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Properties of Helical Polycytidylic Acid*

E. O. AKINRIMISI, C. SANDER, AND P. O. P. TS'O†

From the California Institute of Technology, Division of Biology, Pasadena, California

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The T_m of polycytidylic acid in acidic solutions of various *p*H levels, ionic strengths, and urea concentrations has been investigated by measurements of absorbancy and specific rotation *versus* temperature. Other physicochemical properties of polycytidylic acid in solution were also studied, such as optical rotatory dispersion, viscosity, and sedimentation coefficients. The data support the notion that the polycytidylic acid in acidic solution assumes a helical structure with a hydrogen bonding scheme similar to that of the cytosine-5-acetic acid in crystal. This scheme consists of a pair of hydrogen bonds formed between the amino groups and the keto groups of the two cytosine residues in the helix, with an added proton in between shared by the two ring nitrogen atoms.

Studies of the helical structures formed by synthetic polynucleotides have provided considerable insight into the problem of the specificity and interaction of natural nucleic acids (Doty *et al.*, 1959; Steiner and Beers, 1961). All available homopolymers, *i.e.*, polyadenylic acid, polyinosinic acid, polyuridylic acid, and polycytidylic acid,[1] appear to form regular and helical structures by themselves under certain conditions. Among these, the purine-polynucleotides, especially poly A, are better known (Fresco and Klemperer, 1959; Rich *et al.*, 1961; Ts'o *et al.*, 1962a), while less is known about the pyrimidine-polynucleotides. The structures and the hydrogen-bonding schemes of the homohelices formed by poly U and poly C are of great interest in view of the fact that they are both biochemically active in the synthesis of polypeptides by the ribosomal system of *E. coli* (Nirenberg and Matthaei, 1961; Speyer *et al.*, 1962).

The optical properties of poly C in both neutral and acidic solution have been reported (Ts'o *et al.*, 1962a). The relationship between the optical properties of poly C and its secondary structure was found to be complicated compared to that of poly A and other nucleic acids (Ts'o *et al.*, 1962a; Helmkamp and Ts'o, 1962). This paper contains additional information pertinent to the understanding of the helical structure of poly C in acidic solution. The information is especially relevant in view of the hydrogen-bonding scheme of cytosine-5-acetic acid in the crystal form as recently described by Marsh *et al.* (1962) and the x-ray fiber diagram of helical poly C as described by Langridge and Rich (1963).

MATERIALS

The poly C was purchased from Miles Chemical Co., Clifton, N. J. The polymer was further deproteinized by the detergent–amyl alcohol–chloroform method as previously described (Ts'o *et al.*, 1962a). The polymer was dialyzed against 0.2 M ammonium acetate in the cold for 40 hours and then precipitated with three volumes of cold alcohol. The precipitate was washed twice with alcohol and twice with ether and then dried *in vacuo*. All other compounds were of reagent grade.

INSTRUMENTATION AND METHODS OF ANALYSIS

Optical rotation measurements were made with a Rudolph Model 200S polarimeter equipped with an oscillating polarizer and xenon and mercury arc lamps (Ts'o *et al.*, 1962a). The 20-cm polarimeter tubes utilized glass construction with water jacket and quartz window. The temperature of the polarimeter tube was read directly with a thermometer and held at any desired temperature to ±0.1° by the flow of water through both the compartments and the tube. Measurements of specific rotation $[\alpha]$ were determined at concentrations of 0.4–0.6 mg/ml.

Optical density measurements were made with a Beckman DK-2 recording spectrophotometer fitted with a modified temperature-control device. Quartz cells were fitted with a 20-mm immersion standard taper thermometer for direct reading of solution temperatures to ±0.1°.

*p*H measurements were made with a Radiometer *p*H meter 22, Copenhagen, Denmark, and to an accuracy of ±0.01 *p*H units.

Resistance of the solutions was measured to an accuracy of ±1% at 22.2 ± 0.05° with a portable A.C. Electrolytic conductivity bridge manufactured by Leeds and Northrop Co., New York.

