STUDIES ON POLYNUCLEOTIDES SYNTHESIZED BY POLYNUCLEOTIDE PHOSPHORYLASE

III. INTERACTION AND ULTRAVIOLET ABSORPTION*

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(Received for publication, April 4, 1957)

The discovery of polynucleotide phosphorylase has led to the availability of several types of polymers of ribonucleotides. Polymers of adenylic, uridylic, cytidylic, and inosinic acids[1] and several of the possible copolymers have been prepared (2). Structure studies (3, 4) have shown that the nucleotides are linked by 3′, 5′ diester phosphate bonds and that the polymers are thus similar to ribonucleic acid. Because of this similarity and because of the simplification of structure which results from the presence of a single kind of nucleotide in the polymer chain, there are problems in nucleic acid structure and physical properties that may be illuminated by a study of the biosynthetic polymers. This paper deals with the spontaneous interaction between poly A[1] and poly U with the formation of stable aggregates, as well as with certain features of the ultraviolet absorption spectra of these and other polymers bearing on the problem of polynucleotide interaction.

Methods

The polymers used in this work were prepared as previously described (2),[2] having been furnished in the form of lyophilized sodium salts, free from additional electrolyte. They were dissolved in water and diluted with various buffers as required.

Electrophoresis was carried out at 0° as previously described (5). Sedimentation coefficients were determined with a Spinco model E ultracentrifuge at 16–20°. Diffusion coefficients were measured in a Gouy diffusiometer at 5°, this instrument having been made from a Pearson elec-

* Aided by grants from the National Science Foundation and the Rockefeller Foundation. A preliminary report of this work was presented at the meeting of the American Society of Biological Chemists, April, 1956 (1).

[1] These polymers will be referred to as poly A, poly U, poly C, and poly I, respectively. Poly AU refers to the copolymer of adenylic and uridylic acids and poly AGUC to that of adenylic, guanylic, uridylic, and cytidylic acids. DNA, deoxyribonucleic acid.

[2] Samples of the various polymers were prepared by Miss Priscilla J. Ortiz and by Dr. Sanae Mii.

trophoresis apparatus by the addition of masking system and camera plate holder adapted from those described by Gosting (6). Measurements on the camera plates were made with a two-coordinate comparator modified from a toolmaker's microscope[3] which reads by vernier to 0.0001 inch. Ultraviolet absorption spectra were obtained with a Beckman DK-2 recording spectrophotometer. The spectrum was first recorded between 340 and 220 mμ. The instrument was then reset to a greater sensitivity and the scanning was continued to a wave length of about 205 mμ in order to measure the peak near 208 mμ, which is shown by most nucleotides. The spectrophotometric curves in Figs. 2 to 6 have been replotted on a linear wave length scale and an extinction scale of $\epsilon(P) \times 10^{-3}$ (7). For the latter purpose, the total phosphorus was determined (8) on each stock solution and corrected for traces of inorganic phosphate remaining in the preparation. The polymers were hydrolyzed to mononucleotides by being permitted to stand at room temperature for 18 to 24 hours in 1.0 M sodium hydroxide.

Interaction of Polynucleotides

Evidence for the interaction of poly A and poly U was first obtained by electrophoresis. In a glycine buffer at pH 9.6 the mobility difference between the two polymers is sufficient to permit their resolution in a mixture. However, electrophoresis of mixtures of equal amounts of the two yielded only a single boundary with a mobility intermediate between that of poly A and poly U (Fig. 1). Electrophoretic patterns of the copolymer of adenylic and uridylic acids and of yeast RNA are shown for comparison. A similar result was obtained when the experiment was conducted at pH 8.5. The lack of resolution into separate boundaries clearly shows an interaction resulting in a species with a single electrophoretic mobility. The solutions used in these experiments were kept at 0° at all times to minimize the possibility of hydrolysis. The absence of significant hydrolysis was indicated by the retention of acid precipitability and non-dialyzability of the polymers after completion of the experiments.

