*CONFORMATIONAL PROPERTIES OF POLYFORMYCIN: A POLYRIBONUCLEOTIDE WITH INDIVIDUAL RESIDUES IN THE SYN CONFORMATION**

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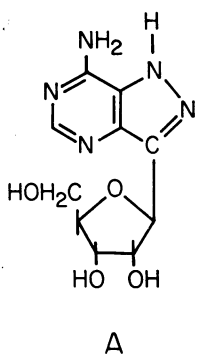
Communicated by Edward L. Tatum, October 9, 1968

The antibiotic formycin (Fig. 1) is a cytotoxic analog of adenosine. As shown previously,[1] formycin nucleotides substitute effectively for adenosine nucleotides in a wide variety of enzymatic reactions, including nucleotide polymerization.

We have reported[1] elsewhere that certain characteristics of formycin nucleotides and polynucleotides are paradoxical. In this paper we describe additional abnormal properties of formycin polymers and present the results of some physical and enzymatic studies. These observations lead to the conclusion that the anomalous behavior of formycin polymers is due to conformational properties thus far unique to formycin residues in enzymatically synthesized polyribonucleotides. We propose that F residues in ribopolymers may exist in either the *syn* or the *anti* conformation. The F units are: (1) *anti* in ordered structures of the Watson-Crick type; (2) probably a mixture of *syn* and *anti* in single-stranded copolymers; (3) entirely *syn* in neutral polyformycin. Furthermore, the behavior of an alternating copolymer of F + ψ suggests that the ψ residues in polymers may undergo analogous conformational transitions.

Materials and Methods.—The preparation of nucleotides, polynucleotides, and polymerizing enzymes and the determination of *Tm* values were performed as previously reported.[1-4] Spleen phosphodiesterase was the gift of Dr. A. Cerami. Phosphodiesterase from rattlesnake venom and micrococcal nuclease were the most highly purified available from Worthington Biochemical Corp. Bovine pancreatic RNase was the gift of Drs. S. Moore and W. Stein. The degradation of poly A and poly F was monitored as follows: (1) by observing spectrophotometrically the decrease in ultraviolet (UV) hypochromism (polymer nucleotide concentration: 5–7 $\times$ 10^{-4} M); (2) by measuring the decrease in acid precipitability of radioactive polymers (10^{-5} M), using a Millipore filter method;[2] (3) by observing the increase in fluorescence of poly F (5 $\times$ 10^{-6} M). The incubation conditions were: for venom phosphodiesterase, 0.05 M Tris-HCl buffer (pH 8.5), 0.01 M Mg^{++}; for spleen phosphodiesterase, 0.01 M phosphate (pH 6.5); for micrococcal nuclease, 0.01 M glycine buffer (pH 9.0), 0.05 M Ca^{++}. Incubations were at 25°.



A

Fig. 1.—Structure of formycin shown in the *syn* conformation. The *anti* conformation is achieved by rotating the chromophore 180° on the glycosyl bond.

Optical rotatory dispersion (ORD) spectra were obtained on a Cary 60 spectropolarimeter that had a thermostated water-jacketed cell with a 1-cm light path. The compounds studied were at a concentration of approximately 10^{-4} M. The observed rotations were corrected with solvent blanks and expressed in terms of M' as defined by Fasman *et al.*[5] and by Ts'o *et al.*[6]

Results.—The paradoxical properties of formycin nucleotides and polynucleotides described in a previous report[1] are as follows:

(1) In view of their identical H-bonding functional groups, the coding properties of F residues in polyribonucleotides

should be the same as those of A with respect to polypeptide synthesis *in vitro*. While this expectation was fulfilled in the case of copolymers containing F, poly F itself failed to code for the synthesis of any polypeptide. This is in contrast to poly A, which is known to direct the formation of polylysine.

