The Helical Conformations of Polycytidylic Acid: Studies on the Forces Involved*

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Polycytidylic acid (poly C) has been shown, by optical rotatory dispersion, to exist in a highly ordered secondary structure at both *p*H 4.1 and 7.0. A direct measure of these secondary structures (probably helical) of poly C at *p*H 4.1 and 7.0 is demonstrated by observing Cotton effects in one of the major ultraviolet-absorption bands. The influence of amino-group hydrogen bonds on the helical stability of poly C at neutral *p*H is negligible, as reacting them with formaldehyde causes no destabilization of structure. The forces responsible for maintaining the helical structure at neutral *p*H have been identified as hydrophobic in nature. This is demonstrated by the use of ethylene glycol which causes the complete collapse of the secondary structure. It is suggested that this helical conformation is a single-stranded structure stabilized by base stacking. The hyperchromicity observed during the loss of the secondary structure of poly C at *p*H 7.0, owing to the unstacking of the bases, is larger than that observed for the collapse of the hydrogen-bonded structure at *p*H 4.1. At *p*H 4.1 the helical stability of poly C is qualitatively and quantitatively different from that observed for the helical structure at *p*H 7.0.

The native helical secondary structure of DNA was originally thought to derive its stability through weak hydrogen bonds that maintain the double strand intact. The suggestion that forces other than hydrogen bonds might exist in nucleic acids is derived from the effects of hydrogen-bond-breaking reagents and reagents influencing hydrophobic forces[1] (Rice and Doty, 1957; Gordon and Jencks, 1963; Levine *et al.*, 1963). Evidence for the importance of hydrophobic forces is obtained from the destabilization of DNA by various organic solvents (Gordon and Jencks, 1963; Levine *et al.*, 1963; Geiduschek and Herskovits, 1961; Herskovits *et al.*, 1961; Helmkamp and Ts'o, 1961; Hamaguchi and Geiduschek, 1962; Ts'o *et al.*, 1962a,b; Herskovits, 1963).

Further support for the implication of hydrophobic forces in stabilization can be derived from the studies of Tinoco (1960a,b, 1961; Tinoco *et al.*, 1963; Devoe and Tinoco, 1962a,b), Zimm and Kallenbach (1962), and Rhodes (1960), who have proposed a theory for the hypochromism observed for polynucleotides. Hypochromism is stated to be mainly due to the dispersion-force interactions between neighboring base pairs because of their proximity and parallel stacking along the chain. This latter interaction could be interpreted as the type of interaction associated with hydrophobic forces. It must be assumed, therefore, that absorbance changes under conditions of denaturation can reflect either hydrogen-bond breakage or the disruption of base-base interactions.

The heterogeneity and variation of composition in different DNA samples is sufficiently complex that studies of the interactions between bases and their effects on secondary structure cannot be made with any degree of certainty. It is for this reason that homopolyribonucleotides provide an ideal model for studying the forces involved in maintaining the secondary structure of polynucleotides.

Studies on polycytidylic acid (poly C), in aqueous solution, have indicated its helical nature under acidic conditions (Ts'o *et al.*, 1962a; Steiner and Beers, 1961; Helmkamp and Ts'o, 1962; Akinrimisi *et al.*, 1963). This conclusion was based upon its hydrodynamic, absorbance, and optical rotatory dispersion properties. The X-ray diffraction studies by Langridge and Rich (1962) of poly C fibers drawn from aqueous solution at *p*H 5.5 were interpreted as showing that poly C forms a two-stranded helical structure. At higher *p*H values, however, no ordered structure was detected. The proposed hydrogen-bonded structure (Akinrimisi *et al.*, 1963; Langridge and Rich, 1962), at *p*H 5.5, is also similar to that suggested for the crystal form of cytosine-5-acetic acid (Marsh *et al.*, 1962). The helical stability is assumed to arise from three hydrogen bonds per pair. A pair of cytosine residues is hydrogen bonded across the center of the helix, sharing two hydrogen bonds involving their carbonyl and amino groups. In addition, at the pK_a of cytosine, a single proton is located between and shared by the same ring nitrogen atoms of the pyrimidines.

The stability of this helical structure, interpreted along these lines, agrees with that proposed by Watson and Crick (1953) for the stabilization of the double-stranded DNA molecule. A recent study employing circular dichroism has indicated that poly C also exists as a helix at *p*H 6.8 (Brahms, 1963).

From the above discussion it is apparent that it cannot be stated categorically that absorbance changes are a reflection of one type of interaction or the other unless specific techniques are employed which can discriminate between them. This report describes experiments in which the secondary structure of poly C is examined by ORD[2] and ultraviolet spectrophotometry under circumstances in which the contribution of hydrophobic forces and hydrogen bonds can be measured independently. These experiments demonstrate that poly C at *p*H 7 exists as a highly ordered asym-

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[1] Hydrophobic forces include van der Waals forces, the interaction of the π-electron systems, and the clustering tendency of nonpolar groups owing to the strong solvent-solvent interaction of water.

[2] Abbreviation used in this work: ORD, optical rotatory dispersion.

metric structure, probably helical, and that the total helicity of this polymer at this *p*H can be maintained in the absence of hydrogen bonding. The helical structure at *p*H 4.1 in aqueous solution, previously implied, is also confirmed.