That this interaction indeed results in the formation of an aggregate of increased molecular size is shown by sedimentation experiments on the same polymers and mixtures. Patterns from one experiment are reproduced in Fig. 1. The sedimentation coefficient, $s_{20,w}$, increased from 2.5 S for poly A and 2.2 S for poly U to 5.3 S for the mixture of equal amounts of the two. These coefficients were obtained at a total polymer concentration of 0.6 gm. per 100 ml. in each case. They increased about 30 per cent over the range of concentration that can be followed by refractive index optics. The average molecular weight of the polymer samples used

[3] Manufactured by Erb and Gray, Los Angeles, California.

for the experiments of Fig. 2 was about 80,000 as determined by end group methods.[4] The results of a similar experiment with different polymer preparations are given in Table I in which approximate, average molecular weights have been calculated. The sedimentation coefficients in Table I were obtained by extrapolation to zero concentration of runs at 0.6, 0.3, and 0.15 gm. per 100 ml. The diffusion boundaries at concentrations of 0.2 to 0.3 gm. per 100 ml. were symmetrical as judged by observation with

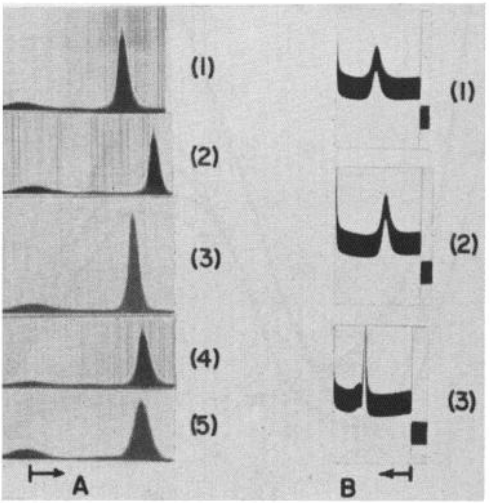

FIG. 1. Electrophoretic and sedimentation patterns of polymers and mixtures. *A*, patterns obtained in a glycine buffer, ionic strength 0.1, pH 9.6, at 0° after electrophoresis for 100 minutes at a field strength of 5 volts per cm. The patterns were enantiographic and only the descending boundaries are shown. (1), poly A, $u = 10.7 \times 10^{-5}$ cm.^{2} volt^{-1} sec.$^{-1}$; (2), poly U, $u = 13.2 \times 10^{-5}$; (3), poly A plus poly U, $u = 11.1 \times 10^{-5}$; (4), poly AU, $u = 12.7 \times 10^{-5}$; (5), yeast RNA, $u = 12.3 \times 10^{-5}$. *B*, sedimentation patterns obtained in a phosphate buffer, ionic strength 0.2, pH 7, at 59,780 r.p.m. (1), poly A, 128 minutes, 2.5 S; (2), poly U, 128 minutes, 2.2 S; (3), poly A plus poly U, 64 minutes, 5.3 S.

the refractive index camera, and concentration dependence of the diffusion coefficient has been neglected. The relative fringe deviations on the type of plot used by Akeley and Gosting (9) were constant over the 10 to 16 hour observation period. The deviations indicate heterogeneity, but were not analyzed to determine the diffusion coefficient distribution. The height-area average diffusion coefficients, D_A, were determined by the extrapolation method of Akeley and Gosting. In Table I this diffusion coefficient, corrected to 20°, has been combined with the sedimentation coefficient and an assumed partial specific volume of 0.58 ml. per gm. to

[4] Unpublished experiments of Dr. J. D. Smith.

