Precipitation of Polynucleotides from Acidic Solution. Effects of Base Composition and Ionic Strength†

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ABSTRACT: Polynucleotides precipitate at well-defined pH values. These pH values are dependent upon both the base composition and the ionic strength of the medium. At low ionic strength, all the polymeric components in solution tend to precipitate together. The pH value at which such coprecipitation occurs can be predicted based upon a simple model which requires charge neutralization between the bases and phosphate residues for insolubility, *i.e.*, precipitation occurs at the net isoelectric point of the solution. Spectrophotometric p*K* values for some common nucleosides were redetermined for use in calculating the isoelectric points (see Appendix). At higher ionic strengths, there were instances of fractional precipitation. Also at higher ionic strengths, several homopolymers (poly(A), poly(G), and to a lesser extent, poly(U)) had a second zone of insolubility near neutral pH values.

Insolubility in acidic media is a familiar and a common property of polynucleotides. We will show that the narrow range of acidity in which a given polynucleotide or mixture of polynucleotides becomes insoluble is a function *inter alia* of its base composition and the ionic strength of the medium. Simple mechanisms of precipitation based upon charge neutralization can rationalize the bulk of the observations.

Materials and Methods

Polynucleotides. Polynucleotides were purchased from the following sources: high molecular weight RNA (from wheat germ, prepared by the method of Glitz and Dekker, 1963) and *E. coli* transfer ("soluble") RNA, Calbiochem, La Jolla, Calif.; DNA from *Escherichia coli*, *Clostridium perfringens*, and calf thymus, Worthington Biochemical Corp., Freehold, N. J.; poly(dA) and poly(dT), Biopolymers, Inc., Pinebrook, N. J.; ^{14}C-labeled poly(A) and poly(U) (0.1–0.3 mCi/mmol), Schwarz BioResearch, Inc., Orangeburg, N. Y.; all others, Miles Laboratories, Kankakee, Ill. All polymer solutions (at concentrations from 0.1 to 5 mg/ml) were treated with phenol and dialyzed extensively as described previously for poly(A) (Zimmerman and Coleman, 1972). Polymer concentrations in the final stock solutions were estimated by total phosphate determinations (Ames and Dubin, 1960).

Precipitation of Polynucleotides. The precipitation of polymers was estimated by the fraction of their ultraviolet absorbance rendered sedimentable under the following conditions. One-tenth volume of a chilled dilution of polymer(s) in water (total concentration, 0.5 μmol of polymer phosphorus/ml) was mixed with 0.9 vol of ice-cold medium. Media were HCl solutions where not specified; the HCl was replaced by a buffer, or NaCl was added where indicated. After 10 min at 0°, the mixtures were centrifuged at 2–4° for 10 min at 8000*g*. The supernatant fluids were decanted. The tubes were drained in an inverted position for a few minutes and then the pellets were redissolved by the addition of 1 vol of 0.1 N NaOH. Shortly thereafter, the absorbances at 260 mμ of the redissolved pellets were determined. The amount precipitated was taken as the fraction of the total absorbance added (measured in alkaline solution) which was found in the redissolved pellet. The data presented have not been corrected for the small amounts of the supernatant fluids which remained with the pellets. A total volume of 2.0 ml was generally employed; however, to conserve some of the polymers, total volumes of 0.6 ml were used where noted. The absorbances in the supernatant fluids were also measured as a check for approximate material balance.

pH, $pH_{1/2}$, and pK Values. All pH values given for solutions of HCl are calculated from the concentration of the HCl stock solution based on the manufacturer's analysis and assuming pH = −log [HCl]. At concentrations of HCl below 1.0 N, measured pH values agreed within 0.1 unit with those calculated from the acid concentration and the appropriate activity coefficients. All pH values for solutions containing buffers are from measurements at room temperature on solutions similar to the experimental mixtures but containing no polymers. The $pH_{1/2}$ value for a polymer in solutions with a given salt concentration is defined as the highest pH value at which one-half of the maximal amount of precipitation has occurred. All p*K* values are apparent values.

Partial Hydrolysis of Homoribopolymers. Solutions of homoribopolymers (1.5 ml, 1.0 μmol of phosphorus/ml) were incubated at 37° in 0.1 M NaOH for 5–45 min in order to form digests containing 15–30% acid-soluble products, the appropriate times being judged from preliminary experiments. The solutions were chilled to 0°, brought to a final HCl concentration of 0.3 N (poly(C), poly(A), and poly(G)) or 2.5 N (poly(U)), and held for 10 min at 0°; then the acid-insoluble fraction was collected by centrifugation for 10 min at 8000*g* at 2–4°, dissolved in 1.0 ml of 0.3 M Tris buffer, pH 8.0, and dialyzed for 1 day *vs.* 300 vol of 1 mM Tris buffer, pH 8.0, containing 0.01 mM EDTA. The recoveries relative to the initial amounts of the polymers were 60–90%. Average chain lengths were determined to be in the range of 30–70, by the amounts of phosphate released upon digestion with *E. coli* alkaline phosphatase (Heppel *et al.*, 1962); the apparent chain length of these samples changed by ≤30% after treatment with acid to hydrolyze cyclic termini (Bock, 1967).

Miscellaneous. Hydrochloric acid was highly purified commercial material (Ultrex grade, J. T. Baker Chemical Co., Phillipsburg, N. J.); reagent grade HCl contains impurities

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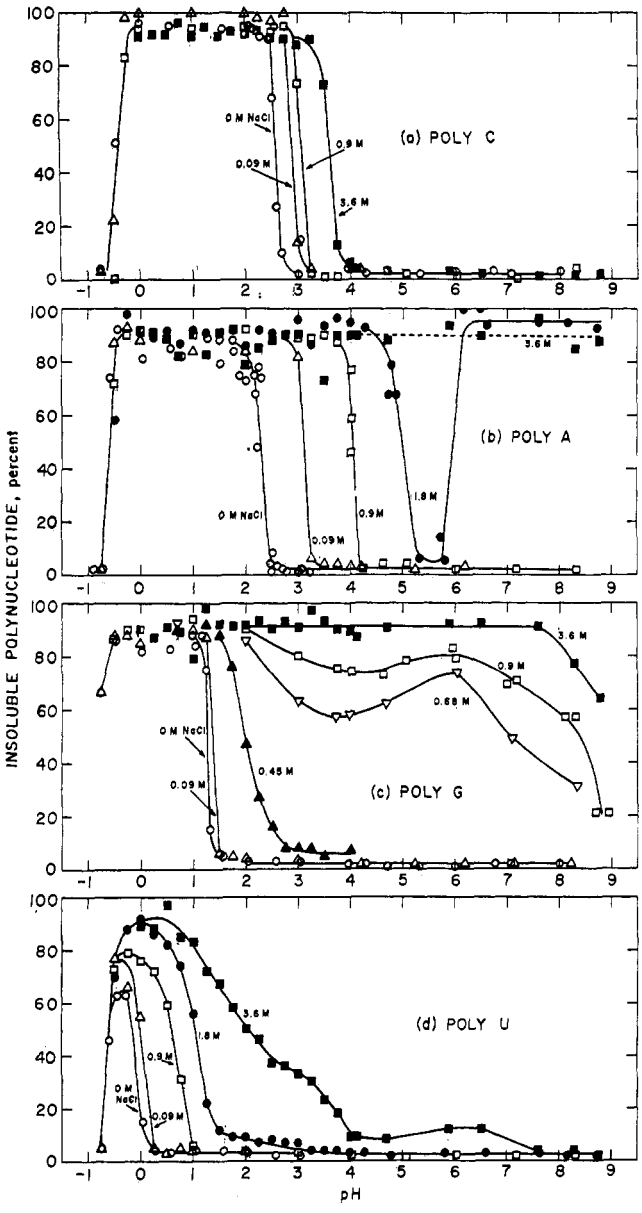