(2) Poly F also differs from copolymers containing F with respect to its synthesis by RNA polymerase. Thus, FTP completely and efficiently replaces ATP for RNA synthesis directed by a variety of naturally occurring and synthetic DNA templates, provided at least one additional nucleotide is being polymerized. Under these conditions, the nearest-neighbor distribution of the products formed with FTP is identical with those synthesized with ATP, and the reactions with FTP proceed at velocities equal to 30–80 per cent of those with ATP. In contrast, the homopolymer (poly F) synthesis directed by denatured DNA proceeds at 0.5 per cent the rate of poly A synthesis.

We have studied in greater detail this interaction of FTP with RNA polymerase during homopolymer synthesis. With poly U or poly dT as template, no poly F is formed under conditions that permit efficient synthesis of poly A. However, with both of these templates, as with denatured DNA (Table 1), FTP

TABLE 1. *Synthesis of poly F, poly A, and poly F,A by RNA polymerase with denatured calf thymus DNA template.*

Substrate Conc. (mμmoles/ml)		Input ratio	Mμmoles NMP Incorporated/ml*		Product ratio
ATP	FTP	ATP/FTP	AMP	FMP	AMP/FMP
—	400	—	—	0.40	—
10	390	1:39	0.98	0.76	1.29
40	360	1:9	1.25	2.20	0.57
200	200	1:1	3.29	2.62	1.26
360	40	9:1	23.2	2.88	8.05
390	10	39:1	58.2	1.88	30.6
400	—	—	84.0	—	—

Synthesis was monitored by following the incorporation of H^3-FMP (3.7×10^6 cpm/μmole) and C^{14}-AMP (9×10^5 cpm/μmole) into acid-insoluble form.

* The reaction mixtures[2] (0.1 ml) containing 20 mμmoles of heat-denatured DNA template were incubated at 37° for 30 min.

is used effectively by the enzyme, provided some ATP is also present, and increasing initial concentrations of ATP promotes increasing incorporation of F. All the above findings demonstrate that the synthesis of poly F with any template appears to be especially difficult for RNA polymerase. Taken together with its failure to function as a template in peptide synthesis, these results suggest some unusual structure for poly F.

Nuclease susceptibility of poly F: A third anomaly of poly F is observed in its interaction with nucleases. Thus, in comparison with the degradation of poly A under identical conditions, the depolymerization of poly F proceeds (*a*) 100-fold more slowly with snake venom phosphodiesterase, (*b*) 1000-fold more slowly with micrococcal nuclease, and (*c*) 10,000-fold more slowly with spleen phosphodiesterase. This behavior of poly F with enzymes known not to possess base specificity is unique among polyribonucleotides and supports the suggestion that the polymer structure is unusual in some respect.

Another unexpected finding, to be described in detail in a later paper,[7] con-

cerns the interaction of poly F with bovine pancreatic RNase. Unlike poly A, poly F is a true substrate for RNase, and is degraded quantitatively to F > p at a rate identical with that found for poly C. This characteristic of poly F, which from the evidence cited above is clearly a purine polynucleotide analog, reflects the unique conformational properties of the polymer (*vide infra*).

Optical and thermal properties of formycin polymers: (*a*) Neutral poly F, like poly A,[8] shows considerable hypochromism (25%) when compared with monomer absorption under similar conditions (Fig. 2). As with poly A,[9] a substantial proportion of this hypochromism is sensitive to elevation of temperature, and the transition is noncooperative, extending over a range of >30°. This suggests that neutral poly F exists in the single-stranded state and not as a multistranded complex. (*b*) The variation of the UV absorption of double-stranded polymers containing formycin residues quantitatively resembles that of comparable polymers containing adenosine.[10,11] In each case the thermal transition is sharp, is associated with a high degree of hypochromism, and is completely reversible on cooling. The thermostability of a series of alternating copolymers of F and different pyrimidines is at least equal to that of the corresponding A compounds (Table 2), with the exception of r(F-ψ), a polymer in which both nucleotide species are C-glycosyls. The exceptional behavior of r(F-ψ) is noteworthy because it stands in contrast to the results obtained for several comparable series of alternating copolymers. We have prepared an analogous series of polymers, using six different purine derivatives together with the pyrimidines listed in Table 2. In all these, the polymer containing ψ as the pyrimidine was the most thermostable. (*c*) The thermostability of polymers containing a single type of base pair is generally relatively insensitive to sequence. For example, under conditions that were identical for the corresponding pairs, the following *Tm*'s have been observed: d(A-T), dA:dT, 61°; r(A-U), 68.5°; rA:rU (1:1), 57°.[10] Therefore, it is of interest that the *Tm* of rF:rU is very much lower (35°) than those of r(F-U) and rA:rU under identical conditions (Table 2). The unusual thermal lability of r(F-ψ) and rF:rU represent additional anomalies of formycin polymers.

