Springgate, C. F., and Loeb, L. A. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 245.
Stanley, J. F., Pye, D., and MacGregor, A. (1975), *Nature (London) 255,* 158.
Stravianopoulos, J. G., Karkas, J. D., and Chargaff, E. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1781.
Weisman-Shomer, P., and Fry, M. (1975), *Mech. Ageing Devel. 4,* 159.
Weissbach, A., Baltimore, D., Bollum, F., Gallo, R., and Korn, D. (1975), *Science 190,* 401.
Wulf, J. H., and Cutler, R. G. (1975), *Exp. Gerontol. 10,* 101.
Zeelon, P., Gershon, H., and Gershon, D. (1973), *Biochemistry 12,* 1743.

Synthesis and Properties of Poly(O^6-methylguanylic acid) and Poly(O^6-ethylguanylic acid)†

Jitendra R. Mehta and David B. Ludlum*,‡

ABSTRACT: The nucleotide analogues, O^6-methyl- and O^6-ethylguanosine diphosphate, have been synthesized and polymerized to high-molecular-weight homopolymers with polynucleotide phosphorylase. The ultraviolet spectra of these polymers show marked hypochromicity, which suggests that they possess considerable secondary structures. Graphs of optical density vs. temperature in 0.15 M NaCl indicate that cooperative melting occurs for both polymers, and that the secondary structure of poly(O^6-methylguanosine monophosphate) is somewhat more stable than that of poly(O^6-ethylguanosine monophosphate). Mixing experiments show that these analogue polymers no longer form helical structures with poly(C), nor do they form helices with poly(U). We would conclude from these data that environmental mutagens and carcinogens which react at the O^6 position of guanine not only disrupt normal base-pairing relationships, but may also affect the secondary structure of nucleic acids.

Many environmental mutagens and chemical carcinogens are known to react covalently with nucleic acids. Individual bases are modified and, in some cases, this leads to an alteration in the informational content of the nucleic acid that contains them. Evidence for these effects has been obtained previously in model template systems (Ludlum and Wilhelm, 1968; Singer and Fraenkel-Conrat, 1970; Ludlum, 1970; Gerchman and Ludlum, 1975; Ludlum, 1975).

It is apparent that these base changes could be causally related to the carcinogenic process. As an essential step in establishing this link, the molecular effects of each base modification must be elucidated. In most cases, the sites of base substitutions have been identified and good reviews of this work have been published recently by several authors (Sarma et al., 1975; Singer, 1975; Lawley, 1972). Current evidence seems to indicate that attack on the O^6 position of guanine is a particularly important molecular event (Loveless, 1969).

A base modification such as the O^6 alkylation of guanine could produce a change in the informational content of a nucleic acid in at least two ways. The specific base-pairing properties of the nucleotide could be altered, or the secondary structure of the macromolecule itself could be changed.

In order to investigate which of these mechanisms might be operating in the case of O^6-alkylguanines, we have synthesized and studied the physical properties of poly(O^6-methyl-) and poly(O^6-ethylguanylic acid). The synthesis of poly(O^6-methylguanylic acid) has been described briefly in a previous communication (Gerchman et al., 1972), and the properties of this base have also been investigated in templates for RNA polymerase (Gerchman and Ludlum, 1973). These studies have shown that the presence of O^6-methyl-GMP[1] in a template leads to misincorporation, and the investigations reported here are designed to help explain the mechanism by which this occurs.

Experimental Section

Synthesis of Monomers. O^6-Methylguanosine was prepared from 6-chloro-2-aminopurine riboside (Waldhof, West Germany) as described previously (Gerchman et al., 1972). Following our earlier procedure, the nucleoside was phosphorylated to m^6-GMP with carrot phosphotransferase. Then m^6-GDP was synthesized by converting the monophosphate to the imidazolidate and displacing the imidazole groups with inorganic phosphate (Gerchman et al., 1972). The corresponding ethyl compounds were synthesized in an analogous fashion from 6-chloro-2-aminopurine riboside and sodium ethoxide as starting materials. All derivatives were chromatographically pure in the systems described below.