Viscosities were determined with a four-bulb dilution-type viscometer (Ubbelohde) designed and constructed by the Cannon Instrument Co., State College, Pa.

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† On leave of absence from Department of Radiological Sciences, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, Md.

[1] Abbreviations: poly A, polyadenylic acid; poly I, polyinosinic acid; poly U, polyuridylic acid; poly C, polycytidylic acid.

The viscometer had a water flow time of 350 seconds for each bulb, and its maximum shear gradient for water varied from 40 sec.$^{-1}$ to 210 sec.$^{-1}$ Measurements were performed at 22.6 ± 0.05°.

Analytical ultracentrifugation was performed in a Model E ultracentrifuge, Spinco Division, Beckman, Inc., with ultraviolet absorption optics. The instrument was provided with a temperature control system. The absorption patterns recorded on the film were traced with a Double-Beam Recording Microdensitometer, Joyce Loebl Co., Newcastle-upon-Tyne, England. All the sedimentation coefficients reported were corrected to 20° but not for effects of solvent viscosity and density.

The molar extinction coefficient per phosphorus of the poly C was determined by hydrolyzing a sample of known absorbancy to cytidylic acid with 0.3 N KOH for 18 and 24 hours at 38°. The molar extinction coefficient of 2'- and 3'-CMP was taken to be 13.0×10^3 at 280 mμ, *p*H 2.0. The maximum molar extinction coefficient of poly C at 25° and 0.1 M salt determined by the above method is 6.6×10^3 (267 mμ) at *p*H 7.5, 7.2×10^3 (274 mμ) at *p*H 4.85, 7.7×10^3 (274 mμ) at *p*H 4.05, and 8.0×10^3 (275 mμ) at *p*H 3.65. All these values are within 5% of those previously reported from this laboratory. The concentration of poly C in all experiments was determined optically with use of these values of maximum molar extinction coefficients.

The recorded T_m is the temperature at the midpoint of the changes of the optical properties of the poly C. The T_m, presumably the midpoint of the helix-coil transition, is in all cases accurate to ±0.5°.

RESULTS

Optical rotation and absorbancy measurements of poly C in 0.1 M sodium acetate, *p*H 4.05, at varying temperatures give essentially the same T_m (Fig. 1). The initial drop of the optical absorbancy before the temperature reaches the T_m can be explained on the basis that protons are being removed from the polymer as the temperature is elevated. This explanation is experimentally substantiated by the following observations: (1) Protons on cytidine-5'-phosphate and on poly C can be removed at elevated temperature (Ts'o *et al.*, 1962a; Helmkamp and Ts'o, 1962). (2) The absorbancy at the minimum of the poly C spectrum increases gradually while the absorbancy at the maximum gradually decreases as the temperature is slowly raised from 30° to 60°. Thus, the maximum/minimum ratio of the spectrum of poly C in Figure 1 is reduced from 2.2 at 30° to 1.95 at 66°. This is consistent with the expectation based on the (maximum, 267 mμ)/(minimum, 248 mμ) ratio of 1.33 obtained at *p*H 7.5 at 25°. (3) There is a slight but notable shift of the minimum position of the poly C spectrum as the temperature is raised, as in the experiment of Figure 1. This red shift of the minimum is indicative of the slight change of the acidic form (minimum at 242 mμ) to the neutral form (minimum at 248 mμ). Thus, these facts all support the suggestion that as the temperature is raised a small number of protons is removed from poly C, although the helix remains intact until the temperature reaches the T_m.

The effect of *p*H on the T_m of poly C in 0.1 M sodium acetate is given in Figure 2. It is clear that the stability of poly C as a function of proton concentration can be classified into two zones, *i.e.*, the proton stabilization zone (*p*H 5.5 and higher to *p*H 4.0) and the proton destabilization zone (*p*H 3.5 and lower). The effects of the ionic strength (expressed as the co-log of the specific conductance) of the medium on T_m are given in Figure 3. At *p*H 4.85, in the zone of proton stabilization, increases in ionic strength of the solution lower the T_m and thus reduce the stability of the helix. At *p*H 3.65, in the zone of proton destabilization, increases in ionic strength of the solution raise the T_m

