
d) poly(dG)
Parameter (A.X)tg(A,X) is plotted vs pH, A : reference spectrum at lowest pH; X : successive spectra. Wavelength range used :
a) and d) 230 to 280 nm
b) and c) 230 to 300 nm
This representation sums up the variations of the spectra in the above wavelength range; the amplitudes of the different transitions are not related to each others (see ref. 8 for details).

single stranded poly(dC) (curve a, fig. 1; fig 2). Poly(dG) is a highly ordered self-structure and is probably four-stranded like poly(I) and poly(G) (ref. 9,10). This structure can be destroyed by alkali treatment. Curve d in fig. 1 shows that the alkaline transition takes place over a wider pH range than the poly(dG).poly(dC) transitions do. Poly(dG) releases protons from pH 11 to pH 12.5; in this pH range the G-rich poly(dG).poly(dC) (sample II) shows two sharp transitions, none of which is comparable with the poly(dG) transition. These results suggest that the poly(dG) fraction in excess in sample II is not organized in the same manner as it is in poly(dG) alone. This fraction is not free in solution, but must be included in another structure, either with poly(dC) or with poly(dG).poly(dC). In order to determine the stoichiometry of this complex we have performed the mixing curves[11] of the system poly(dG) plus poly(dC).

Mixing curves: Two mixing curves are presented: mixing curve I (fig. 3a) has been recorded immediatly after return to room temperature of the eleven samples (see Material and Methods); mixing curve II (fig. 3b) has been recorded 48 hrs. latter. The CD signal plotted versus x_G (x_G = [poly(dG)]/[poly(dG)] + [poly(dC)])(inserts in fig. 3) exhibits two breakpoints around x_G = 0.45 and x_G = 0.66 (the latter being less pronounced in fig. 3a). This demonstrates

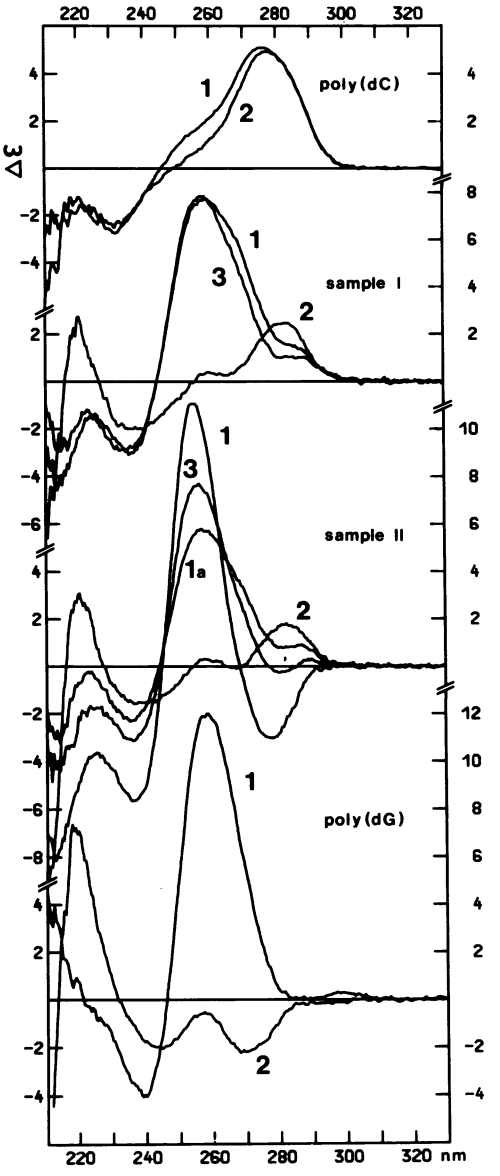

Fig. 2. CD spectra of the same samples as in fig. 1 recorded during the alkaline titrations : 1) pH 8, 1a) pH 11,7, 2) pH 12.5, 3) pH 8 after titration to pH 12.5.

the ubiquitous presence, besides poly(dG).poly(dC), of another complex, the stoichiometry of which is 2G:1C.

