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Poly-2'-deoxy-2'-fluoro-cytidylic acid: enzymatic synthesis, spectroscopic characterization and interaction with poly-inosinic acid

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ABSTRACT The polymerization of 2'deoxy-2'-fluoro-cytidine-diphosphate (dCflDP) by polynucleotide phosphorylase is barely detectable in the presence of Mg^{++} under usual experimental conditions for polymerization of nucleoside diphosphates. High concentrations of enzyme have to be used to accomplish the synthesis. Mn^{++} is a better activator than Mg^{++} for the reaction. dCflDP inhibits the polymerization of CDP and has a $K_m=8.8x10^{-3}M$, six times higher than CDP. - The polymer, poly(dCfl), ressembles in many respects poly(C), but not poly(dC): the acid selfstructure forms at similar pK's; interaction with poly(I) yields a 1:1 complex the CD spectrum of which is similar to that of poly(I).poly(C). Finally, the T_m's of poly(I).poly(dCfl) are comparable to those of poly(I).poly(C).

The structural differences between DNA and RNA have been well established[1] and are due to the difference in sugar puckering. While in the B-form of DNA the sugar shows the S-conformation[2] (3'-exo pucker), RNA always assumes the A-form (which DNA can also adopt under certain conditions[1]) characterized by the N-conformation (3'-endo pucker) of the sugar. The origin of these differences is not yet understood and various proposals have come forward[3-5]. Melcher[5] had suggested that the difference in polarity of the 2'-substituent (OH vs. H) may determine the difference in sugar conformation and thus of the structure of RNA and DNA. Small differences in the N/S equilibrium have been effectively found even on the monomer level[6]: in ribosides this ratio is 60/40, in deoxyribosides 40/60.

We have attempted to attack this problem by the study of the 2'-deoxy-2'-fluoro-nucleosides. Although fluorine is only slightly larger than hydrogen, it is the most electronegative element. The crystal structure of 2'-deoxy-2'-fluoro-uridine-diacetate is effectively in a pure N-conformation[7]. More important, high resolution NMR data showed over 90% N-conformer in three 2'-fluoro-pyrimidine nucleosides[8]. In a comparative study of dinucleoside phosphates[9] containing dUrd, rUrd, or dUfl in the 3'-position, the stacking enthalpies of dUfl-nucleotides showed the same or higher values than the

ribose analogues and much higher ones than the deoxyribo analogues, which are known to show little stacking[4].

On the other hand, 2'-deoxy-2'-chloro-uridine and cytidine have been studied by Hobbs and Eckstein[10,11]. Although the free nucleoside showed the 2'-endo deoxyribose-like conformation in its crystal structure[12], the polymers behaved ribose-like[10].

It was therefore of interest to study polynucleotides and their complexes containing 2'-deoxy-2'-fluoro-nucleosides. Janik et al.[13] had already shown that poly(dUfl) ressembled poly(U) in its complexes formed with poly(A). The case of poly(dCfl) was considered even more interesting. Besides forming complexes with poly(I) and poly(G)[14-16], poly(C) forms a semiprotonated self-structure[17,18], which shows considerable differences with that formed by poly(dC)[3,19,20]. Furthermore, it was interesting to investigate the mechanism of polymerization of dCflDP by polynucleotide phosphorylase.

In this paper the synthesis of poly(dCfl) and some characteristic data on its selfstructure are presented and compared with that of poly(C) and poly(dC). Finally, it is shown that poly(dCfl) interacts with poly(I) in a manner very similar to that of poly(C), but not that of poly(dC).

MATERIAL AND METHODS

2'deoxy-2'-fluoro-cytidine was synthesized as described [7] from dUrdfl[21] using the modification of Hobbs et al.[11].