FIGURE 1: Effect of pH and NaCl concentration on the solubility of poly(C), poly(A), poly(G), or poly(U). The polymers, NaCl concentrations, and pH values are as indicated in the figure; the procedure is described under Materials and Methods. All points for pH ≤ 4 are from dilutions in HCl solutions; at higher pH values, solutions contained 0.1 M buffers (sodium acetate (pH 4.0–5.3)–sodium cacodylate (pH 5.2–7.0)–Tris-chloride (pH 7.1–9.0)).

which have caused variable results in experiments similar to those described here, and accordingly is not recommended. Ultraviolet absorbance measurements were made with a Zeiss PMQII spectrophotometer. A Beckman (Model GS) meter was used to measure pH. Radioactivity was determined with a low-background Geiger counter (Tracerlab Omni/Guard).

Results

Insolubility of Polynucleotides as a Function of pH

Polyribonucleotides. SINGLE HOMOPOLYRIBONUCLEOTIDES. All of the homopolyribonucleotides examined (poly(A), poly(G), poly(U), poly(C), and poly(I)) had qualitatively similar patterns of precipitation from acidic solutions in the absence of

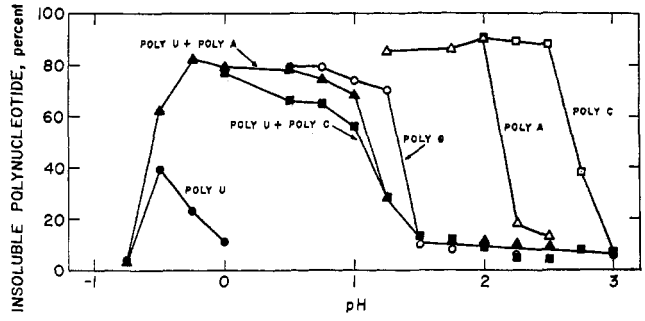

FIGURE 2: Effect of pH on the solubility of shorter chain-length homopolyribonucleotides. The average chain length of these polymers was from 30 to 70. Their preparation by partial alkaline hydrolysis and conditions of their precipitation are described under Materials and Methods. Equal concentrations of polymers were present in the mixtures.

added salt. These polymers were all precipitated from homogeneous solution over a relatively small decrement of pH; as the pH was lowered further, the precipitated polymers once more became soluble (Figure 1). The actual pH at which precipitation occurred was a specific property of each polymer; for purposes of tabulation, the highest pH at which half of the maximal precipitation had occurred is defined as the $pH_{1/2}$, and estimates of this quantity for the homopolyribonucleotides are listed in Table I. Increasing ionic strength raised the $pH_{1/2}$ of the homopolyribonucleotides tested, namely poly(A), poly(G), poly(C), and poly(U). At high salt concentrations, poly(G) and poly(A) precipitated at neutral pH (Figure 1).

With the exception of poly(U), the patterns of precipitation were little affected by holding acidified samples at 0° for as long as 2 hr before centrifugation. The curves were slightly shifted toward higher pH values, but the changes in $pH_{1/2}$ and in the maximum extent of precipitation were relatively small. Poly(U) behaved differently. If centrifuged shortly after lowering the pH, it was largely precipitated; however, if left standing at low pH at 0°, it lost its precipitability rather rapidly and by 2 hr was rendered completely soluble. This change in poly(U) is presumably due to a unique sensitivity to acidic hydrolysis, as suggested by the results of Rammler *et al.* (1965). In any case, the changes in $pH_{1/2}$ values for poly(U) at different times are relatively small.

Precipitation was not readily reversible for at least some of the polymers under the conditions used. Raising the pH sufficiently always caused rapid redissolution, but adjusting it to values slightly above the $pH_{1/2}$ caused no marked tendency to redissolve poly(G) or poly(A) precipitates held up to 1 week at 0°.

The effects of chain length and homopolymer concentration were tested. Polynucleotides of shorter average chain length (30–70 residues/chain) had $pH_{1/2}$ values similar to those for longer chain polymers (>300 residues/chain) (Figure 2 and Table I). The $pH_{1/2}$ values for the homopolyribonucleotides were not affected by several fold changes in polymer concentrations around the level chosen for precipitation in most of these experiments (*ca.* 0.05 μmol of phosphorus/ml). Tests at $^1/_{20}$th of that level (done for poly(A) and poly(U) only) indicated no major change in $pH_{1/2}$ values, but because of broader transitions and higher blank values, the comparisons were less precise (Figure 3). At still lower polymer concentrations, the extent of precipitability decreased drastically. For example, at 0.5 nmol of phosphorus/ml, preparations of ^{14}C-labeled poly(A) or poly(U) were only *ca.* 10% insoluble (over blank values of comparable magnitude), but had maximal extents of

TABLE I: Summary of the pH Dependence for Precipitation of Some Polyribo- and Polydeoxyribonucleotides and Comparison with Predicted pH Values for Precipitation.

