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Protonated polynucleotides structures - 22.CD study of the acid-base titration of poly(dG).poly(dC)

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ABSTRACT

The acid-base titration (pH 8 → pH 2.5 → pH 8) of eleven mixing curve samples of the poly(dG) plus poly(dC) system has been performed in 0.15 M NaCl. Upon protonation, poly(dG)•poly(dC) gives rise to an acid complex, in various amounts according to the origin of the sample. We have established that the hysteresis of the acid-base titration is due to the non-reversible formation of an acid complex, and the liberation of the homopolymers at the end of the acid titration and during the base titration; the homopolymer mixtures remain stable up to pH 7. A 1G:1C stoichiometry appears to be the most probable for the acid complex, a 1G:2C stoichiometry, as found in poly(C^+)•poly(I)•poly(C) or poly(C^+)•poly(G)•poly(C), cannot be rejected. In the course of this study, evidence has been found that the structural consequences of protonation could be similar for both double stranded poly(dG)•poly(dC) and G-C rich DNA's: 1) protonation starts near pH 6, dissociation of the acid complex of poly(dG)•poly(dC) and of protonated DNA take place at pH 3; 2) the CD spectrum computed for the acid polymer complex displays a positive peak at 255 nm as found in the acid spectra of DNA's; 3) double stranded poly(dG)•poly(dC) embedded in triple-stranded poly(dG)•poly(dG)•poly(dC) should be in the A-form and appears to be prevented from the proton induced conformational change. The neutral triple stranded poly(dG)•poly(dG)•poly(dC) appears therefore responsible, although indirectly, for the complexity and variability of the acid titration of poly(dG)•poly(dC) samples.

INTRODUCTION

The acid titration of DNA leads to an intermediate protonated structure prior to the irreversible strand separation. The CD spectrum of this acid form (pH 3.1) is significantly different from the neutral spectrum and is mainly characterized by a positive peak at 255 nm[1,2], the intensity of which depends on the G-C content. This conformational change has been interpreted as the protonation of N^7 of guanosine and the consecutive inversion of the base to the syn conformation leading to the formation of a Hoogsteen G-C pair, including a shared proton between the two bases[2]. Examination of the acid CD spectra of poly(dAC)•poly(dGT) and poly(dGC)•poly(dGC) showed that these poly-

mer complexes do not behave during acid titration according to their G-C content[3], while poly(dAG)•poly(dCT)[3] and poly(dG)•poly(dC)[4] showed an acid titration reminiscent of that of DNA. This suggested that the conformational changes observed in DNA take place preferentially in polypurine•polypyrimidine sequences, including GpG first neighbours.

Our study of the acid titration of poly(I)•poly(C)[5] and of poly(G)•poly(C)[6] had shown that the formation of triple stranded protonated complexes poly(C^+)•poly(I)•poly(C) and poly(C^+)•poly(G)•poly(C) took place. These complexes contain a Watson-Crick pair between the non-protonated pyrimidine and the purine and the second pyrimidine is bound in a Hoogsteen pair to the purine through a shared proton. A similar triple stranded complex is probably formed between oligocytidylates and T7 DNA at acid pH[7]. Morgan and Wells[8] had shown that a hybrid triple stranded complex could be formed between poly(dAG)•poly(dCT) and poly(rCU), where the ribocytidylate would be again bound via a protonated Hoogsteen pair.

Further protonation of poly(I)•poly(C) led to a fully protonated complex poly(I)•poly(C^+) which should be Hoogsteen paired. It was of interest to check whether the acid titration of poly(dG)•poly(dC) would give rise to such protonated complexes. Preliminary results[4] on poly(dG)•poly(dC) showed that this complex was titrated towards acid pH in two steps and that the backward titration did not coincide with the forward titration and a large hysteresis was observed.

We have recently shown[9] that most of the samples containing an excess or even 50% poly(dG) are mixtures of two complexes: poly(dG)•poly(dC) and poly(dG)•poly(dG)•poly(dC). It also has been demonstrated that the ratio of the two complexes depends critically on the past physico-chemical conditions of the sample[9]. In the present paper we analyse the changes in CD spectra upon acid-base titration of poly(dG)•poly(dC) samples synthesized by DNA polymerase and the titrations of the eleven samples that constitute the mixing curve of the poly(dG) plus poly(dC) system.

MATERIAL AND METHODS.

Poly(dG)•poly(dC) was purchased from Miles laboratories, Elkhart, IN, USA. The sample used in this study contained 60% G and 40 % C. pH adjustments were made as before[9].

Mixing curves: The eleven samples were the same as those already used in previous work[9]. For each sample the CD spectrum was recorded for pH values as close as possible to 6.1, 5.0, 4.1, 3.0 and 2.5 for the acid titration and

at pH 3.3 , 4.1, 5.0, 6.1 and 8.3 during the alkaline titration. The actual pH values were kept within ± 0.2 pH unit. Recording of CD spectra was performed as described previously[9].

