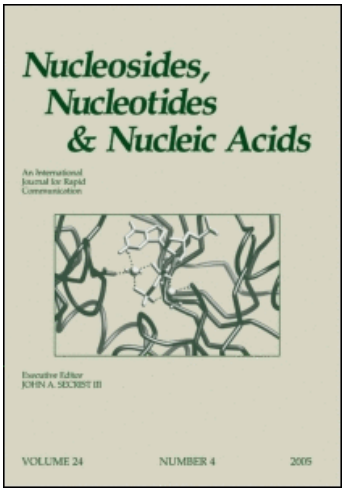

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POLYRIBONUCLEOTIDES. THE ROLE OF ACID/BASE PROPERTIES IN HYDROGEN BOND INTERACTIONS

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Abstract

Three new polyribonucleotides, poly(3-deazauridylic acid), poly(2-fluoroinosinic acid) and "poly(ribavirinic acid)" were synthesized. These compounds were designed to explore the importance and limits of the acidic properties of putative Watson-Crick hydrogen bonding protons in forming duplexes with complementary polynucleotides. None of these polymers formed such duplexes, thus establishing that a limited range of acidities is required.

Introduction

It is by now well-known that base pair formation in nucleic acids involves N-H---N or N-H---O hydrogen bonds. Little is known, however, about the importance of N-H bond acidity in determining the stability of these bonds. No attempt has been reported to determine limits; that is, to identify compounds which meet all obvious structural requirements for Watson-Crick interactions but which have drastically altered N-H bond acidities and do not form such complexes. In order to address this question two polynucleotides, poly(2-fluoroinosinic acid)* and poly

*Abbreviations used in this paper are: poly (fl^2I), poly(2-fluoroinosinic acid); poly(Rv), poly(ribavirinic acid), the polyribonucleotide of 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide-5'-monophosphate; poly-(c^3U), poly (3-deazauridylic acid); fl^2IMP, fl^2IDP, RvMP, RvDP, c^3UMP, c^3UDP, the 5'-mono and diphosphates of the respective nucleosides.

(ribavirinic acid) have been synthesized and relevant physicochemical properties determined.

An additional consideration is whether an enolizable, relatively acidic system in which carbon is isosterically substituted for nitrogen could undergo complex formation with a putatively complementary polynucleotide. As a test of this possibility, the polynucleotide of the antitumor agent 3-deazauridine [1,2] was prepared.

Materials and Methods

Polynucleotide phosphorylase (M.luteus, E. coli, E.C. 2.7.7.8) and acid phosphatase (wheat germ E.C. 3.1.3.2), spleen phosphodiesterase (E.C. 3.1.4.18) and alkaline phosphatase (E. coli E.C. 3.1.3.1.), and venom phosphodiesterase (E.C. 3.1.4.1) were obtained from P-L Biochemicals, Sigma Chemical Co., and Boehringer Mannheim respectively. Thin layer chromatography was carried out using silica gel coated aluminum sheets (E. Merck) in solvent systems ethyl acetate-water-1-propanol (4:2:1 upper layer), glyme-water-NH_4OH (33:3:4), or 2-propanol-NH_4OH-water (7:1:2). Ultraviolet spectra, melting profiles and pKa values were obtained using a Beckman DU8 or a Cary 15 spectrophotometer. Circular dichroism spectra were recorded on a Jasco J40A spectropolarimeter. Sedimentation coefficients were estimated with a Beckman L5-65 Ultracentrifuge using 5 - 20% sucrose gradients and comparing polymer sedimentation velocities with those of poly(I) standards (P-L Biochemicals).

5'-Diphosphates of the Nucleosides (FIG.1).

Ribavirin 5'-phosphate was prepared following the literature[3]. 2-Fluoroinosine[4] or 3-deazauridine[1] (1 mmol) was added to a solution of

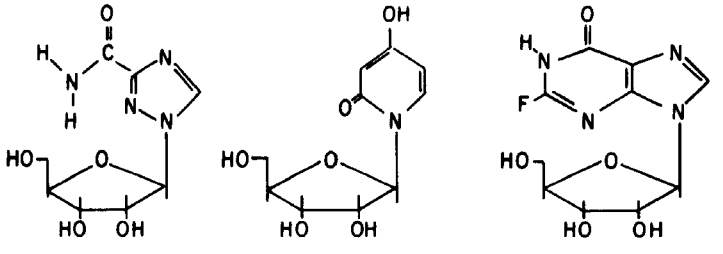

FIG. 1. Nucleosides of polymers.

phosphoryl chloride (0.2 mL; 2 mmol) in triethyl phosphate (4 mL) cooled to -10°C in a dry ice-2-propanol bath. The reaction mixture, after 2 hr at -10°C, was poured into 1:1 ether-pentane (100 mL). The precipitate was filtered, washed with ether (2 x 20 mL) and dissolved in 75 mL of ice-cold water containing 150 mg lithium carbonate. The solution was neutralized with 1 N lithium hydroxide, concentrated to ca 10 mL, and the monophosphate was precipitated as the barium salt. The 5'phosphates of the nucleosides were converted to 5'-diphosphates by Michelson's procedure.[5] The crude products were purified on AG-1 x 4 (chloride) or QAE-Sephadex (chloride) in 35 - 40% yield.