Polynucleotides	Base Composition as Mole Fraction of [a]						Calcd $pH_{1/2}$ for 0 M NaCl [b]	Measured $pH_{1/2}$ at NaCl Concn (M) of			
	Cyt	Ade	Gua	Ura	Thy	Others		0	0.09	0.9	3.6
Polyribonucleotides											
Poly(C)	1.00						2.70	2.6 (2.9[c])	2.9	3.1	3.6
Poly(A)		1.00					2.35	2.3 (2.5[c])	3.1	4.2	*f*
Poly(G)			1.00				1.55	1.3 (1.4[c])	1.4	*f*	*f*
Poly(U)				1.00				−0.1[d] (0.0[e])	0.1	0.6	*g*
Poly(I)						1.00 Hyp		1.3 (1.3[c])			
Poly(C) + poly(A)	0.50	0.50					2.5	2.4	3.0		
Poly(C) + poly(G)	0.50		0.50				1.7	1.4	1.5		
Poly(C) + poly(U)	0.50			0.50			0.9	1.0	*g*		
Poly(A) + poly(G)		0.50	0.50				1.7	1.6	1.9		
Poly(A) + poly(U)		0.50		0.50			0.9	1.0	*g*		
Poly(G) + poly(U)			0.50	0.50				*g*	*g*		
Poly(I) + poly(U)				0.50		0.50 Hyp		*g*			
Poly(C) + poly(A) + poly(G)	0.33	0.33	0.33						*g*		
Poly(C) + poly(A) + poly(U)	0.33	0.33		0.33					*g*		
Poly(C) + poly(G) + poly(U)	0.33		0.33	0.33					1.2		
Poly(A) + poly(G) + poly(U)		0.33	0.33	0.33					1.3		
Poly(C) + poly(A) + poly(G) + poly(U)	0.26	0.24	0.22	0.28			1.3	1.3	*g*	3.1	
C_{66}^{-}	1.00						2.70	2.7			
A_{30}^{-}		1.00					2.35	2.2			
G_{40}^{-}			1.00				1.55	1.4			
U_{33}^{-}				1.00				−0.2[d]			
$C_{66}^{-} + U_{33}^{-}$	0.50			0.50			0.9	1.1			
$A_{30}^{-} + U_{33}^{-}$		0.50		0.50			0.9	1.1			
Poly(C,A)	0.50	0.50					2.5	2.5 (2.5[e])	3.2		
Poly(C,G)	0.48		0.52				1.7	1.8[d] (1.8[d,e])	1.9[d]		
Poly(C,U)	0.50			0.50			0.9	0.9 (0.9[c])	1.2		
Poly(A,G)		0.54	0.46				1.7	1.9	2.3[f]		
Poly(A,U)		0.45		0.55			0.8	0.9	1.0		
Poly(G,U)			0.50	0.50			0.9	0.8	0.9		
Poly(I,U)				0.50		0.50 Hyp		0.8	0.9		
Poly(C,A,G,U)	0.26	0.24	0.22	0.28			1.3	1.6	1.9	2.3	
tRNA, *E. coli*	0.29	0.20	0.32	0.15	0.01	0.02 ψU	1.4	1.6 (1.6[c])	1.9	2.2	2.6
16S RNA, *E. coli*	0.22	0.24	0.32	0.21			1.4	1.6			
23S RNA, *E. coli*	0.21	0.26	0.32	0.21			1.4	1.6			
Wheat germ RNA	0.23	0.22	0.32	0.22		0.02 ψU	1.3	1.4	1.9	2.5	2.9
Wheat germ RNA + poly(C) (1:1)	0.62	0.11	0.16	0.11		0.01 ψU	1.7	1.7		*g*	
Wheat germ RNA + poly(A) (1:1)	0.12	0.61	0.16	0.11		0.01 ψU	1.7	1.8		*g*	
Polydeoxyribonucleotides											
Poly(dA)		1.00					2.35	2.4			
Poly(dT)					1.00			0.4 (0.4[c])			
E. coli DNA	0.26	0.25	0.25		0.25		1.3	1.3 (1.3[c])	1.4	2.0	2.4
Calf thymus DNA	0.21	0.29	0.21		0.29		1.2	1.3 (1.3[c])	1.4	1.9	2.4
C. perfringens DNA	0.15	0.34	0.16		0.35		1.1	1.4 (1.4[c])	1.6	1.9	2.2

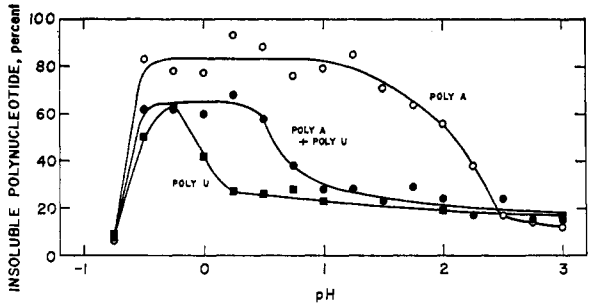

FIGURE 3: Effect of pH on the solubility of some homopolyribonucleotides at relatively low polymer concentrations. Procedure as described under Materials and Methods except that the total polymer concentration was 2.5 nmol of phosphorus/ml (*i.e.*, $^1/_{20}$th the usual level). Poly(A) and poly(U) were added in equal amounts where they were both present.

insolubility similar to those shown in Figures 1b and 1d, respectively, in the presence of unlabeled polynucleotides at a concentration of 0.05 μmol of phosphorus/ml.

MIXTURES OF HOMOPOLYRIBONUCLEOTIDES. The preceding results indicate that, taken singly, the homopolyribonucleotides precipitate reproducibly and generally quantitatively over characteristic narrow pH ranges. This behavior suggested the possibility of fractional precipitation of components from mixtures containing polymers with differing base compositions. However, the results did not generally confirm this expectation. Instead, all the components of the mixture tended to precipitate together at a $pH_{1/2}$ characteristic of the mixture. Such coprecipitation was found to be typical, except in a few cases where salt was added.

In the absence of added salt, the precipitate formed over a narrow range of pH values, and contained the bulk of *all* of the polymeric components of the solution. For example, Figure 4a shows the pH dependence of precipitation for solutions containing equal amounts of various pairs of homopolymers, and Figure 5 shows a summary of similar data for mixtures at varying input ratios of poly(A) and poly(G) or of poly(A) and poly(U). In all these cases, a single zone of precipitation occurred. The $pH_{1/2}$ of the mixtures shifted monotonically as a function of the concentration ratio of polymers between the values for the individual homopolyribonucleotides (Figure 5).

When precipitation experiments were conducted on homopolymer mixtures in solutions containing 0.09 M NaCl, a second type of behavior was seen in several cases. While most pairs of homopolymers gave total precipitation as outlined above for precipitation in the absence of salt, mixtures of poly(A) or poly(C) with poly(U) showed a second zone of insolubility (Figure 4b). The lack of change in $pH_{1/2}$ value and the changes in amounts of precipitate as the ratio of poly(C) to poly(U) was varied suggested that the peak precipitating

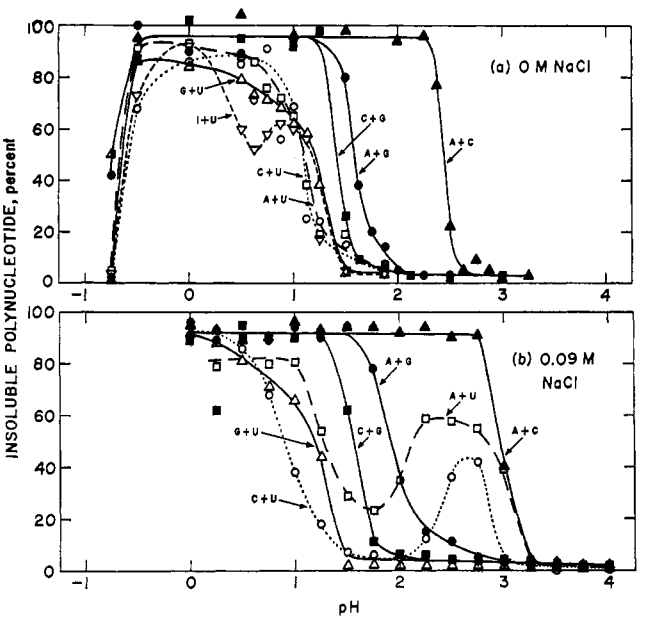

FIGURE 4: Effect of pH on the solubility of pairs of homopolyribonucleotides. The procedure is described under Materials and Methods. Both members of each pair of polymers were present at the same concentration: poly(A) + poly(C) (▲), poly(A) + poly(G) (●), poly(C) + poly(G) (■), poly(I) + poly(U) (▽), poly(G) + poly(U) (△), poly(C) + poly(U) (○), poly(A) + poly(U) (□).

between pH 2 and 3 was largely composed of poly(C) and did not represent a stoichiometric complex of the two polymers (Figure 6). When mixtures of three homopolymers in equal proportions were tested, again in media containing 0.09 M NaCl, poly(A) + poly(C) + poly(U) showed a second peak while the other three combinations did not. When a mixture of all four polymers was tested at that salt concentration, the second peak was again present and in relatively large amounts.