Chromatography and Paper Electrophoresis. Paper chromatography was carried out on Whatman no. 1 paper in the following solvent systems: (A) methanol–concentrated HCl–water (8/1/1, v/v); (B) isobutyric acid–concentrated ammonium hydroxide–water (66/1/33, v/v); (C) isopropyl alcohol–concentrated ammonium hydroxide–water (7/1/2,

† From the Department of Pharmacology and Experimental Therapeutics, University of Maryland School of Medicine, Baltimore, Maryland 21201. *Received November 20, 1975.* This work was supported by Research Grants CA 15696 and CA 16299 from the National Institutes of Health, United States Public Health Service and by American Cancer Society Grants MD 74-19 and NP-74 B.

‡ Present address: Department of Pharmacology and Experimental Therapeutics, Albany Medical College, Albany, N.Y. 12208.

[1] Abbreviations used are: EDTA, (ethylenedinitrilo)tetraacetic acid; GMP, GDP, guanosine mono- and diphosphates; m^6-GMP, e^6-GMP, 6-methyl- and 6-ethylguanosine monophosphates.

v/v). Ascending technique was used with solvent A and descending technique with solvents B and C.

Paper electrophoresis was performed at a constant voltage of 490 V for 150 min in 0.025 M sodium acetate buffer, pH 4.7, on Whatman 3MM paper (12.5 × 23 cm). Thin-layer chromatograms were run on fluorescent silica gel plates, 5 × 20 cm, in chloroform–absolute ethanol (85/15, v/v) and acetone–methanol (20/1, v/v). Nucleotides were also checked for purity on PEI paper in 0.3 M KH_2PO_4.

Preparation of Poly(m^6-GMP) and Poly(e^6-GMP). All polymerization reactions were carried out in an 0.5-ml capillary viscometer at 30.0 °C. In this way, the viscosity of the polymerization mixture could be followed as a function of time and the polymer could be withdrawn at peak viscosity.

Incubation mixtures, approximately 1 ml in volume, contained 40 mM substrate, 75 mM Tris-HCl, and 75 mM sodium cacodylate buffers at pH 9, 8 mM $MgCl_2$, and 10 units of polynucleotide phosphorylase (PL-Biochemicals, type 15). Cacodylate buffer was added to the Tris-HCl in order to prevent bacteria growth over the 1–3-day incubation period.

As noted above, the progress of the polymerization reaction was followed by taking periodic viscosity measurements. Polymerization times varied between 1 and 4 days with 25–45% conversion of monomer to polymer.

At peak viscosity, the reaction mixture was removed from the viscometer and extracted with an equal volume of $CHCl_3$/isoamyl alcohol (3/1, v/v) to remove protein. The aqueous layer was separated and applied to a Sephadex G-200 column (3 × 22 cm). This was eluted with 5 mM triethylammonium bicarbonate solution at a flow rate of 1 ml/min. The effluent was monitored at 254 nm and the polymer that appeared in the void volume was collected in acid-washed glassware and lyophilized. The monomer peak, which was well separated from the polymer on G-200, was collected and reused in subsequent polymerizations.

Ultraviolet Spectroscopy. Ultraviolet spectra as well as temperature–absorbance profiles were obtained on a Beckman DB-G spectrophotometer. Solutions for the absorbance profiles were contained in a thermostated cell with a 10-mm path length (Scientific Cell Co.) Water was circulated through the cell jacket from an external bath whose temperature was increased at about 1°/min. Solution temperature was monitored by a thermistor probe (Yellow Springs Instrument Co.) that was inserted directly into the polynucleotide solution.

Since both the O^6–alkyl bond and the sugar–base bond are acid labile, extinction coefficients for the derivatives were determined by acid hydrolysis to guanine. Solutions of the O^6-substituted nucleosides and nucleotides were scanned at pH 1 (0.1 M HCl), pH 7 (0.05 M sodium cacodylate buffer containing 0.15 M NaCl and 0.1 mM EDTA), and pH 11 (1 mM NaOH). A 1-ml aliquot of each solution was then sealed in an ampule with concentrated HCl: 0.1 ml for the methyl derivatives, or 0.2 ml for the ethyl derivatives, since they were more resistant to hydrolysis. Samples were boiled for 1 h, and the resulting guanine solution was again scanned. After appropriate blank readings were subtracted, molar extinction coefficients were calculated, using a value of 11.4×10^3 for the molar extinction coefficient of guanine in acid solution. Values reported here are the averages of three or more determinations.