We have therefore assumed for this complex a triple stranded structure poly(dG).poly(dG).poly(dC). Examination of the two spectra of the sample

x_G = 0.66 shows that the concentration of this complex is time dependent and is rising from curve I to curve II. In order to study accurately the alkaline transitions and the time dependent changes observed between the two mixing curves, we have computed the spectra of the two complexes from the experimental spectra of mixing curve II.

a) CD spectrum of the double stranded complex. The CD spectra of the samples x_G = 0.0 to x_G= 0.27 define an isosbestic point at 270 nm. This indicates that

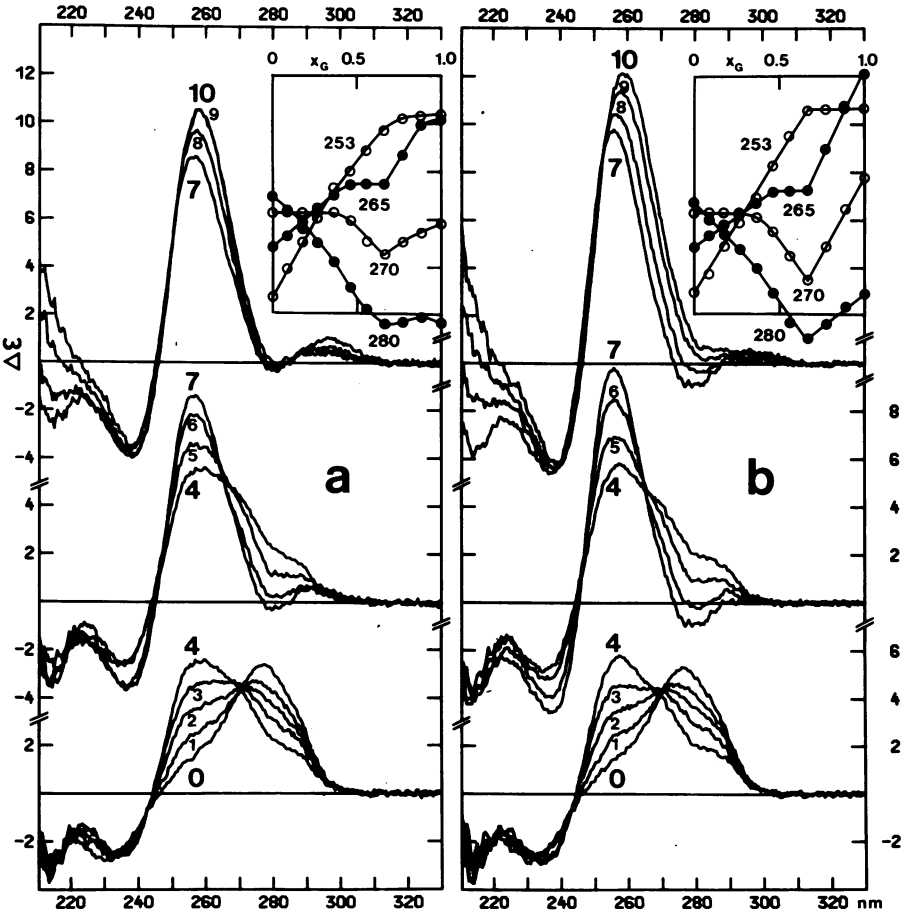

Fig. 3. CD spectra of the different mixtures of poly(dG) and poly(dC) : a) mixing curve I : CD spectra recorded immediately after cooling of heated samples, b) mixing curve II : CD spectra recorded 48 hrs. later . Spectra are numbered from 0 to 10 corresponding to increasing concentration of poly(dG). The mole fractions (x_G = [poly(dG)]/[poly(dG)]+[poly(dC)]) are not exactly in 0.1 increase (see inserts). In order to better evidence isobestic points, spectra 4 (x_G= 0.36) and 7 (x_G= 0.77) are traced twice. Inserts : CD signal plotted vs mole fraction at different wavelengths indicated.

in samples containing up to 27% poly(dG), the formation of the double stranded complex appears to be complete, i.e. there is no free poly(dG) in these samples. This was verified by an orthogonalization of these spectra[8] which showed that only two independent spectroscopic forms were present. The CD spectrum of double stranded poly(dG).poly(dC) (fig. 4) was therefore obtained by the required linear combination of the four spectra of samples x_G= 0.0 to x_G = 0.27; data from the two mixing curves were averaged, since they were virtually identical. This spectrum is in very good agreement with that estimated by Gray and Bollum[4].

