recrystallized twice from 90% ethanol, and then had m.p. 214-215° dec. (lit.[5] 214° dec.), $[\alpha]^{20}D$ +33.9 ± 1.0° (*c* 1, 6.0 *N* hydrochloric acid) [authentic sample isolated from *Lathyrus* seeds (*cf.* ref. 12) $[\alpha]^{20}D$ +32.8 ± 1.0° (*c* 1, 6.0 *N* hydrochloric acid)].

Anal. Calcd. for $C_7H_{16}N_4O_2Cl_2$: C, 32.44; H, 6.18; N, 21.62; Cl, 27.41. Found: C, 32.73; H, 6.04; N, 21.86; Cl, 27.41.

*N,N'-Dicarbobenzyloxy-γ-hydroxy-*L*-lysine Lactone (Xb).* This derivative was synthesized in the same way as described for the preparation of dicarbobenzyloxy-δ-hydroxylysine lactone.[23] The yield of recrystallized lactone was 78%, m.p. 116–118°, $[\alpha]^{20}D$ −2.9° (*c* 1, dimethyl sulfoxide).

Anal. Calcd. for $C_{22}H_{23}N_2O_6$: C, 64.06; H, 5.87; N, 6.79. Found: C, 64.16; H, 5.67; N, 6.90.

(23) N. Izumiya, Y. Fujita, and M. Ohno, *Bull. Chem. Soc. Japan*, **35**, 2006 (1962).

The Tautomeric Form of Helical Polyribocytidylic Acid

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In contrast to the pK of 4.3 seen in the titration curve of cytidine, the polymer of cytidylic acid exhibits two pK values in the acid range, one at 5.7 and the other at 3.0. Each of these involves the uptake of one-half proton for each base. At neutral pH the polymer is single stranded, while below pH 5.7 it is believed to exist as a two-stranded helix in which the bases are held together by three hydrogen bonds, one of which can form only when a proton is added to one ring nitrogen for each pair of bases. Between pH 5.7 and 3.0 the helix is stable, while lowering the pH below 3.0 destroys the structure through the addition of more protons. Infrared studies show that both protonated and nonprotonated cytosine rings are present when the helical form of the molecule is stable. Formation of the helical complex can also be seen by changes in the ultraviolet spectrum as well as in the sedimentation properties of the polymer. Thermal denaturation studies show that the addition of the first half proton per base stabilizes the molecule, while addition of the second half proton per base destabilizes it. These properties are all in agreement with the proposed type of hydrogen bonding.

The synthetic polyribonucleotides represent a large class of polymer molecules which have been studied extensively because of their close relation to the naturally occurring nucleic acids. They have often been used as model compounds for understanding the reactivity of the naturally occurring polymers, and a great deal is known about them at the present time. An X-ray diffraction study has been carried out on oriented fibers of polyribocytidylic acid (poly C) which shows that this molecule exists in a helical form with a pitch of 37.3 Å. and a diameter of approximately 14 Å.[1] The molecule is made of two strands of polycytidylic acid which are helically wrapped round each other in a parallel configuration in which they are related by a twofold rotation axis. An unusual type of hydrogen bonding was postulated as responsible for holding the two chains together. In this form the two strands are held together by sets of three hydrogen bonds between the cytosine bases in the center of the molecule. In order to achieve this type of hydrogen bonding, one of the two cytosine rings must be protonated, as shown in Figure 1. This type of hydrogen bonding has also been found in the crystal structure of cytosine-5-acetic acid.[2]

The present studies were undertaken in an attempt to answer the following question: Does the poly C molecule exist in a helical form in solution and does it maintain the same type of hydrogen bonding between the strands which was suggested by the X-ray diffraction study in the solid state? Akinrimisi, *et al.*,[3] have presented some thermal denaturation and optical rotatory data related to this question. Here we present the results of acid–base titration, infrared and ultraviolet spectroscopy, and thermal denaturation studies from which we conclude that the molecule has a structure in solution which is very similar to that seen in the solid state.

Methods and Materials

Samples. The poly C used in these experiments came from two sources. Part of it was polymerized by using a polynucleotide phosphorylase enzyme obtained from *Micrococcus leisodeikticus*, with methods which have already been described.[4] After the cytidine diphosphate and enzymes had been mixed, they were allowed to incubate at 37° for 4 hr. and were then precipitated by adding two volumes of cold 95% ethyl alcohol. The precipitate was redissolved in water and precipitated a second time with alcohol. The polymer was then dissolved in water again and dialyzed exhaustively against 5×10^{-3} M NaCl at neutral pH to rid the preparation of contaminating residual cytidine diphosphate. The polymer solution was frozen, lyophilized, and stored in the cold in the form of dry fibers. In addition, part of the poly C used in these experiments was obtained from Miles Chemical Company, Clifton, N. J. Although the materials from these two sources differed slightly in

(1) R. Langridge and A. Rich, *Nature*, **198**, 725 (1963).