Two other parameters—polymer concentration and chain length—were also varied. In addition to the usual polymer concentration of 0.05 μmol of phosphorus/ml, $^1/_{20}$th of that level was used (Figure 3); lower molecular weight polymers were also tested (Figure 2). The tendency for all the polymeric components to precipitate together in the absence of added salt was maintained.

HETEROPOLYRIBONUCLEOTIDES. A number of synthetic heteropolymers containing approximately equal amounts of two bases were precipitated in the absence of added salt or in 0.09 M NaCl (Figure 7 and Table I). In all cases the heteropolymers precipitated at $pH_{1/2}$ values intermediate between those of homopolymers containing the same bases. However, the $pH_{1/2}$ for the heteropolymers was not simply an arithmetic average of those for the homopolymers, since, for example, poly(A,U) and poly(G,U) had the same $pH_{1/2}$ value, while

FOOTNOTES TO TABLE I

[a] Sources of base composition data: synthetic polynucleotides, manufacturer's analyses; *E. coli* tRNA, Dunn *et al.* (1960); *E. coli* 16S and 23S RNA, Stanley and Bock (1965); wheat germ RNA, Glitz and Dekker (1963); *E. coli* DNA, Rudner *et al.* (1966); calf thymus DNA, Chargaff and Lipshitz (1953); *C. perfringens* DNA, Spirin *et al.* (1957). [b] The basis of the calculation is described in the Discussion. For these calculations, methylcytidine is taken as equivalent to cytidine, and pseudouridine as equivalent to uridine. [c] Samples held for 2 hr at 0° before centrifugation. [d] Precipitation incomplete. [e] Samples held for 1 min at 0° before centrifugation. [f] Second range of insolubility at higher pH values. [g] $pH_{1/2}$ calculation not meaningful due to broad or complex precipitation pattern.

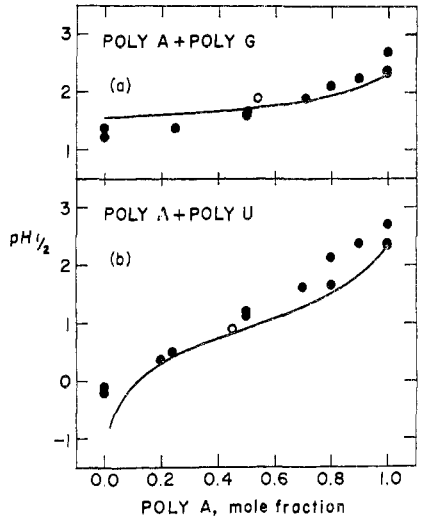

FIGURE 5: Effect of the input ratio of poly(A) to poly(G) or poly(A) to poly(U) on the pH of precipitation. The $pH_{1/2}$ for mixtures of the polymer compositions indicated were determined as described under Materials and Methods; all media for precipitation were HCl solutions lacking NaCl. The extents of precipitation were >85% in all cases except for poly(U) which was *ca.* 65% (*cf.* Figure 1d). The open circles are values for the random heteropolymers (poly(A,G) and poly(A,U) in (a) and (b), respectively) taken from the data in Figure 7. The *solid line* is calculated for the model of isoelectric precipitation detailed in the Discussion.

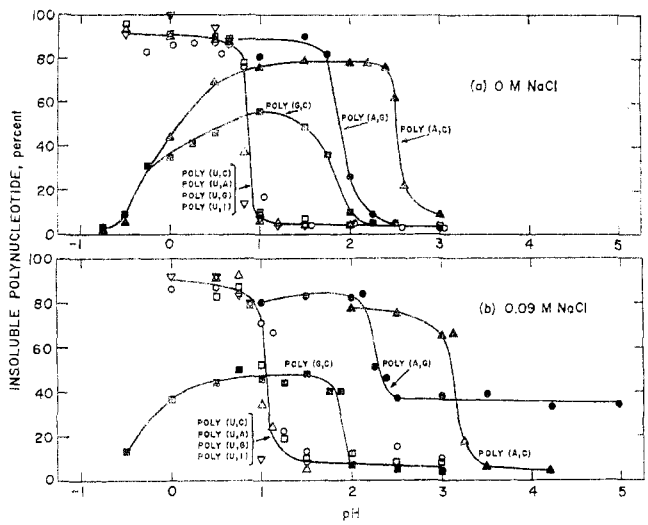

FIGURE 7: Effect of pH on the solubility of random heteropolyribonucleotides containing two types of bases. The base composition of these polymers is listed in Table I; in all cases the bases were present in approximately equal amounts. Procedure is described under Materials and Methods; all media below pH 4 are HCl solutions; above pH 4, 0.05 M sodium acetate buffer was present. Media contained 0.09 M NaCl for b; poly(A, C) (▲), poly(A, G) (●), poly(G, C) (■), poly(U, C) (○), poly(U, G) (△), poly(U, I) (▽) poly(U, A) (□).

poly(A) and poly(G) differ in $pH_{1/2}$ values by a pH unit. A rationalization of this behavior is presented in the Discussion but it should be noted here that the $pH_{1/2}$ values of these heteropolymers are quite close to the values of the equivalent mixtures of homopolyribonucleotides (Table I). For example, a heteropolymer containing equal amounts of adenosine and guanosine residues precipitates with nearly the same $pH_{1/2}$ as does a mixture of equal amounts of poly(A) and poly(G). The patterns of precipitation of several naturally occurring RNAs are shown in Figure 8 ($pH_{1/2}$ values are summarized in Table I). Additions of equal amounts of poly(A) or poly(C) to wheat germ RNA in the absence of added salt caused a relatively small shift in the pH of precipitation of wheat germ RNA (Table I).

Polydeoxyribonucleotides. Because of the greater acid lability of many polydeoxyribonucleotides (Pollmann and Schramm, 1961) and because of the much greater expense of the homopolymers, only a few observations were made to see if any qualitatively different behavior might be expected for the deoxypolymers from that outlined above for the polyribonucleotides. There were no such differences detected.