2'-deoxy-2'-fluoro-cytidine-5'-phosphate (dCflP) was synthesized according to Yoshikawa et al.[22], using the unprotected nucleoside and $POCl_3$ as phosphorylating agent in triethyl-phosphate: 50 mg (0.2 mmol) dCfl were dissolved in 1 ml tri-ethyl-phosphate and 0.05 ml $POCl_3$ added. The solution was stirred for two hours at 0°. A large part of the excess $POCl_3$ was eliminated by evaporation for 20 min.; the remaining solution was poured into 100 ml ether and the centrifuged precipitate redissolved in a minimal quantity of water. Electrophoresis in tri-ethyl-ammonium bicarbonate buffer pH 7.5 showed as the major spot the 5'-phosphate, some unreacted nucleoside and small quantities of the 3',5'-diphosphate. The monophosphate was isolated by chromatography (Whatmann 3M) in isopropanol/tri-ethyl-amine/ water (7/1/2). The major band was eluted with 1 % tri-ethyl-amine, which in turn was evaporated. Electrophoresis after this step showed exclusively the 5'-monophosphate. Yield: 35 to 40 percent.

Synthesis of 2'-deoxy-2'-fluoro-cytidine-diphosphate (dCflDP) was performed according to Hoard and Ott[23], since the methods of Khorana et al.[24] and Michelson[25] did not prove feasible: 32.5 mg (0.1 mmol) dCflMP as tri-ethyl-ammonium salt were dried by repeated evaporation of anhydrous pyridine (3 times 5 ml) and reacted with 2 equivalents N,N'-carbonyl-diimidazole in 1 ml dimethylformamide. The solution was maintained for 30 min. at room temperature. 2 equivalents of tri-n-butyl-ammonium phosphate in 1 ml dimethylformamide were added and the mixture stirred under exclusion of humidity overnight at room temperature. The precipitated imidazolium phosphate was centrifuged and washed 3 times with 4 ml dimethylformamide. The dimethylformamide solutions were evaporated under vacuum and the resulting gum dissolved in a minimum of water and pre-purified by electrophoresis in tri-ethyl-ammonium bicarbonate pH 7.5 from the remaining monophosphate. The 5'-diphosphate was eluted with 1 % ammonia. Yield: 20 to 30 percent.

Purification of dCflDP. The nucleoside diphosphate was still contaminated by monophosphate and by large quantities of inorganic phosphate. The inorganic phosphate was eliminated by chromatography of the crude product on a charcoal column (1000 O.D. applied to 0.6x 1.5 cm Norite). When the phosphate determination of the eluant showed less than 1 nmol/ml of phosphate , a solution ethanol/ 1% ammonia (40/60) was applied to elute the nucleotides. The nucleotides (400 O.D.) were then applied to a Sephadex DEAE-A25 column (0.9 x 2.5 cm) and a step-wise elution with tri-ethyl-ammonium acetate buffer was performed according to Caldwell et al.[26]. The monophosphate was eluted with 0.08 M buffer, the diphosphate with 0.36 M buffer and small amounts of higher phosphates were recovered with 1 or 2 M buffer. After several lyophilizations, this purified dCflDP was used for polymerization studies.

Polynucleotide phosphorylase was purified from E. coli as previously described[27], except that the heating step was replaced by a centrifugation at 100,000 g to eliminate the ribosomes. The enzyme had a specific activity of 700 units/mg.

The polymerization was followed by the liberation of orthophosphate, which was determined by a micro method according to Chen et al.[28]. The polynucleotide obtained was freed from nucleoside diphosphate by filtration on a Sephadex G-100 column and by exhaustive dialysis against Tris-HCl (50 mM) pH 7.5, containing 0.1 M KCl and 1 mM EDTA.

Poly(I) and poly(C) were purchased from Choay, Paris, poly(dC) from Boehringer-Mannheim, Tutzing (Germany).
CD spectra were recorded on a Roussel-Jouan dichrographe III (Jobin-Yvon, France), absorption spectra and thermal denaturation experiments an a Zeiss DMR 10 spectrophotometer with thermostated cuvetted holders.

RESULTS AND DISCUSSION

Enzymatic polymerization of dCflDP

The enzymatic polymerization of dCflDP under usual conditions of synthesis of polynucleotides, i.e. in the presence of Mg^{++} and catalytic amounts of enzyme, was barely detectable. It was necessary to perform the reaction at high concentrations of enzyme (fig. 1). With 8 units of enzyme in 50 µl , the reaction proceeded linearly. The rate of polymerization, however, corresponded only to 0.2 percent of CDP polymerization.

