Polyriboadenylic and Polydeoxyriboadenylic Acids. Optical Rotatory Studies of pH-Dependent Conformations and Their Relative Stability*

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ABSTRACT: The ultraviolet optical rotatory dispersion, circular dichroism, and absorption properties of polyriboadenylic acid, polydeoxyriboadenylic acid, and the corresponding dinucleoside phosphates were examined as a function of pH and dioxane content. The objective of the study was to examine similarities and differences among the structural forms assumed by the ribose and by the 2′-deoxyribose-containing polymers under various conditions. Circular dichroism at neutral pH, conditions under which the polymers form single-strand helices, confirms the optical rotatory dispersion finding of low-magnitude Cotton effects for polydeoxyriboadenylic acid, especially at high wavelength. The lack of dependence upon ionic strength of this ellipticity band implies that phosphate repulsion is not responsible for this reduced rotation. However, the relative stability of neutral polydeoxyriboadenylic acid toward dioxane may indicate a unique base-stacking arrangement. Optical rotatory dispersion of polyriboadenylic acid and of polydeoxyriboadenylic acid, examined as a function of pH, shows that each polymer can exist in two different acidic forms. In both cases, one form (with higher wavelength Cotton effect) is present when the pH equals the pK_a of adenyl residues in the polymer; below this pH there is a gradual conversion to the more acidic structure. Spectrophotometric titrations do not reveal the presence of the two forms. The optical rotation temperature dependence of polydeoxyriboadenylic acid in acid is complicated by depurination. Evidence that the acidic forms of polydeoxyriboadenylic acid are double strand is obtained from the pH dependence of complex formation with polyribouridylic acid. The differences in the acidic forms of polyriboadenylic acid and polydeoxyriboadenylic acid can be seen from the effect of dioxane upon these structures.

The ultraviolet rotatory properties of DNA differ from those of RNA, probably reflecting different geometries for the asymmetric macromolecular structures. These differences affect the related phenomena of optical rotatory dispersion (Samejima and Yang, 1965) and circular dichroism (Brahms and Mommaerts, 1964) of these nucleic acids. Previous work in this laboratory has utilized these methods to study the effect of the pentose configuration upon the structure of cytidine compounds (Adler *et al.*, 1967, 1968). The present work compares poly rA with poly dA as conformational models for the ribo- and deoxyribonucleic acids.

Poly rA, the corresponding dinucleoside monophosphate (rApA), and larger rA oligomers have previously been studied by several optical methods. These are ultraviolet spectroscopy (Fresco and Klemperer, 1959; Leng and Felsenfeld, 1966; Applequist and Damle, 1965, 1966), optical rotatory dispersion (Holcomb and Tinoco, 1965; Warshaw *et al.*, 1965; Sarkar and Yang, 1965; Poland *et al.*, 1966; Michelson *et al.*, 1966), and circular dichroism (Van Holde *et al.*, 1965; Brahms *et al.*, 1966; Hashizume and Imahori, 1967; Bush and Scheraga, 1969). X-Ray diffraction in the solid state (Rich *et al.*, 1961) and in solution (Luzzati *et al.*, 1964), calorimetry (Epand and Scheraga, 1967), and nuclear magnetic resonance (Hruska and Danyluk, 1968; Chan and Nelson, 1969) have also contributed to our knowledge of poly rA and adenylate oligomer structure. There is agreement that at neutral pH poly rA, its oligomers, and even rA(3′p5′)rA exist as single-strand helices with partially stacked bases. In solutions more acidic than the pK_a of the adenyl residues, poly rA and the higher oligomers form double-strand, hydrogen-bonded helices. A recent review (Yang and Samejima, 1969) refers to rotatory research on several synthetic polyribonucleotides, and discusses their relevance to RNA structural interpretation.

Synthetic polydeoxyribonucleotides, among them poly dA, have been studied less extensively. There are data on the ultraviolet absorption (Bollum *et al.*, 1964; Riley *et al.*, 1966; Barszcz and Shugar, 1968), optical rotatory dispersion (Ts'o *et al.*, 1966; Vournakis *et al.*, 1967), and circular dichroism (Bush and Scheraga, 1969) properties of poly dA, some oligo dA's, and d(pA)$_2$.

The present work investigates similarities and differences between poly rA and poly dA, as seen by optical rotatory dispersion and circular dichroism under various solvent conditions. This is a continuation of research on the role of the 2′-hydroxyl group on the polynucleotide structure (Adler *et al.*, 1968). Differences between single-strand poly rA and poly dA are evident from the influence of salt and of dioxane upon their rotatory properties. Evidence is presented showing that poly dA forms a double-strand helix in acidic solution; there has been some controversy over this point (Bollum *et al.*, 1964; Riley *et al.*, 1966; Ts'o *et al.*, 1966; Barszcz and Shugar, 1968). Two acidic forms for each polymer, poly rA

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and poly dA, are demonstrated by optical rotatory dispersion titration.

Experimental Section

Materials. The polyribonucleotides were obtained from Miles Chemical Co.: poly rA, K^+ salt, s = 9.75 S; poly rC, K^+ salt, s = 7.18 S; poly rU, NH_4^+ salt, s = 7.15 S. One sample of poly dA was synthesized by the method of Lee-Huang and Cavalieri (1964) and isolated according to Inman and Baldwin (1962). Another sample of poly dA (having identical optical properties and s = 3.6 S) was a gift from Dr. F. J. Bollum, as was the $d(pA)_2$ (Bollum, 1968). Both rA(3′p5′)rA and rA(2′p5′)rA were purchased from Zellstofffabrik Waldhof, Mannheim, Germany. The rA-2′-*O*-methyl-prAp was a gift from Dr. B. G. Lane (Singh and Lane, 1964). rAMP-5′ was obtained from Sigma, dAMP-5′ from P-L Biochemicals, and standardized 0.01 and 0.1 M HCl and NaOH solutions from Fisher. All salts and buffer components were reagent grade. Glass-distilled water and Matheson Spectrograde dioxane were used.

Solutions and Concentrations. Most measurements were performed in buffered solutions, containing 0.02 M total concentration of buffer components and 0.08 M NaCl, whose pH was adjusted with HCl or NaOH. Titrations and some circular dichroic measurements were done with unbuffered solutions of specified pH and salt concentration. Concentrations were in the range of 0.7–1.3 $\times$ 10^{-4} M adenine residues (corresponding to a maximum absorbance of about one for a 1-cm path length), except for hydrogen ion titrations, where 2 $\times$ 10^{-4} M adenine residues were used. Concentrations were determined by means of the following molar residue extinction coefficients (ϵ_{max} $\times$ 10^{-3}, in l./(mole cm), for neutral pH): poly rA, 10.1 (Holcomb and Tinoco, 1965); poly dA, 9.9 (Ts'o *et al.*, 1966); rA(3′p5′)rA, 13.6 and rA(2′p5′)rA, 12.9 (Brahms *et al.*, 1966); $d(pA)_2$ and rA-2′-*O*-methyl-prAp, both 12.9, determined by the ethylene glycol method of Adler *et al.* (1967); rAMP-5′ and dAMP-5′, both 15.0 (Voet *et al.*, 1963); poly rC, 6.3 and poly rU, 9.6 (Warner, 1957).

Polymer stock solutions were heated briefly to 95° at neutral pH, and then slowly cooled, to ensure formation of the single-strand conformation (Fasman *et al.*, 1964). For salt-dependence circular dichroic investigations, concentrated polymer solutions were dialyzed exhaustively against water; all solutions used in these experiments (polymers, NaF, and water) were adjusted to pH 8.5. For studies of the effect of pH upon optical rotatory dispersion of polymers, the solvent was citrate buffer of 0.1 M ionic strength; for each run the pH of a fresh aliquot was adjusted with 1 M HCl. In experiments with dioxane, the dioxane was added last to the solution, in order to prevent precipitation. Dioxane concentrations are reported as per cent, by volume, in aqueous solution.

Methods. Optical measurements were performed in 1-cm, fused quartz cells. Absorption spectra (usually in the 250–270-nm region) were taken for each solution studied by optical rotatory dispersion or circular dichroism. Data are not presented for solutions which were opalescent or which absorbed light at $\lambda > 310$ nm (*i.e.*, scattered light). The optical equipment (Cary Model 14 spectrophotometer, and Cary Model 60 spectropolarimeter with 6001 circular dichroism accessory) was operated in a manner similar to that reported by Adler *et al.* (1968). The spectrophotometer was flushed with nitrogen for use below 220 nm. All experiments were performed at 22°, unless another temperature is indicated. Large pen periods and slow scanning speeds were required for optical rotatory dispersion and circular dichroism runs at $\lambda < 225$ nm.

Optical rotatory dispersion results are reported in terms of $[m']$ (residue rotation per mole of adenine residue, corrected for refractive index), circular dichroism in terms of $[\theta]$ (residue ellipticity). Both parameters have units of (deg cm^2)/dmole. Formulas used for calculation are $[m'] = (10\alpha_{obsd}/lc)(3/(n^2 + 2))$ and $[\theta] = 10\,\theta_{obsd}/lc$, where α_{obsd} = rotation (degrees), θ_{obsd} = ellipticity (degrees), l = path length (decimeters), c = residue concentration (M), and n = refractive index. The optical rotatory dispersion and circular dichroism curves could be measured to $\pm$0.0005 deg in the wavelength range above 225 nm, and to $\pm$0.001 deg below 225 nm. Signal values at peaks and troughs were greater than 0.01 deg, except in the case of mononucleotides.