HOMOPOLYDEOXYRIBONUCLEOTIDES (poly(dA) and poly(dT)). Poly(rA) and poly(dA) precipitated at virtually the same pH value in the absence of salt (pH 2.3 and 2.4, respectively; Figures 1 and 9). Poly(dT) was of special interest because thymidine and uridine have generally similar acid–base properties and because the extent of insolubility of poly(dT) in strongly acidic solutions is unchanged after 2 hr at 0° (Figure 9), whereas poly(U) becomes totally soluble by that time (see

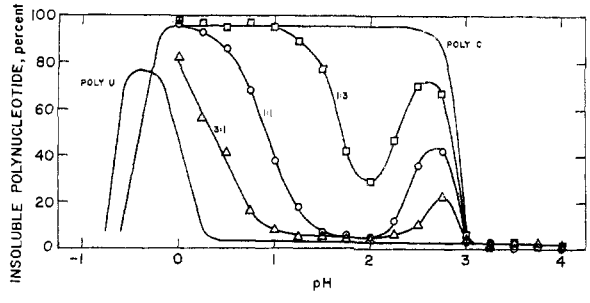

FIGURE 6: Effect of the input ratio of poly(U) to poly(C) on the pH dependence of solubility in HCl solutions containing 0.09 M NaCl. The molar ratio of polymer phosphorus of poly(U) to poly(C) is indicated in the figure. The procedure is described under Materials and Methods; all media were HCl solutions containing a final concentration of 0.09 M NaCl. The curves for poly(U) and poly(C) are taken from Figures 1d and 1a, respectively.

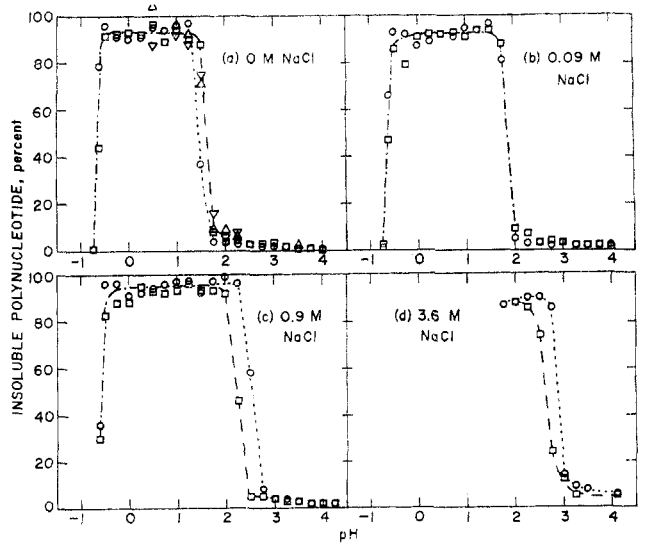

FIGURE 8: Effect of pH on the solubility of several types of naturally occurring RNAs. Procedure is as described under Materials and Methods. The final concentrations of NaCl present in the HCl solutions are indicated in the figure: wheat germ RNA (○), *E. coli* tRNA (□), *E. coli* 23S RNA (▽), *E. coli* 16S RNA (△).

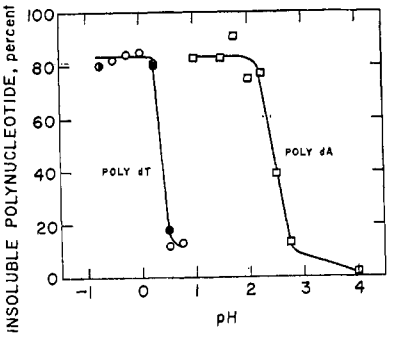

FIGURE 9: Effect of pH on the solubility of poly(dT) and poly(dA). Closed symbols are samples held 2 hr at 0° before centrifugation; open symbols are samples held at 0° for the usual 10-min period. Procedure otherwise is as described under Materials and Methods with a total volume of 0.6 ml. The media are HCl solutions containing no NaCl.

above). In the absence of added salt, poly(dT) had a $pH_{1/2}$ of 0.4 compared to a value for poly(U) of $pH_{1/2} \sim 0.0$.

HETEROPOLYDEOXYRIBONUCLEOTIDES. The precipitation patterns of DNA from *E. coli*, *C. perfringens*, and calf thymus glands were measured at several ionic strengths. The three samples became insoluble at very similar pH values (Figure 10 and Table I).

Titrations of Polynucleotides

In order to estimate the degrees of charge neutralization needed for precipitation to occur, two polymers with relatively high $pH_{1/2}$ values, poly(A) and poly(C), were titrated. The end point of the titration was judged by complete precipitation. Since precipitation of even these two polynucleotides occurs only in relatively strongly acidic solutions, a significant fraction of the total acid needed to reach a given pH value must be added simply to adjust the solvent to that pH value. Therefore, the degrees of charge neutralization determined in this way are only approximate. In order to minimize the relative contribution of the solvent blank, the concentration of the polymers was raised 100-fold over that used above to determine the pH dependence of precipitation. The $pH_{1/2}$ values from such concentrated solutions were 0.6–1.2 units higher than noted in preceding sections. Several conclusions are apparent. For both poly(A) and poly(C) in the absence of added salt, the polymers require over 90% charge neutralization to become insoluble; as the ionic strength increases, the requisite degree of charge neutralization for precipitation decreases significantly (Figure 11).

Discussion

All the polynucleotides tested precipitate as the pH is lowered below some characteristic value. Generally, the total polynucleotide contents of a solution precipitate together at virtually the same pH ("coprecipitation"). In a few cases, one polymeric component was precipitated selectively ("fractional precipitation"). We will develop a simple model for both types of precipitation in which the prerequisite for precipitation is attainment of some degree of charge neutralization on the molecules. The difference between polymers which show coprecipitation as opposed to fractional precipitation is proposed to be the tendency for charge neutralization to occur between different polymer molecules rather than intramolecularly.

Let us consider coprecipitation first. Its hallmark is the precipitation of the bulk of all the polymeric components at a pH

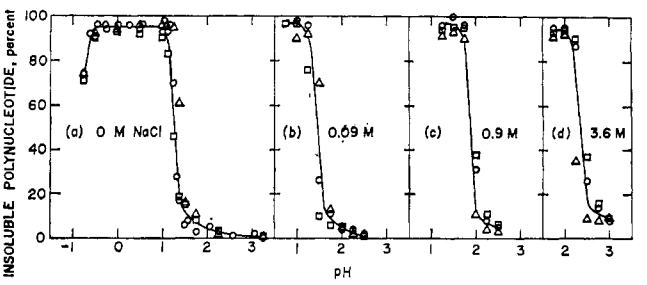

FIGURE 10: Effect of pH on the solubility of DNA from *E. coli*, *C. perfringens*, or calf thymus glands. Procedure as described under Materials and Methods; media were HCl solutions containing the final concentrations of NaCl indicated in the figure: *E. coli* DNA (□), *C. perfringens* DNA (Δ), calf thymus DNA (○).

determined by the total base composition. The total base composition clearly influences the pH of precipitation. A mixture of poly(A) and poly(C) precipitates at a different pH than does a mixture of poly(A) and poly(G), etc. The pH of precipitation is not, however, sensitive to the backbone to which a particular base is attached. A number of examples have been presented where the same pH of precipitation occurs whether a given base composition stems from a mixture of homopolymers or derives from a heteropolymer. For example, in the absence of added salt, a mixture of poly(A) and poly(U) in equal proportions precipitates as a whole at nearly the same