If Mg^{++} was replaced by Mn^{++}, the rate of polymerization was stimulated about two-fold and the yield improved (fig. 2). The rate could be further increased at higher pH (fig. 2, insert), while the yield did not change much. On the contrary, it is not usual to observed a drop of reaction rate at pH 9.5 which is close to the optimal pH for E.coli polynucleotide phosphorylase (about pH 10) using natural nucleoside diphosphates[29].

Even in the presence of Mn^{++} polymerization of dCflDP was far less efficient as compared with that of CDP (fig. 3). This very low efficiency of polymerization was not due to the presence of an inhibitor in the dCflDP. If CDP was added to dCflDP reaction mixture after 120 minutes incubation, the reaction proceeded readily, albeit with a slightly reduced rate. The reduction of CDP polymerization rate was more obvious, when the reaction was started with an equimolar mixture of CDP and dCflDP (fig.4a), In this case, a pronounced lag phase was observed. As substrate dCflDP has clearly lower affinity than CDP, since the apparent K_m values have been determined as 8.8×10^{-3} M and 1.5×10^{-3} M for dCflDP and CDP, respectively.

The reduced rate of CDP polymerization in the presence of equimolar concentrations of dCflDP might be due to partial incorporation of the latter giving rise to a copolymer, poly(C,dCfl). This is suggested by the comparison of the plateau of the kinetic curve for CDP alone with that of the mixture CDP + dCflDP (fig. 4b) which was 16 percent higher. Addition of oligonucleotides, like $(pU)_3$, stimulated about three-fold the rate of dCflDP polymerization; the yield was, however, not improved.

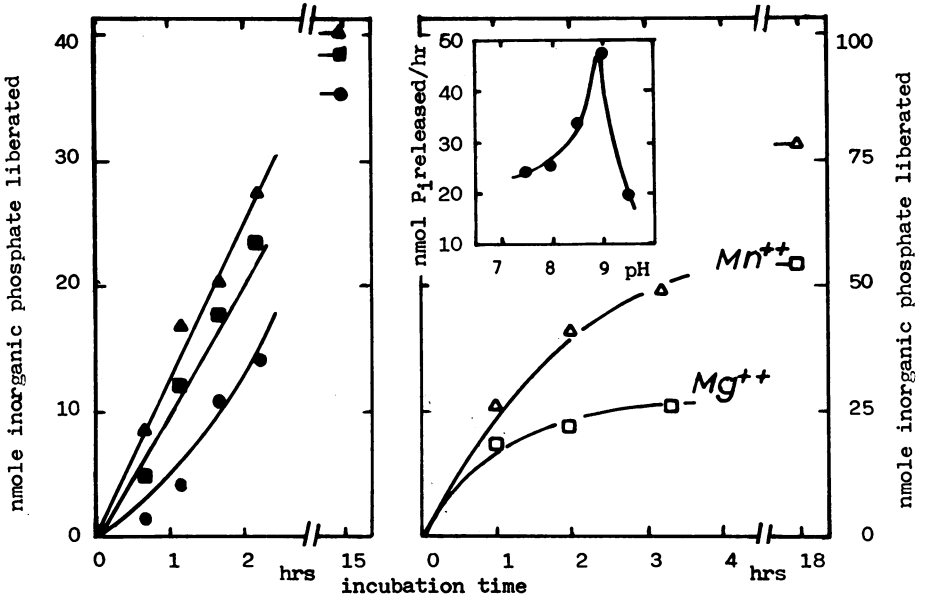

Figure 1: Effect of polynucleotide phosphorylase concentration on polymerization of dCflDP to poly(dCfl) at 37°. Reaction mixture contained: Tris pH 8 100mM, EDTA 0.1 mM, dCflDP 5mM, $MgCl_2$ 2.5 mM and PNPase 2 units (●), 4 units(■) or 8 units (▲) in a total volume of 50 µl. Polymerization expressed as nmol phosphate release in 50 µl.