Polymer hypochromicity was calculated in a similar way. After each polymer solution was scanned and hydrolyzed, the optical density of the original polymer solution was compared to the calculated optical density of a corresponding solution of monomer.

TABLE I: Paper Chromatography and Paper Electrophoresis of O^6-Ethylguanine Derivatives.

Compound	R_f Solv A	R_f Solv B	R_f Solv C	Paper Electrophoresis R_{amp}[a]
e^6-Guo	0.61	0.81	0.74	0.02
e^6-GMP	0.70	0.56	0.26	0.77
e^6-GDP	0.71	0.41	0.18	1.67
GMP	0.33	0.19	0.09	1.00

[a] Migration distances are reported relative to the migration of GMP.

Ultraviolet mixing curves were obtained by adding varying proportions of stock polymer solutions from Lang–Levy pipettes to 0.05 M sodium cacodylate buffer, pH 7, containing 1 M NaCl. Good helix formation between poly(C) and poly(G) occurred when these solutions were incubated in sealed ampules at 60 °C for 2.5 h and allowed to cool slowly to room temperature. The optical density of each solution was measured at 290 nm in a Gilford spectrophotometer. These values were then plotted against the calculated mole fraction of each polymer. The poly(C) and poly(G) used in these experiments came from Schwarz Bioresearch and the poly(U) from Miles Laboratories.

Values of the pK's for the monomers were determined by spectrophotometric titration in a Gilford spectrophotometer. A small amount of each compound was dissolved in 3 ml of 0.15 M NaCl containing 1 mM EDTA at pH 7, and the pH was adjusted by microliter additions of 1 N HCl or 1 N NaOH. The optical density at 252 nm was plotted against pH to determine each pK value.

Sedimentation Analysis. Sedimentation velocity experiments were performed in a Beckman Model E ultracentrifuge equipped with a monochrometer and ultraviolet absorption optics. Band sedimentation (Vinograd et al., 1963) was followed at 250 nm in 0.1 M NaCl–0.05 M sodium cacodylate buffer, pH 7, and 0.1 M NaCl–1 mM NaOH, pH 11. Photographs of the sedimenting bonds were scanned with a Joyce-Loebl recording densitometer.

Sedimentation coefficients at pH 7 for the analogue polymers were similar to those at pH 11. Values of $s^\circ_{20,w}$ at pH 7 varied between 3.6 and 4.9 for poly(m^6-GMP) and between 5.1 and 7.7 for poly(e^6-GMP). This variation in molecular weight did not have any detectable effect on hypochromicity or other physical properties measured here. Poly(C) and poly(G) used in the mixing experiments had sedimentation coefficients of 5.4 and 9.9, respectively.

Results

When adequately purified solvents were used, each chemical step in the monomer synthesis proceeded smoothly and in high yield. Enzymatic phosphorylation of the nucleoside to the nucleotide was also a very satisfactory procedure, resulting in approximately 50% of the desired product. Carrot phosphotransferase was used for this step because of its specificity for the 5′ position; in contrast, chemical phosphorylation frequently produces a complex mixture of products.

Polymerization of the O^6-alkyl GDP's proceeds steadily at 30 °C with polynucleotide phosphorylase, while GDP will not polymerize at all under these conditions. Polymerization of GDP is presumably inhibited by the secondary structure of poly(guanylic acid). Since the poly(O^6-alkylguanylic acids)

TABLE II: Ultraviolet Spectra of O^6-Alkylguanine Derivatives.

Compound	pH 1		pH 7		pH 11	
	λ_{max}	ϵ^a	λ_{max}	ϵ^a	λ_{max}	ϵ^a
m^6-GMP	245	6.9	249	11.2	249	10.5
	288	9.9	280	10.5	281	9.9
e^6-GMP	244	7.1	248	11.4	249	10.8
	288	9.6	280	10.7	280	9.9
poly(m^6-GMP)			249	20–35[b]		
			281	25–45		
			248	35–40		
poly(e^6-GMP)			282	55–60		

[a] Molar extinction coefficient $\times 10^3$. [b] Reported as percent hypochromicity: that is, one minus the ratio of the optical density of the polymer solution to the optical density of the monomer solution.