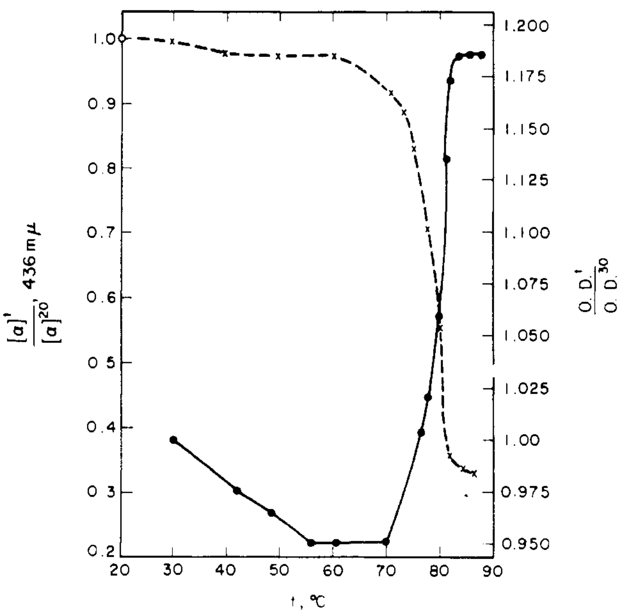

FIG. 1.—Profile of $[\alpha]^t/[\alpha]^{20°}$ (×) and of $OD^t/OD^{30°}$ (●) *versus* temperature of poly C in 0.1 M Na-acetate, *p*H 4.08. OD was measured at 274 mμ, the spectral position of the maximum.

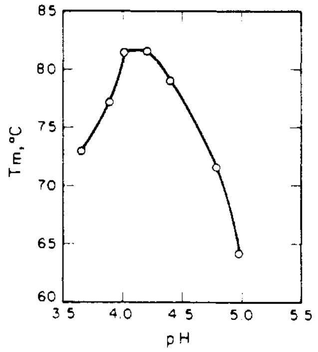

FIG. 2.—Variation of T_m of poly C with the *p*H of Na-acetate buffer (0.1 M Na-acetate) in the range *p*H 3.6 to *p*H 4.9.

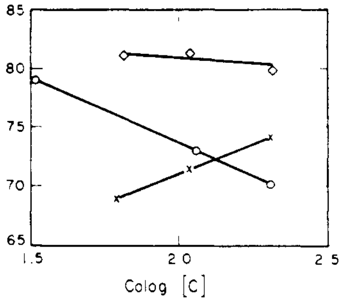

FIG. 3.—Dependence of T_m of poly C in acetate buffer on the concentrations of the electrolytes in solution as expressed as the co-log of the specific conductance: Buffers at *p*H 4.85 (×) and *p*H 4.05 (◇) were prepared from the dilution of 0.2 M Na-acetate to 0.05 M for the variation in conductance. Buffers at *p*H 3.65 (O) consist of 0.2 M NaCl and 0.1 M Na-acetate at the highest conductance, 0.1 M Na-acetate at the medium conductance, and 0.05 M Na-acetate at the lowest conductance.

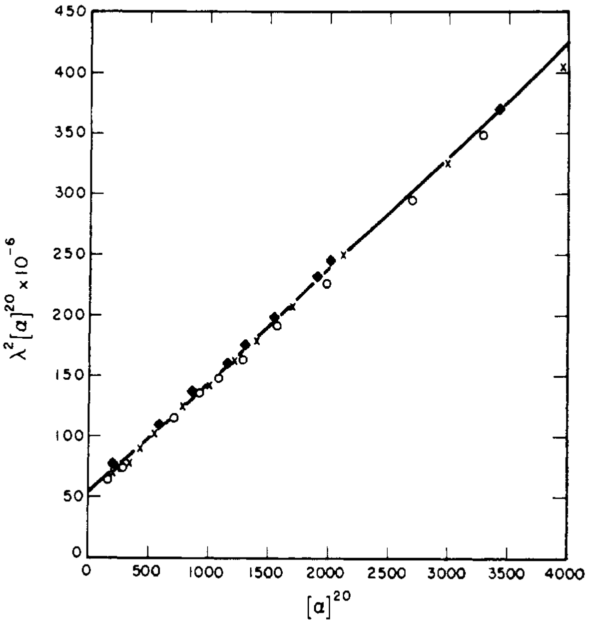

Fig. 4.—Modified one-term Drude plot of poly C in 0.1 M Na-acetate at pH 4.90 (◆), pH 4.05 (×), and pH 3.65 (○). [α] was obtained at 20°.