Detection of the acid complex: If an experimental spectrum (pH and the mole fraction x_G given) was not correctly fitted by the spectra of the known species, it was considered that this indicated the presence of a new complex (this assumes, of course, that the spectrum of any new complex is linearly independent of the spectra of the known species[10]). The four spectra used for least square fit were: the two computed spectra of the double and triple stranded complexes, respectively[9], and the spectra of the homopolymers taken at the same pH as the spectrum to be fitted was recorded. The following criteria were retained to decide upon the correctness of the fit: 1) the difference spectrum (experimental spectrum minus fit) should not display significative deviation from random noise (see fig. 5). 2) Since the coefficients of the least squares fit are concentrations (all spectra used are normalized to molar concentration), the stoichiometry, x_G, can be reobtained from these coefficients ((C^+C) is the concentration of poly(dC^+)·poly(dC), etc.) from

$$x_G = \left[1/2\cdot(GC) + 2/3\cdot(GGC) + (G)\right] \;/\; \left[(C^+C) + (GC) + (GGC) + (G)\right]$$

and must equal the known input stoichiometry, if no other species is present. A deviation greater than 5%, generally accompanied by poor fit, indicated the presence of the acid complex. These considerations can give only a rough indication about the concentration of the acid complex; more accurate data were obtained by orthogonalization of the experimental spectra: for every pH value, the eleven experimental spectra of the mixing curve (recorded at a given pH value) and the spectra computed for the double and triple stranded complexes were collected in the same set; the first four vectors eliminated were chosen as the spectra of the homopolymers and of the two complexes. This gave, for the nine samples x_G=0.08 to x_G=0.89, the component orthogonal to the four spectra eliminated; the norm of this component equals, by an unknown factor, the concentration of the acid complex[10].

Detection of free single stranded poly(dG): Whenever it was suspected that strand separation of the complexes had occurred, giving rise to free single stranded poly(dG), its presence was checked by least squares fit. In the library spectra two different spectra of the same poly(dG) sample, sample x_G=1.00 of the mixing curve,were used; spectrum I was recorded immediately after cooling the poly(dG) sample that had been alkali treated and heated (spectrum 10 of mixing curve I, fig 3a of ref. 9); spectrum II was that of the same sample of poly(dG) recorded at the same pH as the spectra to fit (spectrum 10 of

mixing curve II, fig. 3b, ref. 9) at least two days later. We have interpreted spectrum I as a mixture of single stranded poly(dG) and self-associated poly(dG)$_4$,[11] however in unknown ratio. Nevertheless, spectra I and II can be used to fit the spectrum of a sample containing single stranded poly(dG) and poly(dG)$_4$ in any ratio. In this case, the coefficient of spectrum I has to be positive, while that of spectrum II may be positive or negative (a negative coefficient for spectrum I would be incorrect).

Buoyant density measurements. Poly(dG)•poly(dC) samples used for these experiments were first brought to pH 11.5 in order to increase the amount of double stranded poly(dG)•poly(dC)[9]; the acid treatment was done before adding cesium sulfate. CD spectra were recorded before and after addition of cesium sulfate; small shifts were observed, but the overall shape of the spectra remained the same. The solutions were centrifuged in a Beckmann Spinco model E ultracentrifuge at the Institut de Biochimie, Université Paris-Sud, Orsay.

RESULTS

pH or pK values referring to the acid titration are designated pH_a and pK_a, those of the basic back titration pH_b and pK_b, respectively.

Titration of poly(dG)•poly(dC) synthesized by DNA polymerase: The sample used contained an excess of poly(dG) (60% G, 40% C) and was a mixture of 58% poly(dG)•poly(dC), 37% poly(dG)•poly(dG)•poly(dC) and the rest free homopolymers. In order to increase the GC/GGC ratio, the sample was brought to pH 11.5 before the acid titration[9]. Fig. 1 shows the acid-base titration of this sample. The CD signal at 290 nm of poly(dC^+)•poly(dC) is very important at this wavelength[9,11] compared with that of poly(dG)•poly(dC) or poly(dG)•poly(dG)•poly(dC); this wavelength was retained to follow the appearance of the acid form of poly(dC) and to eliminate as much as possible the variations of the equilibrium poly(dG)•poly(dG)•poly(dC) ⟷ poly(dG) + poly(dG)•poly(dC) during the acid-base titration (this point will be discussed elsewhere[12]). As shown in earlier work[4], the back titration did not coincide with the forward titration. The three cycles in fig. 1 started and ended at pH 8; the pH of re-

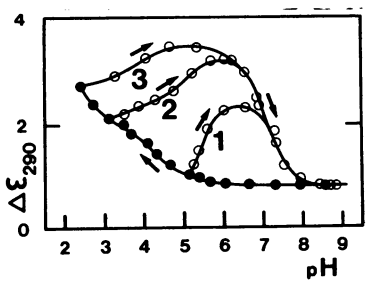


Figure 1: Acid-base titration of alkali treated poly(dG)•poly(dC) sample in three different cycles. CD signal followed at 290 nm.