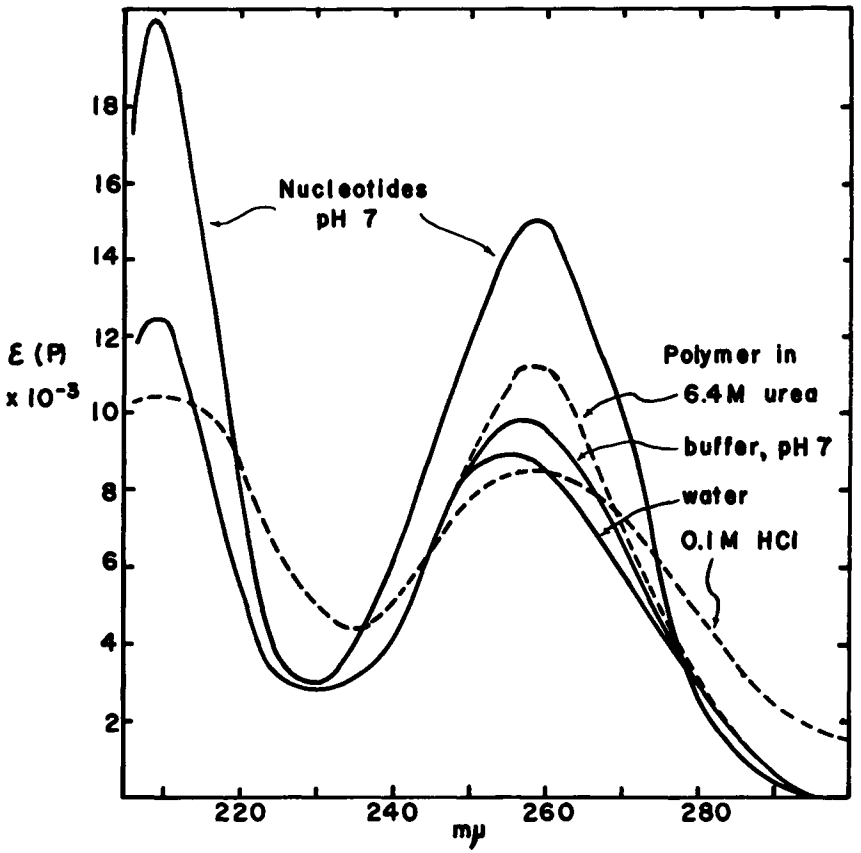

FIG. 2. Absorption spectra of poly A under various conditions and of the mononucleotides obtained by alkaline hydrolysis of the polymer. The ordinate is the molar extinction $\times 10^{-3}$ based on polynucleotide phosphorus.

TABLE I

Molecular Weight of Samples of Poly A, Poly U, and Their Aggregate

Polymer	$D_{A20,w}$	$s_{20,w}$	Mol. wt.
	$cm.^2 sec^{-1} \times 10^7$	*S units*	
Poly A	1.7	5.3	177,000
" U*	7.4	2.2	17,300
" A + poly U	1.3	9.9	450,000

* The low molecular weight of this particular sample of poly U resulted from the use for its preparation of a relatively crude enzyme which was contaminated with nucleases. These are evidently similar in specificity to pancreatic ribonuclease, since the poly A made with the same enzyme was much higher in molecular weight.

give an average molecular weight. The value thus obtained is not properly a weight average value because of the different type of averages entering into the sedimentation and diffusion experiments. It is presented here as an approximate characterization of heterogeneous samples for which distributions have not yet been determined.

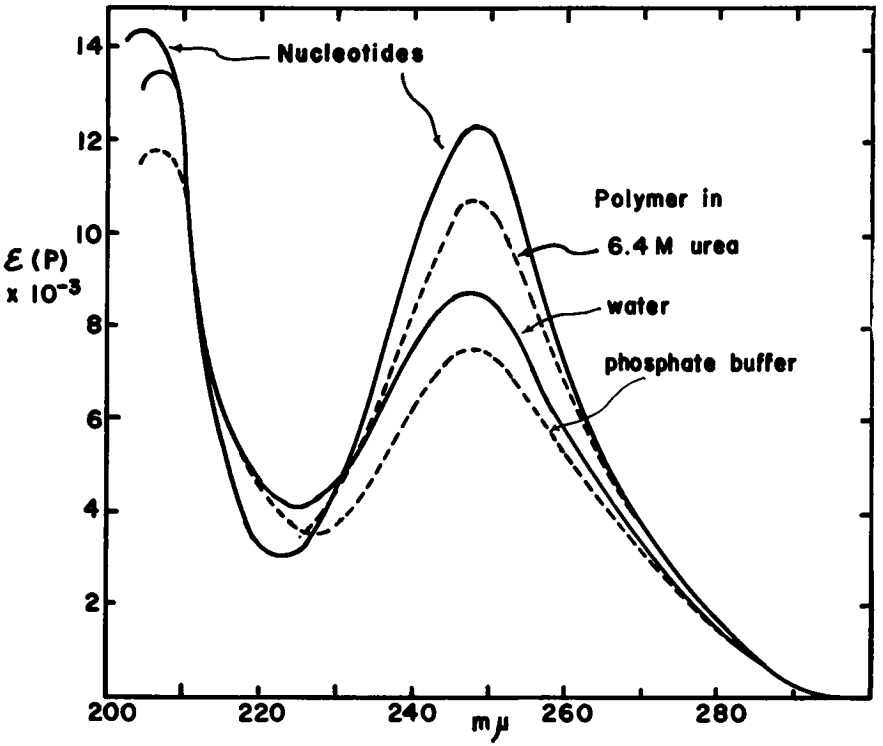

FIG. 3. Absorption spectra of poly I under various conditions and of the mononucleotides obtained by alkaline hydrolysis of the polymer.