Figure 2: Effect of divalent ions on polymerization of dCflDP at 37°. Reaction mixture (100 µl) contained: Tris-Hcl pH 8 100 mM, EDTA 0.1 mM, dCflDP 5mM, PNPase 20 units, and $MgCl_2$ 2.5 mM (□) or $MnCl_2$ 2 mM(△). Insert: Effect of pH on polymerization rate of dCflDP in the presence of $MnCl_2$. Condiationas as above, except 40 units PNPase were used.

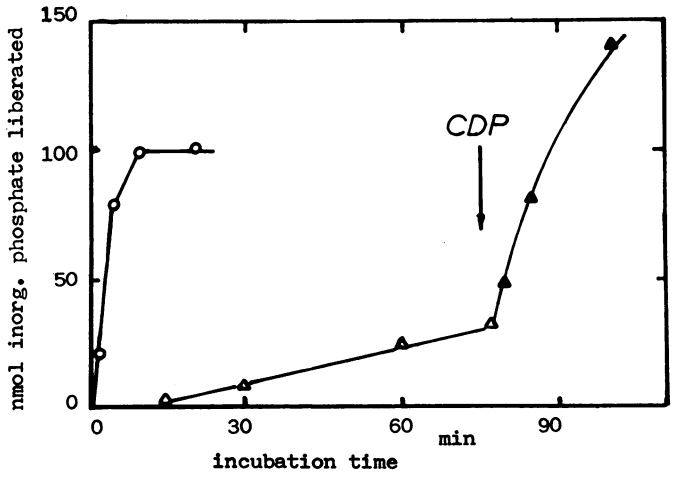

Figure 3: Polymerization of CDP(●) or dCflDP (△), followed by addition of CDP (▲). Incubation mixture contained in 50 µl: Tris-HCl pH 8 100 mM, EDTA 0.1mM, $MnCl_2$ 2 mM, CDP or dCflDP 5mM, PNPase 8 units. After 75 min. incubation of the dCflDP mixture, 5 mM CDP were added (arrow). Temperature:37°

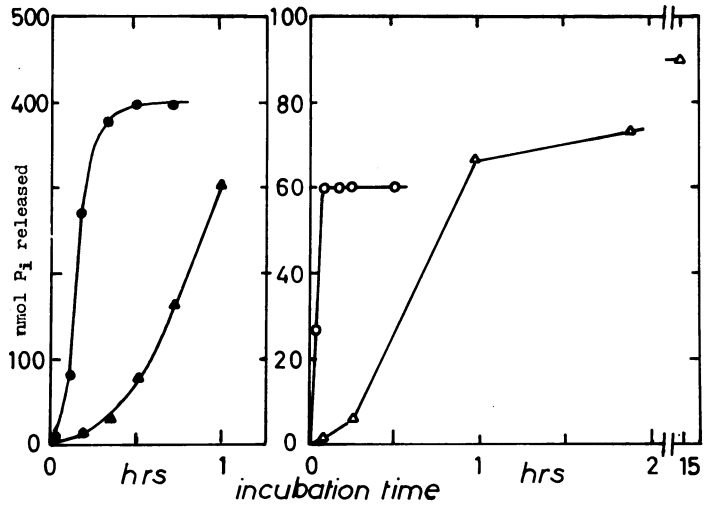

Figure 4: Partial inhibition of CDP polymerization by dCflDP at 37° (left) and copolymerization of CDP and dCflDP (right).
Left: Incubation mixture (100 µl) contained Tris-HCl pH8 100mM, EDTA 0.1 mM, $MgCl_2$ 5mM, PNPase 25 units/ml, CDP 10 mM (●) or CDP 10 mM + dCflDP 5mM (▲). Right: Incubation mixture contained (100 µl) the same constituents, except $MgCl_2$ 1 mM, PNPase 45 units/ml, CDP 2mM (o) or CDP 2 mM plus dCflDP 2 mM (△).

Acid selfstructure of poly(dCfl).