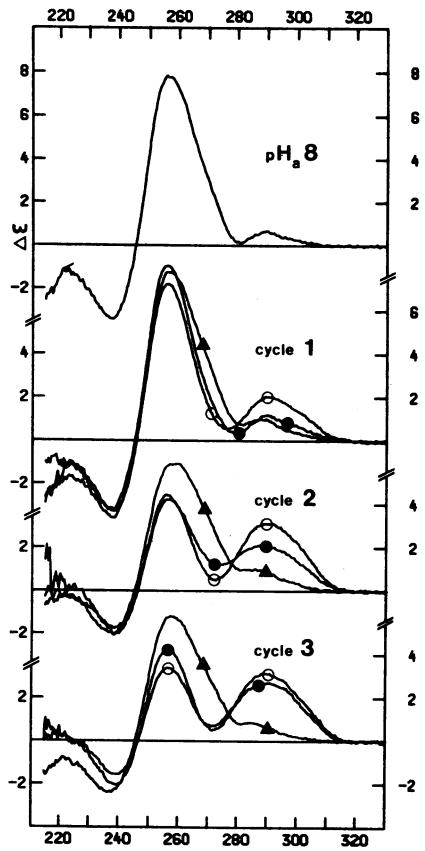
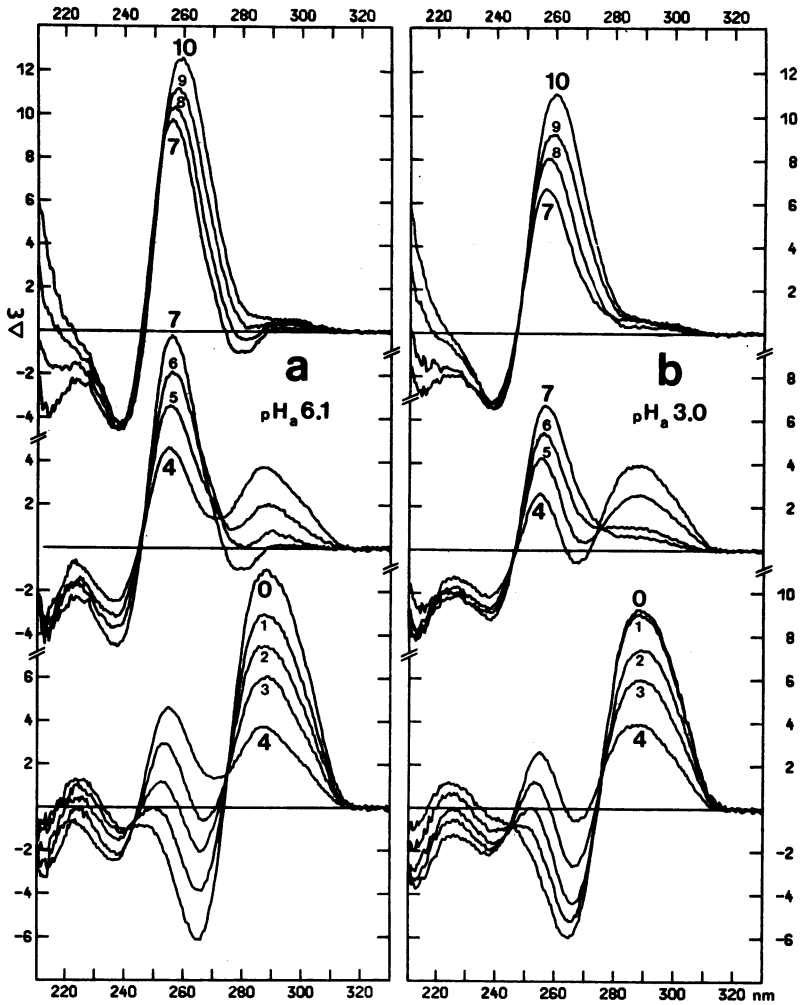


Figure 2: CD spectra recorded during the three cycles of the acid-base titration of an alkali pretreated poly(dG).poly(dC) sample in 0.15 M NaCl. Top: original sample at pH=8 before titration. For all cycles: ● spectra recorded at the lowest pH of the acid titration: cycle (1): pH$_a$=5.1, cycle (2): pH$_a$=3.1, cycle (3): pH$_a$=2.4; ○ pH$_b$=6.0; ▲ pH$_b$=8.3.

turn was 5.0, 3.1 and 2.4, for cycles (1), (2) and (3), respectively. Two steps were observed during acid titration; it was, however, not possible to determine exactly the pK's, since no distinct plateau existed between the two steps: pK$_{a1}$= 5.0±0.25, pK$_{a2}$<2.7. The back titration appears to be more complex; the three cycles show two steps. The second basic pK$_b$=7.3 was the same

TABLE I: Distribution of complexes and homopolymers during acid-base titration of alkali pretreated poly(dG)•poly(dC).

complex	pH$_a$=8	percentage cycle (1) pH$_b$=6	cycle (1) pH$_b$=8	cycle (2) pH$_b$=6	cycle (2) pH$_b$=8	cycle (3) pH$_b$=6	cycle (3) pH$_b$=8
poly(dC)	2	17	14	33	12	35	22
poly(dG)•poly(dC)	56	30	49	5	46	0	32
poly(dG)•poly(dC)••poly(dC)	14	16	7	1	0	3	6
poly(dG)	28	37	30	61	42	62	40



for all three cycles and equaled the pK of the transition 13.14

$$\text{poly(dC}^+\text{)}\cdot\text{poly(dC)} \xrightarrow{-H^+} 2\ \text{poly(dC)}\ .$$

The CD spectra after back titration to pH 8 depended strongly on the pH at which the acid titration was stopped (fig. 2).

Using the spectra of double stranded poly(dG)·poly(dC) and of triple stranded poly(dG)·poly(dG)·poly(dC) previously obtained[9] we have computed the concentrations of the four species present; this computation was only possible at pH=8 and pH=6. Below pH$_a$=6, the spectra were not correctly fitted (see below). Upon alkaline titration, at pH$_b$=6 the fits were again correct, provided that the spectrum of free single stranded poly(dG) was also included in the library spectra (see Methods). Concentrations determined at pH$_b$=6 and pH$_b$=8