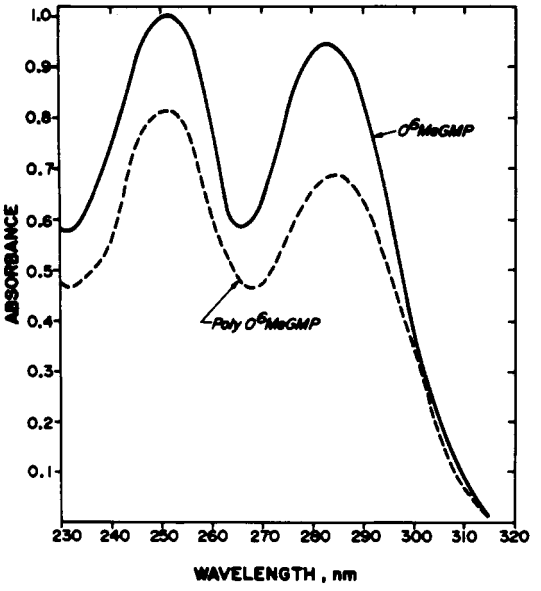

FIGURE 1: Ultraviolet spectra of O^6-methyl-GMP and poly(O^6-methyl-GMP) at pH 7.0 in 0.05 M sodium cacodylate buffer, 0.15 M NaCl, and 0.1 mM EDTA. Spectra are normalized to a peak monomer absorbance of 1.0 after correcting for polymer hypochromicity.

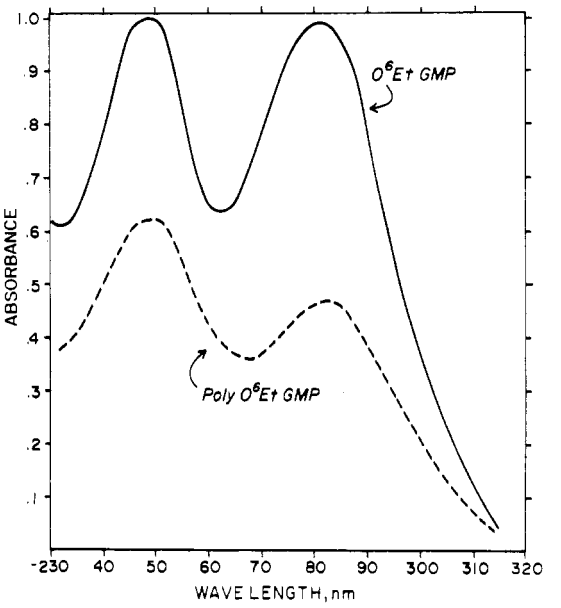

FIGURE 2: Ultraviolet spectra of O^6-ethyl-GMP and poly(O^6-ethyl-GMP). Conditions as in Figure 1.

also have a significant secondary structure as described below, this must either be different or some other factor must be invoked to explain the difference in polymerization behavior.

Chromatographic characterization of the ethyl derivatives is given in Table I. Corresponding data for the methyl derivatives have been published previously (Gerchman et al., 1972) and are similar, but there are small, reproducible differences.

Ultraviolet spectroscopic data are given in Table II and in Figures 1 and 2. Data for the methyl and ethyl monomers are similar, and values for λ_{max} are in agreement with those reported previously for m^6-GMP and e^6-Guo, respectively (Gerchman et al., 1972; Singer, 1972). Extinction coefficients have not been reported previously.

Figures 1 and 2 show the marked hypochromicity of poly(m^6-GMP) and poly(e^6-GMP); the observed range of hypochromicity is shown in Table II. At both 248 and 282 nm, poly(e^6-GMP) had consistently more hypochromicity than poly(m^6-GMP), perhaps indicating a higher degree or exaggeration of the secondary structure possessed by poly(m^6-GMP).

Acid and base titration curves are shown in Figure 3. Corresponding to the fact that the O^6-alkyl GMP's are effectively frozen in the enolized configuration, there is no basic pK for either m^6- or e^6-GMP. The acid pK's of 2.7 for m^6- and e^6-GMP are in fair agreement with the values of 2.4 and 2.5 reported by Singer (1972) for m^6- and e^6-Guo, respectively.

Mixing curves for the various poly(guanylic acids) with poly(C) are shown in Figure 4. The curve for poly(guanylic acid) has a minimum at 0.5 mol percent poly(C), while there is no evidence for interaction of poly(m^6-GMP) or poly(e^6-GMP) and poly(C). We would conclude from this that alkylation of the O^6 position of guanine blocks normal base pairing with cytosine.