Materials and Methods

Optical Rotatory Dispersion (ORD).—Measurements of the optical rotation were made with a Bendix-Ericcson Polarimatic 62 automatic recording spectropolarimeter.[3] This instrument is estimated to read ±0.0002° in the wavelength range 600–185 mμ. The recorder attachment was a Sargent Recorder Model SR. The dispersions were performed with a slit of 1 mm, and water-jacketed fused quartz cells of 0.1 and 0.01 dm length, selected for minimal birefringence, were used.[4] Temperature studies were performed by using a Haake Model F attachment for thermostatic control. The temperature in the cells was also monitored by using a TRI-R electronic thermometer with a bimetallic probe. The molar extinction coefficient per mole of phosphorous of poly C was taken to be $E_{(p)} = 6.3 \times 10^3$ (269 mμ) (Warner, 1957) at *p*H 7.0 in 0.1 M salt and that for cytidine-5′-phosphate (CMP) $E_{(p)} = 6.6 \times 10^3$ (267 mμ) (Beaven *et al.*, 1955). Concentrations were estimated by running a simultaneous standard under the given conditions. Optical density measurements were made on a Cary Model 14 recording spectrophotometer, with the same cells as used for the ORD measurements. The ORD data are expressed in terms of $[m']_\lambda$, the reduced mean residue rotation (Urnes and Doty, 1961; Fasman, 1963), defined as $[m']_\lambda = [\alpha]_\lambda \times (\text{mrw}/100) \times (3/n^2 + 2)$, where $[\alpha]_\lambda$ is the specific rotation at wavelength λ, mrw is the mean residue weight (305.21 for poly C and 323 for CMP), and *n* is the refractive index of the solvent. The refractive indices of the solvents used were obtained from those listed by Fasman (1963). The dispersion of the refractive index of ethylene glycol was not available and the value $n_D^{20} = 1.4306$ was used and the dispersion was assumed to be proportional to that found for water in calculating other values. When mixtures of two solvents were used, the refractive index was calculated to be the average of the respective percentages of each solvent in the mixture.

Preparation of Polynucleotide Phosphorylase.—The polynucleotide phosphorylase was purified and characterized according to procedures already described (Steiner and Beers, 1961).

Preparation of Poly C.—Large-scale preparations of poly C were routinely carried out in Ostwald viscometers (90-second outflow, Scientific Glass Apparatus Co. No. JB 4175). The components of the reaction mixture consisted of the following: 1 ml of a 10% cytidine-5′-diphosphate solution, 0.3 ml of 0.01 M $MgCl_2$, 0.8 ml of 0.5 M Tris buffer, *p*H 9.5, 0.2 ml of enzyme, and water to a total volume of 4 ml. The reaction was followed viscometrically (Grossman, 1963). The poly C was purified according to the procedures described by Steiner and Beers (1961). The purified poly C was then placed on a G-100 Sephadex column (20 × 2 cm) in 0.05 M NaCl and eluted with the same solvent, and the material in the leading peak was concentrated to 2 ml by lyophilization. The concentrated material was dialyzed overnight at 0° against 250 ml of 0.15 M NaCl.

The concentration of poly C was determined spectrophotometrically with the extinction coefficients published by Warner (1957). The sedimentation coefficient of one sample of poly C employed in these experiments was $s_{20} = 5.2$ (0.15 M NaCl, *p*H 7.0).

Absorbance Measurements.—Absorbance was measured with a Zeiss spectrophotometer, Model PMQ II, equipped with a Haake attachment for thermostatic control of the temperature in a block which housed the cuvets. Glass-stoppered 3.0-ml silica cuvets with a light path of 1 cm were used.

Reagents Employed.—(a) Methanol, Spectro quality reagent grade, Matheson, Coleman and Bell; (b) ethylene glycol, Fisher Certified Reagent (cat. E-178); (c) formamide, Fisher Certified Reagent (cat. F-82); (d) citrate buffer, 0.15 M sodium chloride and 0.015 M sodium citrate (*p*H 7.0); (e) sodium acetate buffer, 0.1 M acetic acid–sodium acetate, *p*H 4.1 and 4.5; (f) ribonuclease, stock solution contained 1000 μg RNAase/μl (Worthington) in water and was heat treated for 5 minutes at 55°; (g) formaldehyde solutions (for studies concerning the effects of formaldehyde, stock solutions of this reagent were prepared as: 10% formaldehyde in 0.2 M $KHPO_4$ buffer, *p*H 8.0). The final concentration of formaldehyde employed in these experiments was 1% which is saturating for concentrations of poly C having an absorbance of 1.0 OD unit per cm light path. Measurements of *p*H were made with a Radiometer *p*H-meter 25 SE, Copenhagen, Denmark, and are accurate to ±0.01 *p*H units.

Results and Discussion

The use of ORD as a sensitive probe for determining conformation in polypeptides and proteins has been widely used (Urnes and Doty, 1961; Blout, 1960). More recently the measurement of circular dichroism (Brahms, 1963; Holzworth *et al.*, 1962; Brahms and Spach, 1963) for the same purpose has extended the application of the measurement of the optical rotatory power for establishing macromolecular conformation. The anomalous rotatory dispersion, termed the Cotton effect, is found to occur near optically active absorption bands; thus any chromophore may exhibit a Cotton effect when located in a disymmetric environment (e.g., Kuhn, 1958). Polypeptide helical conformations containing such chromophores in either the backbone (Simmons *et al.*, 1961; Blout *et al.*, 1962, 1963) or in side chains whose rotational freedom is restricted (Goodman *et al.*, 1963) have displayed Cotton effects which are lost when the helical conformation is destroyed. Similar Cotton effects reported herein on poly C also indicate a high degree of asymmetry or of a highly ordered secondary structure, but this does not necessarily imply a helical structure. However, current interpretation of nucleic acid structure makes it difficult to suggest other highly ordered structures for polyribonucleotides with the same degree of asymmetry that would warrant much attention. Thus it is highly probable that the Cotton effect observed for poly C and the loss of this effect represents a helix → random-coil transition and in this paper shall be referred to as such. However, it must be emphasized that ORD cannot prove the existence of helices and such proof can be obtained only by X-ray diffraction studies. Such X-ray proof for fibers of poly C drawn from solution (*p*H 5.5) is available (Langridge and Rich, 1962), so this Cotton effect can undoubtedly be associated with this helical structure.

The technique of ORD has not been widely used with polynucleotides and nucleic acids mainly because of technical problems (Fresco, 1961; Fresco *et al.*, 1961; Ts'o *et al.*, 1962a; Helmkamp and Ts'o, 1962; Akinrimsi *et al.*, 1963). Recently, Brahms (1963) has reported circular dichroism measurements using homo-

[3] Manufactured by Bendix-Ericcson U. K. Ltd. and distributed in the United States by the Bendix Corp., Cincinnati Division, Cincinnati 8, Ohio.

