Chemical Methylation of Synthetic Polynucleotides

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SUMMARY

Polynucleotides were methylated in aqueous solution at 25° with methyl methanesulfonate. The reactions followed second-order kinetics, with relative reactivity of bases decreasing in the order: guanine > cytosine > adenine > uracil. Light scattering and viscosity techniques showed no changes in physical structure of polyuridylic acid, indicating that no methylation of the phosphodiester bond had occurred. Both polyadenylic and polycytidylic acids showed a decrease in molecular dimensions at low levels of methylation, presumably resulting from protonation of the substituted bases. As reported previously, polyadenylic acid showed a tendency to aggregate, but there was no evidence for such aggregation with methylated polycytidylic acid.

INTRODUCTION

The synthetic polynucleotides have provided a new approach to the study of alkylating agents (1–6). Their simple structure compared to that of nucleic acids makes them useful in studying alkylation reactions, while their role as models in protein and RNA synthesis permits the biological significance of such reactions to be investigated. Furthermore, if methylating agents are employed, information may be obtained on the function of naturally occurring methylated bases.

In previous publications, we have reported the methylation of polyadenylic acid by methyl methanesulfonate (1, 5) and have shown the effect of methylation on the secondary structure of poly A[1] (5). Brimacombe *et al.* (2) have methylated poly A and poly C with dimethyl sulfate and poly U with diazomethane; Michelson and Grunberg-Manago (3) have reacted dimethyl sulfate with poly A; and Michelson and Pochon (4) have recently used the same agent to methylate poly G and poly I.

In this paper, the reactions of methyl methanesulfonate with poly A, poly U, poly C, and copoly UG, are contrasted, and the relative ease of alkylation of bases in these polymers is compared with the relative ease of alkylation in the nucleic acids. Light scattering and viscosity measurements have demonstrated the effects of alkylation on the secondary structure of these polymers, and the resulting data show markedly different behavior among the different polymers.

METHODS

Homopolymers were obtained from Miles Chemical Company. Poly A and poly U were used without special purification in most experiments, but poly C was freed of nuclease activity by phenol extraction in the presence of Macaloid (7), followed by ethanol precipitation, dialysis against 0.15 M NaCl–0.015 M sodium citrate, 0.1 M

[1] Abbreviations: poly A, polyadenylic acid; poly C, polycytidylic acid; poly U, polyuridylic acid; poly G, polyguanylic acid; poly I, polyinosinic acid; copoly UG, copolymer of uridylic and guanylic acid; MMS, methyl methanesulfonate.

NaCl, and distilled water, and finally by lyophilization. The poly UG, which contained 30% guanine, was prepared as described previously (8). The known derivatives, 3-methyladenine, 6-methylaminopurine, 7-methylguanine, and 3-methyluracil were kindly supplied by Dr. Gertrude B. Elion, of Burroughs Wellcome & Co.; 1-methyladenine was obtained from the Cyclo Chemical Corporation, and methyl methanesulfonate was purchased from Eastman Kodak Company.

All alkylation reactions, except the physical chemical studies discussed below, were performed in aqueous solution at room temperature (25° ± 2°) and pH 7, maintained in the absence of buffer by a Radiometer pH-stat. After alkylation, polynucleotides were precipitated quantitatively with ethanol, redissolved in water, and lyophilized to dryness.

The polymers were hydrolyzed in 1 N HCl at 100° for 30 min to liberate purine bases and pyrimidine nucleotides. Poly C and poly U were also hydrolyzed by 70% $HClO_4$ at 100° for 2 hr to liberate pyrimidine bases. Derivatives were identified by UV spectra and cochromatography with known compounds on Whatman No. 1 filter paper both in methanol–conc. HCl–water (7:2:1, v/v) and in *n*-butanol saturated with aqueous ammonia. Percentage of alkylation was determined by measuring the optical density of spots eluted in 0.01 N HCl. Column chromatography of methylated poly A, hydrolyzed by HCl, was performed on Dowex 50 × 8 using an exponential 1–2.5 N HCl gradient, followed by 6 N HCl; eluent was monitored continuously at 260 mμ.

Protamine titrations, undertaken to investigate the methylation of phosphate groups, were performed according to the procedure of Reiner and Zamenhof (9).

