POLYNUCLEOTIDES

VI. THE INFLUENCE OF VARIOUS FACTORS UPON THE STRUCTURAL TRANSITION OF POLYRIBOADENYLIC ACID AT ACID pH's*

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SUMMARY

Polyriboadenylic acid undergoes a pronounced structural transition in the vicinity of pH 6. This may be followed by spectral, hydrogen ion titration, or light-scattering methods. An increase in ionic strength serves to extend the region of transition to more acid pH's and also to accelerate the rate of transition at a given pH.

Aggregation of the acid stable form is favored by increasing ionic strength or polymer concentration.

INTRODUCTION

In two preceding papers of this series polyriboadenylic acid (poly A) has been shown to possess a group which binds a hydrogen ion in the vicinity of pH 6[1,2]. The corresponding titration curve was markedly asymmetrical and abnormally steepened. Furthermore, in contrast to the case of adenosine monophosphate (AMP), the monomer, titration of this group was found to be accompanied by a pronounced change in the ultraviolet absorption spectra[2]. Inasmuch as prior blocking of the 6-amino group of adenine by formaldehyde pretreatment served to largely eliminate hydrogen ion binding in this region, the observed pK was attributed to the adenine group itself[2].

Titration of the adenine group has been found to be accompanied by important changes in the physical properties of the polymer, in particular by an increase in sedimentation constant, an increase in molecular weight, and, at low ionic strengths, by a decrease in the radius of gyration[2-4]. FRESCO, DOTY, and coworkers have found hydrodynamic and X-ray evidence that when the transition to an acid pH occurs at

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fairly high polymer concentration the resultant aggregates appear to have an ordered helical structure[4, 5].

It is the purpose of the present paper to explore the effects of several factors upon the structural transition, with a view toward clarifying the conditions under which the formation of helices can occur and establishing the relationship between the optical and structural alterations.

<div align="center">EXPERIMENTAL</div>

A. Materials

Polyriboadenylic acid was prepared by the action upon adenosine diphosphate of the ribonucleotide phosphorylase isolated from *M. lysodeikticus*. The method of enzyme isolation and the details of preparing the polymer have been described elsewhere[6, 7]. The copolymer of polyadenylic and polyuridylic acids was prepared in an analogous manner, the initial mole ratio of monomers being 3 : 1. The composition of the product was computed from the ultraviolet extinction at 255 mμ of its alkaline hydrolysate at pH 12 and pH 7. The ratio $d_{255, \text{pH } 12.0}/d_{255, \text{pH } 7.0}$ is equal to $[14.8\,x + 7.1\,(1 - x)] / [14.8\,x + 9.0\,(1 - x)]$ where $x =$ mole fraction of adenylic residues.

The urea employed was recrystallized twice from ethanol. Analytical grade reagents and glass redistilled water were employed for all investigations.

B. Methods

Measurements of optical density were made employing a standard Beckman model DU ultraviolet spectrophotometer. The latter was equipped with a cell holder possessing coils for circulation of fluid from a thermostat, thereby permitting temperature regulation between 5° and 50°.

The procedure for obtaining hydrogen ion titration data was as follows. 7.0 ml of solvent were introduced into a thermostatted titration cell, stirred magnetically, and equipped with a nitrogen bubbler. The tank nitrogen employed was bubbled through two flasks containing 0.1 M HCl and glass-redistilled water, in that order, to remove any traces of NH_3. The solvent was titrated to pH 3.8, flushed with nitrogen for 30 minutes to remove any dissolved CO_2, and then backtitrated to neutrality with standardized 0.1 M NaOH, using a calibrated microsyringe. The solvent blank thereby obtained was subtracted from the corresponding titration figures for the polymer solution. To the solvent at pH 7–7.5 was added a small volume (0.1–1.0 ml) of concentrated polynucleotide solution, an equivalent volume of solvent having been first removed. The resultant solution was titrated to pH 3.8 with standard 0.1 M HCl and backtitrated to pH 7 with 0.1 M NaOH. pH was measured by means of a Beckman model G battery operated pH meter, equipped with external electrodes. Preliminary standardization of the meter was always made with standard buffers of pH 6.86 and pH 4.00. When titration was performed at an elevated temperature the meter was first restandardized at that temperature.