[4] Optical Cell Co., Brentwood, Md.

polyribonucleotides. The circular dichroism maximum occurs in the region of the strong ultraviolet-absorption bands and agrees with previous studies which indicated that the nature of the electronic transition in purines and pyrimidine is due to an $n \rightarrow \pi^*$ transition which is related to the helical structure (Zimm and Kallenbach, 1962; Brahms, 1963). (However, the contribution of the $\pi \rightarrow \pi^*$ transition cannot be ruled out [Rhodes, 1960].)

The ORD of poly C and CMP at *p*H 7.0 (citrate buffer) and *p*H 4.1 (0.1 M acetate) is seen in Figure 1 for the wavelength range 250–360 mμ. Cotton effects at lower wavelengths are indicated and would also be suggested by the absorption spectra. Such multiple Cotton effects have been reported for DNA (Fresco *et al.*, 1961; Yang and Samejima, 1963). However, due to the high absorption at lower wavelengths these measurements cannot be made with the same degree of accuracy and were not attempted. For the purposes of this paper, however, such measurements are not required as the Cotton effect is used here as a measure of secondary structure and not as a fingerprint of the overall structure. A positive Cotton effect is observed at both *p*H values for poly C, much larger than displayed by the monomer. This would indicate that highly asymmetric structures of poly C, probably helical conformations, exist at both these *p*H values. However, there is a shift in the λ_{max} of the Cotton effect and the relative magnitudes of the peaks and troughs are different, implying that the helical structures of the two species are probably different. At *p*H 7.0 the Cotton effect has a peak at 291 mμ with a mean residue rotation of $[m']_{291} = +34{,}300$, an inflection point at 276 mμ, and a trough at 266 mμ with a mean residue rotation of $[m']_{266} = -35{,}000$. The ultraviolet-absorption-spectrum maximum is at 268 mμ for the polymer at *p*H 7.0 (see insert A, Fig. 1). The CMP Cotton effect has a peak at 286 mμ, $[m']_{286} = 7000$, and an inflection point at 273 mμ. This inflection point agrees with the ultraviolet-absorption-spectrum maximum at 272 mμ. The shift of the inflection point by 3 mμ observed in the polymer over the monomer is indicative of base-base interaction, or the enclosure of the chromophore in a different environment of lower dielectric constant. At *p*H 4.1 the Cotton effect of poly C has a peak at 307 mμ, $[m']_{307} = +26{,}900$, an inflection point at 286 mμ, and a trough at 275 mμ, $[m']_{275} = -66{,}400$. The ultraviolet-absorption-spectrum maximum is at 275 mμ (see insert A, Fig. 1). The CMP Cotton effect at *p*H 4.1 has a peak at $\lambda = 294$ mμ, $[m']_{294} = 3900$, and an inflection point at 278 mμ. The ultraviolet-absorption maximum is at 277 mμ for the monomer at *p*H 4.1.

Poly C at pH 7.0 (citrate buffer).—The $[m']_{291}$ of the peak of the Cotton effect of poly C at *p*H 7.0, in citrate buffer, was found to be a function of its previous history and also varied from sample to sample. Poly C heated to 90° and slow-cooled to 20° had a final $[m']_{291}$ which was greater than the initial value. For example, $[m']_{291} = 23{,}800$ initial, to $[m']_{291} = 34{,}300$ final. If the magnitude of the peak of the Cotton effect is to be considered an index of the degree of asymmetry (helicity) as it is in synthetic poly-α-amino acids and proteins (Simmons *et al.*, 1961) then one must assume that the initial sample is not completely helical; on heating any restraints to the formation of a complete helix are removed, the strands are rearranged, and on cooling a more perfect helix is formed. It was also noted that on storing at 4° the $[m']_{291}$ slowly dropped with time, again indicating that the helicity decreases. The maximal value obtained for $[m']_{291}$ was 49,000. At *p*H 7.0 where no protonation exists, it is evident

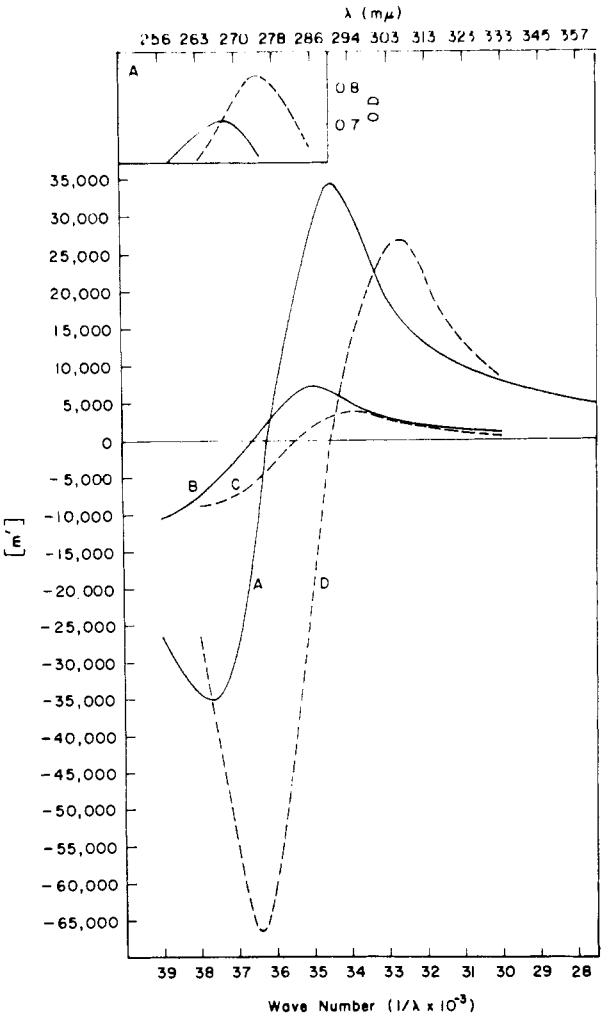

FIG. 1.—Optical rotatory dispersion of poly C and CMP. Poly C: at *p*H 7.1 (citrate buffer), curve A; at *p*H 4.08 (0.1 M acetate), curve D. CMP: at *p*H 7.1 (citrate buffer) curve B; at *p*H 4.08 (0.1 M acetate), curve C. 1-cm cells, $OD_{269} = 0.8$–1.2 per cm path length at *p*H 7.0; $[m']$ is the mean residue rotation. Insert A: Ultraviolet-absorption-maxima spectra of poly C. At *p*H 7.1 (citrate buffer), ———; at *p*H 4.08 (0.1 M acetate), – – – –.

that poly C exists as a helical structure. The average monomer peak residue rotation $[m']_{286}$ for ten experiments at *p*H 7.0 was found to be 7000. Small deviations were observed for this residue rotation with change of *p*H, although the peak wavelength changed in accordance with their ultraviolet spectra. The helical poly C structure at *p*H 7.0 can be destroyed instantaneously by the RNAase-catalyzed depolymerization, yielding the $[m']$ value of the monomer. (Twelve μg of RNAase was added to 46.2 μg of poly C at *p*H 7.0 [citrate buffer].)