(2) R. E. Marsh, R. Bierstedt, and E. L. Eichhorn, *Acta Cryst.*, **15**, 310 (1960).

(3) E. O. Akinrimisi, C. Sander, and P. O. P. Ts'o, *Biochemistry*, **2**, 340 (1963).

(4) G. Felsenfeld and A. Rich, *Biochim. Biophys. Acta*, **26**, 457 (1957).

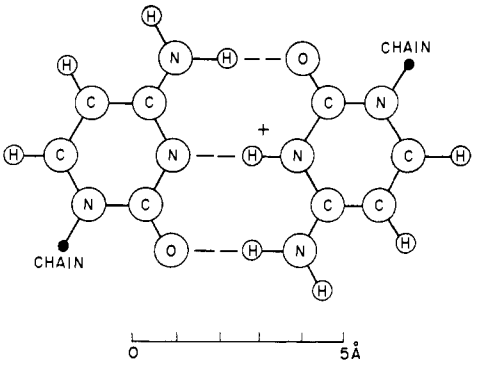

Figure 1. The hydrogen bonding between cytosine residues in the helical form of polyribocytidylic acid (poly C).

their mean sedimentation constant, no other difference in behavior was noted. The sedimentation constant of the polymers was determined by analytical ultracentrifugation in a Spinco Model E ultracentrifuge equipped with ultraviolet absorption optics. Densitometer absorption patterns were made on a Joyce Loebl double beam recording microdensitometer.

Titration Studies. The titration measurements were carried out on solutions which varied in concentration from 0.5 to 3 mM in cytidylic acid. The pH measurements were made with a Radiometer pH meter (Copenhagen, Denmark) and also with a Beckman Model 2 pH meter. The titration curves were obtained by adding aliquots of standardized 0.01 or 0.001 *N* HCl to the solution with a microburet. The solution was stirred continuously with a plastic coated magnetic stirring bar, and the temperature was maintained at 25 ± 0.5°.

Infrared Spectra. The samples for infrared study were dissolved to a concentration of about 6 mg./ml. in a D_2O solution which was 0.1 *M* in NaCl and 0.012 *M* in the sodium salt of trichloromethylphosphonic acid ($Na_2CCl_3PO_3$). This solution provided buffering action and infrared transparency in the regions of interest.

Neutral solutions of the sample were titrated to successively lower pD values. The use of sufficiently strong DCl for the titrations eliminated any significant concentration changes due to dilution. DCl was prepared from benzoyl chloride and D_2O.

A Leeds and Northrup pH meter with miniature electrodes was employed for the infrared studies and the pD of the solution was determined using the expression pD = pH (meter) + 0.4.[5,6]

Aliquots of the sample were removed at the desired pD values and were placed in a 0.025-mm. path length BaF_2 cell (Limit Corporation). The spectrum between 1800 and 1450 $cm.^{-1}$ was then recorded using a Perkin-Elmer Model 421 spectrophotometer with the ordinate scale expanded five times. Solvent compensation was accomplished by placing a matched cell containing the D_2O buffer solution in the reference beam. The instrumental reproducibility under these conditions was better than ±0.0019 absorbance units.

The molar extinction coefficient per mole of phosphorus was determined for poly C by alkaline hydrolysis. Samples were hydrolyzed to cytidylic acid with 0.3 *N* KOH for 24 hr. at 25°. The extinction coefficient was then measured and the concentration of poly C was determined by using as standard the extinction coefficient of cytidylic acid.[7]

Ultraviolet Spectra. The ultraviolet spectra were obtained in a Cary Model 14 recording spectrometer or Beckman Model DK 2. These spectra were normally obtained at room temperature (23°). Spectra were also obtained at other temperatures through the use of the Beckman Model DU spectrophotometer equipped with thermal spacers. In this instrument the cell chamber was equipped with a thermometer for recording the temperature of the cell compartment. The temperature could be maintained within ±0.5°. Melting temperatures were obtained by recording the optical density at each individual temperature after that temperature had been maintained for a 15-min. period. In this way, equilibrium was assured at each point.

Results and Interpretation

The experiments described here were carried out to determine whether or not the type of hydrogen bonding system shown in Figure 1 exists in the helical form of polycytidylic acid. This system of hydrogen bonding implies that a proton is added to a ring nitrogen (N_3) of one of the two cytosine rings. This makes it possible to form three hydrogen bonds to stabilize the helix. However, while such a structure is stabilized by one proton for each pair of bases, we might anticipate that it would be destabilized by the addition of two protons, that is, by full protonation. The results of the X-ray diffraction study thus predict that protonation should be carried out in two distinct steps, and our first experiments were designed to test this in solution.