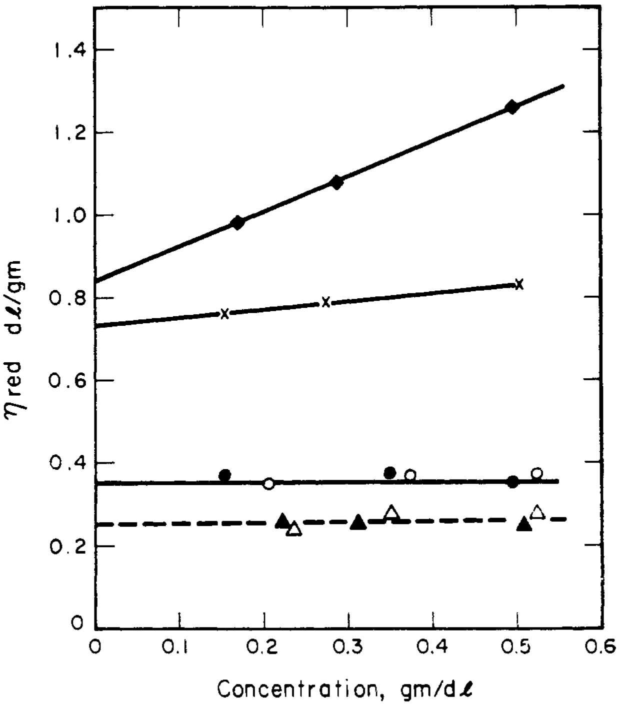

Fig. 5.—Reduced viscosity *versus* the concentration of poly C at different pH and electrolyte concentrations; pH 7.5, 0.01 M Tris, 0.05 M NaCl (◆); pH 7.5, 0.01 M Tris, 0.5 M NaCl (×); pH 3.75, 0.05 M Na-acetate (●); pH 4.85, 0.05 M Na-acetate (○); pH 3.75, 0.5 M Na-acetate (△); pH 4.85, 0.5 M Na-acetate (▲).

and thus enhance the stability of the helix. Therefore, as far as the effect of electrolytes on the T_m is concerned, poly C in the proton stabilization zone behaves like poly A (Ts'o *et al.*, 1962b), while poly C in the proton destabilization zone behaves like DNA (Ts'o *et al.*, 1962b). At the transition between these two zones, that is, at pH 4.0, the T_m of poly C is, interestingly enough, not greatly affected by electrolyte concentration (Fig. 3).

Table I

Optical Rotatory Dispersion Data of Poly C

pH	$[\alpha]^{20°}_{589}$	$[\alpha]^{20°}_{43}$	λ_c (mμ)	$K \times 10^{-6}$	Wave Length Range (mμ)
4.90[a]	225.5	584.4	303	50	320–589
4.05	198.0	531.3	306	50	320–589
3.65	184.3	485.4	305	48	320–589

[a] All in 0.1 M Na-acetate.

The optical rotatory dispersion data on poly C at pH 4.9, pH 4.0, and pH 3.6 are summarized in Table I. The data agree with the previously reported values from our laboratory to within 5–7%. As is indicated in the one-term Drude plot of the rotatory dispersion data (Fig. 4), the conformation of poly C in the zone of proton stabilization, in the zone of proton destabilization, and in the transition between these two zones is essentially the same.