It is possible to draw a base pair between uracil and the enolized form of guanine. Accordingly, we wondered whether poly(m^6-GMP) or poly(e^6-GMP) would form a helix with poly(uridylic acid). However, we were unable to detect any evidence for helix formation with poly(uridylic acid) under the conditions shown in Figure 4.

Figure 5 shows the cooperative melting behavior of poly(e^6-GMP) in the middle panel and poly(m^6-GMP) in the bottom panel. For comparison, the behavior of poly(guanylic acid) under these conditions is shown in the top panel. This type of cooperative melting is similar to that observed for the acid

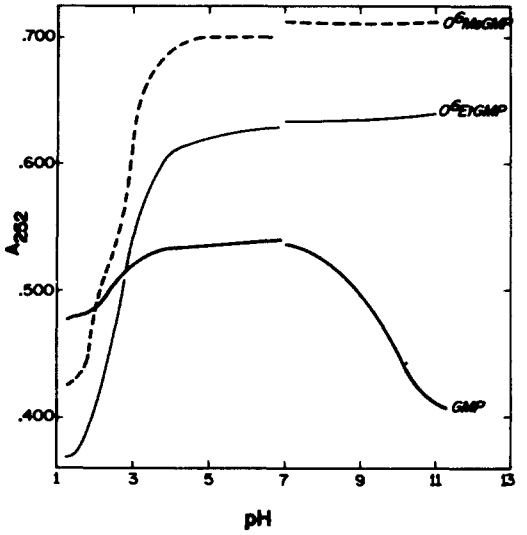

FIGURE 3: Spectrophotometric titration of GMP, O^6-methyl-GMP, and O^6-ethyl-GMP. Monomer was dissolved in 0.15 M NaCl, 0.1 mM EDTA at pH 7, and pH was adjusted with small additions of concentrated HCl and 1 N NaOH. Absorbance at 252 nm was followed as a function of pH.

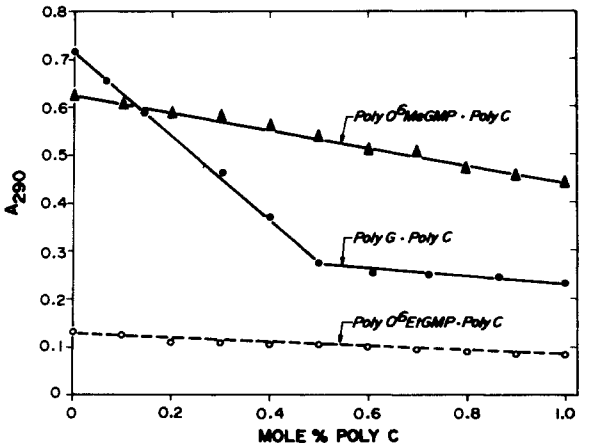

FIGURE 4: Ultraviolet mixing curves at pH 7 in 0.05 M sodium cacodylate buffer and 1.0 M NaCl. Aliquots of polymer stock solutions were mixed together, annealed as described under Methods, and their absorbance was measured at 290 nm.

form of poly(adenylic acid). However, except for the fact that melting temperature increases slightly as the solution is made more acidic, the melting curves for these new polymers remain qualitatively the same over the pH range from 3 to 7.

The ultraviolet spectra and melting curves provide evidence for the existence of two new ordered polymers, poly(m^6-GMP) and poly(e^6-GMP). Of the two, poly(m^6-GMP) is slightly more stable, as shown by its slightly higher melting temperature given in Table III.

In order to confirm that the poly(O^6-alkylguanylic acids) are melted under the annealing conditions used for the mixing experiments in Figure 4, the pH 7 melts were repeated in 0.05 M sodium cacodylate buffer containing 1 M NaCl. Although the additional salt raised the melting point of poly(O^6-methyl-GMP) by 16 °C and the melting point of poly(O^6-ethyl-GMP) by 6 °C, melting was complete at 60 °C. Thus, the polymers would be at least as available as poly(G) to form helices with poly(C) or poly(U) if they did, in fact, base pair appropriately.