A set of experiments was performed in which complex formation between poly rU and the adenyl polymers was monitored by ultraviolet absorption. Solutions for these studies were made of 3 $\times$ 10^{-5} M poly rA and poly dA, and 6 $\times$ 10^{-5} M poly rU, in acetate or formate buffers. The pH was adjusted independently for each solution with 1 M HCl or NaOH. For each pH the adenylate (A) and poly U solutions, at the same pH, were placed in separate 1-cm path-length compartments of a tandem cell, and the ultraviolet absorption spectrum was scanned in the 260-nm region. Then the A and U solutions were removed and quickly mixed, and the mixture was placed in both cell compartments. Spectra were taken at frequent intervals, starting at 2 min after mixing. The kinetics of complex formation were monitored by spectral changes, particularly by a decrease in absorbance at λ_{max}.

A Corning Model 12 pH meter with expanded scale was used for titrations, as was a Manostat mercury-filled 0.1-ml digital readout micrometer buret, read to 0.0005 ml. The volume for titrations was 3.0 ml; the solvent was 0.1 M NaCl; the temperature was 23 $\pm$ 1°. Titrations were stopped when opalescence occurred (pH $\simeq$ 2 for poly dA, and pH $\simeq$ 3 for poly rA). Spectrophotometric titrations were conducted in 1-cm square, stoppered cuvets containing magnetic stirring bars. The titrant was 0.1 M HCl, and volume increases were kept below 1%. Absorbance curves were scanned from 250 to 270 nm at each pH. Potentiometric hydrogen ion titrations were conducted separately; solvent blanks were measured and subtracted, and 0.01 M HCl was used.

Paper electrophoresis was carried out in pH 3.5 sodium formate buffer, on Whatman No. 3MM paper, using 27,000 V. Samples and markers (adenine, deoxyadenosine, dAMP-5′, $d(pA)_2$, poly dA, purine, and deoxyinosine) were located under ultraviolet light.

Results and Discussion

The work will be presented in two parts: (A) data taken at neutral pH, where poly rA is a single-strand helix; (B) experiments performed under acidic conditions, where poly rA is a double-strand helix.

A. Single-Strand Forms

Optical Properties. Optical rotatory dispersion (Holcomb and Tinoco, 1965) and circular dichroism (Brahms *et al.*, 1966) have

TABLE I: Optical Parameters[a] for Various Forms of Adenyl Compounds.

Compound	Form	Conditions[b] pH	Conditions[b] % Dioxane	Absorption λ_{max}	Absorption ϵ_{max}	Circular Dichroism Extrema λ_1	CD $[\theta]_1$	CD Crossover	CD λ_2	CD $[\theta]_2$	Optical Rotatory Dispersion Extrema λ_1	ORD $[m']_1$	ORD Crossover	ORD λ_2	ORD $[m']_2$
Poly rA	Neutral	8.5	0	257	10.1[c]	264	+60.3	256	248.5	−46.0	284	+21.0	268	256.5	−61.4
Poly rA	Acid B	5.81	0	253	8.8						287.5	+36.2	272	253.5	−78.4
Poly rA	Acid A	4.00	0	252	8.7						280.5	+39.4	266	251.5	−89.4
Poly rA	Dioxane neutral	8.5	50	259	13.0[d]						280	−3.7	253	244	+3.0
Poly rA	Dioxane acid	3.76[e]	60	259	9.1						291	+7.9	278	256	−20.3
Poly dA	Neutral	8.5	0	257.5	9.9[c]	281.5	+6.1	261	250	−20.4	287	+3.9	278	258	−5.2
Poly dA	Acid B	4.30	0	261	11.4						282.5	+18.4	267	254	−30.0
Poly dA	Acid A	3.02	0	261	11.5						279.5	+21.6	265	252	−38.1
Poly dA	Dioxane neutral	8.5	60	263	12.7						283	−3.0	260	242	+6.0
Poly dA	Dioxane acid	3.16[f]	60	259	11.0						290	+10.1	269	253	−13.4
rA(3′p5′)rA	Neutral	8.5	0	257.5	13.6[c]	272	+21.3	261.5	251.5	−26.1	282.5	+7.1	275	260.5	−26.3
rA(3′p5′)rA	Dioxane	8.5	80	260	14.9						266	−3.8	254	245	+2.3
rA(2′p5′)rA	Neutral	8.5	0	258	12.9	271.5	+19.0	260.5	252	−16.3					
rA-2′-O-CH_3prAp	Neutral	8.5	0	258	12.9	275	+6.4	264	253	−14.9					
$d(pA)_2$	Neutral	8.5	0	257.5	12.9	272	+12.3	261	252	−19.8	282	+4.9	275	261	−15.8
$d(pA)_2$	Dioxane	8.5	80	259.5	13.6						266	−3.3	254	242	+4.2
rAMP	Neutral	8.5	0	260	15.0[c]	263	−2.6	230	217	−4.5	275	−3.0	257	247	+1.3
dAMP	Neutral	8.5	0	261	15.0[c]	265	−1.3	250			275	−1.6	260	255	+1.0

[a] Only the largest of the highest wavelength features are listed. All wavelengths are in nm; all values of ϵ, $[\theta]$, and $[m']$ are $\times 10^{-3}$. Temperature 22°, ionic strength, 0.1 M. [b] Conditions chosen, when possible, to yield only one molecular form. [c] Literature values (see Experimental Section). [d] Solution slightly opalescent. [e] Aqueous buffer pH. Nominal pH in dioxane mixture = 5.08. [f] Aqueous buffer pH. Nominal pH in d oxane mixture = 4.28.

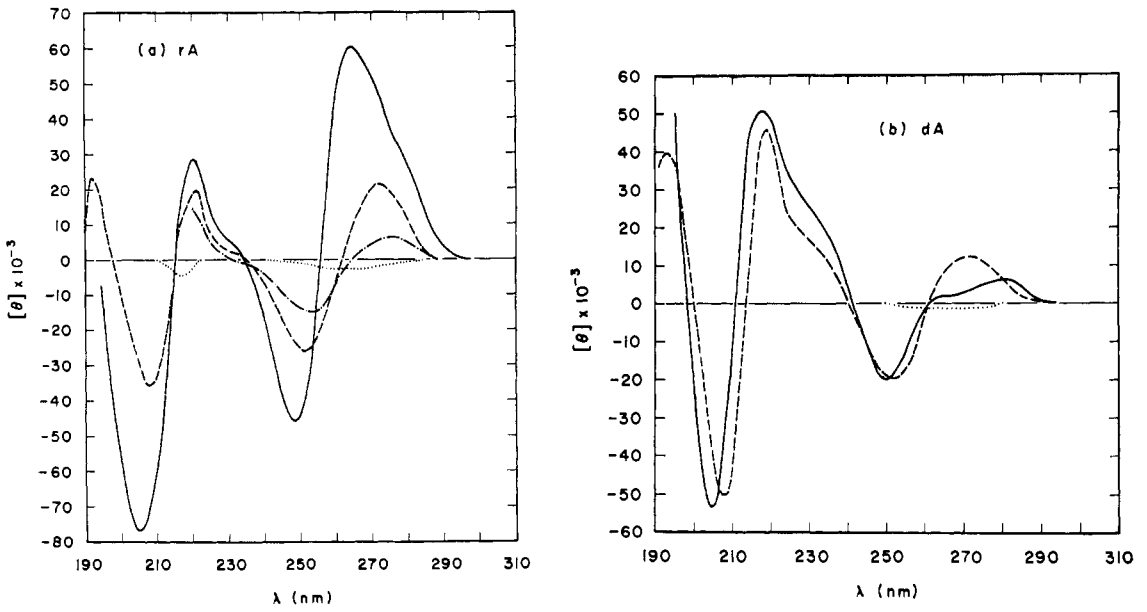

FIGURE 1: Circular dichroism of adenylate compounds at pH 8.5 in unbuffered 0.1 M NaF at 22°. (a) rA compounds: poly rA, ———; rA (3′p5′)rA, ———; rA-2′-*O*-methyl-prAp, —·—·—; rAMP-5′, ·····. (b) dA compounds: poly dA, ———; $d(pA)_2$, ------; dAMP-5′, ·····.

established that poly rA and the corresponding oligomers exist partially as single-strand helices, with a fraction of the bases stacked, when the adenine bases carry no charge. These rA compounds exhibit two high-wavelength Cotton effects, of opposite sign and nearly equal intensity. The optical rotatory dispersion pattern of single-strand poly dA shows a great reduction of the highest wavelength, positive Cotton effect (Ts'o *et al.*, 1966; Vournakis *et al.*, 1967), although the lower wavelength optical rotatory dispersion data are similar for the two polymers. Circular dichroism data for poly rA, poly dA, and for several dinucleoside phosphates and mononucleotides are presented in Figure 1 and in Table I. These data, which extend to 190 nm, confirm the loss in intensity of the first, positive ellipticity peak of poly dA even in comparison with $d(pA)_2$. While this work was in progress, Bush and Scheraga (1969) collected some similar circular dichroism data for these compounds; the two sets of measurements are in substantial agreement. Poly dA is unique among synthetic polynucleotides for the small magnitude of its first circular dichroism band (the other bands being relatively normal); only DNA in ethylene glycol exhibits similar behavior (Green and Mahler, 1968).