Spectra and determinations of optical density *versus* temperature were obtained on a Beckman Model DB spectrophotometer. Samples for the latter determinations were contained in a jacketed cell heated by circulating water; temperature was monitored by a thermistor which was immersed in the solution.

Light scattering experiments were performed with green ($\lambda = 5460$ Å) light in a Brice-Phoenix light scattering photometer in sodium cacodylate buffer (0.2 ionic strength, pH 7). An unjacketed cylindrical cell was used, and temperature averaged about 30° inside the cell compartment. Polymer concentrations were determined from the optical density of a base-hydrolyzed sample; MMS concentrations (approximately 20 mg/ml) were calculated from the known weight of reagent added. The MMS solutions were freed of dust by filtration through Gellman VF filters held in hypodermic adapters; polynucleotide solutions were similarly treated or were clarified by centrifugation.

The intensity of light scattered by the polynucleotide solutions was determined as a function of angle during a control period of 1–3 hr before the addition of MMS solution.

Molecular weights and radii of gyration were calculated in the usual manner by graphing Kc/R_θ *versus* $\sin^2\theta/2$, where K is the light scattering constant, c is the concentration of polymer in g/ml, and R_θ is the reduced intensity of light scattered at angle θ. These parameters were constant during the control period and were determined after the addition of MMS at hourly, or more frequent, intervals. Data were not extrapolated to zero concentration because, in agreement with earlier workers (10), we found that there is little dependence of Kc/R_θ on c at this ionic strength.

Refractive index increments, dn/dc, for polynucleotide solutions in cacodylate buffer, dialyzed against the same buffer, were determined with green light in a Brice-Phoenix differential refractometer. Values of 0.186, 0.171, and 0.187 ml/g were obtained for poly A, poly U, and poly C, respectively.

Viscosities were determined at 25 ± 0.005° in Cannon capillary viscometers with solvent flow times of about 75 sec. These experiments were generally run in parallel with light scattering experiments using the same stock solutions. After control periods to demonstrate the absence of

nuclease activity, MMS solutions were added and relatively viscosities were followed as a function of time.

RESULTS

Chemistry of Methylation

Hydrolysis of the methylated UG copolymers with HCl released 7-methylguanine identified by UV spectrum and chromatographic behavior identical with authentic 7-methylguanine. The only derivative isolated by paper chromatography after HCl hydrolysis of methylated poly A was 1-methyladenine as reported previously (1). However, Lawley and Brookes (11) have found small amounts of 3-methyladenine in addition to 1-methyladenine in methylated RNA; larger amounts in comparison with 1-methyladenine were found in methylated DNA in which the H-bonded 1 position is relatively less available for alkylation. To investigate this further, hydrolyzates of methylated poly A were subjected to column chromatography on Dowex 50 as described above.

There was only a small increase in absorbance in the 3-methyladenine region for hydrolyzates of poly A 10% methylated at 25°. Similarly, little absorbance appeared in this region for samples of poly A treated with MMS in the presence of poly U, where the 1 position of adenine is protected (12). However, larger absorbances in this region (2.4% of total OD_{260}) were found in hydrolyzates of poly A methylated at 37° under conditions sufficient to produce 50% substitution in the 1-position. The optical density recording for the eluent from this sample was qualitatively identical with one obtained by Brookes and Lawley (13) for a hydrolyzate of methylated adenylic acid. The compound eluted in the 3-methyladenine region was identified as such by UV spectrum and cochromatography with known 3-methyladenine.

Hydrolysis of methylated poly C and poly U with HCl released 3-methylcytidylic and 3-methyluridylic acids, respectively, identified by the UV spectra and chromatographic behavior reported by Lawley and Brookes (11). Alkali treatment of 3-methylcytidylic acid converted it to 3-methyluridylic acid. Hydrolysis of the original polymers or the methylated monomers with $HClO_4$ released 3-methylcytosine and 3-methyluracil,[2] respectively, again identified by UV spectra and paper chromatography. Alkali treatment of 3-methylcytosine converted that compound to 3-methyluracil.

Methylated polymers were titrated with protamine, which forms a 1-to-1 complex with unmethylated polynucleotides. Reagents which esterify phosphate groups have been reported to decrease the protamine titer of alkylated DNA (9). No correlations existed between protamine titer and degree of methylation of synthetic polynucleotides, but there was considerable scatter in the data. Physical measurements described below also indicate that there was no esterification of phosphate groups.