Light-scattering measurements were made with a Phoenix light-scattering photometer. The absolute calibration of the latter has been described elsewhere[3]. The relative scattering intensity as a function of angle was determined using a cylindrical cell with planar exit and entrance windows which was furnished by the above company. After determination of the scattering envelope the absolute reduced intensity at 90° was measured in a standard square cell, thereby permitting computa-

tion of absolute reduced intensity as a function of angle. The scattering envelope was corrected for the contribution of the beam reflected by the exit window according to the procedure described elsewhere[3]. Prior to measurement each solution was clarified by centrifugation at 20,000 g in a Sorval centrifuge until constant scattering properties were attained.

Concentration of poly A in AMP units was determined by heating 1 ml aliquots of solution plus 0.5 ml of 1 M NaOH at 100° for 30 minutes, diluting to 25 ml, and reading the optical density at 259 mμ. The value of $1.54 \cdot 10^4$ taken from the Pabst tables was employed for the molar extinction coefficient of adenosine monophosphate (AMP).

Ultracentrifugal measurements were made with a Spinco model L ultracentrifuge equipped with a phase plate.

When the Archibald technique for computing molecular weights was employed, the procedure followed closely that recommended by SCHACHMAN[9]. The lower meniscus was obtained by the use of a layer of silicone oil. To avoid the necessity of an absolute area calibration, total concentration was determined in arbitrary units by means of a separate run with a synthetic boundary cell. The plates were enlarged and projected upon millimeter graph paper, and then traced.

<div align="center">RESULTS</div>

I. TITRATION DATA

A. Effect of ionic strength, concentration, and temperature

Fig. 1 compares the rate of hydrogen ion binding of a high molecular weight sample of poly A at pH 5.9 as a function of ionic strength. The rate is relatively slow at an ionic strength of 0.01 and increases markedly with increasing ionic strength. At an ionic strength of 0.1 the rate is immeasurably fast, equilibrium being attained within the time of mixing.

Fig. 2 gives the equilibrium values of bound hydrogen ion at two ionic strengths. The data cited were attained by titration from acid to alkaline pH's. Under these conditions equilibrium is attained relatively rapidly. It may be seen that an increase in ionic strength has the effect of displacing the characteristically abrupt onset of titration to a more acid pH and thus to extend the pH zone of stability of the alkaline form of the polymer.

An increase in ionic strength has thus a two-fold effect upon the hydrogen ion binding properties of polyadenylic acid. While it displaces the zone of the abrupt onset of hydrogen ion binding to a more acid pH it also greatly enhances the *rate* of binding at a pH acid to this value.

Fig. 2 includes data for poly A at three concentrations for an ionic strength of 0.1. It can be seen that a five-fold variation of concentration had no important influence upon the shape of the titration curve under these circumstances and in particular had no influence upon the position of the transition zone.