Results identical with those in Figure 1 were obtained in pH 8.5 Tris buffer. (See Experimental Section for buffer compositions.) For purposes of comparison with other spectra, the small poly dA positive ellipticity band at 264 nm should probably be considered, rather than the peak at 282 nm. The latter peak is comparable with the shoulder at the same position in poly rA, and may be due to an $n-\pi^*$ transition (Bush and Scheraga, 1969). The 264-nm poly dA peak is much smaller than the 264–272-nm bands of poly rA, rA(3′p5′)rA, and even $d(pA)_2$. (These circular dichroism bands are attributable to the $\pi-\pi^*$ absorption peaks at 257 nm; exciton interactions split these peaks into positive circular dichroism bands at about 270 nm and negative ones at about 250 nm.) The shorter wavelength part of the poly dA circular dichroism spectrum (below 260 nm) is very similar to that of $d(pA)_2$; this finding can be inferred also from the optical rotatory dispersion results of Vournakis *et al.* (1967), when they are calculated on a molar residue basis. This situation is very different from that of cytidine compounds (Adler *et al.*, 1967), where poly rC, poly dC, and the corresponding dinucleoside phosphates all have similarly shaped optical rotatory dispersion curves, and the differences in Cotton effect magnitudes can be explained by varying amounts of base stacking. In the rA series, only the difference between poly rA and rA(3′p5′)rA can be explained on this basis, and only if the poly rA 282-nm circular dichroism shoulder is ignored.

Ultraviolet absorption spectra were obtained for all the adenyl compounds in 0.1 M NaF from 190 to 320 nm. These spectra are all similar. The highest wavelength peaks are listed in Table I. In addition, poly rA and poly dA show shoulders at about 280 nm, and all the compounds have shoulders at about 205 nm (except for the monomers, which have well defined small maxima at this wavelength). The ultraviolet spectrum of poly dA is identical with that of poly rA, to within the experimental error. Therefore, the striking differences in circular dichroism cannot be caused by any differences in electric transition moments. The circular dichroism and absorption spectra of poly dA were analyzed by means of a DuPont 310 curve resolver. Eight gaussian bands were required for an adequate fit of the circular dichroism data above 190 nm. Of these, the bands af 262 and 251 nm both appear to derive from the absorption peak at 257 nm. Similarly, the 217- and 206-nm circular dichroism bands appear to be correlated with a single absorption maximum, thus giving evidence for exciton splitting.

The circular dichroism peaks of rA(2′p5′)rA are only slightly smaller than those of rA(3′p5′)rA at 22° (Table I); this is a result of the choice of temperature. Brahms *et al.* (1967) have shown that the temperature dependence of these two compounds is different. The methylated dinucleoside di-

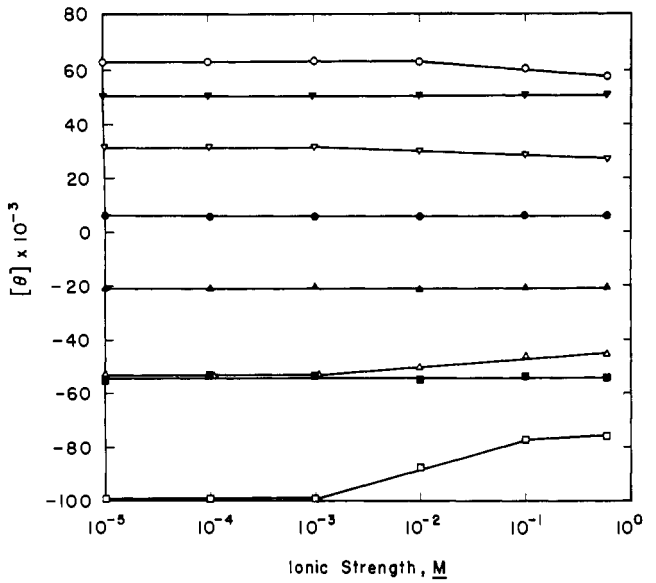

FIGURE 2: Effect of NaF concentration upon circular dichroism peaks of single-strand adenine polymers. Conditions: pH 8.5, 22°. Poly rA peaks (nanometers): 264, ○—○; 248.5, △—△; 221, ▽—▽; 205, □—□. Poly dA peaks (nanometers): 281.5, ●—●; 250, ▲—▲; 218, ▼—▼; 204.5, ■—■.

phosphate, rA-2′-*O*-methyl-prAp, displays greatly reduced ellipticity, even when compared with $d(pA)_2$. A study of Courtauld models shows that the methyl group sterically interferes with base stacking. However, Bobst *et al.* (1969) have recently demonstrated that at neutral pH poly-2′-*O*-methyladenylic acid has a circular dichroism spectrum nearly identical with that of poly rA. In addition, it has been demonstrated that rApAp has a smaller circular dichroism band at 275 nm than does rApA, thus showing that the 3′-phosphate also causes destabilization (Bush and Scheraga, 1969).

Effect of Salt Concentration. The influence of NaF concentration upon the major circular dichroism peaks of poly rA and poly dA was studied (Figure 2). No change in peak wavelengths was observed, indicating that at pH 8.5 the polymers retain their single-strand conformation even at low salt concentration, where the adenine pK_a's rise.

Vournakis *et al.* (1967) showed that lengthening the chain of dA oligomers beyond the dimer does not result in increased rotation. To account for this observation, they postulated a next-nearest neighbor repulsive potential, which might possibly involve the negative charges on phosphate groups. If phosphate repulsion were an important factor in structure destabilization, then the ellipticity of poly dA should increase at high ionic strength, where the charges are shielded. Figure 2 shows that salt has absolutely no effect on poly dA circular dichroism at any wavelength. This is true even at 1°, where increased base stacking causes a 10% increase in circular dichroism peaks, and where destabilization should be revealed more readily. Therefore, phosphate repulsion is most probably not responsible for the weak rotatory properties of poly dA, or for the polymer's apparent inability to form any but pairwise stacks.

The ellipticity bands of poly rA decrease somewhat as the salt concentration is raised. This is opposite to the effect of ionic strength upon poly rC and poly dC (Adler *et al.*, 1968), and indicates that phosphate–phosphate repulsion is unimportant in poly rA. Furthermore, although the salt effect is small, perhaps the data may be used as evidence for possible hydrogen bonds in single-strand poly rA between the 2′-hydroxyl groups and phosphate oxygens. If such bonds exist, then a decrease in salt concentration would increase the effective charge on the phosphate oxygen, thus leading to stronger hydrogen bonds and a greater rigidity of helical structure.

Effect of Dioxane upon Single-Strand Structure. Organic solvents are known to destroy the helical structure of nucleic acids (Geiduschek and Herskovits, 1961) by disrupting base-stacking interactions. Dioxane is a particularly good denaturing solvent (Levine *et al.*, 1963) and has no hydroxyl groups that could possibly hydrogen bond with the polynucleotide. The two ether oxygens of dioxane could serve as possible, weak, hydrogen-bond acceptors, but are not expected to do so in the presence of water. In the present work, dioxane in varying concentrations was used to investigate the base stacking properties of poly rA, poly dA, and the corresponding dinucleoside phosphates. In particular, a comparison of the circular dichroism and the optical rotatory dispersion (Vournakis *et al.*, 1967; Poland *et al.*, 1966) data for these compounds, especially at low wavelength, leads to the expectation that poly dA might be similar to $d(pA)_2$ and to rA(3′p5′)rA in its structural properties (*i.e.*, stabilization factors); poly rA, with its larger optical rotatory dispersion and circular dichroism bands, would perhaps be expected to be different. Furthermore, single-strand poly rC (Fasman *et al.*, 1964) was shown to be more stable than poly dC (Adler *et al.*, 1967) toward ethylene glycol denaturation, presumably because of 2′-OH hydrogen bonding.

The optical rotatory dispersion data collected in dioxane solutions at neutral pH are presented in Figure 3. The optical rotatory dispersion trough at about 213 nm is utilized as well as the one at about 260 nm, since the latter is relatively small in poly dA. All compounds exhibited an increase in extinction coefficient as dioxane was added. Poly rA solutions became opalescent at 45% dioxane; the other compounds were still soluble at 80% dioxane. Monomer rotations were unaffected by dioxane.

Figure 3 shows that it is poly dA, and not poly rA, which differs from the other compounds, and which retains its secondary structure in moderate concentrations of dioxane. Poly rA is similar to the rA and the dA dinucleoside phosphates in that its rotation is sharply reduced by small concentrations of dioxane. Analogous behavior for poly rA was observed in its sensitivity toward ethylene glycol denaturation at neutral pH (Hanlon and Major, 1968). On the other hand, the rotation (throughout the optical rotatory dispersion spectrum) of poly dA is nearly constant until over 25% dioxane has been added. (The possibility that the stability of neutral poly dA toward dioxane may be caused by double-strand helix formation can be excluded on the basis of temperature-dependence experiments (Ts'o *et al.*, 1966; Riley *et al.*, 1966; Vournakis *et al.*, 1967; Barszcz and Shugar, 1968). The noncooperative melting of poly dA, similar to that of poly rA, shows that poly dA is single strand at neutral pH.) The dioxane data indicate that there may be some unique structural property of single-strand poly dA, not present even in $d(pA)_2$, which allows it to maintain its asymmetric conformation. This uniqueness is suggested also by the high-wavelength, positive band in the circular dichroism and optical rotatory dispersion spectra