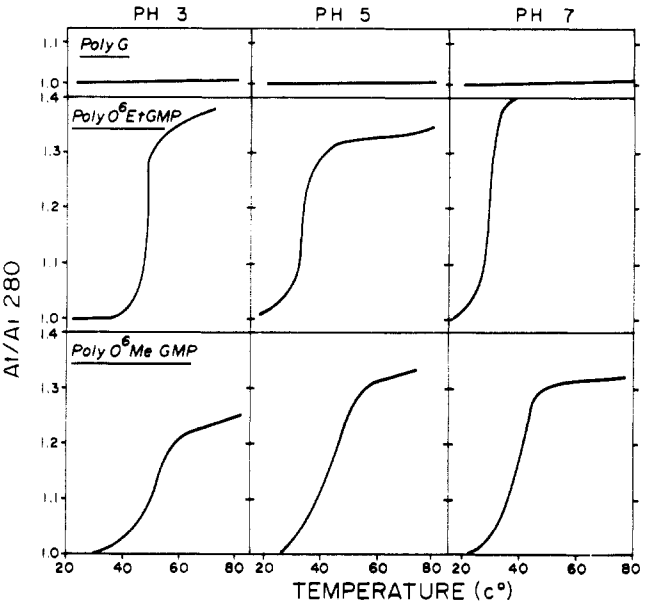

FIGURE 5: Ultraviolet melting curves for poly(G), poly(O^6-methyl-GMP), and poly(O^6-ethyl-GMP). Polymers were dissolved in 0.15 M NaCl, 0.1 mM EDTA, and one of the buffers: 0.05 M sodium, formate, pH 3, 0.05 M sodium acetate, pH 5, or 0.05 M sodium cacodylate, pH 7. Absorbance at 280 nm was followed as a function of temperature.

TABLE III: Melting Temperature of Poly(O^6-alkylguanylic acids).

Polynucleotide	pH 3	pH 5	pH 7
Poly(m^6-GMP)	50	42	38
Poly(e^6-GMP)	48	34	30

Discussion

Following Loveless' lead (1969), several groups have demonstrated the formation of O^6-substituted guanines when alkylating mutagens or carcinogens react with nucleic acids in vitro and in vivo (Craddock, 1973; Kleihues and Magee, 1973; Lawley and Thatcher, 1970; O'Connor et al., 1973; Singer, 1975). In general, O^6-substituted guanines are formed under mutagenic and carcinogenic conditions, particularly when the reaction occurs by an SN1 mechanism.

In an effort to determine the biological significance of m^6-GMP, we incorporated this nucleotide into poly(C, m^6-G), and studied its properties as a template for RNA polymerase (Gerchman and Ludlum, 1973). The presence of m^6-GMP led to the misincorporation of UMP or AMP into the product copolymer.

Such misinformation could result from specific mispairing of O^6-methylguanine with uracil or adenine, but it seems more likely that it represents nonspecific incorporation of UMP and AMP opposite a nonpairing partner, or opposite an aberration in the secondary structure of the template.

The data presented here show that neither poly(m^6-GMP) or poly(e^6-GMP) pairs normally with poly(C). Thus, as would be expected, the normal base pairing relationships have been disrupted by alkylation of the O^6 position of guanine. However, the failure of these polymers to interact with poly(U) suggests that no specific mispairing occurs either.

This focuses attention on the changes produced by O^6-alkylguanines in the secondary structure of polynucleotides that contain them. These effects are clearly evident in the homo-

polymers described here, poly(m^6-GMP) and poly(e^6-GMP).

The spectra studies shown in Figures 1 and 2 and in Table II indicate that these polymers possess a secondary structure with a high degree of hypochromicity. Poly(e^6-GMP) has consistently more hypochromicity than poly(m^6-GMP), and probably represents an exaggeration of its secondary structure.

The curves shown in Figure 5 demonstrate cooperative melting behavior for both poly(m^6-GMP) and poly(e^6-GMP), in marked contrast to the very slight and very gradual increase in optical density shown by poly(G) under these conditions.

Although both analogue polymers depurinate slowly under strongly acid conditions, we were able to obtain reversible melting curves at pH 3. The melting profiles at pH 3, 5, and 7 are all similar, but melting temperature increases as pH is lowered. The melting point is higher at all pH's for poly(m^6-GMP) than for poly(e^6-GMP), indicating that the former polymer has a more stable structure. As pH is lowered below 3, an acid transition is evidenced by precipitation of the polymer.