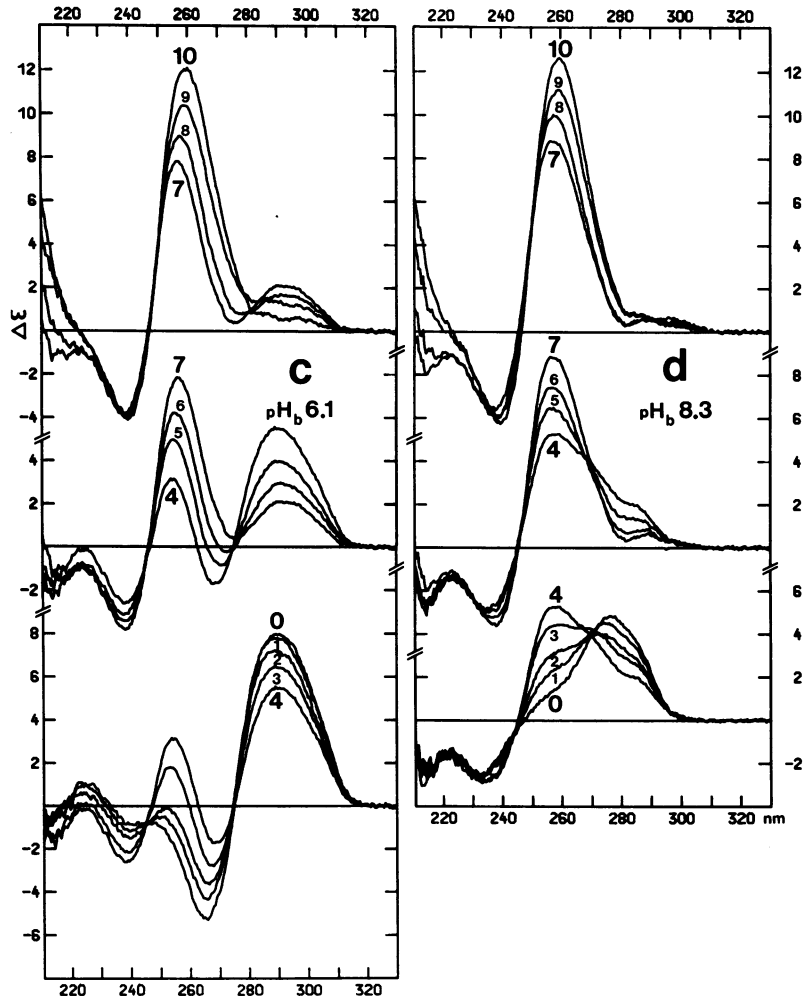


Figure 3: CD spectra of the eleven samples of the mixing curve between poly(dG) and poly(dC) at different pH values:(a) pH_a=6.1, (b) pH_a=3.0, (c) pH_b=6.1, (d) pH_b=8.3. The spectra are numbered from 0 to 10, corresponding to increasing concentrations of poly(dG), as in fig. 3 of ref. 9. Thus, x_G=0.36 corresponds to spectrum (4), x_G=0.45 to spectrum (5), x_G=0.55 to spectrum (6) and x_G=0.66 to spectrum (7) (not 0.77 as erroneously indicated in the legend of fig. 3 in ref. 9).

are given in Table I. The lower the extreme acid pH of the forward titration, the higher was the amount of free homopolymers upon return to pH=6, indicating an increased strand separation of the complexes. In cycle (2), the change of the CD signal at 290 nm (fig. 1) shows that poly(dC^+)·poly(dC) present at pH_b6 was mainly formed during the back titration. This suggested that a new com→

plex was formed during acid titration and that this complex dissociated into homopolymers during back titration. In cycle (3) poly(dC^+)·poly(dC) was already released below pH 3 during the final part of the acid titration. In order to study the mechanism of formation of this complex and to obtain its stoichiometry, we have studied the titration of mixing curves[9] of the system poly(dG) plus poly(dC).

Mixing curves: The eleven samples of the mixing curve (the spectra of which at pH 8.3 have already been presented in fig. 3b of ref. 9) were titrated along the pH cycle 8.3→ 2.5→ 8.3. The spectra of all 11 samples were recorded at various pH's (see Methods), but only those at pH_a=6.1, pH_a=3.0, pH_b=6.1, and pH_b=8.3 are shown in fig. 3. It is important to note that the mixing curves thus obtained are not to be interpreted as usual mixing curves[15], since they were not obtained by mixing the homopolymers at the pH where they were measured. The behaviour of a given sample depends not only on its mole fraction, but also on the initial distribution of the complexes at pH=8.3 (fig. 5b in ref. 9).

pH_a=6.1: Spectra recorded at pH_a=6.1 are presented in fig. 3a. Between pH_a=8.3 and pH_a=6.1, poly(dC) self associates into poly(dC^+)·poly(dC) (pK=7.3). The respective difference spectra between pH_a=8.3 and pH_a=6.1 for the first four samples (x_G= 0.0 to 0.27) were homothetic. The variations of these spectra between these two pH values were therefore only due to the change of the spectrum of poly(dC) into that of poly(dC^+)·poly(dC). Spectra of the last samples (x_G= 0.66 to 1.0) were similar to those obtained at pH_a=8.3 (fig. 3b in ref. 9). The concentrations obtained (fig.4a) were also very close to those obtained at pH_a=8 (fig. 5b in ref. 9).

pH_a=5 to pH_a=2.5: A large change in the CD spectra of nearly all the samples is observed at pH_a=3 (fig. 3b), if compared with those at pH_a=6.1 (fig. 3a). Here again, as found for the polymerase synthesized sample of poly(dG)·poly(dC), the spectra were not fitted correctly with the four known library spectra. This incorrect fit indicated the presence of an additional complex. Fig. 5 shows some of the fits obtained for the spectra of sample (5) (x_G=0.45) at the same pH's as in fig. 3. From pH_a=5 to 2.5 the difference spectrum was not zero and its shape remained unchanged in all the samples at acid pH. This indicated that the same spectrum was missing to fit the acid spectra correctly. The relative concentration of the acid complex as a function of mole fraction and pH is shown in fig. 6. The maximal concentration of this complex was always found at x_G= 0.5. Its concentration was maximal at pH_a=3 and dropped at pH_a=2.5. If the participation of poly(dC^+)·poly(dC) is excluded for the

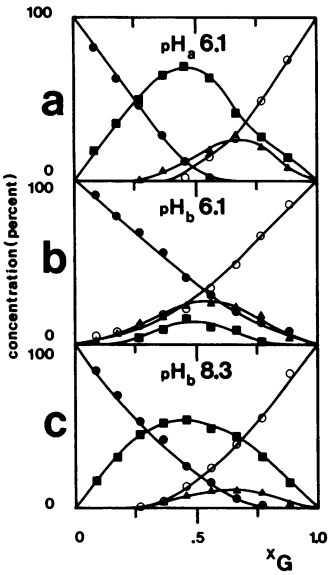


Figure 4: Computed distributions of different complexes as a function of mole fraction in the samples of the mixing curves at (a) pH_a=6.1,(b)pH_b=6.1, (c) pH_b=8.3. ● poly(dC), ○ poly(dG), ▲ poly(dG).poly(dG)•poly(dC), ■ poly(dG)•poly(dC).

formation of this acid complex, the maximal concentration at x_G=0.5 indicates that this complex was only formed from double stranded poly(dG)•poly(dC).