The rate of methylation, R, in this S_N2 reaction can be described by the following equation:

$$R = K_p \cdot P \cdot MMS \qquad (1)$$

where K_p is a constant which depends on the nature of the polymer and P and MMS are the polymer and methyl methanesulfonate concentrations in mg/ml. Since MMS is present in excess and has a half-life of more than 24 hr in water at 25°, its concentration did not vary during the reaction.

Integration of Eq. (1) yields:

$$-\ln F_u = K_p \cdot MMS \cdot t \qquad (2)$$

where $-\ln F_u$ is the negative logarithm of the unmethylated fraction of polymer and t is time in hours. For small degrees of methylation (F_u near 1), $-\ln F_u$ may be approximated by F_m, the fraction of polymer methylated. Thus, the percentage of methylation depends approximately on the product of MMS concentration and time.

[2] Chemical Abstracts numbering system is used here; Lawley and Brookes refer to the above compounds as 1-methylcytidylic acid, 1-methyluridylic acid, 1-methylcytosine, and 1-methyluracil, respectively.

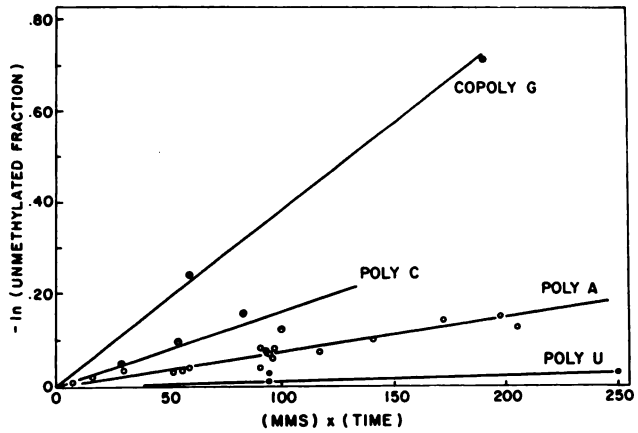

FIG. 1. *Dependence of methylation on reaction conditions at 25°*

Negative logarithm of unmethylated fraction of polymer (proportional to fraction methylated under most conditions) *versus* the product of methyl methanesulfonate concentration in mg/ml and time in hours.

This relationship is demonstrated in Fig. 1, in which we have plotted data on per cent methylation obtained by hydrolysis and chromatography (see Methods section) for polymers methylated at 25° and pH 7. A wide range of conditions is represented for the methylation of poly A, with polymer concentrations ranging between 0.6 and 1.3 mg/ml, MMS concentration between 6 and 45 mg/ml, and time between 1 and 8 hours. Other polymers were methylated at approximately 1 mg/ml, MMS concentration and time being varied to produce different degrees of methylation. The results are described in satisfactory fashion by Eq. 2, with the relative ease of methylating guanine in copoly UG, cytosine in poly C, adenine in poly A, and uracil in poly U decreasing in the order 38:16:7.5:1.

All the methylation reactions reported in Fig. 1 were performed in the absence of buffer, pH being maintained with a pH-stat, except those on copoly UG. The methylation of copoly UG and the studies on changes in physical properties during methylation were performed in the presence of cacodylate buffer; however, control studies in the pH-stat demonstrated that this buffer did not affect the degree of methylation. The somewhat higher temperature in the light scattering compartment in some experiments did, however, result in somewhat more methylation for similar MMS concentrations and time than would be indicated by Fig. 1.

Changes in spectra occurring during methylation of poly A were reported previously (1). Data on the methylation of poly C and poly U, and additional data on the methylation of poly A, were obtained by recording difference spectra on an expanded scale between a sample of the original polynucleotide solution, and samples of the reaction mixture withdrawn at appropriate intervals. Methylated poly A showed complex changes resulting from an overall decrease in absorbance associated with increased secondary structure, combined with the appearance of relatively increased absorbance at approximately 270 mμ associated with the production of 1-methyladenine. Furthermore, it was discovered that the spectrum of the methylated poly A changed over a 5–10 min period following dilution from the reaction mixture to a concentration of 0.01 mg/ml. This suggests that the aggregation of methylated poly A previously reported (5) may be partially reversed by extreme dilution.