Fig. 3 compares the titration curves of poly A at two temperatures in 0.1 M KCl. While the general shape of the curve is not markedly altered at the more elevated temperature there is a perceptible displacement of the steep onset of titration to more acid pH's.
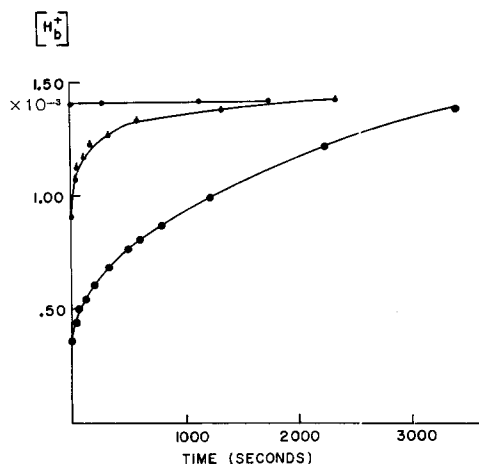
Fig. 1. The change with time in the extent of hydrogen ion binding by poly A XV upon adjusting the pH from 7 to 5.9 at 26°. $1.99 \cdot 10^{-3}$ mmoles of poly A (in nucleotide units) are present in 7.0 ml solution. ● 0.01 M KCl; ● 0.042 M KCl; • 0.10 M KCl.
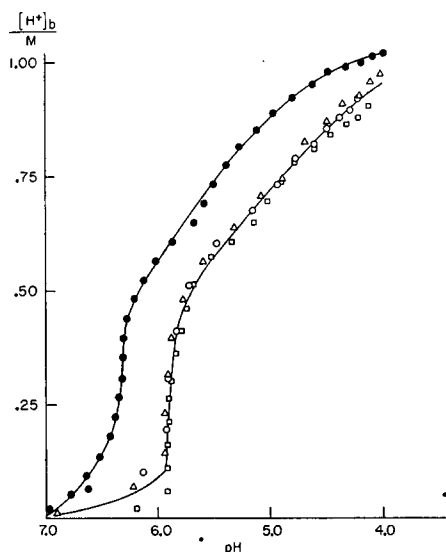

Fig. 2. The titration curve of poly A XV as a function of ioinc strength and polymer concentration. The ordinate gives the number of moles of 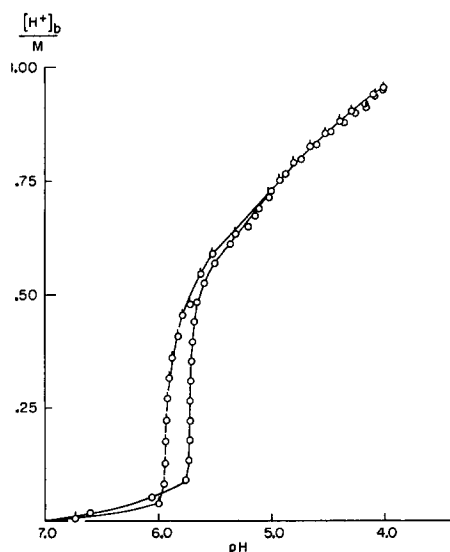hydrogen ion bound per nucleotide unit. The temperature is 26°. ○ 0.1 M KCl, 0.10 mmoles nucleotide/l; △ 0.1 M KCl, 0.27 mmoles nucleotide/l; □ 0.1 M KCl, 0.46 mmoles nucleotide/l; ● 0.01 M KCl, 0.53 mmoles nucleotide/l.

Fig. 3. The titration curve of poly A XIV at two temperatures in 0.1 M KCl. The concentration of poly A is 0.52 mmoles nucleotide/l. ◐ 26°; ○ 40°.
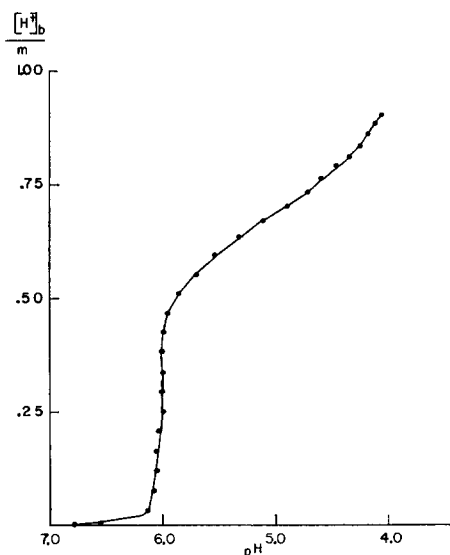

Fig. 4. Titration curve for poly A XIV in 0.1 M KCl and 33% ethanol (by volume) at 27°. The concentration of poly A is 0.57 mmoles nucleotide/l.