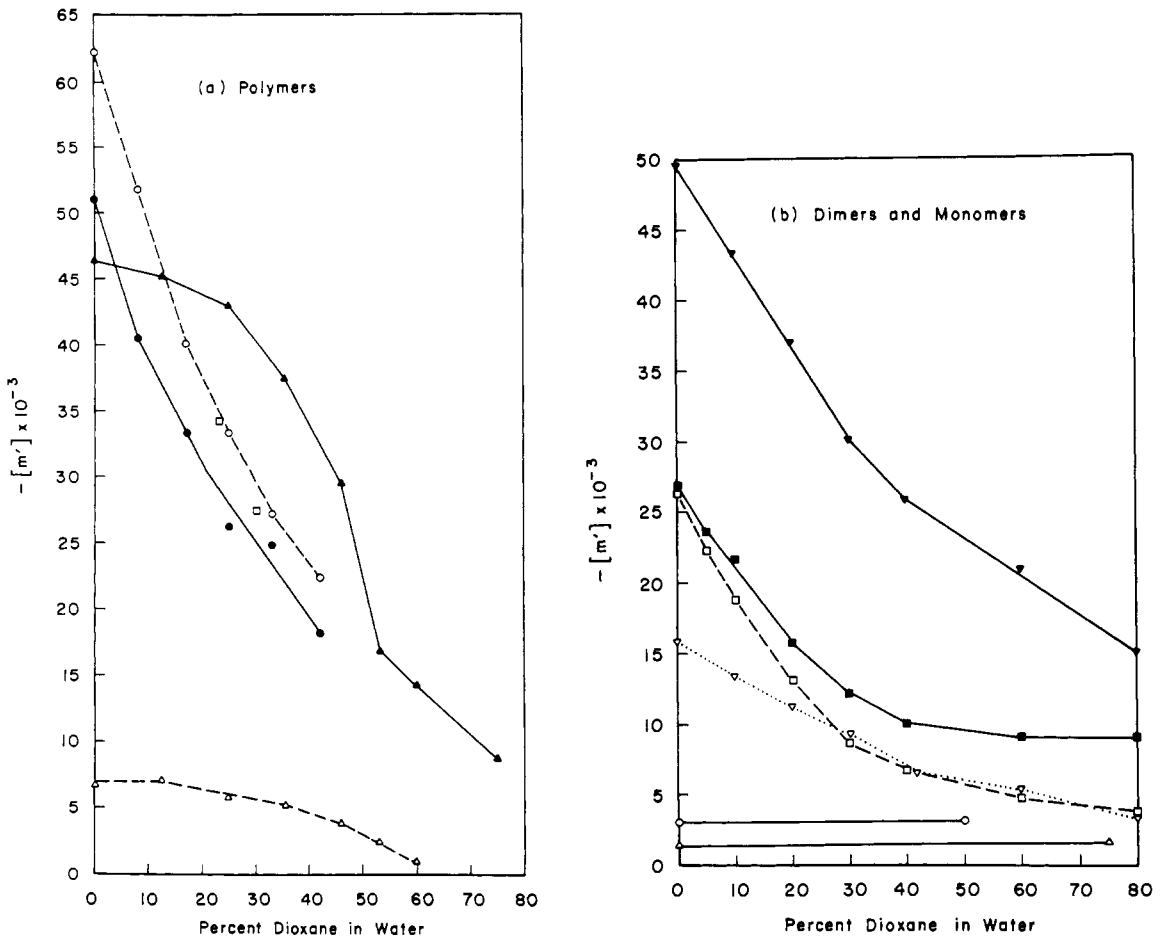

FIGURE 3: Effect of dioxane concentration upon optical rotatory dispersion of adenylate compounds in pH 8.5 Tris buffer. The [m'] values are trough magnitudes. The listed trough positions (in nanometers) are those for aqueous solutions, and usually shifted slightly as dioxane was added. (a) Polymers: poly rA troughs (nanometers) at 257 ○— —○, and 213 ●—●; poly dA at 260 △- -△, and 211 ▲—▲. The □ points are similar to the ○ points, except that the solvent was citrate buffer, initially at pH 5.5. (b) Dimers and monomers: rA(3′p5′)rA at 261 nm □— —□, and 213 ■—■; $d(pA)_2$ at 261 ▽···▽, and 213 ▼—▼; rAMP-5 at 275 ○—○; dAMP-5 at 275 △—△.

of poly dA in the single strand. This additional stability may be caused by a distinctive base-stacking geometry for poly dA, such that the bases would be less available for solvation by dioxane, and such that the 270-nm circular dichroism band would be nearly abolished.

At high dioxane concentrations, the optical rotatory dispersion peaks and troughs of all the polymers and dimers studied gradually shift toward higher wavelength. The optical rotatory dispersion spectra approach the shape and magnitude characteristic of the monomers. This shift is illustrated for rA(3′p5′)rA in Figure 4 and is typical for all the compounds. Optical rotatory dispersion characteristic features in the presence of dioxane are listed in Table I. These shifts do not affect the interpretation of the data in Figure 3, which were always evaluated at actual trough positions.

Structure at Neutral pH. The conformation of poly dA at neutral pH must differ, in some respect, from that of single-strand poly rA. Since these structures have asymmetrically oriented chromophores, the difference influences their rotational properties. The geometry of single-strand poly rA is a helix, in which the fraction of bases which are stacked are oriented perpendicular to the helix axis (Holcomb and Tinoco, 1965; Brahms *et al.*, 1966). The fact that the circular dichroism and optical rotatory dispersion spectra of poly dA are different in shape and magnitude, at high wavelength, from those of poly rA and of $d(pA)_2$, shows that the explanation is not simply a smaller fraction of stacked bases in poly dA (as appears to be true for poly dC compared with poly rC). The difference between the first optically active transitions of poly dA and poly rA cannot be attributed to any change in absorption spectrum. Therefore, the answer probably can be traced to different geometries of helical base stacking in the two polymers.

The stability of poly dA toward dioxane-induced denaturation is further evidence for a unique geometry of base stacking in poly dA. This geometry may involve bases which are tilted with respect to the helix axis (Bush and Scheraga, 1969). Such a tilting could affect to different degrees the rotation arising from different absorption bands, so that poly dA and poly rA could display similar rotatory properties below 240 nm, while showing very different circular dichroism bands in the 240–290-nm region. In DNA (B form) the bases are perpendicular to the helix axis, and the circular dichroism spectrum arising from the 260-nm absorption band is conservative (Brahms and Mommaerts, 1964). In RNA and in several synthetic ribo and deoxyribo polymers, the positive ellipticity band arising from

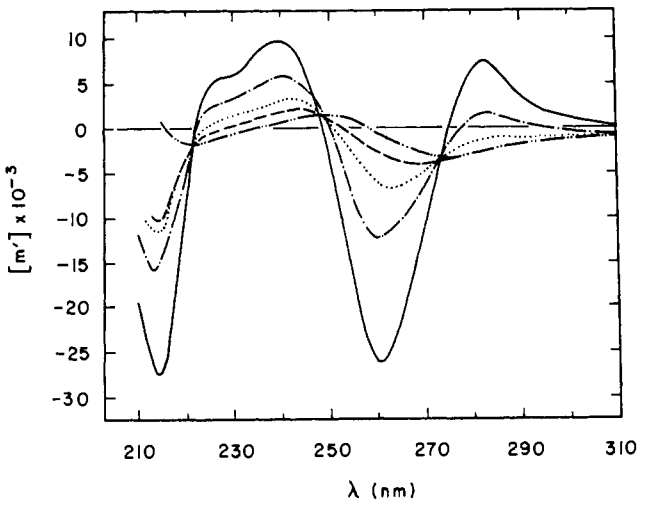

FIGURE 4: Changes in optical rotatory dispersion of rA(3′p5′)rA in pH 8.5 Tris, caused by dioxane. Per cent dioxane: 0, ———; 20, —·—·; 40, ·····; 80, — — —. rAMP-5′ in 0 or 50% dioxane, —··—··.

this absorption band is much larger than the corresponding negative circular dichroism peak. In poly dA, the opposite relationship holds, presumably because of a manner of base tilting distinctive from that in RNA.

The possible role of the 2′-hydroxyl groups of polyribonucleotides in structure stabilization is not obvious (Adler *et al.*, 1968). In the present study, comparison of poly rA and poly dA in dioxane provides no evidence for any 2′-OH hydrogen bond. However, the circular dichroism of poly rA as a function of salt concentration can possibly be explained by a 2′-OH–O-phosphate bond. A 2′-OH–N_3-adenine bond is another possibility (Ts'o *et al.*, 1966).

A next-nearest neighbor repulsive force has been suggested (Vournakis *et al.*, 1967) as the factor preventing poly dA from having a more completely base-stacked structure than $d(pA)_2$. The possibility of phosphate repulsion can be eliminated by means of the salt-effect results. Poor overlap of a third base with the first two stacked bases remains a possibility.

One aspect of polynucleotide structure which is often overlooked is the possible influence of pentose configuration. Both rA and dA residues appear to have the *anti* configuration about the glycosidic bond (Haschemeyer and Rich, 1967; Chan and Nelson, 1969). However, dA (in deoxyadenosine) is the only nucleoside in which any carbon atom is placed exo to the plane of the sugar (Haschemeyer and Rich, 1967). It is not yet clear whether the type of carbohydrate puckering can impose a specific geometry upon a polynucleotide chain. However, molecular models show that the phosphate groups would be closer to the bases in a single-strand helical array of rA than in one of dA (C. D. Jardetzky, 1969, personal communication).