In preparing polymer solutions at concentrations of about 0.5 per cent, an increase in viscosity is evident when poly A and poly U are mixed. At higher concentrations, stiff gels may result. Quantitative determinations of the viscosity changes will be reported elsewhere.

Ultraviolet Absorption Spectra

The ultraviolet spectra of the polymers and their mixtures reveal changes that parallel the direct demonstrations of interaction. Spectra of the homopolymers under various conditions are shown in Figs. 2 to 5. In each figure a comparison is made with the spectrum of the mixture of 2′-

and 3′-mononucleotides obtained by hydrolysis of the polymer with alkali. The extinction coefficients at the wave length of maximal absorption for the polymers and their hydrolysis products are summarized in Table II. The agreement of the latter values with those given in the literature for the 2′- and 3′-mononucleotides demonstrates the absence of other hydrolysis products and confirms the completeness of removal of the nucleoside diphosphates remaining in the reaction mixture at the completion of the enzymatic synthesis.

TABLE II

Extinction Coefficients

The values are given for the polymers and for their constituent mononucleotides prepared by alkaline hydrolysis. The former may vary by several per cent from one preparation to another.

	Polymer		Hydrolysate			
					$\epsilon(P) \times 10^{-3}$ at 260 mμ	
	λ max	$\epsilon(P) \times 10^{-3}$*	λ max	$\epsilon(P) \times 10^{-3}$	From polymer	From literature
	mμ		mμ			
Poly A	257	9.8	259	15.1	15.0	15.0†
" U	261	9.6	261	10.1	10.1	10.0†
" C	269	6.3	271	8.9	7.7	7.6†
" I	247	7.6	248	12.3	7.1	7.1‡
" A + poly U	257	6.3				

* Extinction per mole of polynucleotide phosphorus per liter × 10^{-3} (7).

† 2′- and 3′-mononucleotides (10).

‡ Inosine (11).

The extinction at the wave length of maximal absorption for poly A, poly C, or poly I is less than that of the constituent mononucleotides measured under the same conditions. Hydrolysis of these polymers thus results in a hyperchromic effect similar to that established for RNA and DNA (12). The magnitude of the optical density of the polynucleotide relative to the mononucleotide value varied somewhat with the polymer preparation. Three different poly A samples yielded 66, 67, and 70 per cent. In addition to the reduction in extinction there is a hypsochromic shift of 2 to 3 mμ accompanying the formation of the polymers.

Effect of Urea, Ionic Strength, and pH

The polymers which show a hyperchromic effect on hydrolysis also show marked spectral changes with changes in ionic strength, pH, and urea concentration that are not seen in the mononucleotide spectra. In

each case the optical density is increased in the presence of 6.4 M urea, although not to the mononucleotide level. As compared with the curve obtained in water, the optical density of poly A is increased by salt although those of poly I and poly C are decreased. The entire change is completed when the ionic strength has been increased to 0.01. The pH 7 spectra in