b) CD spectrum of the triple stranded complex. As was expected, the spectra of samples above x_G = 0.36 were very incorrectly fitted if only the three spectra known so far, i.e. those of poly(dC), poly(dG).poly(dC) and poly(dG) were used. In a first step, we have selected a sample of poly(dG).poly(dC) (from Boehringer) that exhibited a large negative peak at 278 nm and directly used its " native " CD spectrum at pH 8 as a fourth sample in our library to fit the mixing curve II data; in this case the fits were very correct. Titration of this G-rich sample to pH 6 showed the absence of free poly(dC)

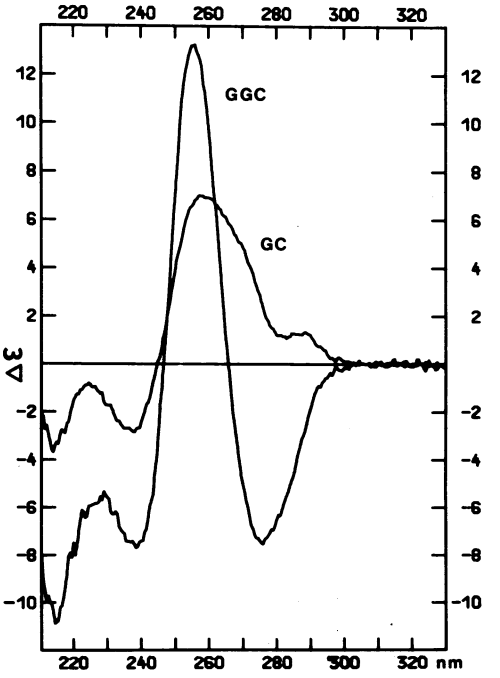

Fig. 4. Computed CD spectra of double stranded poly(dG).poly(dC) and triple stranded poly(dG).poly(dG).poly(dC).

and alkaline titration indicated a negligible amount of poly(dG). We have therefore assumed that this sample contained only double and triple stranded complexes. The triple stranded complex spectrum was obtained taking into account the amount of double stranded complex (fig. 4)

The distribution of the different species along the two mixing curves can thus be computed and is shown in fig. 5. Between the two mixing curves, no change in concentrations was found in samples containing less than 27% poly(dG). For samples of higher G-content, the concentration of triple stranded complex increases (up to a two-fold increase in the sample $x_G = 0.66$) Parallely, the concentration of double stranded and poly(dG) decrease according to the equilibrium:

poly(dG).poly(dC) + poly(dG) $\rightleftharpoons$ poly(dG).poly(dG).poly(dC).

The two spectra of poly(dG) are markedly different in the two mixing curves: in curve II, the usual poly(dG) spectrum is found[4], while in curve I the CD spectrum is intermediate between that of curve II and that of poly($dN_{ac}G$) of Gray and Bollum[4]; this polymer is unable to form the four stranded complex. This supports the idea that the time-dependent changes observed between the two mixing curves for high G-content samples are partly due to the auto-association of poly(dG) to a four stranded structure. This reaction is in competition with the formation of the triple stranded complex. The base-acid titration of poly(dG) showed very rapid kinetics of the formation of the four stranded complex: neutralization after alkali treatment yielded immediatly the

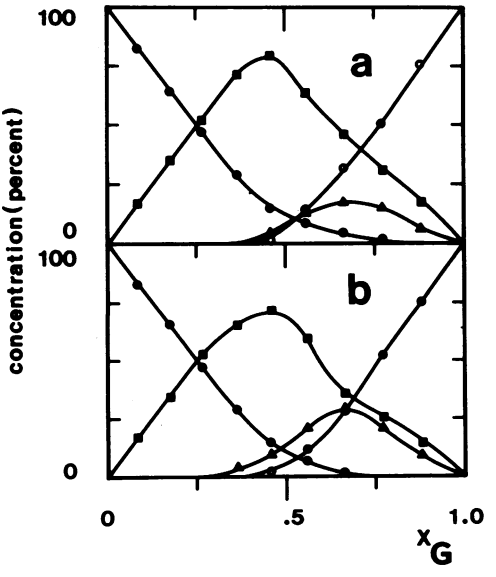

Fig. 5. Computed distribution of :
● : poly(dC),
■ : poly(dG).poly(dC),
▲ : poly(dG).poly(dG).poly(dC),
○ : poly(dG)
as a function of mole fraction in the samples used to perform mixing curves a) mixing curve I
b) mixing curve II.