Titration Experiments. In these experiments, the titration properties of the monomer, either cytidine or cytidylic acid, are compared with those of the polymer. Figure 2a shows the acid titration curve of cytidine in 0.1 *M* NaCl. In the acid range the molecule has a p*K* of 4.3. The curve is characteristic of a one-step process which is noncooperative in that the addition of one proton per base occurs over an extended range of almost 2 pH units. Figure 2b shows the titration curve for poly C in 0.1 *M* NaCl. Instead of a broad, one-step protonation curve, the titration curve of the polymer clearly shows a two-step process. The first step occurs sharply at pH 5.7. At that hydrogen ion concentration there is an abrupt uptake of protons onto the polymer without any resulting change in the pH of the solution. The abruptness of this change is characteristic of a cooperative effect in the polymer in which there is a structural transition involving many residues which are adding a proton. However, the curve does not go all the way up to one proton per nucleotide but rather begins to bend over at approximately 0.4 proton per nucleotide. Further addition of HCl results in the gradual addition of protons to the polymer. When the pH is lowered almost to 3.0, there are still only 0.7 proton taken up per nucleotide. At pH 3.0, there is another abrupt transition in which additional protons are taken up by the polymer with-

(5) N. C. Li, P. Tang, and R. Mathur, *J. Chem. Phys.*, **65**, 1074 (1961).

(6) P. Salomaa, L. L. Schaleger, and F. A. Long, *J. Am. Chem. Soc.*, **86**, 1 (1964).

(7) E. Chargaff and J. N. Davidson, "The Nucleic Acids," Academic Press Inc., New York, N. Y., 1955, p. 191.

out a resulting change in pH. Finally the polymer is fully protonated just under pH 3. A comparison of Figures 2a and 2b shows that the polymerization of cytosine residues into poly C results in the transformation of a noncooperative pK of 4.3 into two cooperative pK values, one occurring abruptly at pH 5.7 and the other at pH 3.0. These results are consistent with the interpretation that the pK at 5.7 is associated with the formation of the helical form of polycytidylic acid. This helical configuration is stabilized by the addition of one proton for every two bases since the titration curve begins to bend over in the region between 0.4 and 0.5 proton per nucleotide. At a pH of 4.5, one proton has been taken on for every two nucleotides, so that at this point the structure is fully stabilized by the addition of the single proton which is important for the hydrogen bonding system shown in Figure 1. The up-take of further protons is resisted, as shown in Figure 2b, since only when the pH is lowered to 3.0 does the helix open abruptly and allow the remaining cytosine residues to become protonated. The protonation is almost complete at pH 3.0, since the normal pK for the pyrimidine nitrogen is 4.3, as seen in Figure 2a.

This interpretation of the titration curve reinforces the observations made in the X-ray diffraction experiments.[1] The highly organized helical form of the poly C diffraction pattern was obtained from specimens in which the pH was lowered slightly below 5.5; however, lowering the pH to 4 resulted in a distortion in the position of the cytosine residues so that they lose the twofold rotation axis which is seen in Figure 1.

Ultracentrifuge Studies. The change in the physical state of poly C which is suggested by the titration curve with an inflection at 5.7 can also be demonstrated by a change in the macromolecular properties of the molecule. This is shown most directly by comparing the sedimentation constant at pH 6 with that at pH 5. When examined in the ultracentrifuge, a preparation of poly C at pH 6.0 had a mean sedimentation constant (s_{20}) of 5.44 S. However, on lowering the pH to 5, the value of s_{20} increased to 9.50 S. These experiments were carried out in 0.1 M NaCl using a 0.01 M sodium cacodylate buffer. Fasman, *et al.*,[8] have shown that the molecule at pH 6 or 7 retains considerable helical character even though it is in a single stranded form at that pH. The large increase in sedimentation rate on lowering the pH below the titration inflection point undoubtedly reflects the aggregation of many polymer strands into the two-stranded helical form with a corresponding increase in molecular weight.

Infrared Spectrum. Infrared spectra of nucleosides and polynucleotides in D_2O solution have been presented and discussed by Miles[9,10] and by Tsuboi, *et al.*[11] Through the use of chemically blocked tautomeric alternatives Miles showed that the cytosine residues in both cytidine and poly C were in the amino form in D_2O solution and that, at low pD, protonation occurred on the ring nitrogen.[10]

For cytidine and poly C at neutral pD infrared bands have been reported at about 1652 and 1618 cm.$^{-1}$, while for cytidine at low pD bands are seen at 1712, 1657, and 1591 cm.$^{-1}$.[9] The large changes observed in the infrared spectrum of cytidine in the protonated *vs.* the nonprotonated form[9,11] lead us to study these changes in both cytidine and poly C as a function of pH.