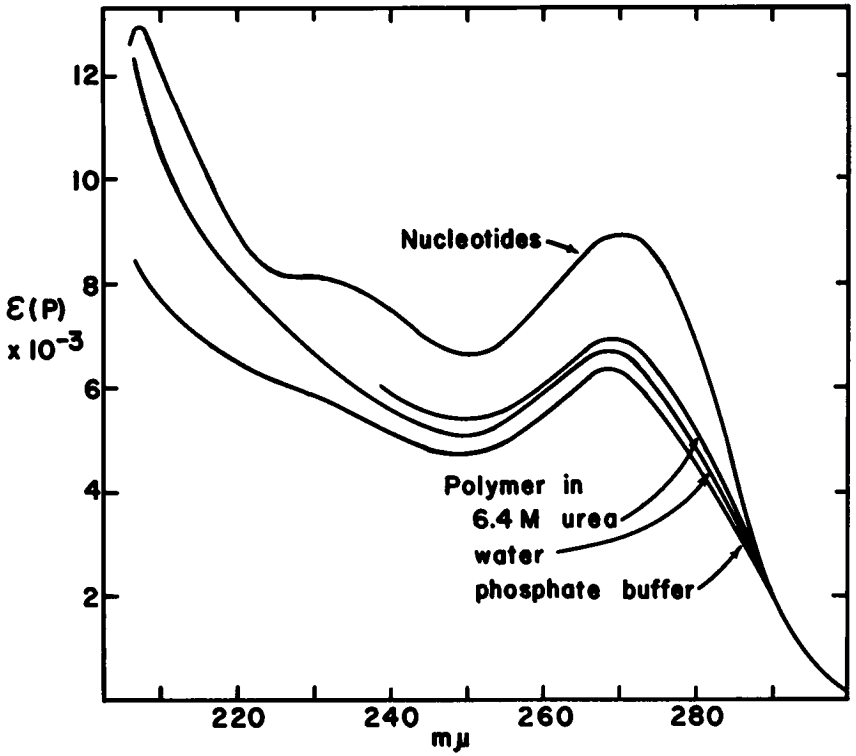

FIG. 4. Absorption spectra of poly C under various conditions and of the mononucleotides obtained by alkaline hydrolysis of the polymer.

Figs. 1 to 6 were obtained in a phosphate buffer with an ionic strength of 0.2.

The spectrum of poly A in 0.1 M hydrochloric acid is shown in Fig. 2. In contrast to the slight change in the spectrum of adenylic acid at pH 1 as compared with pH 7, the poly A curve flattens out considerably and exhibits considerable absorption at 340 mμ. These changes do not occur rapidly and after the polymer is diluted in acid the spectrum shifts slowly over several hours. Similar shifts were observed with poly I and poly C, but because of the insolubility of these polymers in acid the observations were limited to extremely dilute solutions and the curves have not been included in Figs. 1 to 6. The effect of pH has not yet been systematically

explored. However, notable spectral changes are evident upon shifting the pH from 7 to 5.5. At the latter pH the spectra are more sensitive to changes in ionic strength and temperature than at pH 7.

The spectrum of poly U (Fig. 5) shows striking differences from that of the other polymers. The extinction at the maximum is reduced only slightly from that of uridylic acid and the wave length of the maximum is unchanged. The same curve was obtained for the polymer in water,

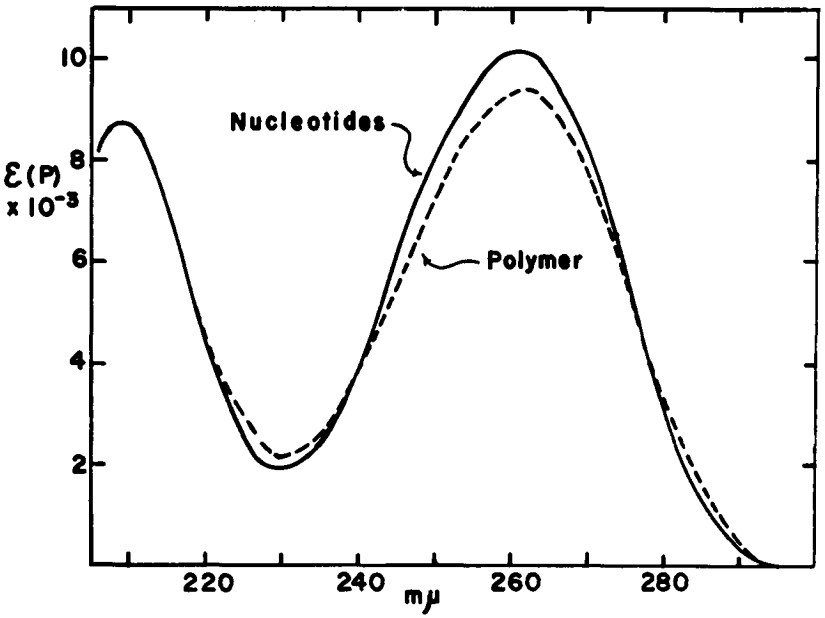

FIG. 5. Absorption of poly U and of the mononucleotides obtained by alkaline hydrolysis of the polymer. The same absorption curve for the polymer (broken line) was found in water, phosphate buffer, and 6.4 M urea.

phosphate buffer, or 6.4 M urea. A small shift in spectrum was observed between pH 7 and pH 1 which corresponds to a similar shift in the uridylic acid spectrum.