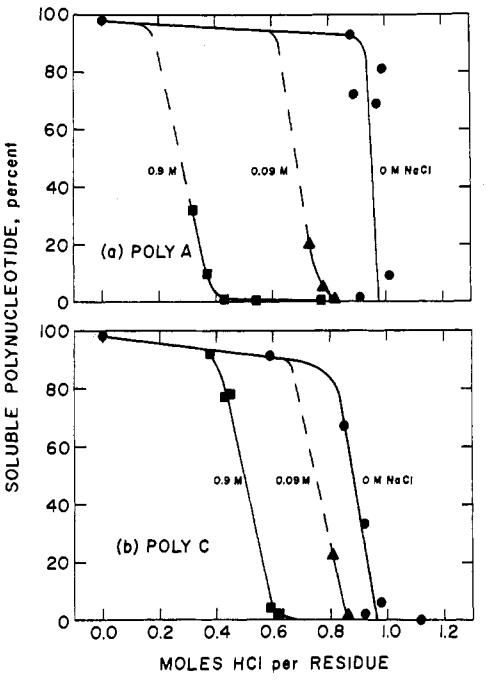

FIGURE 11: Estimation of the amount of acid necessary for precipitation of poly(A) or poly(C). Reaction mixtures containing 1 ml of solutions with poly(A) or poly(C) (5.0 μmol of phosphorus/ml), NaCl to yield the final concentrations indicated, and various amounts of HCl were held for 10 min at 0°, and centrifuged for 10 min at 8000*g* at 2–4°, and the supernatant fluids were decanted. Aliquots of these fluids were diluted and their absorbances at 260 mμ used to estimate the amounts of polymer remaining in the supernatant fluids. The bulk of each supernatant fluid was brought to room temperature and its pH determined. The amount of HCl which served to produce this pH was then corrected for the amount needed to lower the pH of the solution to this value in the absence of polymer; this correction was from 6 to 44% of the uncorrected value. The corrected values were used to calculate the amounts of HCl per residue.

pH as the random copolymer of the same base composition, poly(A,U). If the proportions of poly(A) and poly(U) are varied, the pH of precipitation varies; at the point of precipitation, the *total* contents of the solution precipitate. Clearly the polymer molecules interact in a nonstoichiometric fashion and, to a first approximation, it is immaterial to which phosphodiester backbone a particular base is attached. We will therefore consider that only the total base composition is of importance for coprecipitation.

We propose that formation of a stable precipitate requires the average charge of the polymeric components in solution to be reduced to some specific value which is not sensitive to the particular polymers involved, but which is dependent on the ionic strength of the medium. This is supported by the results of tests with the two polymers with the highest $pH_{1/2}$ values, namely poly(A) and poly(C). Proton uptake by either polymer indicated that >90% neutralization of the charge on the phosphate residues had occurred by the time of precipitation from salt-free media; progressively less complete titration was needed for precipitation as the ionic strength was raised. Assuming that the essentially total neutralization required for poly(A) and poly(C) is representative in salt-free media, several consequences immediately follow. First, it is titration of the final small fraction of uncharged bases which triggers precipitation; this is considered further below. Secondly, the final groups to be titrated occur in a structure which is almost isoelectric, so that we will consider the titrations of these last groups to involve negligible electrostatic work (Katchalsky and Miller, 1954) and we assume each group titrates essentially independently of the others, *i.e.*, the polyelectrolyte nature of these materials is minimized.

In short, the model assumes precipitation will be controlled by titration of groups which ionize independently of each other, and precipitation will occur when some fixed degree of titration has occurred. The expected pH of precipitation for a given base composition then may be calculated from estimates of the pK values of the various types of ionizable groups present and of the degree of charge neutralization required.

The pK values for several nucleosides were determined by spectrophotometric titrations at 2° in the absence of salt (see Appendix). Values for adenosine and cytidine agreed well with published values. There is a range of values for guanosine in the literature; our value lies within the range. Uridine and thymidine also were examined spectrophotometrically for indications of an acidic dissociation, particularly because uracil has been stated to have a p$K \simeq 0.5$ (Cohn, 1955); we found no indications of a dissociation with such a pK value for either uridine or thymidine. For several nucleosides, there were indications of further dissociations occurring below pH 0.

Poly(U) and poly(dT) precipitate from salt-free solutions around pH 0. If these polymers precipitate because of charge neutralization, they must have a titratable group with an acidic pK greater than zero, probably occurring around pH 1–2. Since spectrophotometric titrations of the nucleosides indicated the lack of such a group it seems very likely that it is titration of the charged group resulting from the primary dissociation of the phosphate residues which reduces the charge upon these polymers. The magnitudes of the acidic dissociation constants for analogous model compounds are of the proper order. For example, the pK values of the primary dissociation of CMP, AMP, GMP, and UMP are 0.8, 0.9, 0.7, and 1.0 (Levene and Simms, 1925); dialkyl phosphates, R_2PO_3H, with R = methyl, ethyl, *n*-propyl, or *n*-butyl, have pK_a' values of 1.29, 1.39, 1.59, or 1.72, respectively (Kumler and Eiler, 1943). Again, if precipitation is a consequence of almost complete charge neutralization, use of a monomer pK value is probably a fair approximation. A value was chosen for phosphate, pK = 0.9, which lies within the monomer range and when used in the calculations described below gives reasonable agreement with experimental results.

In summary then, the pK values for the various residues in the absence of added salt which will be used for calculations are cytidine, 4.5, adenosine, 3.8, guanosine, 2.2, and phosphate, 0.9. It is further assumed that precipitation from salt-free media occurs at the net isoelectric point of the solution, *i.e.*, the average charge per residue k, defined by eq 1, is zero,

$$k = \alpha_p + \sum_{i=1}^{n} \alpha_i X_i \qquad (1)$$

where α_p = average fractional charge upon the phosphate residues, α_i = average fractional charge upon a base of the ith type, X_i = mole fraction of a base of the ith type, and n = total number of types of bases present.

Because of the assumption of independent ionization of each base, we may write the fractional charge per residue as in eq 2. For solutions of simple composition, eq 1 and 2 may be

$$pH = pK_p + \log \frac{\alpha_p}{1 - \alpha_p} = pK_i + \log \frac{1 - \alpha_i}{\alpha_i} \qquad (2)$$

solved explicitly and a pH value readily obtained corresponding to whatever value of k is specified. Such solutions rapidly become difficult as the number of bases increases and so we have used the following alternative which allows rapid estimation of the $pH_{1/2}$ values for samples of any base composition for which monomer pK values are assumed. A table[1] was constructed with entries for each type of ionizable group, listing the fractional charge on that group at intervals of 0.1 unit of pH using eq 2. For any given pH, simply multiplying the mole fraction of each type of residue present by the fractional charge upon that residue at that pH value as listed in the table and adding up the resultant positive and negative charges according to eq 1 gives the value of k, the average charge per residue at that pH value. The pH at which precipitation is predicted to occur is then simply that for which the isoelectric condition pertains (k = 0). In Table I, predicted and observed values are compared for a variety of polymers, including heteropolymers, mixtures of homopolymers, and both polyribo- and polydeoxyribonucleotides. For the 31 pertinent entries, the average absolute deviation between observed and predicted values is 0.12 pH unit. We consider this agreement sufficiently good that there is little chance of the basic mechanism of charge neutralization being incorrect. Certainly there are parameters which could be varied and still retain reasonable agreement. Reducing the pK value assumed for guanosine residues by about 0.3 pH unit would still leave the pK value within the range reported in the literature and would improve the agreement between the observed and predicted $pH_{1/2}$ values; such arbitrary changing of the set of self-