Fluorescence of poly F: As reported elsewhere,[12] the fluorescence of formycin is markedly quenched on incorporation into polymers, and still further quenching occurs when the polymer residues are base-paired in helical structures. The *Tm* values and the denaturation profiles obtained by fluorescence coincide closely with those observed by absorption measurements. Denaturation of the

TABLE 2. *Tm values of comparable adenosine and formycin polymers.*

Alternating Polymers				Homopolymer Pairs	
Polymer	*Tm* (°C)	Polymer	*Tm* (°C)	Polymer	*Tm* (°C)
r(A-U)	32	r(F-U)	33	rA:rU (1:1)	57
r(A-$\overline{\text{FU}}$)	32	r(F-$\overline{\text{FU}}$)	35	rF:rU (1:1)	22
r(A-T)	46	r(F-T)	48	rA:dT (1:1)	65
r(A-$\overline{\text{BU}}$)	53	r(F-$\overline{\text{BU}}$)	63	rF:dT (1:1)	37
r(A-ψ)	58	r(F-ψ)	32		

The *Tm* of alternating polymers was determined in 0.001 *M* Na citrate, and that of the homopolymer pairs in 0.01 *M* Tris (pH 7.9)–0.1 *M* KCl.

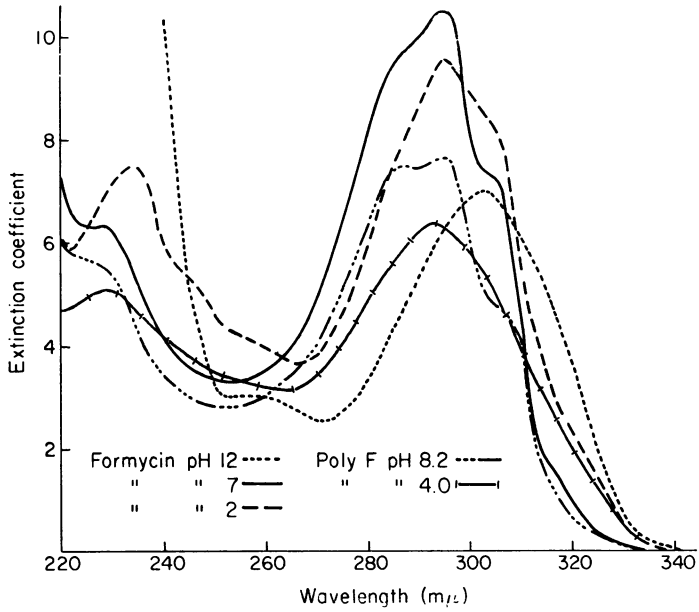


FIG. 2.—Absorption spectra and extinction coefficients of formycin and polyformycin. Neutral and acid poly F are both strongly hypochromic.

ordered structures of rF:rU or r(F-U) leads to a three- to fivefold increase in fluorescence intensity over a narrow temperature interval (3–5°). In contrast, the fluorescence of poly F is increased with temperature only by approximately 25 per cent; this fluorescence transition is noncooperative, extends over a range of 25°, and suggests that poly F exists as a single-stranded structure at neutral pH.