B. Double-Strand Forms and pH Dependence of Poly rA and Poly dA

Upon protonation of adenine residues in acid solution poly rA forms a double-strand helix. X-Ray diffraction studies (Rich *et al.*, 1961) show that this structure is stabilized by three hydrogen bonds per adenine, plus a salt link between the positively charged N_1 and a phosphate on the other strand; the bases are tilted with respect to the helical axis. A sharp structural transition from the neutral, single-strand form of poly rA to the acidic, double-strand form takes place at pH 5.87 in 0.1 M salt solution. This transition is accompanied by striking changes in absorption spectrum, optical rotatory dispersion, and circular dichroism. Analogous changes in optical properties occur with poly dA, under the same conditions, with an apparent pK_a of 4.40. The optical rotatory dispersion (Holcomb and Tinoco, 1965; Sarkar and Yang, 1965; Ts'o *et al.*, 1966) and circular dichroism (Brahms *et al.*, 1966) data from various laboratories do not agree well for acidic poly rA. These data were collected at pH values varying from 4.6 to 5.5, and sometimes give evidence of two positive Cotton effects at high wavelength. Ts'o and coworkers (1966) examined the optical rotatory dispersion and absorption spectra of acidic poly dA under various conditions. In the present study, the pH dependence of the optical rotatory dispersion, absorption spectra, and hydrogen ion uptake for poly rA and poly dA are compared further. Two different acidic structures are found for each polymer. Another problem was to investigate whether the acidic poly dA structures are double strand like those of poly rA. There is some controversy over this point, which cannot be easily resolved by temperature-dependence studies, because of degradation of poly dA upon heating. Several groups (Bollum *et al.*, 1964; Riley *et al.*, 1966; Ts'o *et al.*, 1966; Barszcz and Shugar, 1968) have reached different conclusions concerning poly dA structure on the basis of ultraviolet absorption spectra and other methods.

Effect of pH upon the Optical Rotatory Dispersion of Poly rA and Poly dA. The optical rotatory dispersion spectra in citrate buffer (0.1 M ionic strength) for both adenylate polymers at various pH values are shown in Figure 5. At neutral pH each polymer exhibits the optical rotatory dispersion characteristic for its single-strand form. Then, as the pH is lowered there is a transition to an acidic form, designated as form B (the more basic of the two low pH structures). The curve for poly dA at pH 4.60 shows a mixture of neutral and B forms. At pH 5.81 for poly rA, and pH 4.21 for poly dA, the optical rotatory dispersion indicates pure form B. Then, as the solution is made even more acidic, there is a gradual transition to a still more acidic form, A (see pH 4.00 for poly rA and pH 3.02 for poly dA). For both polymers the form A Cotton effect is at lower wavelength and is of somewhat greater amplitude than that for form B. Parameters for all the forms are listed in Table I. An example of the coexistence of forms A and B is shown for poly rA at pH 5.00; this type of double positive Cotton effect can be seen in previous work (for example, Holcomb and Tinoco, 1965).

It is of interest to compare the acidic forms of the two polymers (Table I and Figure 5): both A and B forms of poly dA have Cotton effects of much smaller amplitudes than the corresponding poly rA Cotton effects. In addition, although the A forms of both polymers have optical rotatory dispersion extrema at approximately the same wavelengths, the B form Cotton effect is at a longer wavelength for poly rA than for poly dA. This shift is opposite to that observed in the absorption spectra; in acid solution poly rA absorbs at 252 nm, and poly dA at 261 nm. Furthermore, the A and B acidic forms of poly rA (as well as the neutral forms of both polymers) have optical rotatory dispersion spectra which show evidence of exciton interaction; the optical rotatory dispersion troughs

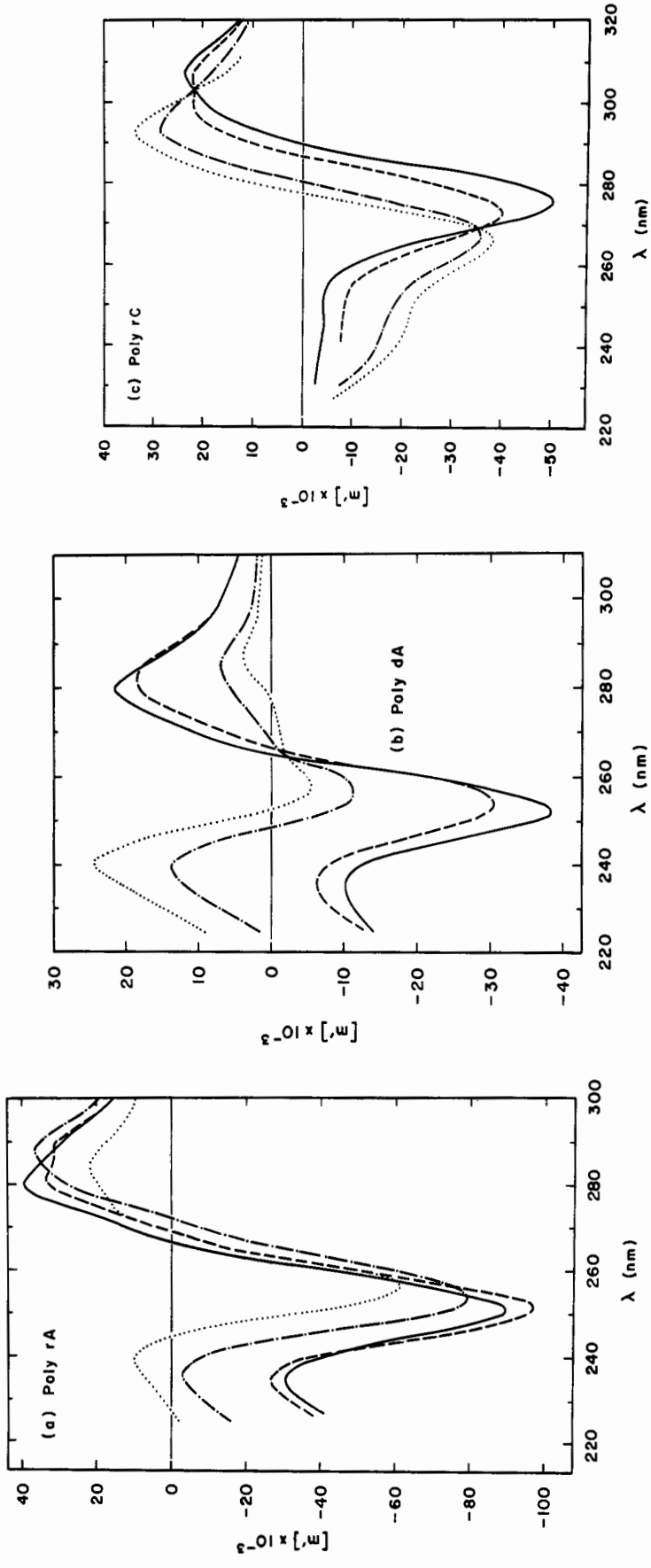

FIGURE 5: Optical rotatory dispersion of polynucleotides in citrate buffer at various pH values. (a) Poly rA: pH 5.91, ·····; 5.81, —·—·—; 5.00, ———; 4.00, ———. (b) Poly dA: pH 7.05, ·····; 4.60, —·—·—; 4.21, ———; 3.02, ———. (c) Poly rC: pH 6.19, ·····; 5.72, —·—·—; 5.70, ———; 5.27, ———. Note that the graphs are drawn to different scales.

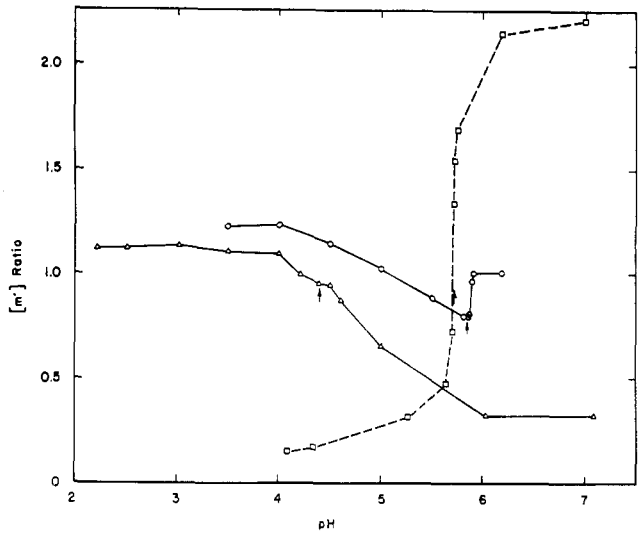

FIGURE 6: Optical rotatory dispersion titrations of polynucleotides; changes in ratios of some peak values as a function of pH in citrate buffer. Poly rA, ratio of $[m']_{280\ nm}$ to $[m']_{257\ nm}$, O—O; poly dA, $[m']_{280\ nm}/[m']_{283\ nm}$, Δ—Δ; poly rC, $[m']_{292\ nm}/[m']_{307\ nm}$, □--□. Arrows indicate pK_a values obtained from spectrophotometric and potentiometric titrations at the same ionic strength (0.1 M).

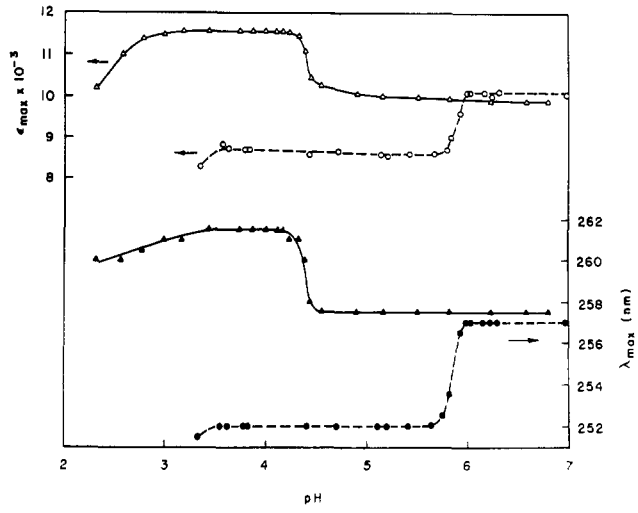

FIGURE 7: Spectrophotometric titrations of adenine polymers in 0.1 M NaCl. Only features of the longest wavelength absorption peak are considered. Poly rA: extinction coefficient, O--O; peak wavelength, ●--●. Poly dA: ϵ_{max}, Δ—Δ; λ_{max}, ▲—▲.

are situated between two apparent maxima and occur at the same wavelength as the absorption peaks. On the other hand, λ_{max} for the acidic forms of poly dA is nearer to the optical rotatory dispersion crossover wavelengths; this is more characteristic for a single, unsplit, positive Cotton effect.