The biological effects of methylation and ethylation are frequently rather different. The data presented here, however, indicate only quantitative differences in physical properties resulting from methylation vs. ethylation of the O^6 position of guanine. Observed differences in biological effects may reflect these quantitative differences or, more likely, result from differences in the sites and extent of methylation and ethylation.

In summary, the evidence presented here indicates that either methylation or ethylation of the O^6 position of guanine changes its normal base-pairing properties. Polymers of these analogue nucleotides, poly(m^6-GMP) and poly(e^6-GMP), have an ordered structure that exhibits cooperative melting; additional studies are in progress to further elucidate this secondary structure.

References

Craddock, V. M. (1973), *Biochim. Biophys. Acta 312,* 202.

Gerchman, L. L., Dombrowski, J., and Ludlum, D. B. (1972), *Biochim. Biophys. Acta 272,* 672.

Gerchman, L. L., and Ludlum, D. B. (1973), *Biochim. Biophys. Acta 308,* 310.

Kleihues, P., and Magee, P. N. (1973), *J. Neurochem. 20,* 595.

Lawley, P. D. (1972), *Top. Chem. Carcinog., Proc. Int. Symp., 2nd, 1971* 237–258.

Lawley, P. D., and Thatcher, C. J. (1970), *Biochem. J. 116,* 693.

Loveless, A. (1969), *Nature (London) 223,* 206.

Ludlum, D. B. (1970), *J. Biol. Chem. 245,* 477.

Ludlum, D. B. (1975), *Handb. Exp. Pharmakol., Part 2, 38,* 6–17.

Ludlum, D. B., and Wilhelm, R. C. (1968), *J. Biol. Chem. 243,* 2750.

O'Connor, P. J., Capps, M. J., and Craig, A. W. (1973), *Br. J. Cancer 27,* 153.

Sarma, D. S. R., Rajalakshmi, S., and Farber, E. (1975), in Cancer, Vol. 1, Becker, F. F., Ed., New York, N.Y., Plenum Press, pp 235–287.

Singer, B. (1972), *Biochemistry 11,* 3939.

Singer, B. (1975), *Prog. Nucleic Acid Res. Mol. Biol. 15,* 219–331.

Singer, B., and Fraenkel-Conrat, H. (1970), *Biochemistry 9,* 3694.

Vinograd, J., Bruner, R., Kent, R., and Weigle, J. (1963), *Proc. Natl. Acad. Sci. U.S.A. 49,* 902.

Conformation of the Extracellular Polysaccharide of *Xanthomonas campestris*†

G. Holzwarth

ABSTRACT: The solution conformation of the extracellular polysaccharide of the bacterium *Xanthomonas campestris* is examined by optical rotation, viscometry, and potentiometric titration. Measurements of optical rotation vs. temperature for solutions of the polysaccharide at low ionic strength reveal a sharp transition to a denatured structure which is reversible if sufficient salt is present. The temperature T_m at the transition midpoint increases as log (Na^+) or log (Ca^{2+}). Viscosity–temperature profiles substantiate a structural change of the polysaccharide at T_m. The intrinsic viscosity of the native molecule at zero shear rate exceeds 5000 ml/g. This high figure is indicative of a stiff chain. The viscosity of the native molecule is relatively insensitive to salt, whereas the denatured molecule collapses if salt is present. Hydrogen-ion titration shows that the pK_{app} of the COO^- groups of the polymer decreases from 3.2 in 0.01 M NaCl to 2.6 in 0.2 M NaCl. All these data suggest that the native polysaccharide possesses ordered secondary structure stabilized by nonionic interactions outweighing the repulsion between adjacent COO^- groups.

The bacterium *Xanthomonas campestris* produces an anionic extracellular polysaccharide which normally provides the organism with a protective slime. The polysaccharide appears to be essential to the pathogenicity of the organism toward its plant host, presumably by blocking the flow of fluids through the xylem (Sutton and Williams, 1970). When grown on a glucose-containing medium in a fermentor, the bacterium produces large amounts of the extracellular polymer (Jeanes

† From the Corporate Research Laboratories, Exxon Research and Engineering Co., Linden, New Jersey 07036. *Received March 29, 1976.*