The physical properties of poly C in neutral and acidic solution were examined by viscosity measurement and ultracentrifugation. The viscosity data of Figure 5 indicate that the intrinsic viscosity of poly C at neutral pH is much higher than that of poly C at pH 4.85. Furthermore, the reduced viscosity of neutral poly C is much more polymer-concentration dependent and electrolyte-concentration dependent than that of the acidic poly C. These observations are in agreement with the previous results of Steiner and Beers (1957) which indicate a reduction in radius of gyration of poly C as it changes from neutral to acidic form. Change of salt concentration from 0.05 M to 0.5 M has a small effect upon the viscosity of acidic poly C. No differences in viscosity of the poly C in pH 4.85, the proton stabilization zone, and in pH 3.75, the proton destabilization zone, under identical preparatory conditions have so far been detected (Fig. 5).

The distribution of sedimentation coefficients of the poly C sample employed is fairly broad. For poly C at pH 7.5, 0.5 M NaCl, 0.005 M Tris, the $S_{20,50\%}$ is 8.4, $S_{20,75\%}$ is 10.5, and $S_{20,25\%}$ is 6.2. For the sedimentation studies of poly C in pH 4.85, the solutions were prepared by dilution in acidic buffer from two different concentrations of poly C in 1 × 10^{-3} M NaCl in order to determine the extent of formation of stable aggregates (Doty and Fresco, 1957). The helix of poly C as formed in low concentration was obtained by diluting poly C in buffer of pH 4.85 to a concentration of 0.1 mg/ml. This preparation yielded a $S_{20,50\%}$ of 10.1, $S_{20,75\%}$ of 13.2, and $S_{20,25\%}$ of 7.7. The helix of poly C as formed at high concentration was obtained by diluting the poly C in buffer pH 4.85 to the concentration of 4 mg/ml, then further diluting it to nearly 1 OD unit/ml for the ultracentrifugation. The S values obtained for the poly C form characteristic of high concentration were not significantly different from those characteristic of low concentration, *i.e.*, $S_{20,50\%}$ was 10.3, $S_{20,25\%}$ was 7.5, and $S_{20,75\%}$ was 12.1.

Urea is moderately effective in lowering the T_m of poly C, as shown in Table II; in contrast, it is ineffective in lowering the T_m of poly A (Ts'o *et al.*, 1962b). It is interesting to note that the effectiveness of urea increases with hydrogen ion concentration of the solution, despite the effect of the proton itself, which can be divided into two zones. Though a direct comparison cannot be made without serious reservation, urea appears to be far less effective in lowering the T_m of poly C than is one of the aromatic ring compounds

TABLE II
EFFECT OF UREA ON THE T_m OF POLY C

pH	Urea Concentration (M)	Expected T_m Without Urea (°C)	T_m Observed in Urea (°C)	ΔT_m (°C)	ΔT_m/M of Urea (°C/M)
4.85[a]	3	71.5	63	8.5	2.8
	5	71.5	53.5	18.0	3.6
4.05	3	81.3	63	18.3	6.1
	5	81.3	44	37.3	7.6
3.65	3	73	39.5	33.5	11.2
	5	73	<15[b]	>58	>12

[a] 0.1 M Na-acetate buffer. [b] Solution became cloudy at lower temperature. Beginning of the transition was observed.

in lowering the T_m of poly A or DNA. Interestingly, the effectiveness of urea (ΔT_m per M urea) increases with concentration of urea employed, a phenomenon opposite to that found with the aromatic ring compounds (Ts'o *et al.*, 1962b). Since the fundamental mechanism of urea denaturation of poly C is uncertain, less can be said about why the more extensively protonated form of poly C is more susceptible to the attack of urea than the less protonated form.

DISCUSSION

Three lines of evidence presented here and in previous publications (Ts'o *et al.*, 1962a; Helmkamp and Ts'o, 1962) suggest that poly C in acidic solution assumes the conformation of a regular, compact, and perhaps helical structure: (1) The abrupt transition of the optical properties of poly C over a narrow range of temperature; (2) the hypochromicity and the increase of dextrorotatory power of poly C as compared with monomeric cytidine phosphate in acidic solution; and (3) the reduction of viscosity and dependency of viscosity on polymer and electrolyte concentration as the neutral form of poly C changes to the acidic form. Comparable changes in physical and optical properties have been observed in the helix-coil transition of poly A, poly A-poly U, and poly I. Hence the compact and regular structure of acidic poly C is likely to be helical in nature. The observation reported previously (Ts'o *et al.*, 1962a; Helmkamp and Ts'o, 1962) should be reiterated: because of the complexity of the spectra, comparisons of the optical properties of the neutral form of poly C with those of the acidic form do not yield simple interpretations.