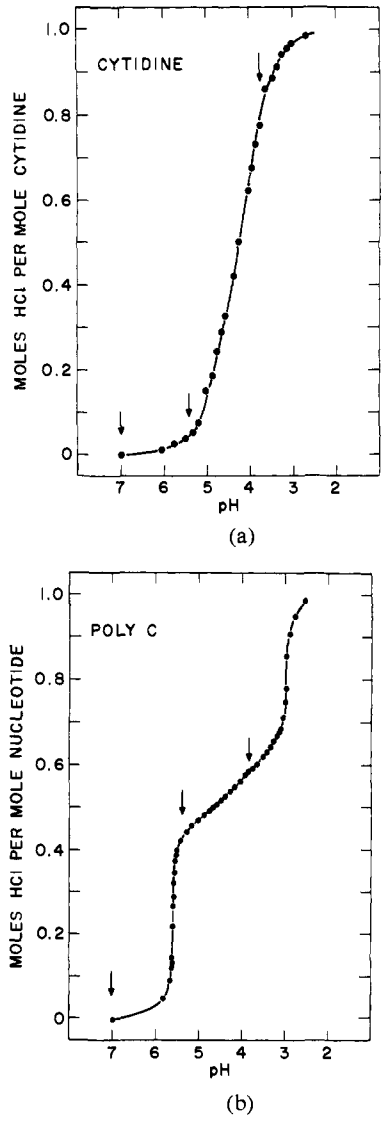

Figure 2. Acid–base titration curves of (a) cytidine and (b) poly C in 0.1 M NaCl at 25°. The cytidine curve shows a single pK of 4.3. The titration curve of poly C shows that on adding acid protons are taken on abruptly at two points with pK values of 5.7 and 3.0. Near pH 3, the solution of poly C becomes turbid, suggesting that the molecule becomes insoluble when it is fully neutralized. Almost half the bases are protonated just below pH 5.7. The vertical arrows in a and b indicate the pH values at which infrared spectra are presented in Figure 3.

The infrared spectra of D_2O solutions of cytidine and poly C in the region of 1800 to 1450 cm.$^{-1}$ are shown in Figures 3a and 3b. The absorption bands in these spectra arise from vibrations of the cytosine ring atoms and the external oxygen and nitrogen atoms. At pD 7 the bases are not protonated and bands are seen at 1652, 1616, 1582, 1522, and 1502 cm.$^{-1}$ for cytidine. At pD 3.8 nearly all the bases are protonated and bands now appear at 1709, 1655, 1588, and 1523 cm.$^{-1}$. The explanation of these changes lies in the fact that protonation of the ring nitrogen atom of the cytosine residue will change the spatial distribution of the π-electron

(8) G. D. Fasman, C. Lindblow, and L. Grossman, *Biochemistry*, **3**, 1015 (1964).
(9) H. T. Miles, *Biochim. Biophys. Acta*, **35**, 274 (1959).
(10) H. T. Miles, *Proc. Natl. Acad. Sci. U. S.*, **47**, 791 (1961).
(11) M. Tsuboi, Y. Kyogoku, and T. Shimanouchi, *Biochim. Biophys. Acta*, **55**, 1 (1962).

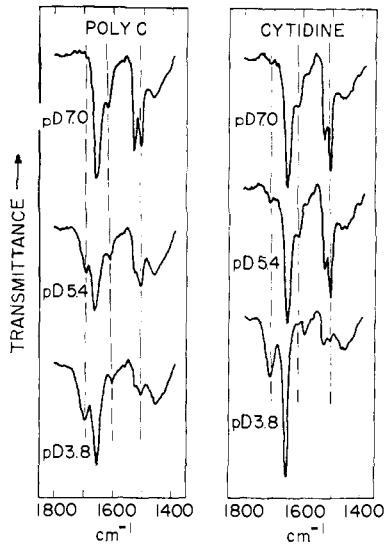

Figure 3. Infrared spectra taken in D_2O solutions with the pD fixed by 0.12 *M* sodium trichloromethylphosphonic acid buffer. The three pD values shown are indicated by vertical arrows on the titration curves in Figure 2. (a) Spectrum of cytidine; (b) spectrum of poly C.

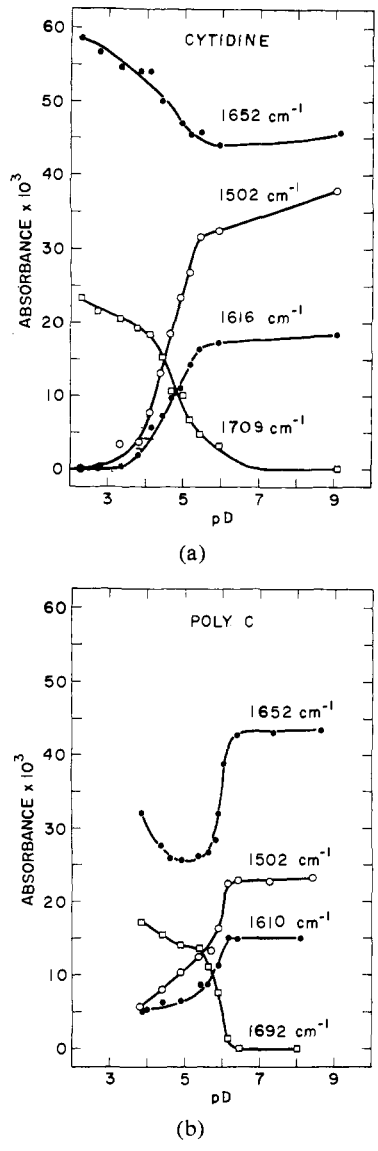

Figure 4. Absorbance of various infrared bands as a function of pD for (a) cytidine and (b) poly C. The band at 1652 cm.$^{-1}$ shows a strong hypochromism in the helical form of the molecule. The other bands confirm the existence of both protonated and nonprotonated forms at pD values in which the helical form is stable.

system; this changes the force constants and therefore alters the resulting normal modes of vibration which arise. Although some assignments of the bands have been attempted, existing knowledge is tenuous and such knowledge is unnecessary for the purpose of this paper. The question of exact assignments will therefore not be discussed, and attention will be restricted to the use of the intensities of the bands as indicators of the extent of titration.