Alkaline back titration: pH_b=2.5 to pH_b=6.1: It is noteworthy that samples (4) to (7) (x_G=0.36 to 0.66) show considerable variations upon back titration to pH_b=6.1. (fig. 3c). In particular the region around 290 nm increased considerably, indicating an increase in concentration of poly(dC^+)•poly(dC) (compare also with figs. 1 and 2). The concentration of the acid complex decreased further upon back titration and reached undetectable amounts at pH_b=5. The concentrations of the different species as a function of mole fraction at pH_b=6.1 (fig. 4b) show an increase of the concentrations of both homopolymers compared with pH_a=6.1 (fig. 4a): poly(dC^+)•poly(dC) increased from 15% to 40%, poly(dG) from 3% to 21% in sample (5) (x_G=0.45). In samples below x_G=0.66, the triple stranded complex had increased (e.g. at x_G=0.27,27%, while no detectable amount was found at pH_a=6.1) and in all samples it exceeded the double stranded complex.

Return to pH_b=8.3: At this pH the CD spectra (fig. 3d) showed reversibility at the extreme mole fractions, but not in the middle range (compare with fig. 3a in ref. 9). Double stranded complex reformed up to 54% in sample (5) (x_G=0.45) (fig. 4c), although the initial amount found before the whole titration cycle, was 72%[9]. Examination of the variations of concentrations between pH_b=6.1 and pH_b=8.3 indicates that poly(dG)•poly(dC) should be reformed at the pK of poly(dC^+)•poly(dC) by two mechanisms:

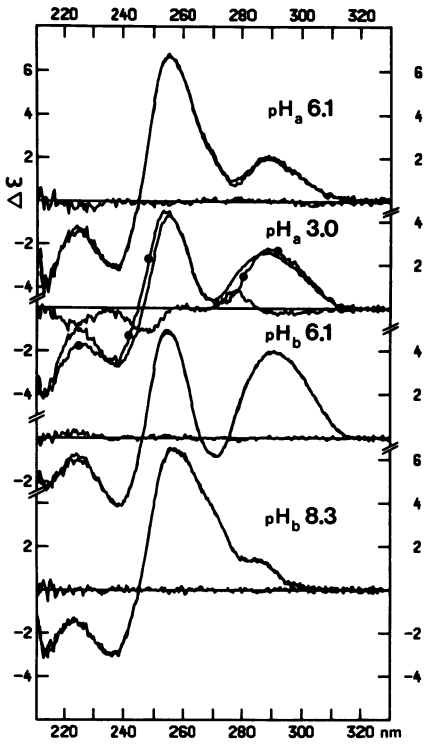


Figure 5: Least squares fit of CD spectra recorded during acid-base titration of sample (5) (x_G=0.45). In all samples the experimental CD spectrum and the fit are superimposable, except at pH$_a$=3 (Dots indicate fitted spectrum). Difference curve (experimental minus fitted spectrum) is also plotted.

poly(dC) + 1/4 · [poly(dG)$_4$ ⇌ 4 poly(dG)] ⟶ poly(dG)·poly(dC)

poly(dC) + poly(dG)·poly(dG)·poly(dC) ⟶ 2poly(dG)·poly(dC) .

These reactions imply that poly(dC) could destabilize both poly(dG)$_4$, reformed during base titration, and the Hoogsteen poly(dG) strand of the triple stranded complex poly(dG)·poly(dG)·poly(dC). This latter reaction would account for the drop of concentration of triple stranded complex at pH$_b$=8.3.

Acid-base titration of individual samples of the mixing curve: Fig. 7 shows the acid-base titrations of some poly(dG) plus poly(dC) samples used for the mixing curves. From sample 0 (x_G=0.0, pure poly(dC)) until sample (3) (x_G= 0.27), the acid and the base titrations had the same pK value (7.2). In sample (3), the great variation of the poly(dC) spectrum could hide any other possible transition. An important feature of these cycles is the hysteresis in nearly all samples, even in pure poly(dC), between pH 7 and pH 2.5. It has been reported[13,14] that poly(dC) probably exists in two different structures at acid pH, one between pH 7.3 and pH 5, the other one below pH 5. At pH 2.4 no precipitation was observed and a T_m = 70° was obtained at pH 2.6 with a cooperative transition[12], which indicates an ordered hydrogen bonded complex. The

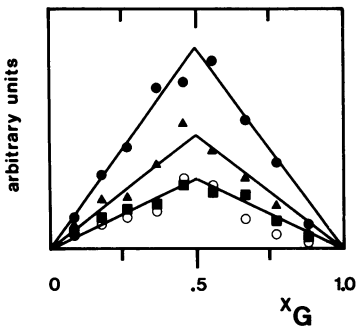


Figure 6: Concentration of the acid complex as a function of mole fraction in the samples of the mixing curve at different pH values. ○ pH_a=5.0, ▲ pH_a=4.1, ● pH_a=3.0, ■ pH_a=2.5. The parameter plotted as a function of x_G is the norm of the component of spectra of samples (1) to (9) orthogonal to the spectra of poly(dC$^+$)•poly(dC), poly(dG)•poly(dG)•poly(dC), poly(dG)•poly(dC) and poly(dG). This presentation gives the concentration of the acid form times an unknown factor (see ref. 10 for details.)