poly(dG)$_4$ spectrum. It is possible that the chain length of the poly(dG) used for the mixing curves was shorter than that used for the alkaline titration. This point does, however, not affect the above conclusions.

Buoyant density measurements. In order to evidence the triple stranded complex, buoyant density centrifugations were performed on poly(dG).poly(dC) before and after alkali treatment. The results are shown in Table I. As found by Wells et al.[5], only one peak was observed for every centrifugation, but the density varied between 1.51 and 1.48 g/ml, depending on the pH treatment. This suggests that poly(dG).poly(dG).poly(dC) does not exist as an independent complex, but would be rather a double stranded poly(dG).poly(dC) with additional poly(dG) randomly bound to it.

DISCUSSION

The existence of a three stranded complex evidenced by alkaline titration and by the mixing curves may clarify many experimental results so far unexplained. The spectra of sample x_G = 0.66 and of poly(dG) exhibit a slight negative peak at 280 nm after cooling (fig. 3a). Poly(dG) reanneals to poly(dG)$_4$ within a few hours and its CD signal becomes positive at 280 nm (spectrum 10 in fig. 3b). On the contrary, 48 hrs. later , the negative peak observed for sample x_G = 0.66 has increased (spectrum 7 in fig. 3b). Only the formation of poly(dG).poly(dG).poly(dC) with its large negative peak at 275 nm (fig. 4) can explain the increase of negative signal around this wavelength. One of the two spectra of Green and Mahler[3] ressembles the spectrum of our sample II (fig. 2).

Gray and Bollum[4] have studied samples which all contained an excess of poly(dC) and therefore have not observed spectra with this negative band at 280 nm. One of their samples, however, after heating and cooling gave a decrease of the CD signal around 275 nm, indicating a partial formation of triple stranded complex.

Table 1: Buoyant density values of poly(dG).poly(dC) samples after various pH treatment (in Cs_2SO_4).

pH variation before centrifugation	pH of centrifugation	buoyant density g/ml
7	7	1.510
7 → 9.5	9.5	1.506
7 → 11.5 → 9	9	1.480

We have computed the concentrations of the complexes and homopolymers present in the samples used for alkaline titrations. Least square fits with the spectra of the homopolymers and those computed for the two complexes yielded perfectly the experimental data. Results are shown in table II.

It is now obvious that the first transition observed in sample II (curve c in fig. 1) corresponds to the dissociation of the third strand of the triple stranded complex according to the scheme :

$$\text{poly(dG).poly(dG).poly(dC)} \xrightarrow{OH^-} \text{poly(dG).poly(dC)} + \text{poly(dG)} \quad (1)$$

This transition gives rise to double stranded complex and the negative peak at 278 nm disappears (spectrum 1a in fig. 2). The second transition of sample II and the unique transition of sample I reflect the alkaline dissociation of the double stranded structure :

$$\text{poly(dG).poly(dC)} \xrightarrow{OH^-} \text{poly(dG)} + \text{poly(dC)} \quad (2)$$

For both samples, the spectra above pH 12 can be expressed as the sum of the spectra of the homopolymers under the same conditions.