The three spectra shown for poly C and cytidine were taken at pD values indicated by the arrows on the titration curves of Figures 2a and 2b. As expected from the titration curves, little change in the infrared spectrum of cytidine is observed between pD 7.0 and 5.4. By pD 3.8, however, the spectrum of the protonated species is obtained. We can take the strong band at 1502 cm.$^{-1}$ and the shoulder at 1616 cm.$^{-1}$ as indicative of the nonprotonated cytosine residue and the band at 1709 cm.$^{-1}$ as indicative of the protonated species. Less prominent band shifts and intensity changes will not be discussed.

Although the spectrum of poly C is nearly identical with that of cytidine at pD 7, a striking difference is observed at lower pD values. At pD 5.4 the poly C spectrum resembles a superposition of the neutral and acid spectra of cytidine with bands at 1692, 1658, 1610, 1523, and 1502 cm.$^{-1}$. This situation persists at pD 3.8 where a larger fraction of the protonated species is evident from the increased intensity of the 1692 cm.$^{-1}$ (1709 cm.$^{-1}$ in cytidine) band and the decreased intensity of the 1610 and 1502 cm.$^{-1}$ bands. This behavior is consistent with the chemical titration curve (Figure 2b).

Figures 4a and 4b present the quantitative variations of the absorbance of these bands as a function of pD for cytidine and poly C, respectively. Again differences in behavior are striking. In proceeding to lower pD, the curves for cytidine bend over at about pD 5.5 and smoothly decrease (or increase) until protonation is complete below pD 3. Besides a smooth decrease in the intensity of the bands at 1502 and 1616 cm.$^{-1}$ and an increase of the 1709 cm.$^{-1}$ band, it is seen that protonation also increases the intensity of the 1652 cm.$^{-1}$ band. A slight increase in the frequency of this band is also observed.

In proceeding to lower pD, the curves for poly C break more sharply than those for cytidine and the break occurs at about pD 6.2 as opposed to pD 5.5 for cytidine. The absorbance of the 1502 and 1610 cm.$^{-1}$ bands decreases rapidly below pD 6.2 and then become more level below about pD 5.5. The curve for the band at 1692 cm.$^{-1}$ increases sharply at pD 6.2 but also becomes more level below pD 5.5. The behavior of the absorbance of the 1652 cm.$^{-1}$ band of poly C is in even greater contrast with that of cytidine. The poly C band absorbance is seen to decrease sharply between pD 6.2 and 5.5, become level, and then turn upward at about pD 4.5. This drop in absorbance upon helix formation may be called a "hypochromic effect," although this lowering of the extinction co-

efficient probably does not arise from the same sort of interactions that produce hypochromism in the ultraviolet absorption spectrum.

The behavior of the infrared absorbance bands shown in Figures 4a and 4b should be compared with the chemical titration curves of Figures 2a and 2b. The cytidine curve (Figure 4a) shows the behavior expected for protonation of a species with a single pK in D_2O of about 4.6. The curve for poly C (Figure 4b) shows that there exists a pK in D_2O of about 6.0. The second pK shown in Figure 2b is not observable by infrared means since the poly C begins to precipitate just before this pK is reached, and spectra cannot be obtained. The displacement of the pK in D_2O to higher values than the pK in H_2O is expected.[12]

It should be noted that if the absorbance value at pD 5 is divided by the absorbance value at pD 7 for poly C bands, one obtains 0.47 for the 1610 cm.$^{-1}$ band and 0.48 for the 1502 cm.$^{-1}$ band. Since these bands are indicative of the nonprotonated cytosine residues and assuming that concentration is proportional to absorbance, we may say that these ratios provide evidence that the bases of poly C are indeed about one-half protonated near pD 5 in D_2O solution, in complete agreement with the chemical titration results.

A similar ratio cannot be constructed for the 1692 cm.$^{-1}$ band, since an estimate of protonation based on this band would involve knowledge of the absorbance value at pD 3 which is unobtainable. The 1652 cm.$^{-1}$ band also fails to provide a quantitative estimate of protonation since it is not directly correlated with the protonated or nonprotonated bases. Interpretation is further complicated here by the observed hypochromism, and this restricts the band to a qualitative indicator of helix formation.