The physicochemical data presented above provide some information on the hydrogen-bonding scheme which the helical poly C may assume. These data indicate that the hydrogen-bonding scheme of the acidic poly C in solution may be similar to that of cytosine-5-acetic acid in crystal form as determined by Marsh *et al.* (1962). This scheme (Fig. 6) consists of a pair of cytosine residues hydrogen bonded together across the center of the helix. The amino group of first cytosine residue (N_7) is hydrogen bonded to the carbonyl oxygen (O_8) of the second cytosine residue, and the amino group (N_7) of the second cytosine residue is hydrogen bonded to the carbonyl oxygen (O_8) of the first cytosine residue. In addition, a single proton is located between and shared by the N_1 atoms of two cytosines across the center. Thus, the helix is stabilized both by hydrogen bondings of amino to keto groups and by the charge effect of the additional proton on the two ring nitrogens. This structure involves protonation of only half of the cytosine and is impossible in the unprotonated as well as in the fully protonated form of poly C.

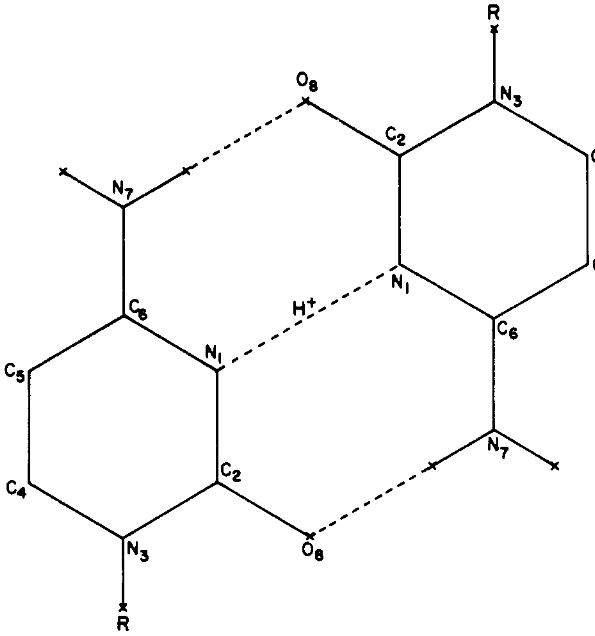

FIG. 6.—Diagram of the proposed scheme for the pairing of cytosines of helical poly C.

In support of this scheme, it is shown that the initial addition of protons to the N_1 nitrogen of the cytosine tends to stabilize the helix. In this zone of proton stabilization, addition of electrolytes weakens the helix by reducing the electrostatic attraction. This electrostatic shielding will tend to reduce the affinity of poly C for protons and thus decrease the stability of poly C helix in this zone of insufficient protons. After a certain number of protons has been added, further addition of protons to the helix tends to destabilize it. In this zone of proton destabilization, addition of electrolytes strengthens the helix by reducing electrostatic repulsion. This reduction of affinity of poly C for protons by electrostatic shielding therefore stabilizes the poly C in this zone of excess protons. The transition of poly C from the proton stabilization zone to the proton destabilization zone does not involve any gross change of structure and conformation, as indicated by the optical rotatory dispersion data, the viscosity measurements, and the ultraviolet spectral data.

Langridge and Rich (1962) have recently and independently arrived at the same hydrogen bonding scheme for the helical poly C from data based on x-ray fiber diagrams, titration, and infrared spectroscopy measurements It appears, therefore, that the scheme proposed above provides the correct interpretation of our physical and chemical measurements of helical poly C in solution.