Upon acid titration of poly(C), an acid (semiprotonated) double-stranded selfstructure is formed, which manifests itself by characteristic changes in absorption, ORD and CD spectra, which show a red shift and large intensity changes. In an analogous manner, poly(dCfl) undergoes an acid transition with a red shift in the absorbance and CD spectra. Upon heating, these changes are reverted, characteristic of the deprotonation of the semiprotonated structure, yielding the neutral single strand. These changes are cooperative and reversible, similar to those observed for poly(rC).

Comparison of the CD spectra of poly(dCfl), poly(rC) and poly(dC) (figure 5) shows some striking similarities and differences. While the intensity of the high wavelength band of the acid form is the same in these three polymers, the negative band around 265 nm is highest in poly(dC) and lowest in poly(dCfl). On the other hand, the neutral spectra, although similar in shape, show great differences in their amplitudes: the positive band around 275 nm is about three times as large in the case of poly(rC)

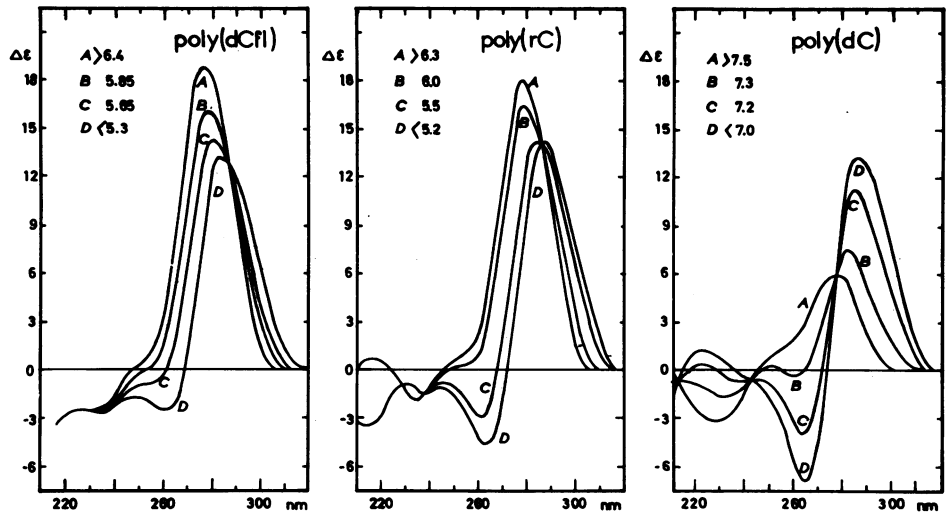

Figure 5: CD spectra in 0.1 M NaCl at pH values indicated of poly(dCfl) (left), poly(rC) (center) and poly(dC) (right).

and poly(dCfl) than in poly(dC). The latter polymer is known to be very little stacked[3,17], while poly(rC) and poly(dCfl) appear to have considerably more secondary structure.

Another characteristic feature of the selfstructure of poly(rC) is the strong ionic strength dependence of its pK; poly(dC) in turn shows no change in its pK as a function of ionic strength (fig.6); furthermore, its pK's are more than one unit higher than those of poly(rC). The ionic strength dependence of the acid pK of poly(dCfl) is compared with that of its analogous in figure 6. The slope of pK *vs.* log(Na^+) of poly(dCfl) is about that of poly(rC). Also the pK values themselves are very similar between these two polymers and do not at all ressemble those of poly(dC). Poly(dCfl) thus ressembles in many respects the ribopolymer, but not the deoxyribopolynucleotide.

Interaction of poly(dCfl) with poly(I).

Poly(C) forms a 1:1 complex with poly(I)[16]; contrary to the complex formed with poly(G), the interaction is rapidly obtained[30] in reasonable salt conditions where poly(I) does not form a selfstructure[31] (below 0.6 M Na^+). Figure 7 shows the titration of poly(dCfl) with poly(I) followed by CD and UV absorbance. The negligible optical activity of poly(I) under these conditions[32] compared with that of poly(dCfl) and of the complex, permits the unequivocal determination of the equivalence point (fig. 7a).