[1] Supplementary material consisting of this table will appear following these pages in the microfilm edition of this volume of the journal. Single copies may be obtained from the Business Operations Office, Books and Journals Division, American Chemical Society, 1155 Sixteenth St., N.W., Washington, D. C. 20036. Remit check or money order for $3.00 for photocopy or $2.00 for microfiche, referring to code number BIO-73-2916.

consistent spectrophotometric pK values described in the Appendix was resisted in the present context but may be justified for other purposes. Also, precipitation with a partial negative charge on the polymers (*i.e.*, values of k less than zero) may well occur, and presumably generally does in media of higher ionic strength. Precipitation at slightly negative k values may also occur for those polymers (poly(U) and poly(dT)) where the base has no group which can be protonated in the pertinent pH range. In general, calculations of $pH_{1/2}$ for values of $k < -0.1$ give much poorer fit with the $pH_{1/2}$ values observed in the absence of added salt.

The model outlined for coprecipitation fails to consider the influence of secondary structure upon polymer precipitation. Polymer pK values may be greatly altered because of the participation of bases in "acid" structures (protonated or hemiprotonated bases) (*e.g.*, Steiner and Beers, 1958; Hartman and Rich, 1965). But the model fits reasonably well in spite of this, probably for a number of reasons. Even though the bulk of the bases may participate in some given ordered "acid" structure and accordingly have a shift in pK value, a significant fraction of the bases are likely not to participate in the ordered regions because of mismatching (frayed ends, looping out, intermolecular linking strands); the bases on these mismatched regions would probably have pK values like those in an amorphous structure. If the charged bases stabilize the "acid" structure (*e.g.*, poly(A), see below), bases in the ordered fraction should titrate at higher pH values than those in the amorphous fraction. Hence the last residues to titrate before precipitation might have a pK relatively unaffected by the "acid" secondary structure. It is just this last fraction of the bases to titrate which controls the degree of neutralization required for precipitation from media of low ionic strength. Secondly, in at least one case (poly(C)), an "acid" structure is destroyed in a pH zone before precipitation occurs (Hartman and Rich, 1965). Finally, there were several instances in which coprecipitation did not occur, and in these cases the effects of secondary structure may indeed have been dominant.

Fractional precipitation differs from coprecipitation in that some component of a solution can be precipitated selectively rather than all components interacting and precipitating together. If we assume that charge neutralization must precede precipitation of any polynucleotide, then this difference in behavior may simply be a reflection of the probability of intermolecular *vs.* intramolecular charge neutralization for a given component. Intramolecular charge neutralization in this acidic range of pH implies stable "acid" structures, and so we might ask whether fractional precipitates contain relatively well-ordered secondary structures. In one case this is certainly true; poly(A) fractionally precipitated from HCl solutions containing 0.09 M NaCl in the presence of 1 equiv of poly(U) forms fibers with an X-ray diffraction pattern unmistakably that of the "acid" structure of poly(A) (unpublished results). In the model for the "acid" structure proposed by Rich *et al.* (1961) it has been suggested that, when protonated, the N-1 of adenine will interact with the negatively charged phosphate residue in the adjoining chain. This may be the intramolecular charge neutralization needed for fractional precipitation. Fibers from the fractional precipitate formed by poly(C) under similar conditions have so far given patterns indicating little or no ordering. The semicrystalline hemiprotonated form of poly(C) described by Langridge and Rich (1963) would presumably have been destroyed before reaching the pH of precipitation. The fractional precipitates of both poly(A) and poly(C) redissolve as the pH is lowered somewhat further, implying the "acid" structures in the fractional precipitates are unstable beyond some degree of protonation, possibly because the titration of the phosphate residues causes stereochemical changes in the backbone or because a net positive charge is being built up as the phosphate residues are titrated.

An additional number of precipitates, including those of other homoribopolymers, of DNA, and of tRNA, have been examined as powders by X-ray diffraction. The poly(A) precipitate had unmistakable ordering; the others had much less or none. A technique of freezing acidic solutions of poly(A) to form partially oriented solids has been described (Zimmerman and Coleman, 1972). We used this approach on some of the other homopolymers; poly(G) clearly formed a highly ordered structure in such samples while poly(C) did not. In short, there is no evidence for a *generally* occurring well-ordered structure in these "acid" precipitates.

Dore *et al.* (1972) have recently demonstrated that DNA goes through a sharp transition at pH values below the pH value where it normally denatures. If this transition is carried out in very dilute solution, they found a tendency for the DNA to collapse intramolecularly; in more concentrated solutions aggregation was apparent. The acidity at which they observed the intramolecular transition is within a few tenths of a pH unit of that at which we find precipitation of DNA of similar base composition under fairly similar conditions. Hence their results and ours taken together suggest that at sufficiently low polymer concentrations, polynucleotides may generally undergo an intramolecular "isoelectric" collapse (Katchalsky and Miller, 1954; Alfrey and Morawetz, 1952; Alfrey *et al.*, 1952) at pH values similar to those at which they show precipitation, at least in media of low ionic strength. Under some circumstances, Dore *et al.* (1972) were able to bring native DNA through the transition and then reneutralize it to yield material with the density of native DNA. They consider this behavior to indicate that the duplex structure is not necessarily disrupted upon going through the transition. We suggest that similar behavior might result even if denaturation did occur, as long as the sister strands were held in propinquity by mutual charge interaction so that upon reneutralization they could efficiently re-form their native structure.

There are a number of useful applications of the present work. For example, poly(U) is generally considered to be "acid-soluble." The tendency to be soluble in acidic solution seems to be at least partially a result of the absence of a group on uridine which is titratable in the proper pH range. This can be readily circumvented by addition of a polymer such as poly(A) or poly(C) which (in the absence of added salt) will give quantitative precipitation with poly(U). Of course, it is not necessary to use a polynucleotide; a carrier of bovine plasma albumin will also allow complete precipitation of poly(U), presumably for similar reasons. As another example, it may be useful to increase the pH at which precipitation of a polymer occurs, either to decrease hydrolytic changes, to preserve specific secondary structures with limited ranges of pH stability or, for other reasons. This increase in pH of precipitation may be effected by adding another polymer with a high isoelectric point (under conditions where they give coprecipitation) or by increasing the ionic strength. Similarly, the pH of precipitation could be depressed by admixture with the proper polyanion. In addition to precipitation at acidic pH, several of the homopolymers (poly(A), poly(G), and poly(U)) became insoluble at more neutral pH values if concentrations of NaCl were high. There is a possible use of these