B. *The effect of ethanol*

Fig. 4 shows a titration curve in 33% ethanol. The general features of the normal poly A titration curve appear to be retained in this mixed solvent, although the *apparent* region of transition appears to be displaced slightly to the alkaline. However, because of inevitable uncertainties in the behavior of the glass electrode in this mixed solvent, little quantitative significance can be assigned to this latter observation.

II. ULTRAVIOLET EXTINCTION

A. *The effect of ionic strength*

At a pH acid to the transition region for the entire range of ionic strengths the effect of ionic strength is to markedly accelerate the rate of the optical change, until, at an ionic strength of 0.1, the change is complete within the period of mixing. This results, which is illustrated by Fig. 5, is in accordance with what would be anticipated from the titration results.

On the other hand, an increase in ionic strength also serves to displace the region in which the rate of transition becomes appreciable to more acid pH's. Thus the curve of the variation of rate with pH is both steepened and displaced, until at an ionic strength of 0.1 the rate changes from immeasurably slow to immeasurably fast over a very small pH interval ($<$ 0.1 pH unit). This point is illustrated by Fig. 6.
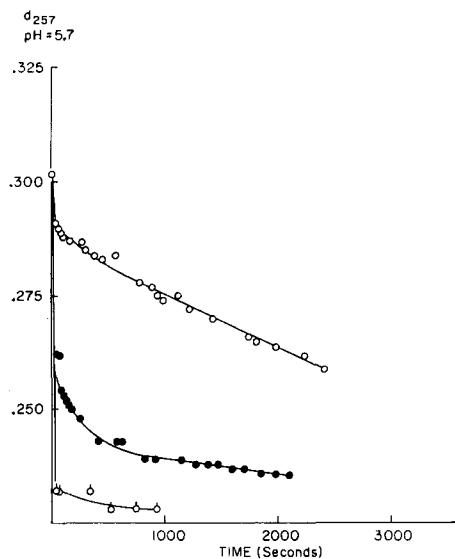
Fig. 5. Variation in d_{257} with time at pH 5.7 for poly A XVI (0.033 mmoles nucleotide/l) at three ionic strengths. ○ 0.01 M NaOAc; ● 0.01 M NaOAc, 0.01 M KCl; ◐ 0.01 M NaOAc, 0.03 M KCl.
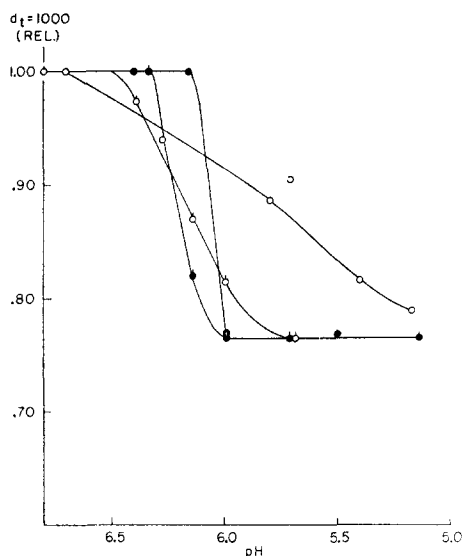
Fig. 6. Relative variation in d_{257} with pH for poly A XVI (0.033 mmoles nucleotide/l) 1000 seconds after adjustment to the given pH from pH 7. ○ 0.01 M NaOAc; ◐ 0.01 M NaOAc, 0.01 M KCl; ◖ 0.01 M NaOAc, 0.03 M KCl; ● 0.01 M NaOAc, 0.1 M KCl.

B. *The effect of temperature and urea*

Figs. 7 and 8 show the variation in extinction at 257 mμ with pH at two temperatures in the presence and absence of 7 M urea. At the ionic strength considered

(0.1) an increase in temperature from 26° to 45° serves to displace the zone of transition to more acid pH's, but leaves the general shape of the curve unaltered, in accordance with the titration data. While the extinction of the alkaline form is appreciably sensitive to temperature, that of the acid is essentially unaltered by the temperature increase.