Polymerization of 5'-Diphosphates.

The solution (10 mL) containing 0.2 M Tris,HCl (pH 9.0 for RvDP and pH. 7.0 for c^3 UDP and fl^2IDP), 5 mM $MgCl_2$ ($MnCl_2$ for fl^2IDP), 0.5 mM EDTA, 20 mM 5'-diphosphate, and M. luteus polynucleotide phosphorylase (1,2, and 5 mg/mL for RvDP, c^3 UDP, and fl^2IDP respectively) was incubated at 37°C. The reaction was followed by estimating the inorganic phosphate released.[6] The rate of polymerization of fl^2IDP was too slow to follow. The reaction mixture (after 7, 38, and 120 hr respectively for poly(Rv), poly(c^3U), and poly(fl^2I)) was diluted 5 times with water and extracted with 5:2 chloroform-isopentyl alcohol (8 x 25 mL). The aqueous solution containing the polymer was dialyzed against 0.1 M NaCl + 5 mM EDTA (pH 7.5; 8 L) and water (8 L) in a Dowex b/HFD-1 beaker dialyser and lyophilyzed. The yields were 40% for poly(Rv) and poly(c^3U) and only 10% for poly(fl^2I); the results for the latter were the same whether the M. luteus or E. coli enzyme was used.

Poly(Rv) was degraded in solution containing 0.2 M Tris-HCl (pH 9.0), 15 mM $MgCl_2$, 0.1 mg polymer, venom phosphodiesterase (1.5 units) and alkaline phosphatase (0.3 unit). The degradation of the other two polymers was accomplished in the same volume of solution containing 0.2 M acetate (pH 4.8), bovine spleen phosphodiesterase (0.03 unit) and acid phosphatase (0.02 unit). At 37°C the polymers were degraded within a day.

The formation of complex was attempted by mixing component polymers in an appropriate buffer containing 0.10 M NaCl and 0.001 M $MgCl_2$, heating to 70°C for 1 hr and cooling overnight at 4°C. The ultraviolet spectrum of the mixture was compared with the spectra of the individual polymers.

Results

The ease of polymerization of the three nucleoside diphosphates was related to the ionic state of the aglycones at the polymerization pH. Ribavirin 5'-diphosphate was readily polymerized; FIG.2 shows comparative polymerization curves for RvDP and IDP. The triazole moiety undergoes no ionization in the pH range (7-10) at which polynucleotide phosphorylase exhibits reasonable polymerizing activity. Polymerization was carried out at pH 9.0 (the optimum pH for the M luteus enzyme). Under these conditions, however, no polymerization of either c^3UDP or $f1^2$IDP took place.

The pKa values for c^3UDP and $f1^2$IDP were determined to be 6.5 (4-OH) and 4.6 (N^1-H), respectively. Since at pH 9 both heterocyclic bases were essentially completely ionized, polymerization was undertaken at pH 7. As seen in FIG. 3, c^3UDP was effectively, albeit slowly, polymerized

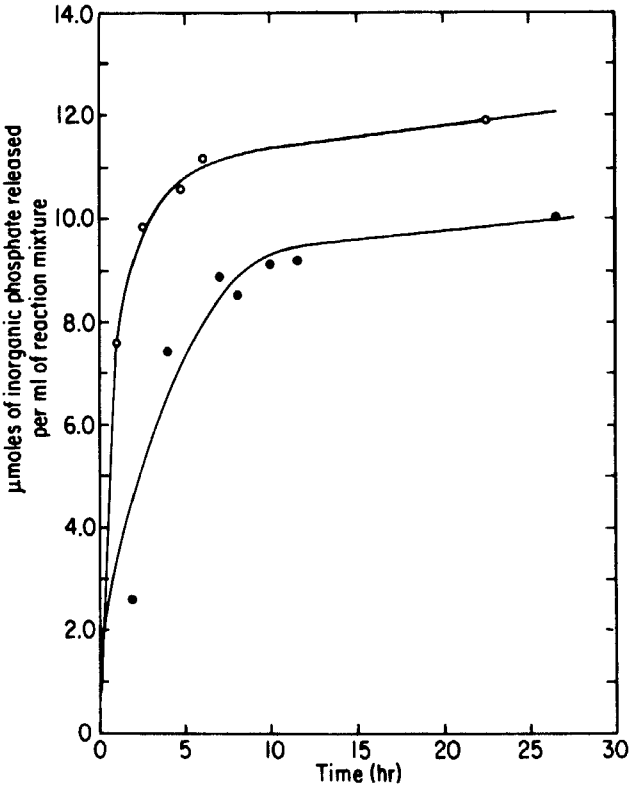

FIG. 2. Rate of polymerization by polynucleotide phosphorylase of IDP (o) and RvDP (●)