Spectral changes observed during methylation of poly C can be attributed to the increased absorbance of 3-methylcytosine

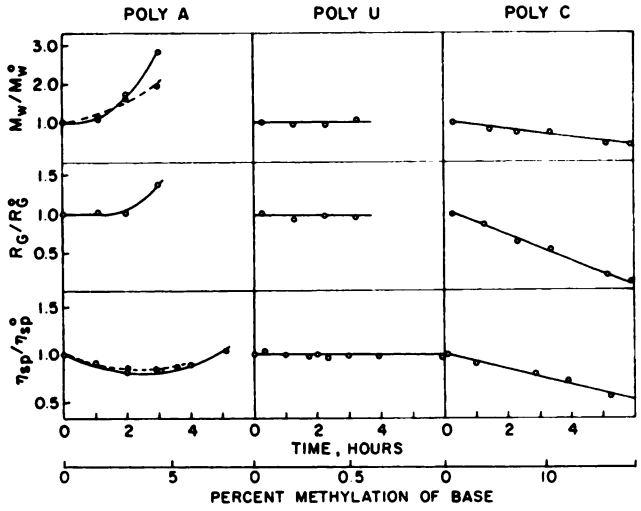

FIG. 2. *Physical chemical effects of methylation at pH 7*

Ratio of molecular weight to initial molecular weight, M_w/M_w°, ratio of radius of gyration to initial radius of gyration, R_G/R_G°, and ratio of specific viscosity to initial specific viscosity, $\eta_{sp}/\eta_{sp}^\circ$, *versus* time of methylation for poly A, poly U, and poly C. Methyl methanesulfonate concentrations were 19 mg/ml and polymer concentrations were between 0.6 and 0.9 mg/ml. Original molecular weight for poly U and poly C was 100,000; molecular weight for poly A: open circles, 360,000; closed circles, 36,000. Percentage methylation is indicated on a separate scale.

in the 280 mμ region. Minimal changes were observed during methylation of poly U since only about 1% of the base residues were substituted.

Physical Chemical Effects of Methylation

Typical changes in molecular weight, radius of gyration, and specific viscosity which occur during methylation of poly A, poly U, and poly C are shown in Fig. 2. This figure, in addition to showing the effects of methylation, illustrates one of the advantages of light scattering and viscosity measurements for this purpose. Physical properties are observed during methylation, and interpretation is not complicated by changes which may occur in the isolation process when samples are withdrawn for study.

In comparing results obtained on the different polymers in Fig. 2, differences in rates of methylation must be taken into account. After 3 hours, there would be approximately 4.5% methylation of poly A, 0.5% methylation of poly U, and 9.5% methylation of poly C under the conditions of this figure. Average results from at least 2, and usually 4 or more runs, are compared at equal levels of methylation in Table 1; the extreme range of values obtained is indicated.

The meaning of the parameters given in this table and in Fig. 2 should be considered briefly. The molecular weight obtained by light scattering is a weight average value independent of any assumptions as to shape of the polynucleotide. The radius of gyration measures overall size and, although an exact interpretation depends on the shape assumed for the polymer, a fall in this parameter indicates that the molecule has become more compact. Specific viscosity, defined as relative viscosity minus 1 (i.e., $\eta_{sp} = \eta_{rel} - 1$) is sensitive to changes in both size and shape, but in general it decreases as molecules decrease in molecular weight and become more compact.

The data shown in Fig. 2 and in Table 1 indicate clearly that there are no detectable changes in the physical properties of poly U under rather strenuous methylating conditions. We know from chemical studies that uracil is resistant to methylation, but

TABLE 1
Effects of methylation on physical properties of polynucleotides

Property	Per cent methylation	Poly A	Poly U	Poly C
M_w/M_w°	1	1.02 ± .05	1.00 ± .05	1.00 ± .05
	5	1.40	—	0.94
	8	2.13	—	0.90
R_G/R_G°	1	0.98 ± .05	1.00 ± .05	0.99 ± .05
	5	0.94	—	0.88
	8	1.17	—	0.78
$\eta_{sp}/\eta_{sp}^\circ$	1	0.89 ± .05	1.00 ± .05	0.97 ± .05
	5	0.86	—	0.85
	8	1.15	—	0.78

as discussed below, this is good evidence against the methylation of phosphate groups in the polymer.