It has long been known that the stability of nucleic acids and polynucleotides increases as the ionic strength is increased owing to the tendency of counterions to minimize the repulsion of the charged phosphate groups (Steiner and Beers, 1961). A similar effect is found for poly C at *p*H 7.0. The $[m']$ of the peak increases from +11,300 to +27,000 on increase of the ionic strength from 0.002 to 1.0 in 0.001 M phosphate-citrate buffer (Gomori, 1955), *p*H 7.0, in which the ionic strength is adjusted with NaCl. The λ_{max} shifts from 303 to 291 mμ on increase of the ionic strength. The $[m']_{286}$ of the peak for CMP = 6000 and the slight variation found is probably within experimental deviation. The difference in the monomer value previously cited

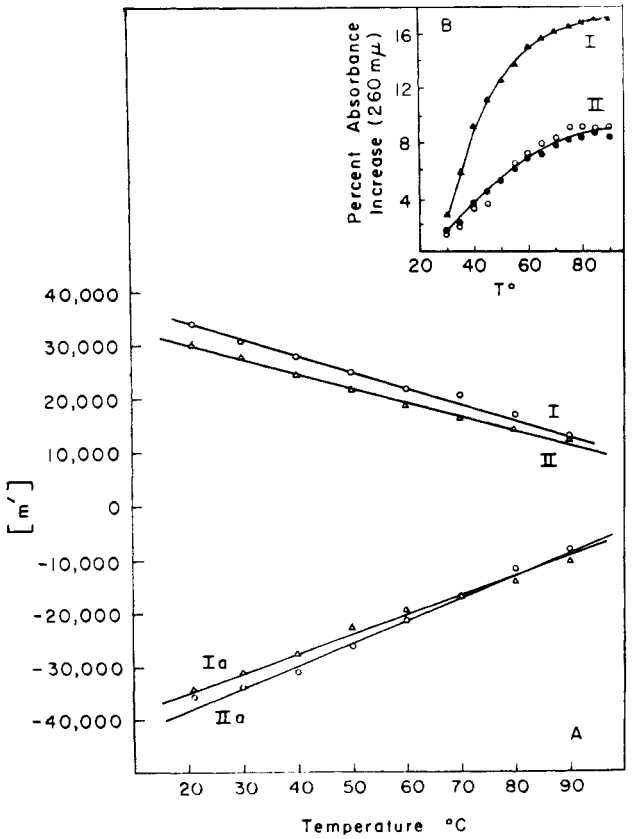

FIG. 2.—Temperature-ORD and temperature-absorbance profiles for poly C. A, $[m']$ as a function of temperature for poly C with and without formaldehyde. Poly C in citrate buffer: curve I, peak $[m']_{291}$; curve Ia, trough $[m']_{265}$. Poly C in citrate buffer and 10% formaldehyde: curve II, peak $[m']_{296}$; curve IIa, trough $[m']_{269}$; 1-mm cell; OD_{269} = 0.8–1.25 per mm path length. B, temperature-absorbance profile for poly C. Curve I, poly C heated in the presence of 1% formaldehyde–citrate buffer; curve II, O-O-O, poly C heated in the presence of citrate buffer; ●-●-●, poly C previously heated in the presence of formaldehyde and slow-cooled, recycled. All samples of poly C were previously heated and slow-cooled. Path length, 1 cm; OD_{260} = 0.6–0.65 per cm path length; $[m']$ is the mean residue rotation.

(7000) is probably due to the different buffer employed.

The structural transformation of poly C during the *p*H change from 4 to 7 is interpreted as a structural transition from that having a conformation involving three shared hydrogen bonds at *p*H 4 to a structure at *p*H 7.0 which has the maximal capacity to share two hydrogen bonds. Previous studies have suggested a disordered conformation at *p*H 7 (Akinrimisi *et al.*, 1963). However, the Cotton effect observed at this *p*H indicates the existence of a helical structure as did circular dichroism studies (Brahms, 1963). Since a decrease in the number of potential hydrogen bonds did not result in a corresponding decrease in helicity, the contribution of hydrogen bonding to helicity was further investigated. Formaldehyde has proved to be a useful reagent for reaction with the exocyclic amino groups capable of hydrogen bonding. Formaldehyde has been shown not to react with the amino groups of native DNA which are presumably hydrogen bonded; however complete reaction occurs with denatured DNA at elevated temperatures. Hydroxymethylated denatured DNA does not undergo renaturation during the slow cooling process (Grossman *et al.*, 1961; Stollar and Grossman, 1962; Haselkorn and Doty, 1961).

The product of the reaction of formaldehyde with these amino groups has not been characterized. It has been suggested that either a hydroxymethyl group (Grossman *et al.*, 1961; Stollar and Grossman, 1962) or a Schiff base (Fraenkel-Conrat, 1954; Hoard, 1960) is the product. As it has been demonstrated that formylated DNA cannot undergo renaturation (Grossman *et al.*, 1961; Stollar and Grossman, 1962; Haselkorn and Doty, 1961), the product does not allow the correct hydrogen bonding to occur or eliminates the hydrogen-bonding capacity of the amino groups.