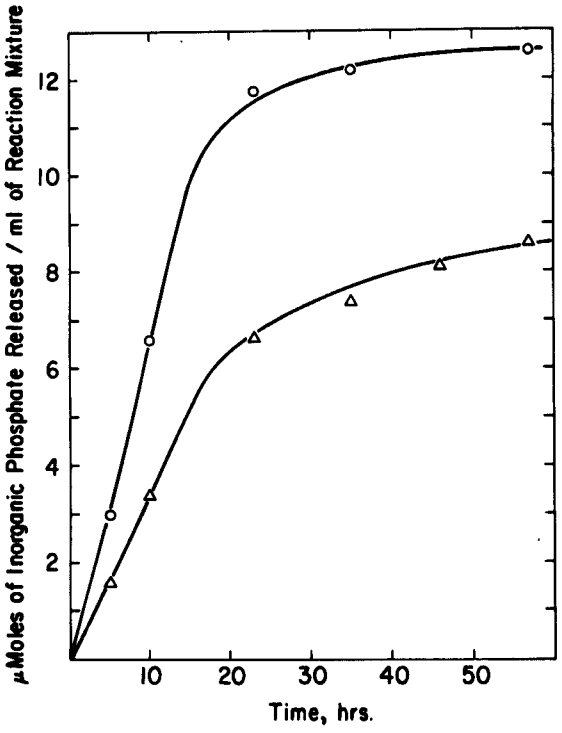

FIG. 3. Rate of polymerization by polynucleotide phosphorylase of UDP (o) and c^3UDP (Δ)

to give a 40% yield of polymer having a pKa of 8.5 (it should be remembered that the polynucleotide phosphorylase reaction is an equilibrium one, so that yields in excess of 40 - 50% are seldom obtained). However, fl^2IDP was still mostly ionized even at pH 7 so that very slow reaction, low yield (ca. 10%) and low polymer molecular weight were observed. No advantage resulted from lowering the pH below 7, since enzyme activity fell off very sharply at lower pH. The pKa of poly(fl^2I) was found to be 4.8.

The molecular sizes of the polymers were estimated by ultracentrifugation and found to correspond to $s_{20,w}$ of 5 for poly(Rv) and 10 for poly(c^3U). Poly(fl^2I) was of much lower molecular weight; it sedimented less rapidly than the smallest available poly(I) marker ($s_{20,w}$ <2.5). The average size of the fl^2I oligomer was determined to be about 8 by subjecting it to gel filtration chromatography on Sephadex G50 and noting the elution volume. A partial alkaline digest of poly(I) was chromatographed under the same conditions. The corresponding

fractions were found to contain primarily I_8 using the poly(C)·oligo(I) melting profiles developed by Ts'o.[7]

The effect of temperature upon uv absorbance was studied for each polymer. Neither poly(fl^2I) nor poly(c^3U) showed any significant hyperchromicity in the temperature range 2 - 80°C. Poly(Rv), on the other hand, underwent a marked hypochromic, bathochromic shift (FIG. 4) as the temperature was changed from 8 to 68°C. This unusual behavior was also observed with the free nucleoside. Measurement of the circular dichroism spectrum at 10°C gave virtually no ellipticity, supporting the lack of any significant secondary structure at low temperature.

Each of the polymers was subjected to conditions which should favor complex formation with complementary polynucleotides. Attempts were made to complex poly(Rv) and poly(fl^2I) with poly(C) and poly(A) and poly(c^3U) with poly(A) and poly(G). Under no circumstances was any evidence for duplex formation obtained.

Discussion

It is apparent from the data presented above that the adaptability of polynucleotide phosphorylase to a variety of aglycones does not extend to anions such as those of c^3UDP and fl^2IDP. This was somewhat

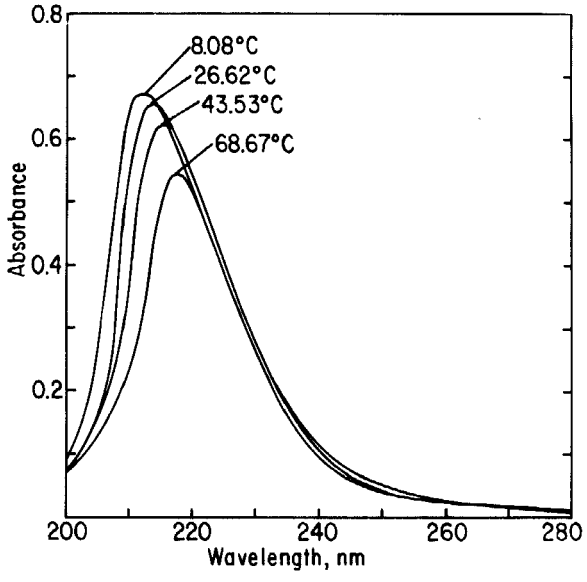

FIG. 4. Ultraviolet spectra of poly(Rv) in 0.005 M Mops (pH 7.2) + 0.1 M NaCl at various temperatures.