Effect of Temperature

Preliminary measurements of the effect of temperature on the spectra indicate a rise in optical density when poly A, poly C, or poly I is heated in phosphate buffer above 65°. Upon cooling, the optical density returns essentially to its initial value, although in the case of poly I it may do so only slowly.

Spectral Changes with Polynucleotide Interaction

The spectral changes accompanying the mixing of poly A and poly U in phosphate buffer are shown in Fig. 6 (*cf.* also Table II). The calculated spectrum for the sum of the constituent mononucleotides for a mixture of poly A and poly U is shown in the upper curve. The polymers were pres-

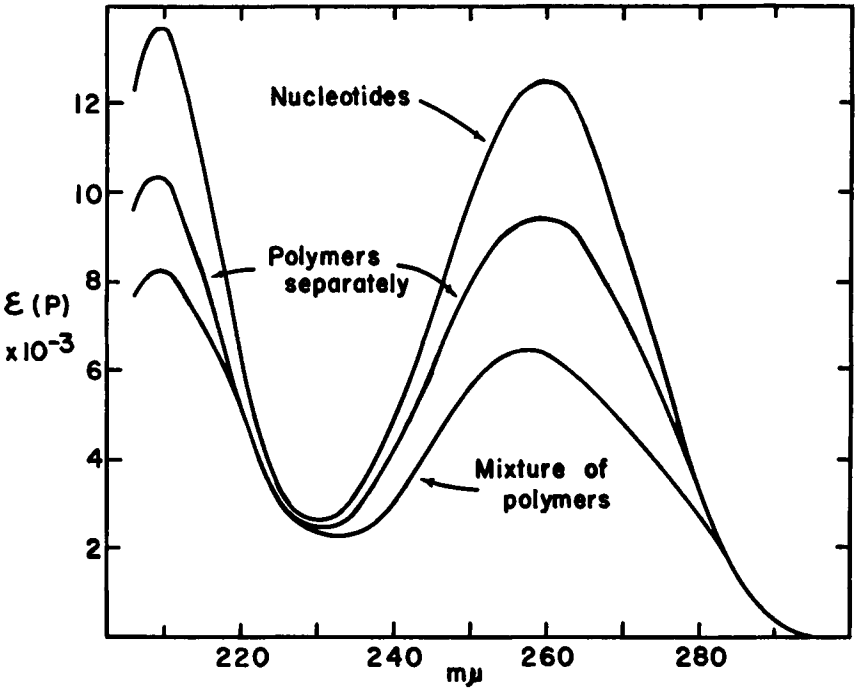

FIG. 6. Absorption spectra of an equimolar mixture (phosphorus basis) of poly A and poly U. The upper curve refers to the mononucleotides obtained by alkaline hydrolysis of the mixture. The middle curve is that calculated for the sum of the separately measured polymer spectra. The lower curve is the measured curve for the mixture of polymers.

ent at equimolar concentrations calculated on a mononucleotide basis. The same curve was obtained after alkaline hydrolysis of the polymer mixture. The middle curve is the calculated spectrum for the sum of the contributions of the separately measured polymers, and the lower curve is that observed for the mixture. The extinction at the maximum for the mixture is thus 68 per cent of that expected on the basis of additivity and 51 per cent of that for the constituent mononucleotides. In addition to the reduction in extinction, there is a hypsochromic shift of about 2 mμ

upon mixing. Similar mixture experiments were performed with all of the other possible pairs of polymers, but in each case the spectra were additive.[5] At a concentration of 6.4 M urea a spectrum intermediate between the two lower curves of Fig. 6 was obtained, indicating a partial dissociation. Raising the temperature of a solution of the aggregate in phosphate buffer results in dissociation at a temperature of about 60°, as shown by a sharp increase in optical density. This effect is reversible since the density drops again upon cooling.