Thus, optical rotatory dispersion shows that both poly rA and (less obviously) poly dA can exist in two different acidic forms. The optical rotatory dispersion patterns are not simply related to the absorption spectra. The A and B acidic forms for each polymer are structures with different periodic, asymmetric arrangements of residues, perhaps with differently tilted bases.

The optical rotatory dispersion of poly rC at various pH values is included in Figure 5 for comparison. This polymer exhibits only a single transition, a simple change from neutral to acid form. All the optical rotatory dispersion curves meet at isosbestic points at 269 and 302 nm. Two approximately equal peaks appear at the pK_a (5.70, determined by hydrogen ion titration; Hartman and Rich, 1965). This simple behavior of poly rC shows that the complex pH dependence of the adenylate polymers is not attributable to instrumental or buffer artifacts. Furthermore, there is no evidence here for the structural collapse of poly rC noted by Guschlbauer (1967) at pH slightly above the pK_a.

The changes in optical rotatory dispersion patterns as a function of pH are plotted as "optical rotatory dispersion titrations" in Figure 6. For each polymer two optical rotatory dispersion peaks were chosen, whose ratio is particularly sensitive to changes in pH. In this way, the changes in spectral shape accompanying interconversions between structures can be visualized quantitatively. The poly rC data indicate a single titration curve for the transition from single- to double-strand forms. On the other hand, for both poly rA and poly dA, lowering the pH from the neutral range produces two discrete changes in peak ratio, with a plateau section between the steps. This plateau region corresponds to acidic form B. Form B is seen to be the only structure present at a pH very near the pK_a's (determined by hydrogen ion and spectrophotometric titrations), where there is partial protonation of adenine. Then, as the pH is further lowered, there is a gradual conversion of form B into form A (at pH range 5.8–4.0 for poly rA, 4.4–3.0 for poly dA). Within these pH ranges all the optical rotatory dispersion spectra can be fitted quite well to curves calculated from various mixtures of forms A and B, using the appropriate parameters of Table I. For example, the data for poly rA at pH 5.0 agree with the curve for 60% A and 40% B. This is additional evidence that the broad transitions at acid pH are attributable to interconversions between two forms. At very low pH values, only form A is found in solution; this results in another plateau (Figure 6). The optical rotatory dispersion titrations are reversible when the pH is raised; this indicates that a real equilibrium between A and B exists throughout the pH regions indicated. (Below pH 2.5 at 22° poly dA shows a slow decrease in optical rotatory dispersion amplitude because of degradation; however, the curve shape and the peak ratio remain constant.)

In the next sections other types of titration data will be presented and compared with the optical rotatory dispersion results.

Spectrophotometric Titrations. The ultraviolet absorption spectra of both poly rA (Fresco and Klemperer, 1959) and poly dA (Bollum *et al.*, 1964) undergo large changes upon protonation of the adenine residues. Spectrophotometric titration curves for these polymers in unbuffered 0.1 M NaCl are presented in Figure 7. Identical results were obtained in the same citrate buffer as was used for optical rotatory dispersion. The titrations were begun at neutral pH and were continued until the solutions became opalescent or scattered light. The poly dA data are in agreement with those of Ts'o *et al.* (1966). Both polymers exhibit sharp titration steps. The pK_a for poly rA is 5.87; that for poly dA is 4.40. Both pK_a's are higher than that of adenine in the monomers (both about 3.8). Upon protonation, both λ_{max} and ϵ_{max} decrease for poly rA and increase for poly dA. This difference may reflect preferential

protonation at N_1 of poly rA and at N_3 of poly dA (Ts'o *et al.*, 1966).

It is noteworthy that there is no spectral change in the pH region of the form A $\rightleftarrows$ form B equilibrium, which was shown to exist by optical rotatory dispersion. For each polymer both acid forms have the same ultraviolet spectrum, different from that of the neutral form. Even the prominent 288-nm absorption shoulder of poly rA is unchanged from pH 5.7 to 3.5. The two acidic forms are distinguishable only by their rotatory properties.

At very low pH (below 3.5 for poly rA, below 3.0 for poly dA) both polymers show decreases in λ_{max} and in ϵ_{max}. This effect has been noted for poly dA by Ts'o *et al.* (1966) and may be related to increasing aggregation. In any case, these spectral changes occur in a pH region where form A has reached a plateau concentration (see Figure 6) and are thus not relevant to the optical rotatory dispersion results.

Hydrogen Ion Titrations. These titrations were performed (in 0.1 M NaCl) in order to seek evidence related to the two acidic forms. The results are shown in Figure 8. The pK_a values, obtained from the steep increases in H^+ uptake, agree fairly well with the spectrophotometric values. However, for both poly rA and poly dA, there are regions of gradual H^+ uptake, which do not appear in the sharp spectrophotometric titrations. These regions (pH 5.7–4.0 for poly rA and 6.0–4.5 for poly dA) correspond to pH ranges where gradual structural transitions occur, as seen by optical rotatory dispersion (Figure 6). These transitions are from acidic form B to form A for poly rA and neutral form → form B for poly dA. Thus, optical rotatory dispersion is sensitive to slight changes in protonation, whereas absorption spectroscopy, in this case, can detect only gross single-strand → double-strand helical transitions.

In careful hydrogen ion titrations of poly rA, Holcomb and Timasheff (1968) noted asymmetric titration curves similar to ours. Their explanation was that, after a certain fraction of protonation (about 0.5 under the conditions used here), the single-strand form becomes unstable and converts to the double-helical structure. Full protonation is not a structural requirement for the acidic form of poly rA determined by X-ray data (Rich *et al.*, 1961); the positive charge on the adenine contributes only to a salt bridge.

When the hydrogen ion titrations are compared with the optical rotatory dispersion titrations, it is seen that acidic form B is the only stable structure for both poly rA and poly dA in the region of half-protonation (pH range 5.8–5.9 and 4.4–4.5, respectively). Thus, form B is likely to be a half-protonated double helix.

At very low pH (below 3.6) both adenylate polymers are seen to take up a second equivalent of hydrogen ions, with an apparent pK_a of about 3.2. This titration may be correlated with the absorption changes seen in Figure 7 and noted for poly dA by Ts'o *et al.* (1966).

Comments on the Two Acidic Forms. An attempt will be made to correlate data relating to forms A and B of poly rA and poly dA. In the pH range above 3.5, absorption spectroscopy indicates only a single change in form for each polymer upon protonation. However, both optical rotatory dispersion and hydrogen ion titrations show anomalies which can be explained by two acidic forms. For each polymer, form B is stable near half-protonation, and form A is stable at complete protonation of adenine. At intermediate pH values an equilibrium is present between the two forms. The two acidic forms have optical rotatory dispersion spectra more similar to each other than to that of the neutral forms; therefore, form B is probably double strand.

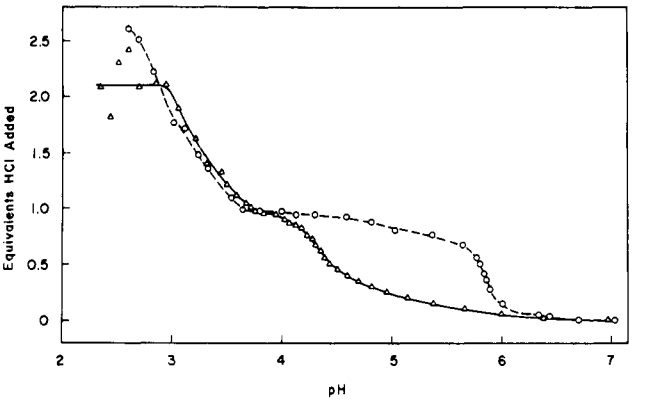

FIGURE 8: Hydrogen ion titrations of adenine polymers in 0.1 M NaCl: poly rA, O--O; poly dA, Δ—Δ. Curves are corrected for titration of corresponding salt blank.

Evidence from X-ray studies of poly rA fibers reinforces this interpretation. At pH 5.5 a mixture of two double-helical forms is found (A. Klug, 1967, personal communication). At a somewhat higher pH pure form B can be obtained, in which protons may go onto alternate bases in the structure to minimize the repulsion. At somewhat lower pH pure form A is found, with a larger base separation; in this form the protons may be on adjacent bases, probably causing some untwisting of the helix. It is probably these same two forms which are observed to have different optical rotatory dispersion patterns.

Both poly rA (Holcomb and Tinoco, 1965) and poly dA (Ts'o *et al.*, 1966) aggregate at low pH. However, form A cannot be merely an artifact due to aggregation of form B, for the following reasons. First, the A and B forms are evident as independent structures by X-ray diffraction of poly rA. Second, the B → A transition occurs in the same pH region (relative to the pK_a) for poly rA and poly dA. For example, for both polymers form B is stable at half-protonation. This would be a remarkable coincidence if caused only by aggregation. Finally, aggregation has a pH dependence different from that of the B → A transition. This transition, for both polymers, begins at a much higher pH than at which aggregation can first be detected. Furthermore, the conversion to form A is complete in a pH region where aggregation steadily increases as the pH decreases.