It seemed appropriate therefore to determine whether poly C (at *p*H 7.0), having reacted with formaldehyde, was capable of existing as a helical structure. The Cotton effect, with a peak in the 290-mμ region, remained essentially unchanged after heating in the presence of formaldehyde and cooling (see $[m']$ value, Fig. 2A), indicating retention of the helical conformation. However there was a 14% increase in absorbance and a shift of the λ_{max}, of 4–5 mμ, to a longer wavelength. Such hyperchromism has usually been associated with complete reaction of the exocyclic amino groups, eliminating hydrogen bonding of the amino group if a Schiff base is formed, or changing the geometry of the hydrogen bonding now possible for a hydroxymethyl product, and therefore destroying the helical conformation. Figure 2A indicates that the peak $+[m']_{291}$ and trough $-[m']_{265}$ for poly C, and the peak $+[m']_{296}$ and trough $-[m']_{269}$ for poly C plus formaldehyde are essentially identical.

As the formaldehyde-treated poly C exists in a helical conformation, a comparison of its stability to temperature with that of a structure capable of the correct hydrogen bonding at *p*H 7.0 was made. A temperature-ORD and a temperature-absorbance profile are seen in Figure 2A,B.

The temperature-absorbance profile (Fig. 2B), similar to those reported by Ts'o *et al.* (1962a), reveals two important features of structure. The first point is that the poly C, whether preheated in the presence or absence of formaldehyde, shows the same per cent absorbance changes with temperature although the original absorbances are different because of the formaldehyde reaction. Another feature of the temperature profile (not shown) is that the absorbance changes are reversible on cooling. The conclusion drawn from these observations is that the masking of the potential hydrogen-bonding amino groups does not influence the relative absorbance changes. This would imply that the two structures are similar since their temperature profiles are identical, and that hydrogen bonding of the amino groups probably plays no role in stabilizing this helical structure. The third feature of these heating curves involves the interpretation of the reaction of formaldehyde with the poly C during the heating reaction. This curve shows a greater relative change in absorption because of the reaction of formaldehyde with the amino groups plus the hyperchromicity associated with destruction of conformation. A misleading interpretation of this curve is that the poly C is being denatured at a more rapid rate in the presence of formaldehyde. However, the ORD studies, to be discussed, carried out under identical conditions, do not reflect such behavior. The ORD-temperature profiles performed under the same conditions as the absorbance-temperature profile are shown in Figure 2A. The change in Cotton effects, peak $+[m']_{291}$ and trough $-[m']_{265}$ for poly C, and peak $+[m']_{296}$ and trough $-[m']_{269}$ for poly C plus formaldehyde follow the same dependence on temperature increase or decrease. On temperature recycling the ORD behavior shown in the figure is repeated. The change in the poly C Cotton effect at *p*H 7.0, citrate buffer, as a function of tempera-

ture, is depicted in Figure 3. The $[m']$ values for the cooling curve for poly C are higher than those found in the initial heating curve because of the previously discussed instability of poly C on standing at *p*H 7. The monomer rotation was found to be temperature independent. The fact that the two heating profiles are the same in the presence or absence of formaldehyde indicates that the rate of reaction of formaldehyde with the amino groups in no way influences the rate and extent of helical change during temperature changes. The cooling curves are similar to those observed during heating, which shows that the hydrogen-bonding capacity of the amino groups does not contribute to the observed helical changes. From both the absorption and ORD temperature curves it can be concluded that hydrogen bonding plays no role in the stability or helical content of poly C at *p*H 7.0. The gradual transition observed for the helix → random-coil transition on raising the temperature, as observed by both ORD and absorbance change, is in contrast to the sharp transitions observed for many DNA species, as well as for polyribonucleotides (Steiner and Beers, 1961) and poly C at *p*H 4.1 (Ts'o *et al.*, 1962a; Akinrimisi *et al.*, 1963) (*vide infra*). This would indicate that the forces responsible for conformational stability are not acting in a cooperative fashion at *p*H 7.0 and that it is possible to have short helical regions dispersed along the polymer chain. It can be inferred that this helical structure is not necessarily a completely rigid one, but an interrupted helix made up of many smaller helical regions. Since amino hydrogen bonding is not necessary in the helical structure of poly C at *p*H 7.0, it must be concluded that other forces, probably hydrophobic in nature, are responsible for the helical conformation. The temperature profile observed must therefore be that expected for the disruption of hydrophobic interactions. Such broad transitions due to hydrophobic interactions have been theoretically predicted (Scheraga, 1963).

The Effect of Organic Solvents on the Helicity of Poly C at pH 7.0 (citrate buffer).—In order to distinguish between the contributions of hydrogen-bonding and hydrophobic forces to the structure of poly C at *p*H 7.0, organic solvents were used. Hydrogen bonds should become more stable in weakly protic organic solvents, as the dielectric constant of the media is reduced (Singer, 1962). This is demonstrated for synthetic polyamino acids, where poly-L-glutamic acid in the helical conformation is stabilized by organic solvents (Fasman *et al.*, 1964). However, if hydrophobic forces are the predominant stabilizing feature, then the addition of organic solvents reduces conformational stability. Such a solvent is ethylene glycol, which is active in destroying such hydrophobic forces (Sage and Singer, 1962). By using organic solvents many investigators (Gordon and Jencks, 1963; Levine *et al.*, 1963; Geiduschek and Herskovits, 1961; Herskovits *et al.*, 1961; Helmkamp and Ts'o, 1961; Hamaguchi and Geiduschek, 1962; Ts'o *et al.*, 1962a,b; Herskovits, 1963; Marmur *et al.*, 1963) have implicated hydrophobic forces in DNA stabilization. As it has been demonstrated that the correct amino-group hydrogen bonds play no role in helical stabilization of poly C at *p*H 7.0, the role of hydrophobic forces was investigated by the use of ethylene glycol, methanol, and formamide. Figure 4 depicts the per cent $[m']$ change (helicity) and absorbance change as functions of ethylene glycol concentration (ionic strength = 0.15). A 90% ethylene glycol solution reduces the $[m']_{291}$ to the residue rotation found for the monomer, CMP, in this solvent, indicating complete collapse of structure. The temperature study in citrate buffer had previously