ORD of formycin polymers (Figs. 3–6): Because the main UV absorption maximum of formycin is at 295 mμ (remote from the absorption bands of other nucleotides), it was possible to study the ORD of F polymers in the hope that this might provide some insight into the suspected structural abnormality of poly F. Several representative spectra are presented in Figures 3–5, together with those of comparable adenosine polymers. The salient points are as follows: (*a*) In *native alternating copolymers and native homopolymer pairs*, the Cotton effect corresponding to the F transition shows (with decreasing wavelength) a positive and then a negative component. The native F polymers therefore resemble, in this respect, the corresponding native A polymers.[6] (*b*) Denaturation of r(F-U) completely eliminates the positive rotation due to the F residues. (*c*) *The Cotton effect in neutral single-stranded poly F is inverted* relative to that seen in native two-stranded polymers. (*d*) On denaturation of the homopolymer pairs the positive rotation due to F becomes inverted and thus identical with that found in poly F itself. This inversion of rotation is cooperative, coincides exactly with the optical transitions induced by denaturation in any ionic environment (Fig. 6), and is entirely reversed on renaturation. (*e*) In the single-stranded

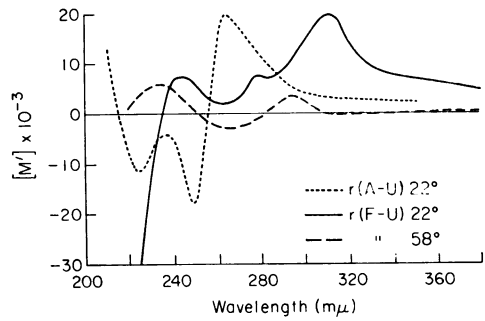


FIG. 3.—ORD curves of native r(A-U) and native and denatured r(F-U) in 0.01 *M* Tris buffer, pH 7.9.

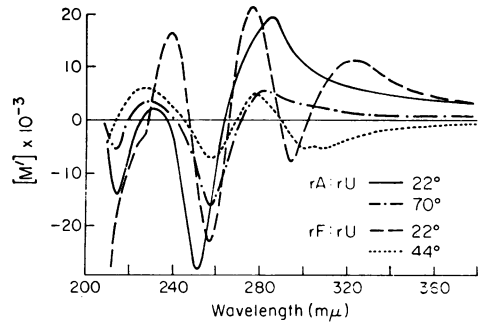


FIG. 4.—ORD curves of native and denatured rA:rU (1:1) in 0.01 *M* Tris buffer (pH 7.9)–0.1 *M* KCl (*Tm* = 59°); and rF:rU (1:1) in 0.01 *M* Tris (pH 7.9)–0.2 *M* KCl (*Tm* = 30.5°).

copolymers (spectra not shown) r(F,A), r(F,G), r(F,U), r(F,C), r(F,I), the optical activity due to F is much decreased in rotational strength, is variable in sign, and depends on the base composition of the polymer. Thus, the F transition is feebly positive in r(F,U) and r(F,C) and weakly negative in r(F,I) and r(F,G).

The following interpretation is proposed: (*i*) the disposition of F residues in ordered structures and the corresponding ORD spectra reflect the single conformation found in double helices with Watson-Crick geometry, namely a right-handed helix with the *anti* conformation at the glycosyl bond.[13] (*ii*) Denaturation of ordered structures leads to conformational changes which are most marked in the case of homopolymer structures. In other single-stranded polymers the presence of adjacent residues, such as U in denatured r(F-U), acts to restrict the conformational freedom of some F residues and thus limits the proportion of those that exist in either conformation at a given time. (*iii*) The individual residues in single-stranded poly F exist in a single conformation that differs from that maintained in ordered structures. (*iv*) The individual F residues in single-stranded copolymers exist as a mixture of two conformational states. These are, respectively, the conformation occurring in double-stranded structures and that found in single-stranded poly F.

Discussion.—The results described above show that the anomalous behavior of poly F in enzymatic systems has its counterpart in several physical properties. On the basis of the ORD spectrum we infer that a single structural peculiarity underlies the entire spectrum of unusual characteristics, and propose that the structural anomaly is represented by the *syn* conformation of individual residues in poly F.

The UV hypochromism, the fluorescence properties, the thermostability, and RNase susceptibility of poly F show that under the conditions of our experiments poly F exists as a loosely ordered, single-stranded polymer. The peculiarities of poly F therefore reflect a structural parameter of the single-stranded state.