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The Reaction of Methylamine with Periodate-Oxidized Adenosine 5′-Phosphate

JOSEPH X. KHYM

From the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee*

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The periodate oxidation product of adenosine 5′-phosphate reacts with methylamine to form a cyclic, hemialdal type of structure (I) that releases inorganic phosphate in neutral or acid solution. Reduction of the cyclic compound with sodium borohydride gives rise principally to 6-adenyl-5-hydroxy-4-methyl-2-hydroxymethylphosphate morpholine (IV), in which the methylamine residue is firmly incorporated and which is hydrolyzed by acid to *N,N,N*-(ethanolal-methyl-2-hydroxypropyl-3-phosphate) tertiary amine (V). A small amount of a trialcohol monophosphate (III), free of added amine, is also formed.

The release of inorganic phosphate from periodate-oxidized nucleotides was first studied by Whitfeld (1954) and also by Brown *et al.* (1953, 1955). Several independent investigations (Hakamori, 1959; Ogur and Small, 1960; Yu and Zamecnik, 1960) have shown that the release of phosphate occurs by a more complex mechanism than the earlier reports indicated. Many amino compounds react with periodate-oxidized 5′-nucleotides to form addition complexes whose stability is dependent upon *p*H (Khym and Cohn, 1961). Inorganic phosphate is released slowly at $pH > 8$ but is released rapidly and quantitatively at $pH < 8$, the rate depending upon the amino compound used. A typical example of these interactions is given in Reaction 1 for oxidized AMP and methylamine (partial structures are used for the nucleotide derivatives). The addition compounds formed by primary amines above *p*H 8 can be stabilized with respect to phosphate released by reduction with sodium borohydride.

This paper describes the compounds formed when methylamine-treated periodate-oxidized adenosine 5′-phosphate is reduced with sodium borohydride. This reduction step sheds considerable light on the over-all reaction of periodate-oxidized nucleotides with amines, and, in particular, clarifies the relation of *p*H to stability of the amine complexes formed. The reduction, as demonstrated in Reaction 2, yields two compounds, both of which have been isolated in crystalline form. One is a morpholine derivative (IV), isolated in 77% yield, containing one nitrogen atom more than the five initially present in the adenine moiety. The other compound (III), isolated in 15% yield, contains no additional nitrogen and was found to be identical with the trialcohol monophosphate that results when oxidized adenosine 5′-phosphate is reduced with sodium borohydride in the absence of methylamine.

The compounds were identified from the fragments produced by acid hydrolysis. The trialcohol derivative (III) yields adenine, glycolaldehyde, and α-glycerophosphate. The morpholine derivative (IV) yields adenine and a compound which does not absorb ultraviolet light and which contains N and P in a 1:1 ratio. This latter compound, to be designated here as compound V, has been identified as a six-carbon amine having either structure Va or structure Vb of Reaction 3, which shows the products obtained when V is oxidized with periodate.

EXPERIMENTAL

Methylamine, Formic Acid, and Phosphorus Measurements.—The nitrogen still described by Steyermark (1951) was used to determine both *N*-methylformamide (as methylamine) and formic acid. Authentic *N*-methylformamide hydrolyzes readily to yield formic acid and methylamine under the conditions used here to determine either of these compounds. Methylamine was detected by paper chromatography (Block *et al.*, 1955) only after steam distillation of *N*-methylformamide solutions from concentrated alkali into dilute boric acid solutions. The amine was determined quantitatively by titrations of the distillates (Jones, 1944). Methylamine was determined similarly from periodate-oxidized V by the addition of excess ethylene glycol before transfer of the sample to the nitrogen still. If a volume reduction was necessary, excess acid was added after the ethylene glycol and the sample was concentrated by vacuum distillation before transfer of the sample to the nitrogen apparatus. In some cases, methylamine was determined in the effluent from a Dowex-1 column that had removed IO_3^-, IO_4^-, and phosphate derivatives and that contained only methylamine and formaldehyde. The average yield of methylamine found in oxidation mixtures was about 65%, and this value did not change significantly when samples were withdrawn for analysis any time from 6 to 72 hours.

Formic acid was determined in the same steam still used for the methylamine determinations by a method similar to that described by Bell *et al.* (1944). The oxidized solution was vacuum distilled almost to dryness

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