The presence of 7 M urea has a pronounced hyperchromatic effect upon the alkaline form of poly A. The increase in extinction at the higher temperature also persists in this medium. However, the general nature of the variation in extinction with pH is unaltered by the presence of urea except for a slight apparent displacement of the zone of transition to more acid pH's. In particular, the abrupt nature of the transition remains, as well as the displacement of the maximum to lower wave lengths (252 mμ).

The important and somewhat surprising conclusion follows that the poly A optical transition is not inhibited to any important degree by the presence of urea in high concentrations.
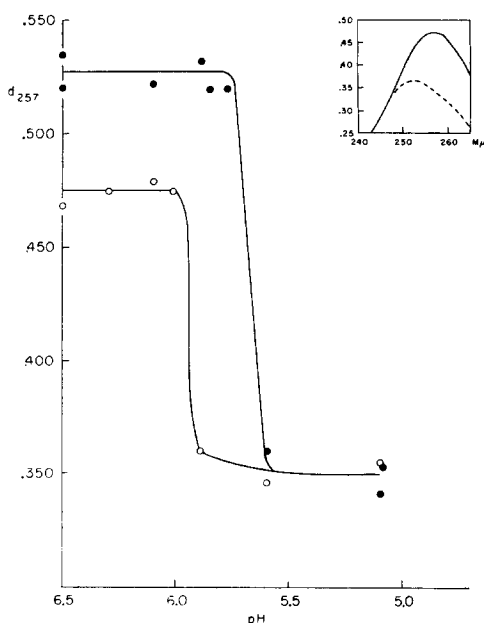

Fig. 7. Variation in d_{257} with pH for poly A XIV in 0.1 M KCl. The concentration of poly A is 0.057 mmoles nucleotide/l. The insert compares spectra at 28° for pH 6.5 (————) and pH 5.0 (-------). O 28°; ● 49°.
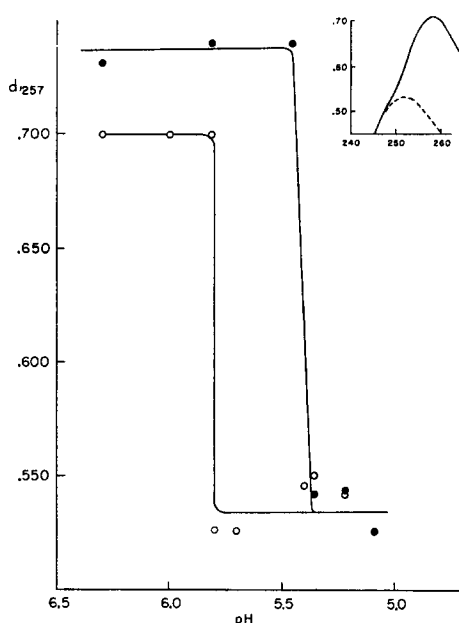
Fig. 8. Variation in d_{257} with pH for poly A XIV in 0.1 M KCl and 7 M urea. The concentration of poly A is 0.057 mmoles nucleotide/l. The insert compares the spectra at 28° for pH 6.5 (————) and pH 5.1 (-------). O 28°; ● 49°.

C. The effect of alkaline degradation

Fig. 9 compares the variation of the extinction at 257 mμ with pH for poly A at several stages of alkaline degradation.

To study the behavior of relatively low molecular weight samples of poly A a acid pH's, a high molecular weight sample was subjected to alkaline hydrolysis as

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follows. A 0.5 % solution of poly A in 0.1 M KCl was titrated to pH 10.8 and heated to 100° C. Aliquots were withdrawn at regular intervals and neutralized.