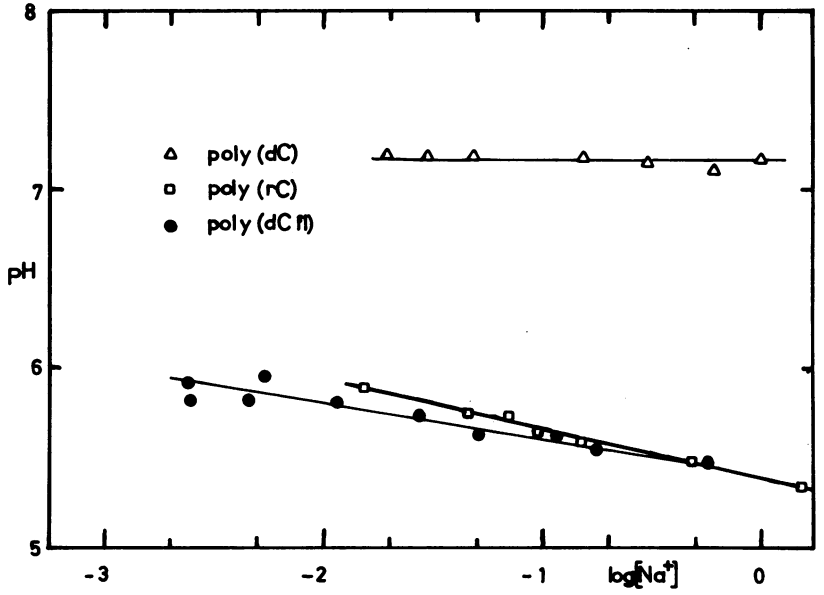

Figure 6: Ionic strength dependence at 25° of pK for the double helix formation of poly(dC) (△), poly(rC) (□) and poly(dCfl (●).

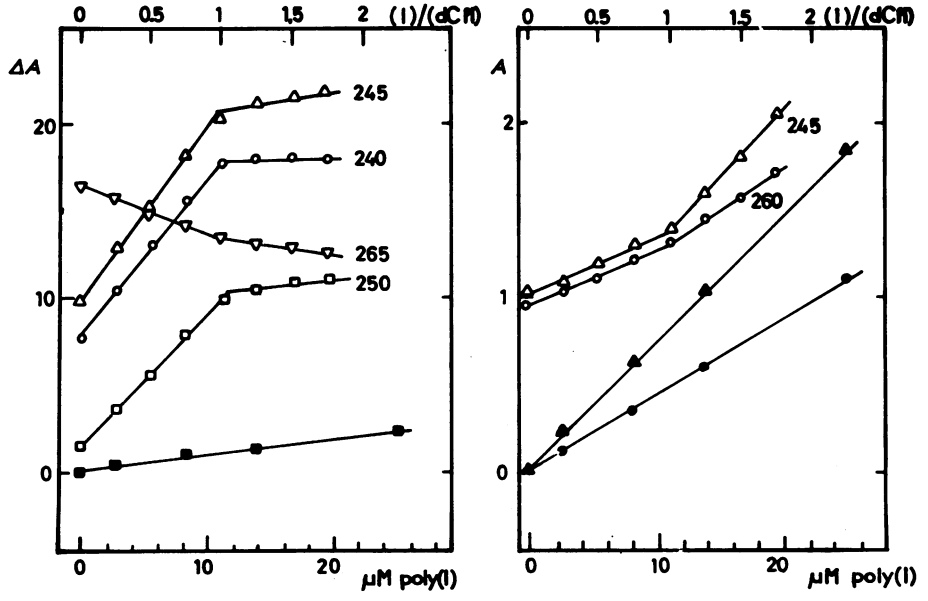

Figure 7: Titration of poly(dCfl) with poly(I) in 0.05 M Na-cacodylate. left: followed by CD at wavelengths indicated; right: followed by absorbance at wavelengths indicated. Filled symbols: CD (or absorbance) of poly(I) (in the absence of poly(dCfl)).

On the other hand, in absorbance measurements, above the equivalence point, the absorbance at a given wavelength will increase with the same slope as free poly(I) (filled symbols in fig. 7b).