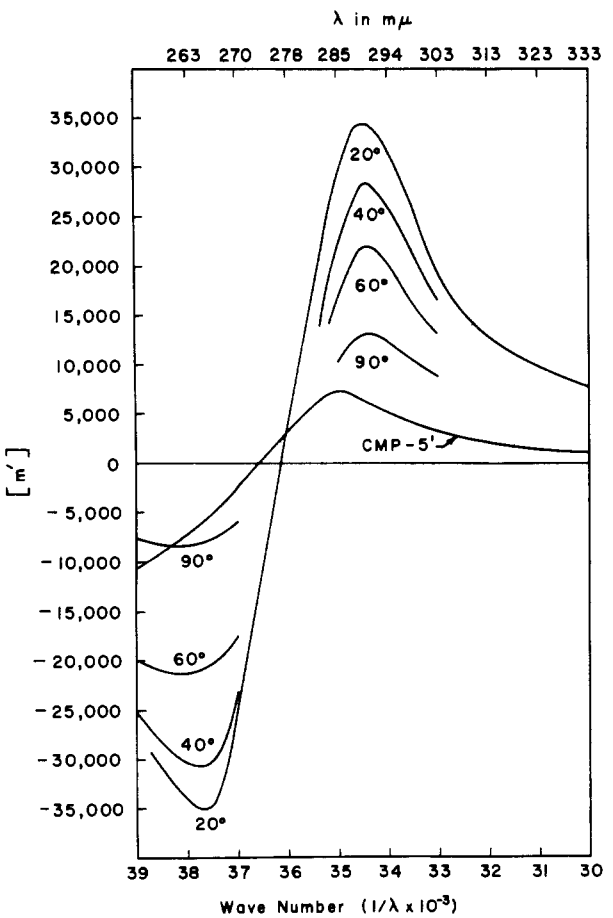

FIG. 3.—Optical rotatory dispersion of poly C as a function of temperature at *p*H 7.0 in citrate buffer. Path length, 1 mm; OD_{269} = 0.71; $[m']$ is the mean residue rotation. Lower curve shows monomer CMP.

shown that the $[m']_{291}$ at 90° was +12,500, indicating that some residual helical structure was maintained. Thus it is obvious that the hydrophobic reagent, ethylene glycol, is extremely efficient in destroying the forces responsible for the maintenance of helicity. Figure 4 also shows that the per cent absorbance change under these conditions directly reflects the helical content of poly C. It should be noted that there is a 45% hyperchromic effect on disruption of the helical structure at *p*H 7.0 as compared to a 25% hyperchromic effect on destruction of the helix at *p*H 4.0. The hypochromism observed in the helical conformation at *p*H 7.0 is caused only by the stacking of the bases. The gradual transition from a helical structure to a random coil, as seen in Figure 4, again demonstrates the noncooperative effect of the forces involved, inferring the presence of regions of random and of helical structure.

Other organic solvents were investigated. As the concentration of methanol is increased, the $[m']$ of the peak also decreases. The $[m']_{291}$ values observed for this poly C sample at 0%, 20%, and 40% methanol (ionic strength = 0.15 M) are, respectively, +28,300, +24,700 and +18,600. At a 40% methanol concentration a 46% decrease in $[m']$ was observed. Higher concentrations of methanol caused the poly C to precipitate out of solution. Thus it is seen that methanol also causes extensive disruption of the hydrophobic forces and consequently the helicity. A 90% formamide solution (ionic strength = 0.15 M) also affects the ORD, resulting in a 79% drop in helical content. Thus it can be concluded that formamide is not as ef-

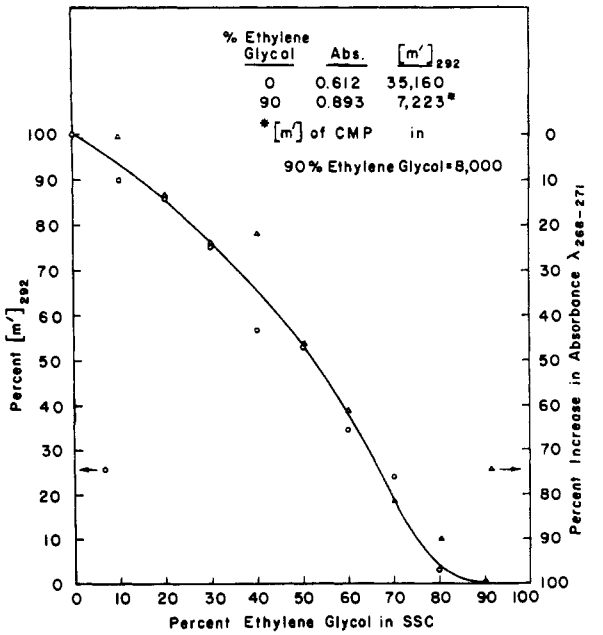

FIG. 4.—Per cent $[m']_{292}$ change and per cent absorbance change of poly C at *p*H 7.0, (citrate buffer), as a function of ethylene glycol concentration. Per cent $[m']$, O—O—O; 100% in 0% ethylene glycol and 0% in 90% ethylene glycol. Per cent increase in absorbance, △—△. The absorbance changes were calculated from the absorbance maximum of the spectrum of each ethylene glycol concentration. The λ_{max} was 268 mμ in citrate buffer and shifted gradually to 271 mμ as the ethylene glycol concentration increased. All measurements in a 1-cm path length.