Fig. 9 compares the behavior of two such degraded samples with a high molecular weight preparation. It may be seen that the sepctral change persists at quite low degrees of polymerization ($\sim$ 30). However, the pH of onset is displaced to the acid. In addition, the abrupt character of the change is lost, the transition becoming much more gradual. The molecular weight of the degraded samples was determined by the ARCHIBALD method.
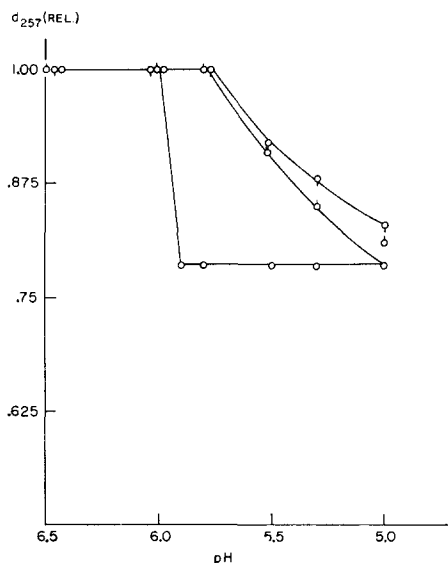

Fig. 9. Relative variation of d_{257} with pH for poly A XI (0.058 mmoles nucleotide/l) at three stages of alkaline degradation in 0.1 M KCl. ◯ mol. wt. = 3.2·10⁵; ◇ mol. wt. = 1.5·10⁴; ◯ mol. wt. = 1.2·10⁴.

III. MOLECULAR PARAMETERS

A. Effect of pH and ionic strength for polyadenylic acid

The ultraviolet spectrum of the acid form of poly A is remarkably independent of conditions, suggesting that the environment of the chromophore is similar in all cases. However, as Table I shows, the size and shape of the acid form are highly sensitive to the manner in which the transition is carried out.

The pronounced increase in molecular weight displayed by poly A at low pH's at an ionic strength of 0.1 suggests the occurrence of aggregation to an important degree below pH 5. This is borne out by the detailed Zimm type grids for these conditions, as Fig. 10 and Table I show.

At an ionic strength of 0.1 and pH < 5 the predominant molecular effect is clearly aggregation. An interesting point is that, despite the demonstrated reversibility of the effect, the aggregates show no sign of any pronounced dissociation upon dilution. This presumably reflects the very low rate of the dissociation process.

When the pH is adjusted to an acid value in water, followed by addition of KCl

TABLE I

MOLECULAR WEIGHTS AND DIMENSIONS OF POLYRIBOADENYLIC ACID AT ACID AND ALKALINE pH'S

Preparation	pH	Solvent	Maximum concentration*	$Mw^{**} \times 10^{-6}$	$\langle R_g^2 \rangle^{1/2}$***
Poly A XIV	6.5	0.01 M NaOAc, 0.1 M KCl		2.2	840
	4.1	0.01 M NaOAc, 0.1 M KCl	0.044	13.3	1410
	4.1	0.01 M NaOAc, 0.1 M KCl, 7.7 M urea	0.046	6.6	
Poly A XV	6.5	0.01 M NaOAc		2.50	1090
	4.7	0.01 M NaOAc	0.028	3.7	690
	4.7	0.01 M NaOAc	0.47	4.2	760
Poly A U III	6.5	0.01 M NaOAc, 0.1 M KCl		0.48	450
	3.3	0.01 M NaOAc, 0.1 M KCl	0.083	0.52	240
Poly A XVII	6.5	0.01 M NaOAc, 0.1 M KCl		2.4	770
	5.6	0.1 M NaOAc	0.115	2.7	830
	5.6	0.1 M NaOAc	0.69	5.5	1620
Poly A XX	6.5	0.01 M NaOAc, 0.1 M KCl		0.44	400
	4.0	0.01 M NaOAc, 0.1 M KCl	0.065	1.54	680
	4.0	pH altered in H_2O, then electrolyte added to 0.01 M NaOAc, 0.1 M KCl	0.065	1.02	570