hysteresis took place probably because the second acid structure remained stable upon back titration and dissociated only above pH 7.3. Sample (2) (x_G=0.18) showed perfect reversibility (fig. 7) of the acid-base titration, contrary to sample (3); in this case pK_a and pK_b were the same, although hysteresis was observed. Sample (5) (x_G=0.45) showed two acid pK's: pK_{a1}= 5.8, pK_{a2}< 3 and one pK_b=7.2. In this sample 15% free poly(dC) were found at pH_a=8.3 (i.e. before the acid-base titration). Since only one measure was made between pH_a=8 and pH_a=6.1, the first acid transition observed was in fact a combination of two transition, the protonation of poly(dC) and of poly(dG)•poly(dC), respectively. This gave rise to a pK_a=6.0, different from the pK_a measured in fig. 1, where no poly(dC) was free. The second acid pK_{a2}< 3.0 corresponds to partial strand separation.

At pH 8 in sample (8), 21% poly(dG)•poly(dG)•poly(dC) and 20% poly(dG)•poly(dC) are formed[9]. The acid titration of this sample showed only a very small first acid transition and strand separation near pH 3. The ratio GGC/GC was about 1 and the presence of triple stranded complex stabilized the poly(dG)•poly(dC) molecules so that only very little acid complex was formed.

Buoyant density studies: Table II shows the results of some buoyant density experiments, performed with DNA polymerase synthesized poly(dG)•poly(dC), which was alkali treated, and with the constituent hompolymers. After acid treatment, poly(dC) had a density value of 1.40, as found in the literature[16,17]; poly(dG) had, on the contrary, a much higher density value (ρ = 1.61), indicating some aggregation; after light alkali treatment, the density decreased to 1.56. Acid treatment enhanced the density of poly(dG)•poly(dC), compared with the density obtained at neutral pH after alkali treatment. Two bands were observed when the pH was decreased to 4.6 and back to 5.0: one of them had a density of 1.50, close to that of the neutral product, the second had the same value as the single band obtained when the pH was decreased to 3.0 (ρ = 1.54-1.55).

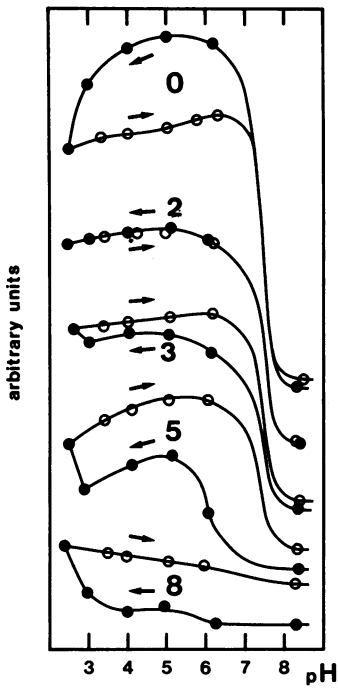


Figure 7: Hysteresis cycles of individual samples of the mixing curves. Samples are numbered as in fig. 3. The parameter plotted as a function of pH is $(\bar{A}\cdot\bar{X})\cdot\tan(\bar{A},\bar{X})$, where $\bar{A}$ is the reference spectrum at pH_a=8, and $\bar{X}$ the spectra at the pH's to be compared with (see ref. 10 for details.).

We know from the mixing curve experiments that the various complexes began to dissociate when the pH was decreased to pH=3 and reformed again only above pH=7; only one band was observed, however. This indicates that single stranded poly(dG) released, partially formed four stranded $poly(dG)_4$, remaining bound together to some extent with double stranded $poly(dC^+)\cdot poly(dC)$. Taking these results into account, the best description of poly(dG)·poly(dC) below the first acid transition and during alkaline back titration below pH 7 would

TABLE II : Buoyant density values of poly(dG)·poly(dC) samples and their component homopolymers as a function of various pH treatments (in cesium sulfate).

polymer	pH variation before centrifugation	pH of centrifugation	buoyant density (g/ml)
poly(dC)	2.9	5.0	1.40
poly(dG)	2.9	5.0	1.61
	10.0	8.0	1.56
poly(dG)·poly(dC)	11.5	9.0	1.48
	11.5 → 3.0	3.0	1.54
	11.5 → 4.6	5.0	1.50 / 1.54
	11.5 → 2.9	5.0	1.55

be a high density molecule containing several of the structures described corresponding to the different complexes, i.e. a "polycomplex". This would account for the relatively easy reformation of poly(dG)•poly(dC) after neutralization without the need of alkali treatment, as is necessary when poly(dG) and poly(dC) are mixed at pH=8[9].

DISCUSSION

The study of the acid-base titration of the system poly(dG) plus poly(dC) led us to the following conclusions:

1) Along the first acid step an acid complex is formed at the expense of double stranded poly(dG)•poly(dC); upon back titration this complex dissociates into poly(dC^+)•poly(dC) and poly(dG).

2) The second acid transition corresponds to a partial strand separation of the acid complex and possibly of the remaining poly(dG)•poly(dC) and poly(dG)•poly(dG)•poly(dC).

3) The triple stranded complex does not participate directly in the formation of the acid complex.

4) During back titration, at the pK=7.3 of poly(dC^+)•poly(dC), double stranded poly(dG)•poly(dC) is formed again by reannealing of the homopolymers and at the expense of poly(dG)•poly(dG)•poly(dC).