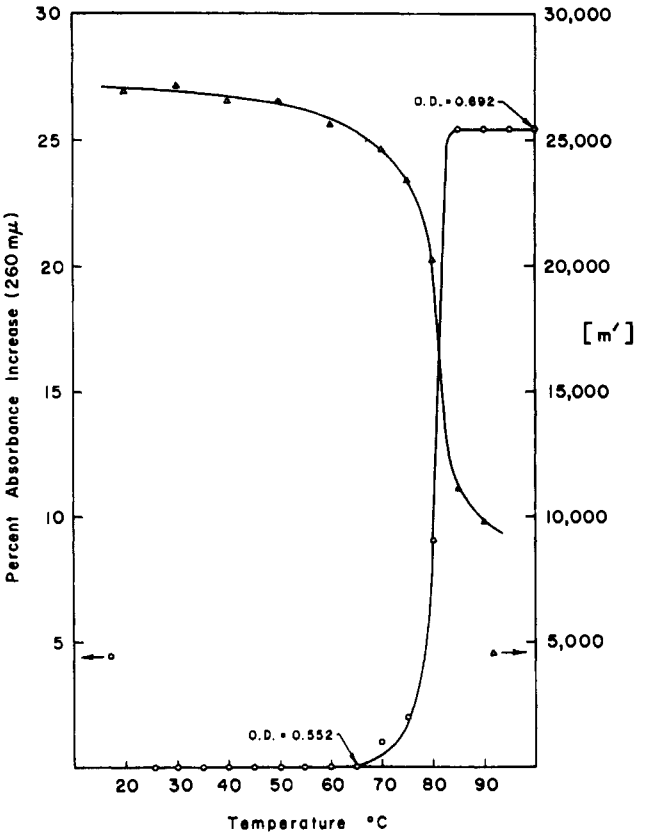

FIG. 5.—Absorbance temperature profile and $[m']$ for poly C at *p*H 4.08 (0.1 M acetate). Per cent increase in absorbance, O—O—O, at 260 mμ, 1-cm path length; $[m']$, △—△—△, at temperatures 20–70°, at λ_{max} = 299 mμ; at higher temperatures λ_{max} = 307 mμ; 1-mm path length; OD_{269} = 0.83.

fective in weakening hydrophobic forces as is ethylene glycol. Previously it has been shown that formamide is an effective denaturant of DNA (Marmur and Ts'o, 1961), and recently it has been stated that ethylene glycol causes denaturation of DNA (Herskovits, 1962, 1963). As these reagents, which have been demonstrated to disrupt hydrophobic forces and strengthen hydrogen-bonds, cause extensive destruction of the helical poly C structure at *p*H 7, it can probably be concluded that hydrophobic forces play the major role in the stabilization of the helical conformation of this homopolyribonucleotide.

At *p*H 7.0, under conditions where hydrogen bonding appears to be absent and hydrophobic forces are the predominant stabilizing factor, it seems unlikely that a two-stranded helical poly C structure is present. This leads to the suggestion of a single-stranded helix, in which the stability is due to intrastrand stacking of the pyrimidine bases.[5] This could account for the relative instability on storage at 4° (hydrophobic forces being less effective at lower temperatures).

Poly C at pH 4.1.—Ts'o *et al.* (1962a) and Akinrimisi *et al.* (1963) have demonstrated that poly C at *p*H 4.0 has a completely different temperature profile than that observed at *p*H 7.0. The temperature-absorbance and temperature-ORD curves for poly C at *p*H 4.1 (Fig. 5) are in direct contrast to those observed at *p*H 7, which show a gradual transition rather than the sharp transitions with T_m of 81° seen at the lower *p*H. At *p*H 4.1 the stability of the helix, whether judged by ORD or by absorbance, is probably dependent on factors other than hydrophobic forces and must in part be due to the interaction of the two strands by ionic or hydrogen bonds, thus accounting for the cooperative phenomena observed. These data agree with those reported previously by Ts'o *et al.* (1962a) and by Akinrimisi *et al.* (1963) and is similar to that found for DNA (Marmur *et al.*, 1963). Prior studies of the poly C structure at its pK_a indicate that it is a double-stranded hydrogen-bonded structure (Ts'o *et al.*, 1962a; Akinrimisi *et al.*, 1963; Langridge and Rich, 1962). The data of Ts'o *et al.* (1962a) and of Akinrimisi *et al.* (1963) and those reported herein support the conclusion that poly C in aqueous solution, at *p*H 4.1, exists as a double-stranded species. This structure at *p*H 4.1 is less hypochromic than the structure at *p*H 7.0 (see Fig. 1A).

On lowering of the *p*H to a point where the pyrimidine bases are essentially all protonated (*p*H 3.42), a further decrease in peak $[m']$ occurs; $[m']_{301}$ = +12,900 from that observed at *p*H 4.0, $[m']_{302}$ = +17,900. The monomer rotation did not change during this *p*H change. This would indicate a loss of asymmetry which might be expected because of the repulsion of neighboring, positively-charged cytosine residues. These helical variations are completely reversible on neutralization.

CONCLUSIONS

Poly C has been shown to exist as a highly ordered asymmetric structure at both *p*H 4.1 and 7.0. Both these structures are probably helical, although different. The stability of these two structures is quite different as indicated by their temperature-ORD and temperature-absorbance profiles. At *p*H 4.1 the helical

[5] Preliminary ultracentrifuge studies were performed on poly C in citrate buffer at *p*H 4.0 and 7.0. It was found that the polymer at *p*H 4.0 had a higher sedimentation coefficient than at *p*H 7.0. This would support the idea of a single strand at the higher *p*H. (We thank Dr. E. Seaman for these studies.)

structure is dependent on hydrogen bonding for stabilization. At *p*H 7.0 the influence of hydrogen bonding on helical stability is negligible and the forces responsible for maintaining the secondary structure have been suggested to be hydrophobic in nature. It is implied that the helical structure at *p*H 7.0 is single stranded, stabilized by base stacking. Hyperchromicity has been demonstrated upon destruction of the secondary structure of both poly C species. Thus both hydrogen bonding and base stacking (hydrophobic interactions) can contribute to hypochromism in ordered structures. It therefore might be difficult to interpret the case when hydrogen bonds are broken, causing hyperchromism, while hydrophobic interactions could maintain secondary structure, if absorbance measurements alone are used as an indication of secondary structure.

Although the importance of hydrophobic forces is demonstrated for this synthetic polyribonucleotide, an unequivocal statement relating this to naturally occurring nucleic acids cannot be made. It is apparent with nucleic acids, under different structural conditions and the involvement of sequence varieties of pyrimidine and purine bases interacting with one another, that the relative contribution of hydrogen bonding and hydrophobic forces must be determined with a variety of experimental techniques and reagents. A recent study (Tikchonenko *et al.*, 1963) on DNA reported that the stability of the double-stranded helix is maintained in a large part by hydrophobic interactions. These results are in full agreement and would be anticipated by the work reported herein.