* When cited this refers to the concentration at which the pH was altered to an acid value.
** Weight-average molecular weight.
*** Weight-average radius of gyration, computed assuming a "random" distribution of particle sizes.
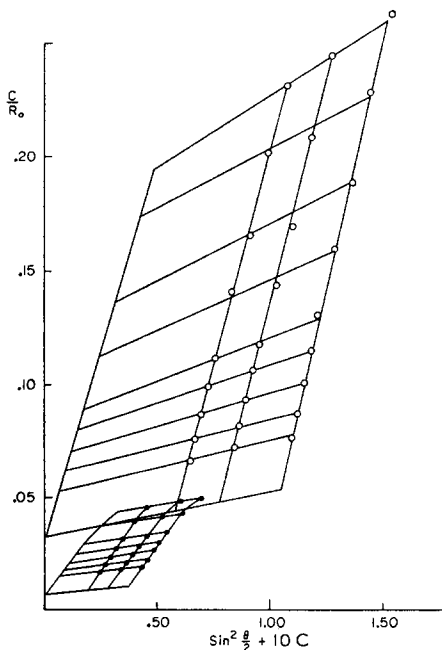

Fig. 10. Zimm type grids for poly A XIV at two pH's and ionic strengths. ◯ 0.01 M NaOAc, pH 6.6; ● 0.01 M NaOAc, 0.1 M KCl, pH 4.1.

to an ionic strength of 0.1, the increase in molecular weight is less, and the relative extension greater, than when the pH is adjusted directly in 0.1 M KCl (Table I).

The increase in molecular weight at pH's < 5 likewise proceeds to a high value in 7 M urea, as Table I shows. Thus the intermolecular aggregation process as well as the spectral change can proceed in this medium.

At the lower ionic strength of 0.01 aggregation occurs to a much smaller degree for comparable poly A concentrations. In addition there is also a considerable shrinkage in radius of gyration, as Table I shows.

B. Behavior of an adenylic–uridylic copolymer

In the case of the adenylic–uridylic copolymer, the variation in R_{90} with pH is gradual, paralleling the spectral variation. Furthermore, the increase in R_{90} does not proceed to an extent comparable to that observed for poly A, suggesting that aggregation occurs to a much lesser degree. This latter is borne out by the detailed computation of the molecular parameters, as Table I shows. No important increase in molecular weight is observed even at pH 3.3, in 0.1 M KCl, under conditions which in the case of poly A itself are accompanied by pronounced aggregation. A considerable shrinkage in the radius of gyration is however observed and it is presumably to this intramolecular process that the variation in R_{90} is to be attributed.

Thus the "dilution" of the poly A chain by uridylic residues to the extent of 25 % serves to eliminate the aggregation process, at least at concentrations < 0.1 g/l.

C. Effect of poly A concentration

Table I includes data for the molecular parameters of the acid form of poly A as a function of the concentration of poly A at which the transition occurred. It can be seen that the molecular weight of the product increased pronouncedly with increasing poly A concentration, in accordance with the findings of FRESCO AND DOTY[4,5].

At pH 5.6 and an ionic strength of 0.1 only about a 10 % increase in molecular weight occurred if the transition was carried out at a concentration of 0.03 g/l. If it was carried out at a concentration of 0.69 g/l a more than two-fold increase occurred.

From the preceding it can be seen that the degree of aggregation of poly A at pH's < 5 is a sensitive function of conditions and is favored by increasing ionic strength or poly A concentration.

<div align="center">DISCUSSION</div>

There appears to be substantial evidence that polyriboadenylic acid, in aqueous solution at pH's > 6.5, exists as largely unorganized Gaussian coils. This follows from the available light scattering and molecular kinetic data and from the accessibility of the adenine base to hydrogen ions, formaldehyde, and various dyes, as well as from the absence of any thermal denaturation effects[1-5].

Both the hydrogen ion titration and spectral studies are consistent with the occurrence of a marked structural transition in the vicinity of pH 6, depending upon the ionic strength. Perhaps the best tentative explanation of the seemingly inconsistent influence of ionic strength upon the kinetics and equilibria of the process is as follows. An increase in ionic strength serves to reduce the pH at which the transition occurs by

minimizing the electrostatic term in the free energy of binding of hydrogen ion, thereby reducing the effective pK of the adenine group. On the other hand it also serves to reduce electrostatic repulsion between different poly A molecules as well as different segments of the same molecule, thus accelerating the structural transition once a critical fraction of the adenine groups has been titrated.