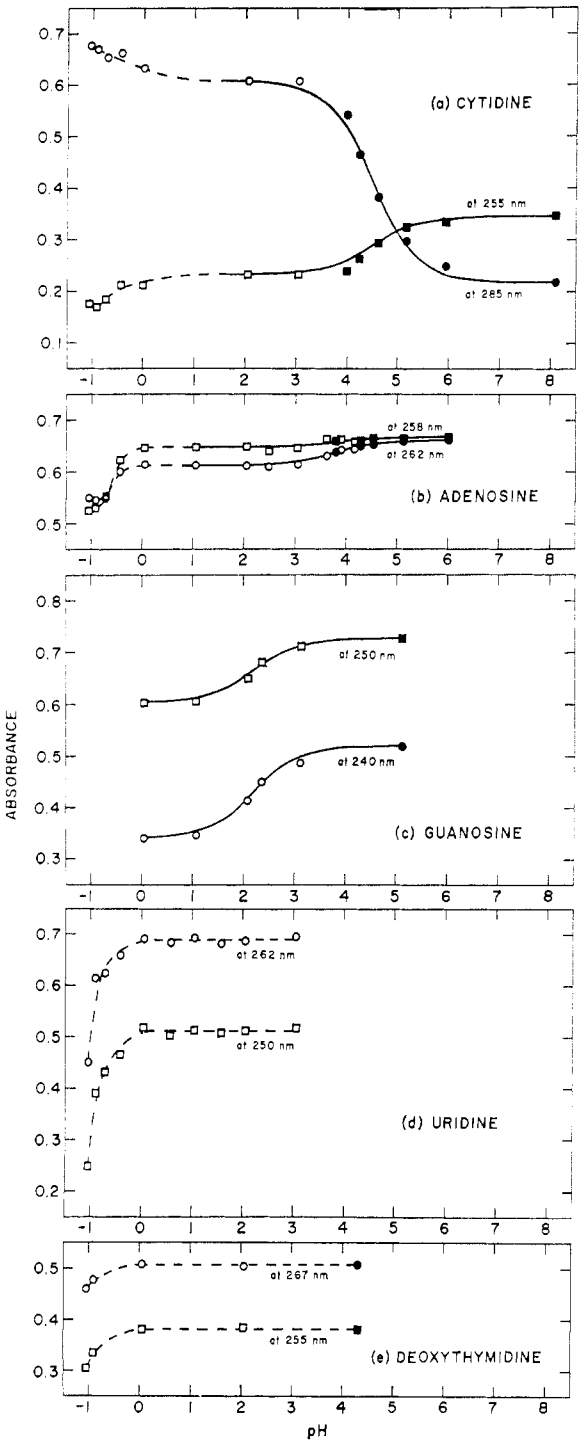

FIGURE 12: Ultraviolet absorbance of several nucleosides in acidic solutions. Stock solutions of nucleosides (A grade, Calbiochem) were diluted to 50–70 nmol/ml in HCl solutions (open symbols) or in 0.01 M buffer solutions (closed symbols) of sodium acetate for pH 3.8–6.0 or Tris-Cl for pH 8. The pH conventions are described in the main paper. Spectra were recorded after equilibration to 2 ± 0.2° in a thermostated cell holder of a Cary Model 14 spectrophotometer. The solid lines were calculated with the Henderson–Hasselbach equation for the stated pK values.

phase separations of poly(G) or poly(U) for molecular weight fractionation in a manner similar to that previously described for poly(A) by Eisenberg and Felsenfeld (1967).

Recently Drysdale and Righetti (1972) have demonstrated electrofocusing of several di- and trinucleotides. These materials formed bands at pH values generally corresponding to their isoelectric points; tRNA behaved in a complex fashion in this system. While, in principle, electrofocusing is an excellent way to take advantage of base compositional differences of nucleic acids, the lack of "carrier ampholytes" with a range extending below pH 3 restricts its application.

All the polynucleotides tested redissolve at values of pH below 0, probably due to further titration and to hydrolytic degradation. This and a number of other aspects of acid precipitation with specific application to poly(A) have been discussed elsewhere (Zimmerman and Coleman, 1972).

Precipitation measures massive molecular interactions. It seems to be adequate for the present survey, in large part because the differences being considered are not subtle. Study of interactions such as those described here by light scattering or by hydrodynamic methods would yield more information about the earlier molecular interactions, perhaps occurring several pH units above the point of precipitation and on occasion overlapping into a relatively neutral zone of pH. Early interactions may also show specificities different from those deduced from patterns of precipitation, since the latter seem to be a reflection of gross charge neutralization phenomena.

Acknowledgment

It is a pleasure to acknowledge the excellent participation of Alan Nadel in many of these experiments.

Appendix: Spectrophotometric Titrations of Cytidine, Adenosine, Guanosine, Uridine, and Deoxythymidine in Acidic Solutions

By Patricia McKinley and Steven B. Zimmerman

In order to perform the calculations described in the main paper, we needed estimates for the magnitude of any dissociation constants corresponding to pK values between 0 and neutrality for cytidine, adenosine, guanosine, uridine, and deoxythymidine. The ranges of pH covered in the literature generally do not extend to sufficiently acidic values and there also is disagreement between pK values of various authors for some of the nucleosides. Hence, we did spectrophotometric titrations under conditions similar to those employed for the precipitation experiments; the aim was to obtain a self-consistent set of apparent pK values under these conditions. Highest accuracy was not necessary; estimation of pK values to within 0.1 unit was sufficient. Compilations of pK values of nucleic acid derivatives may be consulted for references to the extensive literature (Kochetkov and Budovskii, 1971; Sober, 1970); a recent careful study of cytidine and CMP should also be noted (Wrobel *et al.*, 1970).

Two procedures were used to check for degradation of the nucleosides in the strongly acidic media used for some spectra. First, the spectra at the lowest pH values recorded in Figure 12 were tested over periods of at least 30 min and were found not to change. Secondly, a sample of each nucleoside was tested by paper chromatography (solvent A of Fuller *et al.* (1972) for cytidine, adenosine, guanosine, and uridine; solvent system No. II of P-L Laboratories (1970) for deoxythymidine). After exposure to 12 N HCl for 30 min at 0°, cytidine, adenosine, uridine, and deoxythymidine showed $\leq 3\%$ formation of the corresponding free bases. Similar amounts of each of these nucleosides were recovered at the appropriate nucleoside R_F values whether or not the nucleosides were exposed to acid. Recoveries upon elution from paper were 56–74% of

the amounts added to incubation mixtures based upon ultraviolet absorbance. Guanosine did form large amounts of a material with the R_F of guanine after incubation in 12 N HCl for 30 min at 0°; after similar incubation in 1 N HCl, 6% of guanine was found.

The results of the spectrophotometric titrations are shown in Figure 12. Spectral changes for cytidine, adenosine, and guanosine correspond to pK values of 4.5, 3.8 and 2.2, respectively. Uridine and deoxythymidine spectra were unchanged from pH 0 to at least 3, giving no indication of a pK value within this range. These pK values are in agreement with those in the literature for cytidine and adenosine after they are extrapolated to 2°; the value for guanosine agrees with many prior determinations but differs from some others which cluster at pK = 1.6 (Kochetkov and Budovskii, 1971). The nucleosides showed further spectral changes in strongly acidic media (1–12 N HCl); while these changes are probably not due to degradation for the reasons outlined above, they do not necessarily arise from further protonations since the character of the solvent is changing in these highly concentrated solutions of HCl. Spectral changes under similar circumstances have been noted for a series of cytosine derivatives (Wempen *et al.*, 1961).

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