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References

Akinrimisi, E. O., Sander, C., and Ts'o, P. O. P. (1963), *Biochemistry 2*, 340.
Beaven, G. H., Holiday, E. R., and Johnson, E. A. (1955), *in* The Nucleic Acids, Vol. I, Chargaff, E., and Davidson, J. N., eds., New York, Academic, p. 493.
Blout, E. R. (1960), *in* Optical Rotatory Dispersion, Djerassi, C., ed., New York, McGraw-Hill.
Blout, E. R., Carver, J. P., and Gross, J. (1963), *J. Am. Chem. Soc. 85*, 644.
Blout, E. R., Schmier, I., and Simmons, N. S. (1962), *J. Am. Chem. Soc. 84*, 3193.
Brahms, J. (1963), *J. Am. Chem. Soc. 85*, 3298.
Brahms, J., and Spach, G. (1963), *Nature 200*, 72.
Devoe, H., and Tinoco, I., Jr. (1962a), *J. Mol. Biol. 4*, 500.
Devoe, H., and Tinoco, I., Jr. (1962b), *J. Mol. Biol. 4*, 518.
Fasman, G. (1963), *Methods Enzymol. 6*, 928.
Fasman, G. (1964), *Federation Proc. 23*, 218.
Fasman, G., Lindblow, C., and Bodenheimer, E. (1964), *Biochemistry 3*, 155.
Fraenkel-Conrat, H. (1954), *Biochim. Biophys. Acta 15*, 307.
Fresco, J. R. (1961), *Tetrahedron 13*, 185.
Fresco, J. R., Lesk, A. M., Gorn, R., Doty, P. (1961), *J. Am. Chem. Soc. 83*, 3155.
Geiduschek, E. P., and Herskovits, T. T. (1961), *Arch. Biochem. Biophys. 95*, 114.
Gomori, G. (1955), *Methods Enzymol. 1*, 141.
Goodman, M., Felix, A. M., Deber, C. M., Brause, A. R., Schwartz, G. (1963), *Biopolymers 1*, 371.
Gordon, J. A., and Jencks, W. P. (1963), *Biochemistry 2*, 47.
Grossman, L. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 657.
Grossman, L., Levine, S., and Allison, W. S. (1961), *J. Mol. Biol. 3*, 47.
Hamaguchi, K., and Geiduschek, E. P. (1962), *J. Am. Chem. Soc. 84*, 1329.
Haselkorn, R., and Doty, P. (1961), *J. Biol. Chem. 236*, 2738.
Helmkamp, G. K., and Ts'o, P. O. P. (1961), *J. Am. Chem. Soc. 83*, 138.
Helmkamp, G. K., and Ts'o, P. O. P. (1962), *Biochim. Biophys. Acta 55*, 601.
Herskovits, T. T. (1962), *Arch. Biochem. Biophys. 97*, 474.
Herskovits, T. T. (1963), *Biochemistry 2*, 335.
Herskovits, T. T., Singer, S. J., and Geiduschek, E. P. (1961), *Arch. Biochem. Biophys. 94*, 99.
Hoard, D. E. (1960), *Biochim. Biophys. Acta 40*, 62.
Holzworth, G., Gratzer, W. B., and Doty, P. (1962), *J. Am. Chem. Soc. 84*, 3194.
Kuhn, W. (1958), *Ann. Rev. Phys. Chem. 9*, 417.
Langridge, R., and Rich, A. (1962), *Nature 198*, 725.
Levine, L., Gordon, J. A., and Jencks, W. P. (1963), *Biochemistry 2*, 168.
Marmur, J., Rownd, R., and Schildkraut, C. L. (1963), *Progr. Nucleic Acid Res. 1*, 232.
Marmur, J., and Ts'o, P. O. P. (1961), *Biochim. Biophys. Acta 51*, 32.
Marsh, R. E., Bierstedt, R., and Eichhorn, E. L. (1962), *Acta Cryst. 15*, 310.
Rhodes, W. (1960), *J. Am. Chem. Soc. 83*, 3609.
Rice, S. A., and Doty, P. (1957), *J. Am. Chem. Soc. 79*, 3937.
Sage, H., and Singer, J. (1962), *Biochemistry 1*, 305.
Scheraga, H. A. (1963), *Proteins 1* (2nd ed.), 551.
Simmons, N. S., Cohen, C., Szent-Gyorgyi, A. G., Wetlaufer, D. B., and Blout, E. R. (1961), *J. Am. Chem. Soc. 83*, 4766.
Singer, J. (1962), *Advan. Protein Chem. 17*, 1.
Steiner, R. F., and Beers, R. F., Jr. (1961), Polynucleotides, Amsterdam, Elsevier.
Stollar, D., and Grossman, L. (1962), *J. Mol. Biol. 4*, 31.
Tikchonenko, T. I., Perevertaylo, G. A., and Kisseljov, F. L. (1963), *Biochim. Biophys. Acta 76, 167.*
Tinoco, I., Jr. (1960a), *J. Am. Chem. Soc. 82*, 4785.
Tinoco, I., Jr. (1960b), *J. Chem. Phys. 33*, 1322.
Tinoco, I., Jr. (1961), *J. Chem. Phys. 34*, 1067.
Tinco, I., Jr., Woody, R. W., and Bradley, D. F. (1963), *J. Chem. Phys. 38*, 1317.
Ts'o, P. O. P., Helmkamp, G. K., and Sander, C. (1962a), *Biochim. Biophys. Acta 55*, 584.
Ts'o, P. O. P., Helmkamp, G. K., and Sander, C. (1962b), *Proc. Natl. Acad. Sci. U. S. 48*, 686.
Urnes, P., and Doty, P. (1961), *Advan. Protein Chem. 16*, 401.
Warner, R. C. (1957), *J. Biol. Chem. 229*, 711.
Watson, J. D., and Crick, F. H. C. (1953), *Nature 171*, 737.
Yang, J. T., and Samejima, T. (1963), *J. Am. Chem. Soc. 85*, 4039.
Zimm, B. H., and Kallenbach, N. R. (1962), *Ann. Rev. Phys. Chem. 13*, 171.