Ultraviolet Spectroscopy. Changes in the configuration and structure of polynucleotides have often been followed by changes in the ultraviolet absorption spectrum. Some spectroscopic data for poly C have been presented previously.[3,13] Here we present in somewhat greater detail the spectrum of poly C and cytidylic acid as a function of pH. The results are shown in Figure 5a,b. In Figure 5a the ultraviolet absorption spectrum of cytidylic acid is plotted as a function of pH in the acid range. It can be seen that lowering the pH from 6.4 results in a shift of the absorption maximum from 270 to 278 mμ near pH 3. Accompanying this red shift there is also an increase in the intensity of the band. These spectra all pass through a single isosbestic point near 260 mμ. The spectrum of cytidylic acid in the acid range thus has the typical appearance of a chromophore undergoing a single transition such as would be found in the change associated with the addition of a proton with a pK of 4.3. This can be shown more clearly by plotting the absorbance as a function of pH at different wave lengths. In Figure 6a the absorbance of cytidylic acid as a function of pH is plotted for three different wave lengths. At 270 and 275 mμ, there is a gradual rise in the intensity of the absorbance as the pH is lowered. At 243 mμ there is a fall in the absorbance. The vertical dashed line

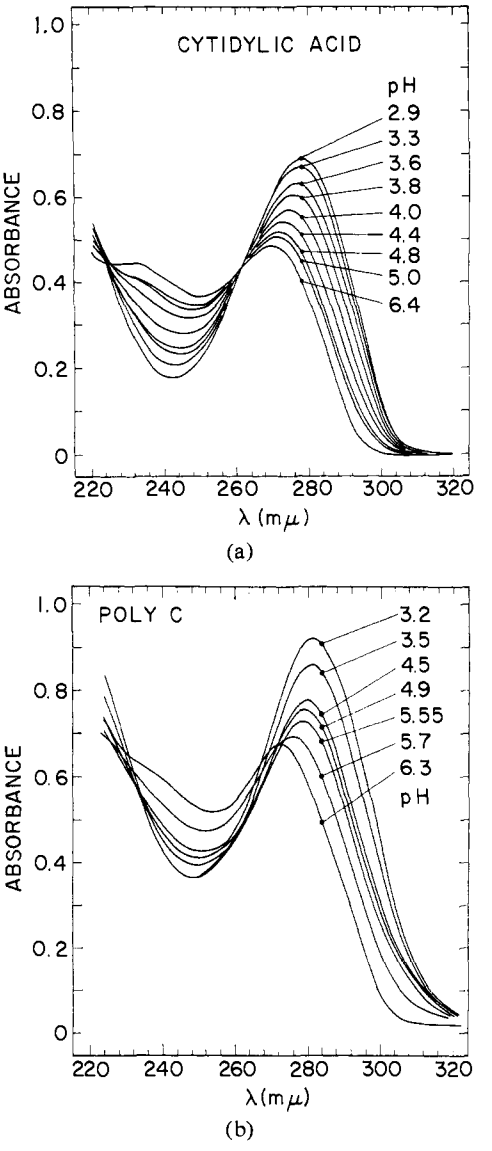

Figure 5. Ultraviolet absorption spectra of (a) cytidylic acid and (b) poly C in 0.1 M NaCl as a function of pH. Cytidylic acid has an isosbestic point at 262 mμ while the spectral changes in poly C are more complex.

in Figure 6a indicates the position of the pK at 4.3 which was determined in Figure 2a. The analogous ultraviolet absorption spectra for poly C are shown in Figure 5b. When the pH is lowered from 6.3, there is also a red shift and an associated rise in the absorption maximum. On changing the pH from 6.3 to 5.5, however, there is a rather abrupt red shift which is somewhat greater than that observed with cytidylic acid under analogous conditions. In contrast to what is observed for cytidylic acid, no isosbestic point is observed in the acid spectrum of poly C. The processes which take place when the polymer is protonated are thus rather more complex than those associated with protonating the monomer. An indication of this can be seen in Figure 6b in which the absorbance of poly C at three different wave lengths is plotted as a function of pH. The wave lengths chosen are the same as those which are shown for cytidylic acid in Figure 6a. It can be seen that there is an abrupt change in the absorbance which occurs at pH 5.7. The absorbance at 270 and 275 mμ rises sharply, while at 243 mμ it falls

(12) R. P. Bell, "The Proton in Chemistry," Cornell University Press, Ithaca, N. Y., 1959.

(13) P. O. P. Ts'o, G. K. Helmkamp, and C. Sander, *Biochim. Biophys. Acta*, **55**, 584 (1962).