Since conformational transitions generally require the rotational freedom

provided by single bonds, the site of the conformational modification in poly F must be confined to one of two regions: the first region includes the phosphodiester structure and is represented by the system of five single bonds which are located between the 4′-C of one pentose and the 3′-C of the adjacent residue; the second region of potential rotational freedom is at the glycosyl bond. Conformational modifications in the former may be expected to affect the sense (or other gross properties) of the helix, whereas rotation at the latter independently determines the conformation of the individual nucleosides in the polymer. In assigning a specific probable structure for single-stranded poly F, we reason as follows: (1) A right-handed helix with individual units in *anti* (i.e. a structure such as that found in double helices with Watson-Crick geometry,[13] or in neutral poly A[8]) is excluded for poly F by the ORD spectra. (2) Studies of molecular models reveal that it is very difficult to construct a left-handed helix with *syn* residues. Such a structure is (*a*) fully extended with minimal overlap of adjacent bases, and thus appears inconsistent with the UV-spectral and fluorescence properties of poly F, and (*b*) appears otherwise grossly dissimilar to the organization of all other polynucleotides. For these reasons, it seems an unlikely structure for poly F. (3) The remaining alternatives are: left-handed helix with *anti* and right-handed helix with *syn* residues, either of which might conceivably account for the ORD spectrum of poly F. The weight of evidence and of plausible arguments is entirely in favor of the latter alternative: (*a*) We consider that the sense of the polynucleotide helix reflects the packing of the atoms which comprise the pentose phosphate backbone. This backbone with its 3′-5′ phosphodiester bridge accounts for over two thirds of the mass of polymers and, like the right-handed helical pattern, is common to all polynucleotides studied to date. The sugar-phosphate structures of poly A and poly F are identical. We therefore assume that both exist as right-handed helices; we assign the conformational difference between them to the glycosyl bond, and propose the *syn* structure for individual nucleosides in poly F. (*b*) The major structural difference between adenosine and formycin, and therefore between poly A and poly F, is localized at the glycosyl bond. Moreover, this difference is of particular stereochemical

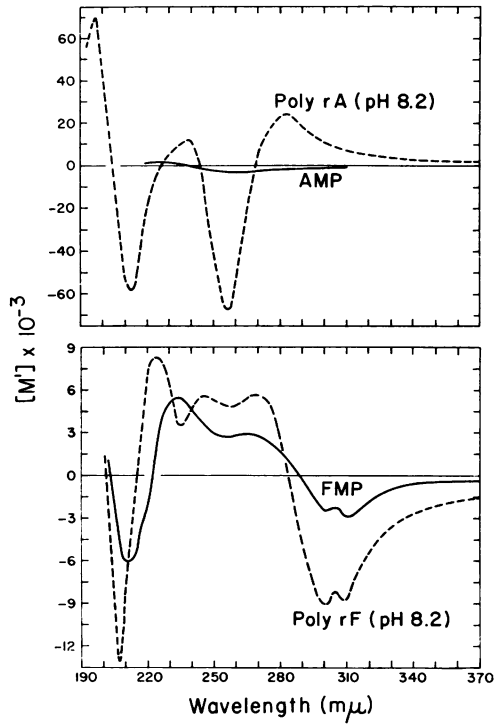


FIG. 5.—ORD curves of 5′-AMP, poly A, 5′-FMP, poly F, in 0.01 *M* Tris (pH 8.2).