5) The different complexes and homopolymers appear to be tied together in a kind of "polycomplex", so that it is not possible to obtain discrete bands in a cesium sulfate density gradient corresponding to the individual complexes.

In a preliminary study[4], we had proposed the existence of an acid complex which would have been stable until pH 7 upon back titration and responsible for the observed hysteresis. It is now established that the system is much more complicated and that what we had considered as the acid complex, was in fact a mixture of several complexes tied together in a "polycomplex" structure.

The behaviour of the poly(dG) plus poly(dC) system therefore differs from that of related polynucleotide systems, like poly(I) plus poly(C)[5] and poly(G) plus poly(C)[6] by the presence of a stable neutral triple stranded complex poly(dG)•poly(dG)•poly(dC) and by the probable absence of an intermediate protonated triple stranded complex, analogous to poly(C^+)•poly(G)•poly(C). Although the existence of a triple stranded structure (dI)•(dI)•(dC) has been reported[18-20], a third hydrogen bond between the basic N^2-amino group of guanine with the N^7 of its neighbour would greatly increase the stability of the analogous (dG)•(dG)•(dC).

In the two related systems mentioned above[5,6], the formation of the triple stranded protonated complex is indicated in both cases by a break at x_I

(or x_G, respectively) = 0.33 on the mixing curves titrated to pH=5 (3.8, respectively) in 0.03 M NaCl. In both cases and again in the present study, mixing had been performed at a pH favorable for the formation of the neutral double stranded complex, after which the pH was lowered equally in all samples. If one considers the formation of protonated poly(C^+)•poly(I)•poly(C), what does the break at x_I=0.33 indicate? If the only reaction leading to this complex were

2 poly(I)•poly(C) ⟶ poly(C^+)•poly(I)•poly(C) + poly(I)

the break should be observed at x_I=0.5, since in this case the concentration of poly(I)•poly(C) is the limiting factor and its maximum is at the mole fraction x_I=0.5. On the contrary, it can also be considered that the homopolymer complex poly(C^+)•poly(C) participates in the formation of the triple stranded protonated complex, either directly

poly(C^+)•poly(C) + poly(I)•poly(C) ⟶ poly(C^+)•poly(I)•poly(C)+poly(C)

or indirectly

poly(C^+)•poly(C) ⟶ 2 poly(C) + H^+

2 poly(C) + H^+ + poly(I)•poly(C) ⟶ poly(C^+)•poly(I)•poly(C) + poly(C).

Whatever are the reactions into which poly(C^+)•poly(C) enters to form the protonated triple stranded complex, the mole fraction at which its maximal concentration is found and consequently the break in the mixing curve should be at x_I=0.33. This is actually observed; therefore the formation of both poly(C^+)•poly(I)•poly(C) and poly(C^+)•poly(G)•poly(C) must imply the participation of poly(C^+)•poly(C).

In 0.03 M NaCl, poly(C) is titrated at a pK=5.9[21,22], while poly(C^+)•poly(I)•poly(C) is formed at a pK=5.3[5] and poly(C^+)•poly(G)•poly(C) at pK=4.2[6]. The respective T_m values of poly(C^+)•poly(C) at these pH values are 63° and 75° (maximal T_m=80° at pH=4.3 in 0.03 M NaCl). Poly(dC) is titrated at much higher pH values than the ribopolymer: pK=7.3 in 0.015 M NaCl. The stability of poly(dC^+)•poly(dC) increases rapidly at lower pH values; at pH=6.2 its T_m=70°[17]; this corresponds to the mean T_m of poly(C^+)•poly(C) at the pK of formation of the triple stranded protonated complexes with poly(I) or poly(G). At this pH the complete protonation of N^7 of guanosine may not be possible; at pH=5, the T_m>100° of poly(dC^+)•poly(dC)[17]. This does not exclude, however, that the acid complex could be a triple stranded poly(dC^+)•poly(dG)•poly(dC), but in this case it would be formed by a mechanism different from that encountered in the ribo-series[5,6].

We have therefore retained three possibilities for the formation of the acid complex:

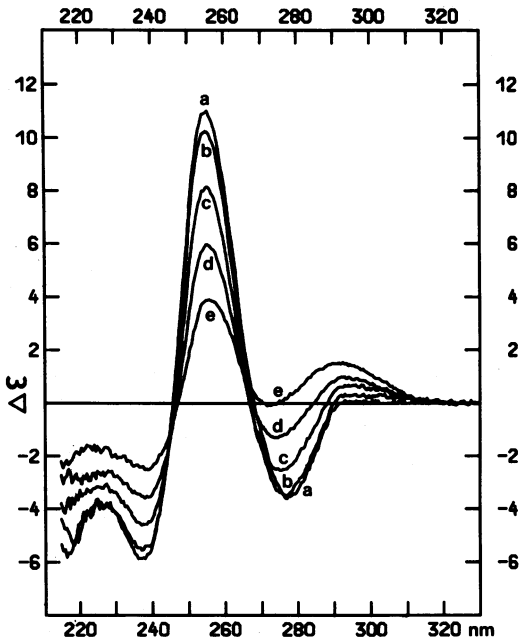


Figure 8: CD spectra recorded during the acid-base titration of a "native" poly(dG)•poly(dC) synthesized by DNA polymerase (without alkali treatment). a: pH=8.5, b: pH=5.1, c: pH=4.6, d: pH=3.6, e: pH=2.7.

1) A double stranded poly(dC^+)•poly(dG) with Hoogsteen pairing is formed by internal rearrangement within the double helix poly(dG)•poly(dC).

2) A double stranded Hoogsteen paired acid complex is formed by the interaction of two helices poly(dG)•poly(dC).

3) A triple stranded complex poly(dC^+)•poly(dG)•poly(dC) is formed by the interaction of two double stranded poly(dG)•poly(dC).