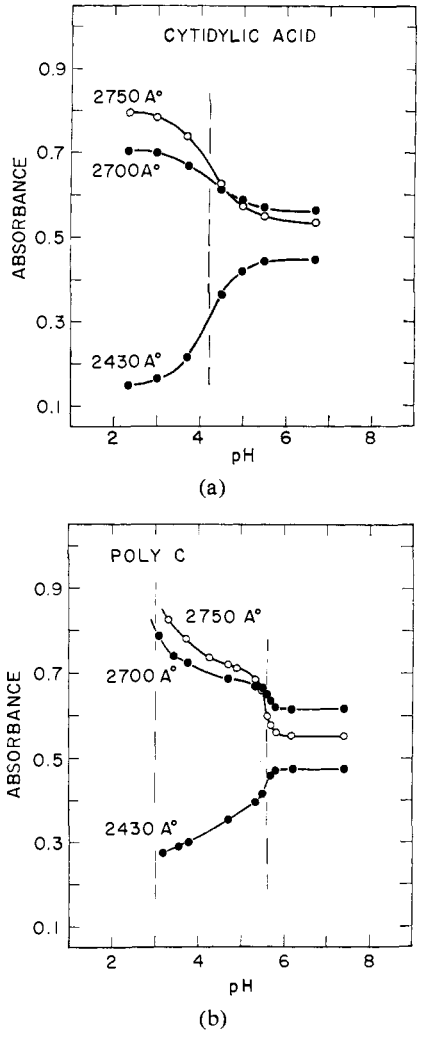

Figure 6. The ultraviolet absorbance at three wave lengths of (a) cytidylic acid and (b) poly C in 0.1 *M* NaCl as a function of pH. The dashed vertical lines represent the position of the acid-base p*K* values which were shown in Figure 2. The absorption changes gradually in cytidylic acid but abruptly at pH 5.7 in poly C.

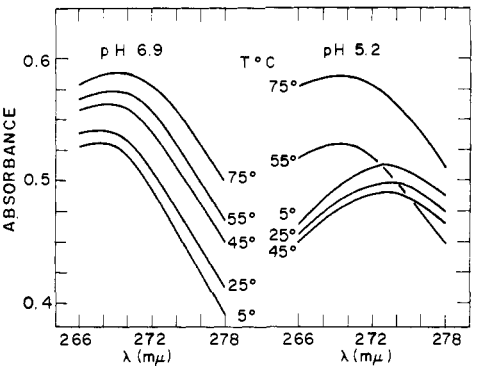

Figure 7. The ultraviolet absorption spectrum of poly C in 0.15 *M* NaCl at pH 6.9 and 5.2 as a function of temperature. Sodium cacodylate (0.01 *M*) was used as buffer. While the absorption increases steadily with increasing temperature at pH 6.9 the behavior at pH 5.2 is more complex. An abrupt change in the spectrum, which is associated with the breaking up of the helix, occurs at 49° at pH 5.2.

abruptly. The two vertical dashed lines in Figure 6b indicate the positions of the two p*K* values which are found in poly C as shown in Figure 2b. Unfortunately, poly C begins to come out of solution when the pH is lowered below 3, and the turbidity in the solution makes it impossible to record the spectrum. However, there is a slight indication in Figure 6b that the absorbance at 270 and 275 mμ may be going through another change as pH 3 is approached, since the curves start to rise rapidly. At any event, a careful study of the change in the absorption spectrum of poly C provides evidence for the structural discontinuity which occurs as the pH is lowered below the p*K* of 5.7.

Thermal Denaturation Studies. Multistranded polynucleotide structures break up in a cooperative manner at elevated temperatures and this "melting" behavior is usually characteristic of the molecule. Akinrimisi, *et al.*[3] have reported some thermal denaturation studies on poly C. Here we present further details of the spectrum of poly C as a function of temperature and pH. Figure 7 shows the absorption spectrum of poly C near its absorption maximum as a function of temperature for both the neutral molecule and the helical form at pH 5.2. At pH 6.9, the absorption maximum of poly C increases gradually with increasing temperature. Since poly C has some hypochromicity at neutral pH when it is single stranded, the effect of increasing temperature is to reduce the parallel stacking of bases which is believed to be associated with hypochromism. In the acid form of the molecule the spectral changes are more complex (Figure 7). On going from 5 to 45°, there is a decrease in the absorption maximum. A similar effect is seen in the monomer itself,[13] and it is probably associated with the loss of protons as the temperature is raised. The same effect probably occurs in the polymer resulting in a corresponding decrease in the absorption maximum. However, at 49° an abrupt transition occurs, and the absorption maximum shifts from 273 to 269 mμ. As the temperature is raised further, there is a rise in the absorption maximum which is similar to that seen in the poly C at pH 6.9. Thus, upon heating, the spectrum of the helical form of poly C at pH 5.2 undergoes three sequential changes: a lowering of the maximum at 273 mμ; a blue shift of the maximum so that the spectrum now is similar to that seen at neutral pH; and finally a rise in the optical density as the temperature is raised further. At 75° and pH 5.2 the absorption spectrum is very similar to that of the heated neutral form of poly C. We can use the abrupt shift in the position of the maximum as an indication of the melting of poly C. It occurs very sharply at a definite temperature and has the abruptness characteristic of a cooperative melting such as that seen in the absorption spectrum of DNA upon heating. As can be seen in Figure 7, a melting curve for poly C measured at a fixed wave length would have an initial component in which the optical density falls as temperature is increased. This is followed by an abrupt rise, and the break in the curve is taken as the melting temperature. For poly C, the break in the curve is much sharper than the corresponding transition in DNA or other complementary polynucleotides. In Figure 8 we have plotted the melting temperature as a function of pH for two ionic strengths. The results agree approximately with those reported by Akinrimisi, *et al.*[3] At ionic strength 0.15 *M* NaCl there is no melting temperature which can be observed