surprising in view of the successful polymerization of xanthosine diphosphate by Michelson.[8] The pKa of xanthosine is 5.75 and that of poly(X) is about 6.6[9]; nonetheless, polymerization of XDP proceeded readily at pH 8.2.[10] The only obvious difference in the cases is in the position of negative charge on the heterocycle; in X it is at N^3-O^2 enolate[9] whereas in $f1^2I$ and c^3U it is localized in the Watson-Crick hydrogen bonding region. Presumably, then, polynucleotide phosphorylase has a negative charge or dipole within the active site situated in such a way that electrostatic repulsions can hinder binding of anions such as $f1^2I$. If a substantial amount of neutral aglycone can be generated by reducing the pH to the lowest level tolerated by the enzyme, as is the case with c^3UDP, facile polymerization to a high molecular weight polymer can occur. If, on the other hand, even at pH 7 the aglycone of the nucleoside diphosphate is almost entirely anionic, low or no levels of polymerization will result; this is the case with $f1^2$IDP. RvDP, with no aglycone ionization in the pH range studied, was a very good substrate for the enzyme.

Once the polymers were obtained, albeit with small molecular weight in the case of poly($f1^2I$), the self-association and duplex-forming properties were studied. No self-association, as demonstrated by an increase in absorbance upon heating could be demonstrated for any of the three polymers. Indeed, poly(Rv) showed a marked reversible hypochromicity upon heating from 8 - 68°C (FIG. 4), exactly the opposite of the behavior ordinarily observed. The cd spectrum contained only very low ellipticities and gave no evidence for secondary structure. The same temperature study was carried out on ribavirin, the nucleoside itself, and its uv spectra exhibited identical behavior. The origin of this anomaly is not understood.

Since poly(I) is well-known to form a multiple-stranded self-association helical structure[11], it was initially surprising that poly($f1^2I$) gave no evidence for such a structure, even at a pH well below its pKa and in 1.0 M salt. However, a sample of poly(I) having similar chain length was subjected to the same conditions and also failed to form a self-association complex, suggesting that there is a critical molecular weight greater than 3000 for poly(I) self-association to occur.

It was intriguing to discover that none of these polymers formed complexes with potentially complementary polynucleotides. In the case of poly(c^3U) this was not unexpected, since the CH (or - CH_2) at position 3 would be more likely to hinder than to participate in hydrogen bond formation. Ribavirin, however, seems so structurally related to inosine that Watson-Crick type hydrogen bonding was expected. The major difference lies in the acidity of the amide N-H (N^1-H of inosine), for which there is no ionization below pH 11 in ribavirin. Similar lack of complementary hydrogen bonding has been reported for 5,6-dihydrouracil derivatives, but this was attributed primarily to disruption in stacking resulting from conversion from planar to half-chair pyrimidine conformations.[12] That ambiguity is removed in the case of poly(Rv), leaving reduced hydrogen-bonding capacity as the probable cause for the observed lack of duplex formation.

The reverse situation was observed in the case of poly(fl^2I). The relatively short chain length minimized the usual marked polymerization increase in acidic pKa and the polynucleotide was essentially fully anionic in both base and phosphate moieties even at pH 7. Poly(C) was expected to be the complementary polynucleotide, but protonation and duplex formation below pH 5.5 have been reported for poly(C).[13] As might have been expected, then, no complex formation could be demonstrated across a pH range of 4.8-6 other than the protonated double helix formation of poly(C) below pH 5.8. Since the pKa of poly(br^5C) is lower (4.5)[14] than that of poly(C), attempts were made to complex it with poly(fl^2I) over the pH range 3.8 - 4.8. Again, however, either no complex formation or poly(br^5C) double helices were observed. Since it was possible that the lactim form of poly(fl^2I) might predominate in the neutral purine, mixtures with poly(U) were studied at pH 3. No complex formation could be demonstrated. This is not simply a function of chain length, since hexainosinate forms a duplex with poly (C) melting at 16.8°C.[7]

These studies have established that limits exist on the degree of N-H bond acidity required for the formation of complementary double stranded helices. If the acidity is too great (fl^2I) the polymer acts only as a proton donor; if not great enough (Rv) the putative hydrogen bonds are too weak to initiate double helix formation.

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