Perhaps the most crucial aspect of the study of polyriboadenylic acid at acid pH's is the determination of the conditions favorable to the transition to a helical structure and in particular to a doubly stranded helix of the type postulated by FRESCO AND DOTY and by CRICK et al.[4,8]. While a final answer can only come from X-ray diffraction data it is reasonable to suppose that an intermolecular transition to a doubly stranded helix would be reflected by an increase in molecular weight and that only conditions for which this is observed are consistent with the formation of such structures by this mechanism. A double strand could also be formed by an intramolecular process[2].

From the present results it appears that at initial concentrations < 0.1 g/l the formation of multistranded aggregates is feasible below pH 5 at an ionic strength of 0.01 and at an ionic strength of 0.1, and that in either case their formation is favored by an increase in poly A concentration at the pH of transition. At a ten-fold higher concentration of poly A the formation of multistranded aggregates is feasible at a pH as high as 5.6 at an ionic strength of 0.1.

The markedly reduced extent of aggregation when the transition is carried out at an ionic strength of 0.01 and a concentration < 0.1 g/l, suggests that a competitive formation of helices by an intramolecular process may also be important here[2]. The decrease in radius of gyration under these conditions probably reflects the presence of numerous gaps in the ordered structure, which endow the molecule with sufficient flexibility to shrink upon the relief of electrostatic stress through titration of adenine groups.

It is of particular interest that both the spectral transition and the subsequent aggregation can occur in the presence of 7 M urea. This finding serves to accentuate the importance of electrostatic forces in accounting for the stability of the acid forms, as the latter would be relatively uninfluenced by this unfavorable medium for hydrogen bond formation. With regard to the acid stable aggregates it is of interest in this connection that the doubly stranded helical structure suggested for the latter by CRICK et al.[8], invokes the close juxtaposition of an adenine and a phosphate group. If the positive charge on the adenine is localized at either the 6-amino or the 1-nitrogen positions, electrostatic forces should make a strong contribution to the stability of the structure.

The evidence presented to date would appear to implicate the 6-amino and the primary phosphate groups in both the spectral transition and aggregation effects, in view of the effect of formaldehyde pretreatment and the presence of divalent cations[1].

The observation that an adenylic-uridylic copolymer does not undergo the aggregation process characteristic of poly A at comparable degrees of neutralization appears to be consistent with the presence of an ordered doubly stranded structure for the latter, as the presence of uridine residues to this extent would be expected to disrupt seriously the cooperative bonding essential for the stability of such a structure.

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THE DIFFUSION CONSTANT OF *CYPRIDINA* LUCIFERASE

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SUMMARY

The diffusion constant, D, of *Cypridina* luciferase was measured by the porous disk method of NORTHROP AND ANSON[3], and a value of $7.4 \cdot 10^{-7}$ cm²/sec was found. This would indicate an upper limit of about 80,000 for the molecular weight of this enzyme.

INTRODUCTION

Cypridina luciferase is the enzyme which catalyzes the blue luminescent reaction of the ostracod crustacean, *Cypridina hilgendorfii*. The animal ejects the enzyme and its substrate, luciferin, into the surrounding sea water where, in the presence of dissolved oxygen, the luminescence occurs[1].

The purpose of this paper is to record the diffusion constant of this enzyme and to describe the measurements upon which it is based. Because the luminescent reaction in *Cypridina* is first order[2] and requires no other components than luciferase, luciferin, water and oxygen, the activity, and hence the concentration, of luciferase can be determined from the reaction velocity constant with a high degree of precision, even in the presence of protein and other impurities. Consequently, NORTHROP AND ANSON's method[3] of measuring the diffusion constant by observing the rate of passage through a porous disk is particularly applicable and yields data of a high order of reliability. It is, indeed, the only method applicable at the present time since *Cypridina* luciferase is not available in any quantity or in a pure form.

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