Comparison of the CD spectra of poly(I).poly(dCfl) and poly(I).poly(C) with that of poly(I).poly(dC) shows the same two characteristic positive bands around 245 and 275 nm. This suggests that the two complexes, poly(I).poly(C) and poly(I).poly(dCfl), must be very similar, possibly isomorphic in the 11 fold A helix,[32] but different from poly(I).poly(dC), which is a 12 fold A'helix in the fibrous state[33]. The difference with the CD spectrum of poly(dI).poly(dC)[34] (figure 8) is even more striking. This polynucleotide complex is known to be in the B-form[35]. The CD results are not very surprising, since not only the pyrimidine, but also the purine strand will influence the conformation. Still, as in the case of the semiprotonated selfstructure, poly(dCfl) ressembles more poly(C) than poly(dC).

A further illustration of this proposal is furnished in figure 9,

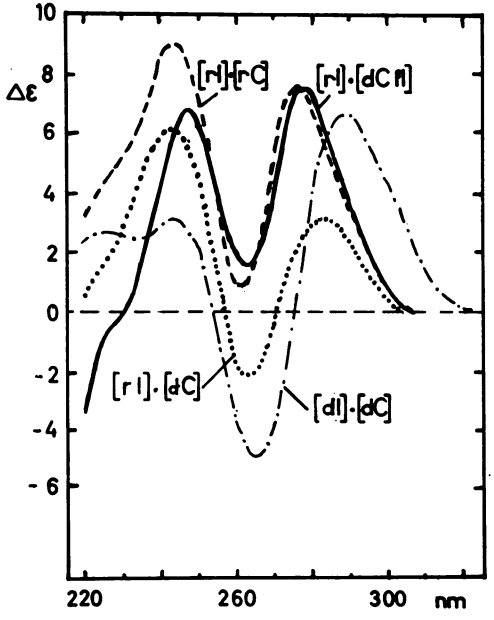

Figure 8: CD spectra of poly(I).poly(C) (- - -)[30], poly(I).poly(dC)(•••••), poly(dI).poly(dC) (-·-·-)[34], and poly(I).poly(dCfl) (_____) in 0.15 M Na^+, pH 8.

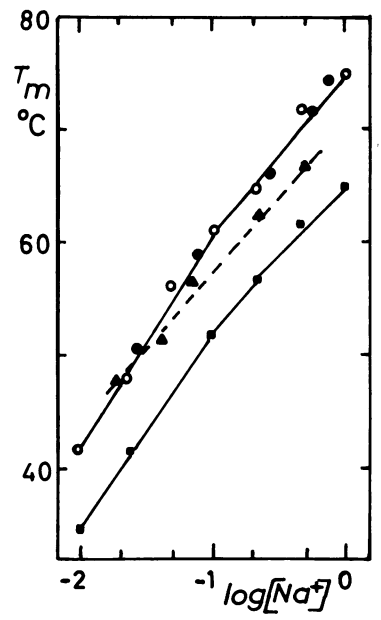

Figure 9: Ionic strength dependence of melting points (T_m) of poly(I).poly(C) (o ref.14 , ● ref. 15), poly(I).poly(dC) (■ ref.14) and of poly(I).poly(dCfl) (▲) at pH8.

where the published T_m values of poly(I).poly(C)[14,15] and poly(I).poly(dC)[14] are compared with those observed for poly(I).poly(dCfl). The latter complex shows T_m's 2 to 3° lower above 0.1 M Na^+ and the same ones below this ionic strength as the all-ribo complex, while interesting enough the T_m of poly(I).poly(dCcl)[10] was slightly higher in 0.1 M Na^+. As already noted by Chamberlin and Patterson[14], the T_m's of poly(I).poly(dC) are about 10° lower than those of poly(I).poly(C). Here again, the fluorine containing polymer complex behaves very similar to the all-ribo complex. Also, the rather large differences in T_m between poly(I).poly(C) and poly(I).poly(dC), which probably reflect the differences between two rather similar A-type helices[32], as well as their optical differences, suggest that such variations in nucleic acid fine structure may become accessible by optical methods.

Further characterization of such 2'-fluoro substituted polynucleotide complexes by enzymatic approaches as well as by physico-chemical methods are under study.

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