Effect of Heat on Poly dA in Acid. The remaining sections of this paper will concentrate on the problem of whether poly dA is a double-strand helix below its pK_a. The titration curves of poly dA were seen to be as sharp as those of poly rA, not broad, like that of AMP (Alberty *et al.*, 1951). The titration data of poly dA are, therefore, indicative of a cooperative transition to a double helix. However, further data would be desirable to verify this hypothesis. A sharp loss of structure in a narrow temperature range has been shown to result from a helix → coil transition in poly rA. This change has been measured by hypochromicity (Fresco and Klemperer, 1959) and optical rotatory dispersion changes (Holcomb and Tinoco, 1965; Sarkar and Yang, 1965). Heating of poly dA in acid is

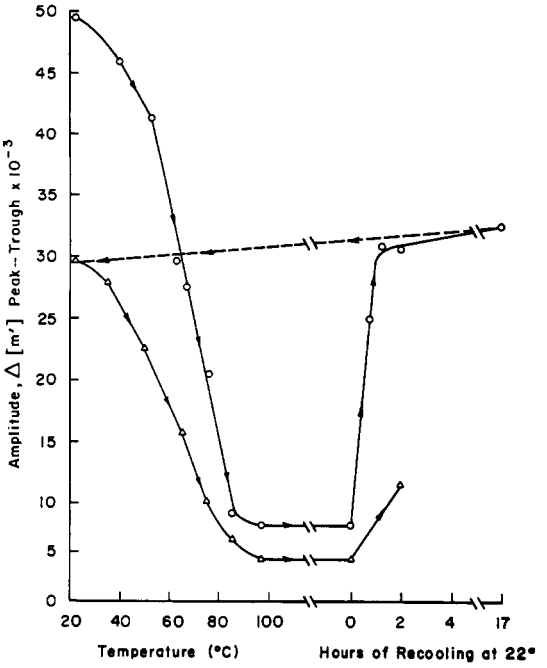

FIGURE 9: Effect of heating and recooling upon the optical rotatory dispersion of poly dA in pH 4.3 acetate buffer. The ordinate is the amplitude of the first Cotton effect, *i.e.*, $[m']_{peak, \sim 282\ nm} - [m']_{trough, \sim 253\ nm}$. First cycle of heating and recooling, O—O; second cycle, Δ—Δ. The time course of the experiment is shown by the arrows. In each cycle, the solution reached about 80° after 1 hr of heating, reached 97° after 1.2 hr, and was recooled starting at 1.5 hr. All points are corrected for volume expansion.

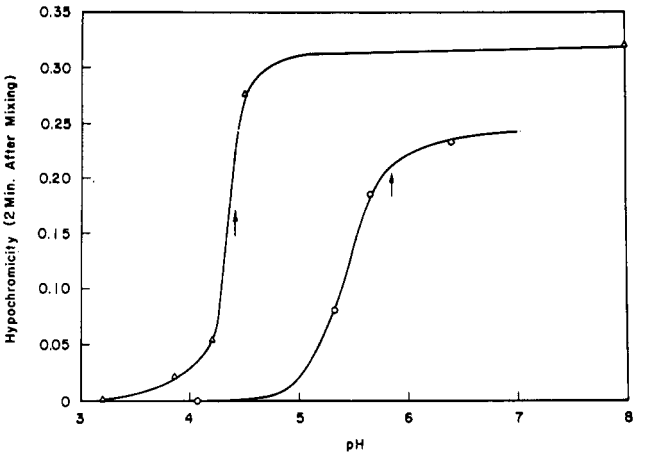

FIGURE 10: Ability of adenine polymers to form complexes with 2 equiv of poly rU, as a function of pH. Poly rA, O—O; poly dA, Δ—Δ. Ordinate equals the decrease in OD_{max} (at λ ~260 nm) observed 2 min after mixing the A and U solutions, divided by the total OD_{max} of the separated A and U solutions. Arrows indicate titrimetric pK_a values. Buffers: acetate at pH > 4, formate at pH < 4.

accompanied by hydrolysis (Ts'o *et al.*, 1966) and depurination (Barszcz and Shugar, 1968). Nevertheless, it was of interest to examine this thermal transition by means of optical rotatory dispersion.

The optical rotatory dispersion temperature profile results for poly dA at pH 4.3 are given in Figure 9. The arrows indicate the time course of the experiment. Heating from 22 to 97° also causes a 14% increase in ϵ_{max} and a shift in absorption λ_{max} from 261 to 258 nm, with the changes beginning at 60°. (All results are corrected for volume expansion.) The optical rotatory dispersion and absorbance heating curves are only partially reversible. They are not as sharp as those for poly rA but are considerably sharper than those for single-strand neutral forms of polynucleotides. Both optical rotatory dispersion heating cycles display the same temperature dependence. Neutralization to pH 7 of the solution after both heating cycles showed a 40% decrease in the characteristic poly dA optical rotatory dispersion spectrum.

The degradation accompanying thermal treatment of poly dA was investigated by paper electrophoresis (see Experimental Section). The monomers, dAMP-5′ (which showed irreversible spectral changes upon heating at pH 2.5) and rAMP-5′ (which displayed no such changes), were examined similarly. Both heated poly dA and heated dAMP yielded the same negatively charged compound. In addition, some poly dA (or high oligomers) remained at the origin. Thus, during heating in acid, poly dA undergoes degradation, probably depurination and chain scission, in common with other heat-labile dA compounds.

The temperature dependence of acidic poly dA is difficult to interpret, because of degradation. However, the relative sharpness of the heating curves, together with the consistent melting temperature (65°) for both heating cycles, show that a double-strand helical form is likely for poly dA in acid. For comparison, poly rA at a pH (5.45) slightly below its pK_a melts at 43°; this sharp double-helix to single-strand transition is completed within three degrees. The slow time course of optical rotatory dispersion reversibility upon recooling acidic poly dA may be additional evidence for annealing to a double helix.

Reaction of Adenylate Polymers with Polyuridylic Acid. When poly rA is in the neutral, single-strand form, it reacts readily with 2 residue equiv of poly rU to form a triple-strand helix at moderate salt concentration (Barszcz and Shugar, 1968). When poly rA is in its acidic, double-strand form, it is not available for combination with poly rU. It was anticipated that the ability of poly dA to form a similar complex with poly rU could be used as an index of poly dA structure and its variation with pH. A poly dA·2 poly rU triplex can form in solutions considerably more basic than the pK_a of poly dA (Riley *et al.*, 1966; Barszcz and Shugar, 1968), but no studies have been reported at lower pH. If poly dA were double strand at low pH, then its behavior would be expected to be similar to that of poly rA at low pH; that is, poly rU would not be able to compete with the poly dA duplex for a strand of poly dA, and no dA·rU complex should form.

Complex formation with poly rU for both poly rA and poly dA was monitored by observing changes in the ultraviolet absorption spectra when the polymers were mixed. (See Experimental Section for details.) In particular, the change in OD_{max}, as a function of time, was followed.

In Figure 10 is seen the fractional decrease in OD_{max} (*i.e.*, the hypochromicity) 2 min after mixing poly rU with either poly rA or poly dA, as a function of pH. As time increased, further absorbance decreases were noted at pH values near the pK_a's. At lower pH (for example, 3.20 for poly dA and 4.07 for poly rA) no change in spectrum was observed at all, even 20 hr after mixing.

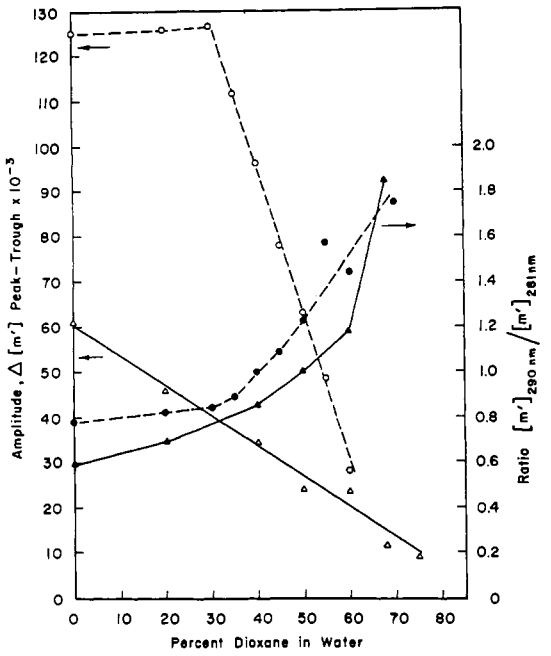

FIGURE 11: Effect of dioxane concentration upon optical rotatory dispersion of acid forms of adenine polymers in formate buffer. Poly rA, initial pH 3.76 (in aqueous solution): $\Delta[m']$, O--O; ratio of $[m']_{290\text{ nm}}$ peak to $[m']_{281\text{ nm}}$ peak, ●--●. Poly dA, initial pH 3.16: $\Delta[m']$, △—△; $[m']_{290\text{ nm}}/[m']_{281\text{ nm}}$, ▲—▲. The amplitude of the first Cotton effect, $\Delta[m']$, was calculated using the peak at 290 or 281 nm (whichever was larger), and the trough value at about 252 nm.