The maximum concentration of the acid complex encountered at x_G=0.5 would account for all three mechanisms. Mechanisms (2) and (3) suffer from the necessity to change the conformation of deoxycytidine from anti to syn, a rather improbable event in a polypyrimidine chain (see ref. 5 for a detailed discussion of this point). Mechanism (1) calls obligatorily for the B-form and for an antiparallel structure for the acid complex.

The acid-base titration of the poly(dG)•poly(dC) sample (fig. 1 and 2) has been performed also without prior alkaline treatment. The spectra recorded during this acid-base titration (fig. 8) can nearly be fitted correctly with the usual library spectra, indicating formation of only very small amount of the acid complex, if at all. We can therefore classify three types of poly(dG)•poly(dC) samples, according to their ability to give rise to the acid complex: 1) sample x_G=0.45 from the mixing curve, 2) polymerase synthesized poly(dG)•poly(dC) after alkali treatment, 3) the same "native" sample, i.e. without alkali treatment.

In the previous paper[9] we have proposed that native samples of dG-rich poly(dG)•poly(dC) are in fact long chains of double stranded poly(dG)•poly(dC) with a third strand of poly(dG) intermittently bound in the large groove. If the predominant mechanism of protonation is the anti to syn change of deoxyguanosine and the formation of a Hoogsteen pair with a shared proton (mechanism (1))[2], the classification given above appears consistent. Triple stranded regions are obligatorily in the A-form[23] and double stranded regions, if not too long, embedded in the alternating double-triple stranded structure would also be maintained in the A-form, and therefore could be protected from a proton induced conformation change from anti to syn. Furthermore, the presence of a second guanine in Hoogsteen binding would shield the main protonation site of the Watson-Crick guanine and thus prevent its interaction with the protonated cytosine. On the contrary, samples from mixing curves and alkali treated poly(dG)•poly(dC) have a structure that can be regarded as a "polycomplex", where the same molecule includes several and probably all possible structures. Y-like branching of a Hoogsteen poly(dG) strand from one poly(dG)•poly(dC) double helix to another is probable. Upon return to pH_b=6, still 10% of poly(dG)•poly(dC) are present in the x_G=0.45 sample; this could represent the fraction of double stranded poly(dG)•poly(dC) protected from protonation.

We come finally to the problem whether the mechanism of protonation of double stranded poly(dG)•poly(dC) is the same as that of the G-C pairs in DNA. As already mentioned, the exact determination of the pK of the acid complex is extremely difficult, since the formation of this complex is never complete. Nevertheless, the acid complex becomes detectable at pH_a=5, is formed down to pH_a=3 in increasing amounts, and dissociates below pH_a=3. This is quite similar to what is observed during the acid titration of DNA[1,2]. When titration is monitored by the variation of the CD signal at 260 nm the titration appears very cooperative. At pH<3 in 0.15 M NaCl, a steep transition is observed due to irreversible strand separation. For high G-C content DNA the first significant changes in the CD spectrum take place around pH 5; for such DNA's, the orthogonalisation of the CD spectra recorded along the acid titration shows at least three intermediate spectroscopic forms[24], besides the spectra at pH 6 and pH 3. This may reflect the different protonation of GC pairs embedded in different sequences.

A straightforward computation of the CD spectrum of the acid form is not possible, however, but we have scanned a range of feasible concentrations for the different species present at pH =3 in sample (5) (x_G=0.45) of the mixing curve. The spectrum obtained for the acid complex always presented two peaks

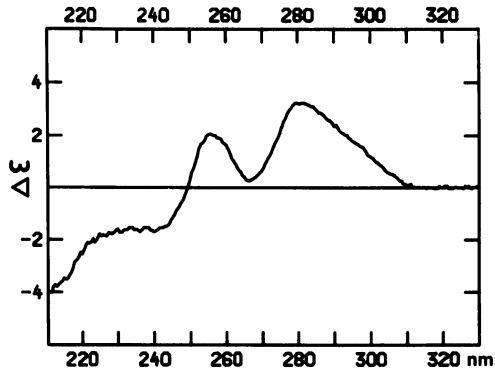


Figure 9: Estimated spectrum of the acid poly(dC^+).poly(dG) complex. It was obtained from sample x_G=0.45 from the mixing curve at pH_a=3.0 (fig. 3b) assuming the following (reasonable) distribution of complexes: poly(dC^+).poly(dC) 11%, poly(dC^+).poly(dG) 49%, poly(dG).poly(dC) 22%, poly(dG).poly(dG).poly(dC) 15%, poly(dG) 4 %.

at 255 and 280 nm and a trough at 265 nm (fig. 9). This spectrum shows striking ressemblance with the CD spectra of G-C-rich DNA's at pH 3[1,2,24].

CONCLUSION

This paper is the result of a long effort of the authors to clear up an old problem of polynucleotide structure. The variability of the structure is clearly due to the past and present conditions of study. The equilibrium poly(dG).poly(dG).poly(dC) $\rightleftharpoons$ poly(dG).poly(dC) can be shifted to the right by alkali treatment. The problem of the "polycomplex" containing all possible structures between poly(dG) and poly(dC) is extremely critical. A very similar structure appears to be formed between poly(C) and oligo(dG)[25], which is used as template for reverse transcriptase. Similarly, poly(dG).poly(dC) right out of the bottle or treated with slightly acid buffer will be a poor template. This has been demonstrated for E. coli RNA-polymerase[26].

A reasonable estimate for the purity of the double stranded structure of poly(dG).poly(dC) can be obtained by recording a CD spectrum after alkali treatment and comparing it with the CD spectrum we had presented[9]. Any sample showing a first negative band around 280 nm should be disregarded as double stranded poly(dG).poly(dC) for any enzymatic studies.

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