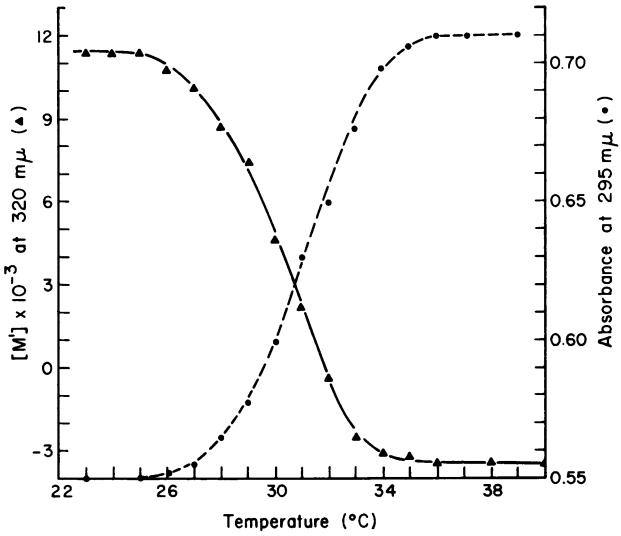


FIG. 6.—Optical rotation and absorbance of rF:rU (1:1) as a function of temperature. Solvent: 0.01 *M* Tris (pH 7.9)–0.2 *M* KCl. The mid-point of both transitions is at 30.8°.

significance, since the C—N bond in adenosine, with a length of 1.47 Å, is replaced by the C—C bond in formycin, which is 1.55 Å in length. While this increase need not by itself produce a conformational inversion, it will certainly facilitate one, given a suitable driving force. In addition, C-8 H of A is absent in F, thus further lowering any potential barrier to rotational changes.[14] Consequently, it is reasonable to conclude that the conformational difference between poly A and poly F is in fact localized at the site which is structurally modified. (*c*) That a driving force exists which favors the *syn* conformation for formycin is shown by the crystal structure of the nucleoside itself. Of the crystalline nucleosides the structure of which has been analyzed by X-ray diffraction, only formycin exists in *syn*.[15] (*d*) The strongest evidence for the proposed conformation for poly F derives from its interaction with pancreatic RNase. In a subsequent paper we demonstrate that poly F can function as a hybrid of poly U + poly C with respect to certain H-bonding functions provided that F residues exist in the *syn* conformation.

We therefore propose that individual formycin residues in polynucleotides may exist in either *anti* or *syn* conformation. The tendency of F to adopt the *syn* conformation satisfactorily accounts for all the anomalies of poly F structure and function. The conformational determinants for individual F residues include the nature of the adjacent nucleotide sequence, the presence of absence of base-pairing and helical structure, and the ionic environment. Formycin residues can therefore act as centers for conformational switches and provide an excellent model for analyzing comparable behavior in the case of the minor bases that may function to regulate the activity of DNA and RNA *in vivo*.

The *anti* conformation is indispensable for ordinary base-pairing, and the lowered thermostability of rF:rU, compared with r(F-U), r(A-U), and rA:rU,

shows that the *syn-anti* conformational equilibrium can act as one important determinant of helix stability, and perhaps account for some of the known effects of sequence on stability.

Our observations are significant also as a likely guide to the physical basis for pseudouridine function. Like formycin, ψ is a C-glycosyl and this can be expected to lower markedly the energy barrier to rotation at the glycosyl bond and thereby account for the lower *Tm* of r(F-ψ) in comparison with both r(F-U) and r(A-ψ). The possibility that ψ may normally function in this way to provide a conformational switch mechanism is the subject of current experimental tests.

A more detailed discussion of the implications of this work will be presented elsewhere.

We are indebted to Dr. G. Perlmann for generously providing the facilities for spectropolarimetry, to R. K. Robins and H. Umezawa for gifts of formycin, to A. Kapuler for discussions and suggestions, and to E. L. Tatum for his interest.

Abbreviations: A, $\overline{FU}$, F, T, $\overline{BU}$, ψ, U, G: adenosine, 5-fluorouridine, formycin, thymine riboside, 5-bromouridine, pseudouridine, uridine, guanosine; NMP, nucleoside 5′-monophosphate; r and d denote polyribonucleotide and polydeoxynucleotide, respectively. Polymers are identified as in the following examples: r(A-U), alternating copolymer; rA:rU, homopolymer pair; r(F,A), random copolymer. FMP, FTP: formycin-5′-mono- and triphosphate, respectively. F > p, 2:3 cyclic FMP. ATP, adenosine triphosphate.

* This investigation was supported in part by a grant (CA 08290-02 and 03) from the National Institutes of Health.

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