The formation of stable aggregates, demonstrated directly by electrophoresis and sedimentation, is, therefore, seen to be accompanied by changes in the ultraviolet spectra. The interaction thus involves a modification of the properties of the bases and can be most simply accounted for by assuming that hydrogen bonds are formed between the adenine and uracil moieties in adjacent chains. Hydrogen bonds provide the only available forces of sufficient magnitude to overcome the considerable electrostatic barrier to association of the chains. Their presence is indicated by the effect of urea and temperature on the aggregates. The similar response of DNA to these variables, together with other evidence, has led to the assumption that extensive hydrogen bonding is of importance in maintaining the specific structure of DNA. A regular sequence of hydrogen bonds between two DNA chains is a central feature of the Watson and Crick model (13). The base pairing involved in the poly A-poly U aggregation is equivalent to the adenine-thymine pairing assumed in the Watson and Crick structure, since a corresponding pair of hydrogen bonds can be formed in the two cases. The experiments presented here provide for the first time direct experimental evidence for the hypothesis that a set of cooperative hydrogen bonds can stabilize the interaction between two polynucleotide chains and that only specific pairs of bases will permit such stabilization.

The observation of the ultraviolet spectra provides a rapid means of determining some of the characteristics of the interaction between poly A and poly U. Descriptions of a few such experiments follow.

When an experiment similar to that shown in Fig. 6 was made in the absence of salt instead of in phosphate buffer, the spectrum of the mixture was that expected for additivity. In such a mixture, interaction accom-

[5] The action of polynucleotide phosphorylase on guanosine 5′-diphosphate leads to a much slower liberation of orthophosphate than is the case with other nucleoside diphosphates, and it stops when about 10 per cent of the maximum has been reached (2). From such reaction mixtures it was possible to isolate a product which had a different absorption spectrum from guanosine diphosphate. However, this product was obtained in much smaller yield than expected from the phosphate liberation (the remainder being dialyzable) and has as yet not been characterized. For this reason no work has so far been possible with polyguanylic acid.

panied by a rapid drop in optical density could be brought about by making the solution 10^{-4} M in magnesium sulfate or by increasing the ionic strength to 0.01 with either sodium chloride or phosphate buffer.[6] Further increase in these concentrations did not produce any further change in optical density.

When a mixture of poly A and poly U in water was made to an ionic strength of 0.002 with phosphate buffer or when the polymers were prepared separately in such a buffer and then mixed (Solution A), only a slight drop in optical density took place. The same final concentrations of polymers and salt may be achieved by mixing concentrated stock solutions of the polymers in a buffer at an ionic strength of 0.02 and then diluting (Solution B). When this was done, the spectrum of aggregated poly A plus poly U was obtained. The optical densities of the two solutions were stable for several hours, but that of Solution A gradually decreased over several days. When the final ionic strength was 0.006, this subsequent decrease was more rapid and amounted to 10 per cent in 24 hours. The dependence of the rate of the reaction on the ionic strength probably results from an energy of activation determined in part by the electrostatic repulsive forces between chains.

A similar experiment was performed in urea. Poly A plus poly U containing just sufficient buffer or $MgSO_4$ to permit interaction were mixed and urea was added to a concentration of 6.4 M. A spectrum intermediate between the two lower curves of Fig. 6 was obtained. However, when the polymers were made up separately in urea and buffer and then mixed, the spectrum was additive for poly A and poly U in urea; *i.e.*, it was above the middle curve of Fig. 6. The final buffer concentration in this experiment was 0.01 ionic strength or sufficiently high to permit the aggregation of poly A and poly U in the absence of urea. The aggregate once formed was thus not dissociated by urea. However, under the same conditions it was not formed in the presence of urea. Equilibrium was not attained and there was no indication under these conditions of whether equilibrium lies in the direction of aggregation or dissociation.

DISCUSSION

The possibility of interaction based on hydrogen bonding between bases exists in a solution of single polymers as well as in mixtures of poly A and poly U. Evidence for hydrogen bonding in both cases can be derived from the spectra obtained under various conditions. In each case a high con-

[6] Our first samples of polymers were apparently contaminated with magnesium, and when this experiment was performed with them, a drop in optical density was found upon mixing aqueous solutions. The effect of Mg^{++} was first observed by Dr. J. D. Smith (personal communication).

centration of urea increased the optical density, as would be expected for competition with the hydrogen bonds between bases. In no case did the optical density rise to the mononucleotide level, although urea prevented interaction under conditions in which it otherwise would have taken place. An increase in optical density also occurs on raising the temperature. This may be correlated with a thermal dissociation of hydrogen bonds and in the case of poly A plus poly U should result in dissociation of the two polymers, although there is no direct evidence from this experiment that this has taken place.