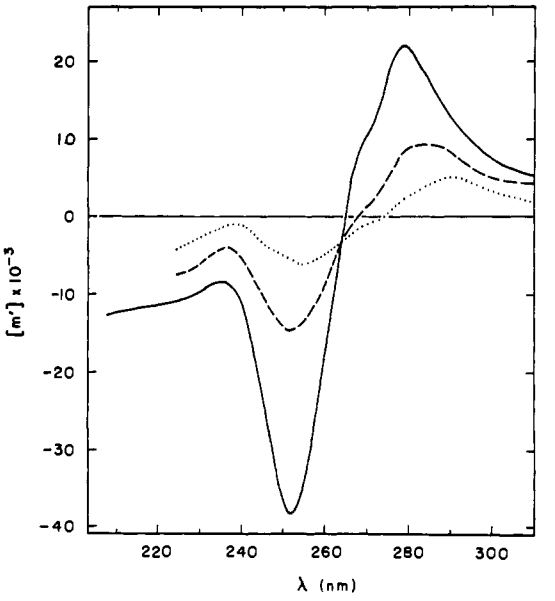

FIGURE 12: Changes in shape of poly dA optical rotatory dispersion in pH 3.16 formate, caused by dioxane. Per cent dioxane: 0, ——; 50, – – – –; 68, · · · ·.

Poly dA, like poly rA, is seen to be unable to complex with poly rU if the pH is well below its pK_a. This finding may be taken as evidence that poly dA forms a double-strand helix, similar to that of poly rA, in acidic media; consequently, poly rU is then unable to compete successfully for poly dA. The pH dependence of complexing ability (Figure 10) relative to the pK_a, is similar for the two adenylate polymers. The A and B acidic forms of the polynucleotides cannot be differentiated by this spectrophotometric method of complex formation.

The pH dependence (fairly broad at low pH) of poly rA and of poly dA complex formation with poly rU is very similar to the pH dependence of the reaction of poly rA with formaldehyde at 44° (Fresco and Klemperer, 1959). The pH dependence of the kinetics of these reactions are similar, as well. The effectiveness of all these reactions depends upon the availability of the adenine amino groups on the polymers. All of the reactions proceed, to some extent, in the pH range where the adenylate polymers exist in acidic form B as well as in single strands. This finding may indicate that the interactions between the two strands in form B are weak enough to allow competition from poly rU and from formaldehyde. Form A of poly rA and of poly dA is apparently a more stable double helix and does not react at all.

Effect of Dioxane upon Acidic Poly dA and Poly rA. In an attempt to detect similarities and differences between the acidic structures, the optical rotatory dispersion spectra of double-helical poly rA and poly dA were measured in various concentrations of dioxane.

Upon the addition of dioxane to poly rA in citrate buffer at pH 5.5, no change was observed in the optical rotatory dispersion until 23% (by volume) dioxane was added. At this concentration there was an abrupt change to the neutral form optical rotatory dispersion spectrum. At this point and at all higher dioxane concentrations, the optical rotatory dispersion results were identical with those obtained at neutral pH (see Figure 3). The apparent pH of the citrate buffer in 23% dioxane was 5.9. Hydrogen ion titration showed that the pK_a (*i.e.*, the apparent pH of the transition) for poly rA in 25% dioxane was 5.85, identical with that in water. Therefore, the sharp double-strand → single-strand transition was caused not by any effect of dioxane *per se* upon the structure of the polynucleotide but rather by the increase in apparent pH of the solvent. All additional experiments were performed at much lower pH, in order to avoid this effect.

The changes in optical rotatory dispersion of poly rA (initial pH 3.76 in aqueous formate buffer) and of poly dA (initial pH 3.16) are summarized in Figure 11. The concentration for each polymer was 1.0×10^{-4} M. Experiments were terminated when precipitation occurred. All data for poly rA and all but the last two points for poly dA were taken at apparent pH below the pK_a's. The corresponding mononucleotides at low pH showed no changes of optical rotatory dispersion in 75% dioxane. The acidic polymers show little or no hyperchromism in dioxane (see Table I).

The double-strand structure of poly rA is resistant to concentrations of dioxane up to 30%, as is seen from the amplitude of the first Cotton effect. This finding agrees with the stability of acidic poly rA toward ethylene glycol found by Hanlon and Major (1968), which they attributed to the organic solvent's role in strengthening the internal ion-pair bond of poly rA. At dioxane concentrations above 30%, the Cotton effect of poly rA decreases in a steep linear manner, similar to that of double-strand DNA (Geiduschek and Herskovits, 1961). In contrast, poly dA begins its loss of structure at lower amounts of dioxane, and its melt-out is not as sharp as with poly rA. This relative instability of acidic poly dA to-

ward dioxane may indicate that the 2′-hydroxyl group of poly rA strengthens the poly rA double helix through hydrogen bonding; however, such a conclusion could be reached only if base-stacking interactions were identical in the two polymers.

An additional observation is that both poly rA and poly dA shows changes in the position of their optical rotatory dispersion spectra; as the dioxane concentration is increased, the first peak shifts from 281 to 290 nm for both polymers. This is illustrated for poly dA in Figure 12. The ratio of the two peaks is shown in Figure 11 for both polymers. This wavelength shift is not related to any conversion from acidic form A into form B. Although the apparent pH values of the solutions are raised (for example, to 4.06 for poly dA at 50% dioxane), neither the pH dependence nor the shapes of the optical rotatory dispersion curves can be explained by an A → B transition. Furthermore, the optical rotatory dispersion spectra do not approach those of the monomers, as was the case at neutral pH. Hanlon and Major (1968) did not observe a red-shifted optical rotatory dispersion spectrum for acidic poly rA in ethylene glycol.

Conclusions from pH Dependence. A comparative study of poly rA and poly dA in acidic solution has shown that poly dA exists as a double-strand helix. The best evidence for this is the inability of acidic poly dA to form a complex with poly rU. Other supporting data are the sharpness of the poly dA titration curves and temperature dependence (which is complicated by degradation)

The optical rotatory dispersion results show that both adenylate polymers can form two different acidic structures. One form predominates at half-protonation, the other at complete protonation. The two structures are in equilibrium at intermediate pH values.

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The Amino Acid Sequence of Bovine Carboxypeptidase A. II. Tryptic and Chymotryptic Peptides of the Cyanogen Bromide Fragment F_{III}*

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ABSTRACT: An 81 amino acid residue fragment (F_{III}) of bovine carboxypeptidase A, obtained by cleavage with cyanogen bromide and isolated by gel filtration on Sephadex G-75, has been subjected to tryptic and chymotryptic hydrolysis. The resulting peptides have been isolated and characterized by Edman degradations and hydrazinolysis, and by hydrolysis with carboxypeptidase A and B and leucineaminopeptidase. Alignment of these peptides yields a tentative structure for the entire fragment, which is in exact agreement with the amino acid composition deduced from acid hydrolysates.

As the first step in the complete sequence analysis of bovine carboxypeptidase A, the three residues of methionine have served as cleavage sites in the cyanogen bromide reaction to produce four fragments. These fragments account satisfactorily for the molecular weight and amino acid composition of the native enzyme (Nomoto *et al.*, 1969). Two of these fragments, termed F_N and F_C, have been examined in detail and their complete primary structure has been elucidated (Bargetzi *et al.*, 1964; Sampath Kumar *et al.*, 1964). One of the remaining two fragments, F_I, contains 198 amino acids and is attached in the sequence to the carboxyl-terminal peptide F_C, while the other, F_{III}, containing 81 amino acids, has been positioned adjacent to the amino-terminal fragment, F_N (Nomoto *et al.*, 1969). This fragment is devoid of half-cystine and possesses a single amino-terminal residue of aspartic acid.

Sequence analysis of F_{III} has been initiated by isolation and characterization of the tryptic and chymotryptic peptides. These data are sufficient to produce a tentative structure of the fragment. The additional information necessary to provide the complete structure has been obtained from peptides produced by hydrolysis of F_{III} with thermolysin and is reported in the accompanying paper (Bradshaw, 1969).

Experimental Procedure

Materials. Carboxypeptidase A (Anson) was purchased in 10-g lots as twice-crystallized material from Worthington Biochemicals and used without further purification.

Fragment F_{III} was prepared by cyanogen bromide cleavage and purified as previously described (Nomoto *et al.*, 1969)

Trypsin, chymotrypsin, carboxypeptidase B, and leucineaminopeptidase were obtained from Worthington Biochemicals.

Pyridine and *N*-ethylmorpholine were redistilled from solid ninhydrin (1 g/l.) before use.

Phenyl isothiocyanate and trifluoroacetic acid were purchased from Eastman Organic Chemicals and redistilled before use.

Hydrazine was obtained from Matheson Coleman and Bell.

Methods. Tryptic and chymotryptic peptides were prepared in the following manner. Lyophilized F_{III} was suspended in water (10 mg/ml) and dissolved by the addition of 1 N NaOH. When all of the protein had dissolved, the solution was readjusted to pH 8.5–9.0 with 1 N HCl to yield a uniform suspension. Trypsin or chymotrypsin, prepared as a stock solution (10 mg/ml) in 10^{-3} M HCl, was added to a final concentration of 1% (w/w) relative to the protein substrate. The pH of the reaction mixture (37°) was held constant with a Radiometer pH-Stat equipped with an Ole Dich recorder. At the completion of the reaction (3–6 hr), the hydrolysate was adjusted to pH 2.0 with 6 N HCl. The insoluble material formed was removed by centrifugation.

The soluble peptides from both digests were fractionated on a column (2.0 × 25 cm) of Dowex 50-X8 (Spinco amino acid

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