In sample I we found that 12% poly(dG) were free; this could be the reason for the slight displacement observed between the second pK of sample II and the single pK of sample I (fig. 1). On the other hand, in sample II, the release and immediate titration of the single stranded poly(dG) (reaction (1)) takes place in a narrow pH range. Less than one percent of free poly(dG)_4 is detected in this sample before titration. The existence of a plateau (pH 11.5 to pH 11.9) between the two transitions, coinciding with the pK of poly(dG) (fig. 1), is a further indication of the absence of free self-associated poly(dG) in this sample II before alkaline titration. After reneutralization less than one third of the initial amount of triple stranded complex is refor-

Table II: Distribution of complexes and homopolymers during alkaline titration and back titration (percent)

	sample I		sample II		
	pH 8	pH 8 return	pH 8	pH 11.7	pH 8 return
C	14	8	-	-	-
G•C	76	86	48	86	77
G•G•C	-	6	52	3	19
G	12	-	-	11	3

med. The concentration changes in sample I after reneutralization are less drastic since this sample contained no triple stranded complex : the concentration of double stranded complex has increased and a slight amount of triple stranded complex is formed. The results of the alkaline titration may explain why reproducible results can be obtained with poly(dG).poly(dC) samples after alkali treatment. This procedure had already been used empirically by other authors (Dr. C. Zimmer, personnal communication).

Only DNA-polymerase seems to be able to form maximal amounts of triple stranded complex (up to 60% of the bases in this structure) without the occurence of free homopolymers. If this complex is destroyed by alkali treatment, it will not reform completely upon neutralization. Mixing of the homopolymers leads also to a lesser extent of triple stranded complex (30%). This difference can be explained by the competition of the two reactions in which poly(dG) enters : 1) the autoassociation of poly(dG) into a four stranded structure, 2) the formation of a three stranded complex by the addition of one poly(dG) strand to the double stranded complex poly(dG).poly(dC).

The only reasonable possibility to obtain a triple stranded structure with two purines and one pyrimidine is the addition of an Hoogsteen bound poly(dG) strand to the double stranded poly(dG).poly(dC). Arnott and collaborators[12,13] had shown that poly(dG).poly(dC) is in the A-form in fibers and that it was very difficult to obtain a B-form with this complex. It would be interesting to know the G·G·C / G·C ratio of the samples used, to determine whether this is an intrinsic property of double stranded poly(dG).poly(dC). It could be due to the presence of non negligible amounts of triple stranded complex in the sample used which would be obligatorily fixed in the A-form.

A stoichiometry of 2G/1C had already been observed by Pochon and Michelson[14] for the interaction between oligo(G) and poly(C). A similar stoichiometry has been observed for the inverse system poly(G) plus oligo(C) in our laboratory[15], but a triple stranded structure has been disregarded since it did not reappear upon neutralization after acid titration. A four stranded poly(G), interrupted by oligo(C).poly(G) sequences was then proposed to explain this stoichiometry; however, the present work would favour the former hypothesis. Finally, a triple G·G·C has been shown to exist in t-RNA$_{phe}$, with a reverse Hoogsteen pair G·G and a Watson-Crick pair G·C[16].

CONCLUSION

Poly(dG).poly(dC) samples synthesized by DNA-polymerase may contain, in various amounts, triple stranded complex poly(dG).poly(dG).poly(dC). Our main

arguments for the presence of this complex are the following:

1) The excess of poly(dG) in G-rich samples of poly(dG).poly(dC) does not behave like self-associated poly(dG) does during alkaline titration.

2) Destabilized poly(dG) (alkali treatment plus heating) reanneals into $poly(dG)_4$ upon cooling; the negative band at 278 nm already observed by Green and Mahler[3] is not explained by the presence of free single stranded poly(dG) as asserted by Gray and Bollum[4].

3) Mixing curves display the formation of a 2G/1C complex upon cooling of alkali treated and heated mixtures of the homopolymers.

The data presented in this paper are all consistent with the experimental results obtained so far by other workers. We interprete the variability of many physico-chemical and enzymatic properties observed for different samples of "poly(dG).poly(dC)" as the result of two surimposed phenomena :

1) The ability of poly(dG) and poly(dC) to form two heterocomplexes : poly(dG).poly(dC) and poly(dG).poly(dG).poly(dC). The latter complex would be formed by the more or less complete addition of a poly(dG) strand in Hoogsteen binding to the double stranded complex.

2) The ratio between these two complexes is not only dependent on the intrinsic G/C ratio in a given sample, but also on the past and present physico-chemical conditions.

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