The changes in spectra at different concentrations of salt are consistent in most cases with the expected effect of ionic strength on electrostatic interaction. The spectrum in a phosphate buffer of 0.2 ionic strength has been taken as the basic spectrum because this ionic strength is well above the level at which the absorption is sensitive to ionic strength at pH 7. The increased extinction for poly C and poly I in the absence of salt and the lack of interaction of poly A and poly U under the same conditions may be attributed to electrostatic repulsion between chains or between parts of the same chain. This effect is screened by buffer salts and disappears at an ionic strength of 0.01. Magnesium salts are effective at a much lower concentration in the poly A plus poly U system probably because magnesium ion will form complexes with the polyelectrolyte chains and partially neutralize their charge. Magnesium salts show no effect on the spectra of the other polymers beyond that due to their contribution to the ionic strength. These changes are in the same direction as those produced by low ionic strength on DNA (14). The effect of ionic strength on poly A is anomalous in that the extinction in water is below that in buffer. The behavior of poly U is exceptional. The spectrum was unaffected by urea, ionic strength, pH, or temperature. It thus appears that hydrogen bonds are not formed although the necessary pairings are among those listed by Donohue (15).

The chain interaction of the single polymers may be on either an inter- or intramolecular basis. The evidence presented here for interaction in solutions of homopolymers is based on comparison of the ultraviolet spectra with those of the constituent mononucleotides and does not permit a decision between these alternatives. Other physical properties which will reflect the varying consequences of these two possibilities are under investigation.

Our evidence provides a direct demonstration of the spontaneous formation of a stable aggregate from two kinetically independent polynucleotides and of the association of changes in the ultraviolet spectrum with this phenomenon. The aggregates that are formed bear resemblance to certain features of DNA. The formation of hydrogen bonds between successive pairs of bases along the chain may be assumed to be responsible for the

stability of the structure in both cases. The alterations in the spectrum of DNA in response to urea, pH, and temperature are largely irreversible (16, 17), whereas those of the poly A-poly U aggregates are reversible. This probably results from the fact that every apposition of a poly A chain and a poly U chain provides matching pairs of bases, although in DNA only certain specific appositions will presumably permit pairing of the bases. Whether the similarity in structure extends further cannot be decided from the present data. Rich and Davies, however, have suggested a DNA-like, double-stranded helical structure for extended fibers prepared from mixtures of poly A and poly U (18).

Another consequence of the interaction of poly A and poly U has been found (19) in the reduction in the rate of phosphorolysis of the mixture. Poly A and poly U alone are readily phosphorolyzed by polynucleotide phosphorylase. The mixture was phosphorolyzed at about 20 per cent of the rate of the separate polymers. Poly AGUC and several RNA preparations were also phosphorolyzed slowly. The formation of aggregates thus inhibits phosphorolysis, perhaps by making the sensitive bond less available to the enzyme. This effect should place a limitation on the reversibility of the over-all reaction when copolymers are being synthesized and may be an important effect in the biosynthesis of RNA.

SUMMARY

1. Enzymatically synthesized polyadenylic and polyuridylic acids interact spontaneously in dilute neutral salt solutions with the formation of a stable aggregate of higher molecular weight and lower extinction coefficient. The aggregate migrates electrophoretically as a single component with a mobility intermediate between those of its constituent polynucleotides.

2. Polyadenylic, polycytidylic, and polyinosinic acids have lower molar extinctions in the ultraviolet region than the respective mononucleotides. Their spectra show characteristic changes with change in ionic strength, urea concentration, pH, and temperature. The spectrum of polyuridylic acid does not show these changes.

3. The aggregation and the accompanying spectral changes are believed to be a result of the formation of hydrogen bonds between the purine and pyrimidine bases in adjacent polynucleotide chains. Hydrogen bond formation between bases in homopolymers may also be responsible for the spectral changes associated with polymerization.

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