at a pH higher than 5.7. However, as the pH is dropped below 5.7, there is an abrupt rise in the melting temperature and a plateau is reached between pH 3.8 and 4.4. As seen in Figure 2b, this is the region in which approximately one proton has been added for every two cytosine bases. Lowering the pH further results in the addition of more protons, and these can only be added by opening up the pairing seen in Figure 1. This is accompanied by a destabilization of the helix as shown by a drop in the melting temperature. When the ionic strength of the solution is raised to 1 *M* NaCl there is a shift in the melting temperature. The helix does not begin to form until pH 5.4 and, in addition, the melting temperature is decreased. The poly C helix is thus destabilized by the addition of added electrolyte in sharp contrast to the enhanced stability of DNA and other polynucleotides. This difference is readily explained by the fact that the helical form of poly C with the hydrogen bonding shown in Figure 1 is stabilized somewhat by forming an inner salt. The positive charge centrally located on the pyrimidines partially neutralizes the two negative charges on the ribose phosphate chains on the outside of the helix. The molecule is stabilized by this electrostatic interaction. Raising the ionic strength reduces the magnitude of the interaction and this results in a destabilization of the molecule as shown by the lower melting temperature. The same electrolyte effect is seen in the helical form of polyadenylic acid[14] which forms at acid pH. In that structure the proton is also attached to the centrally located purine bases and it neutralizes the charge on the externally located phosphate groups.[15]

Several types of experimental investigations have been described above which suggest that the poly C molecule has the same form in the solution as that which

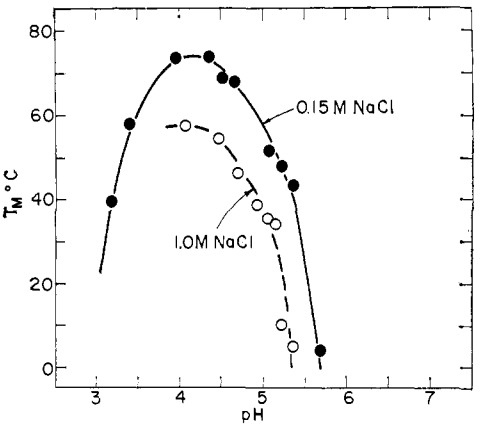

Figure 8. The melting temperature of poly C as a function of pH and ionic strength. In 0.15 *M* NaCl no melting is observed above pH 5.7 where the molecule is single stranded. Increasing ionic strength decreases the melting temperature down to pH 4.

was observed by X-ray diffraction studies of fibers in the solid state.[1] The titration experiments have shown that the single p*K* which is observed in cytidine or cytidylic acid at pH 4.3 has been modified in poly C so that half of the protons are titrated in a cooperative manner at pH 5.7, while the other half are titrated cooperatively at pH 3.0. In the region between pH 5.7 and 4, vibrations from both protonated and nonprotonated cytosine rings are seen in the infrared spectrum which also imply that the polymer is half protonated. Further evidence for helix formation at pH 5.7 is seen from the changes in the ultraviolet absorption spectra. Finally, thermal denaturation experiments are also consistent with the interpretation that the helical form of poly C is held together by pyrimidine rings which are only half protonated.

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(14) P. O. P. Ts'o, G. K. Helmkamp, and C. Sander, *Proc. Natl. Acad. Sci. U. S.*, **48**, 686 (1962).
(15) A. Rich, D. R. Davies, F. H. C. Crick, and J. D. Watson, *J. Mol. Biol.*, **3**, 71 (1961).

Studies on Peptides. II. Synthesis and Physiological Properties of D-Histidyl-D-phenylalanyl-D-arginyl-D-tryptophylglycine, an Optical Antipode of an Active Fragment of α-Melanocyte-Stimulating Hormone[1,2]

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Synthesis of the pentapeptide D-*histidyl*-D-*phenylalanyl*-D-*arginyl*-D-*tryptophylglycine, an optical antipode of an active fragment of α-melanocyte-stimulating hormone, is described. This synthetic peptide has been shown to inhibit the action of* L-*histidyl*-L-*phenylalanyl*-L-*arginyl*-L-*tryptophylglycine in frog melanocyte, in vivo and in vitro.*

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(2) Preliminary report of the biological observations described herein has appeared in *Biochim. Biophys. Acta*, **90**, 201 (1964). The authors wish to express their appreciation to Dr. K. Hano and Mr. M. Koida, University of Osaka, for the biological assays which were performed according to the method of K. Shizume, A. B. Lerner, and T. B. Fitzpatrick, *Endocrinology*, **54**, 553 (1954).