The initial fall in viscosity of poly A and also of radius of gyration (although this does not show clearly in Fig. 2) indicate that this molecule becomes more compact when it is methylated. This has been explained (5) by charge neutralization which follows conversion of adenine to the protonated form of 1-methyladenine. The picture is further complicated, however, by an aggregation reaction which leads to an increase in molecular weight[3] and radius of gyration, and eventually to an increase in specific viscosity. It is interesting to note that specific viscosity may be below the original value at times when both molecular weight and radius of gyration have increased. Such an effect could be explained, for example, by the conversion of a relatively long, rod-shaped molecule of poly A to shorter, heavier, aggregates of methylated poly A.

[3] In order not to disturb the reaction mixtures, solutions were not diluted and light scattering data were not, therefore, extrapolated to infinite dilution. When methylated poly A was isolated and light scattering measurements were performed over the concentration range 0.4–1.2 mg/ml, the same independence of Kc/R_θ on c was observed which was reported at this ionic strength for the unmethylated polynucleotides (10). Thus, the aggregation reaction is not readily reversible by dilution and the average molecular weights reported include the aggregated form.

Finally, the data show that there is a fall in viscosity and radius of gyration for poly C, presumably resulting from a decrease in charge following conversion of cytosine to protonated 3-methylcytosine. There is no tendency for poly C to aggregate under these conditions, however, and molecular weight actually falls slightly during methylation. This may indicate an increased susceptibility of the methylated polymer to hydrolysis.

Since poly U and poly C were available at molecular weights of only 100,000, and earlier studies of poly A (5) utilized polymer of approximately 400,000 molecular weight, control experiments were performed on poly A which had been partially degraded by alkaline treatment. The physical effects reported in Table 1 were found to be independent of molecular weight for samples of poly A in the region 30,000 to 500,000; results from one experiment with low molecular weight material are plotted in Fig. 2 for comparison.

The effect of methylation on the physical properties of copoly UG has not been as extensively investigated. As reported elsewhere (8), however, there was no change in sedimentation coefficient or light scattering molecular weight accompanying methylation of 50% of the guanine residues in a copolymer containing 70% uracil and 30% guanine.

In separate experiments investigating the effects of methylation on complex

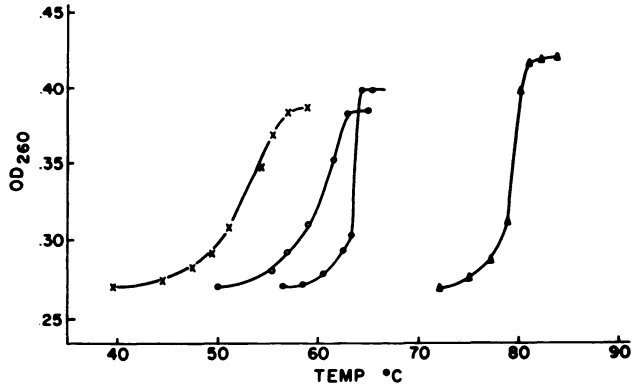

FIG. 3. *Melting curves for poly A–poly U complexes*

Optical density at 260 mμ for equimolar mixtures of poly A and poly U in 0.15 M NaCl–0.015 M sodium citrate, pH 7, *versus* temperature. X, 13.5% Methylated Poly A–Poly U; ○, Poly A–3.4% methylated Poly U; ●, Poly A–Poly U; △, Poly A–Poly 5-methyl U. Data of Szer *et al.* (14).

formation, melting curves were obtained as described above. Results of such determinations are shown in Figs. 3 and 4, in which we have added data obtained by Szer, Swierkowski, and Shugar (14) on complexes of poly A and poly 5-methyl U, and by Szer and Shugar (15) on complexes of poly I and poly 5-methyl C. For ease of comparison, the initial optical densities have been normalized to the same value for each complex studied. In contrast to the effects of methylation in the 5 position observed by the other authors, substitution in the 3 position, which is involved in H-bonding of the pyrimidines, decreased the stability of the complex.

DISCUSSION

The derivatives isolated from polynucleotides after methylation are those that would be expected from previous studies (1–6, 11). The identification of 3-methyladenine as a minor derivative in heavily methylated poly A is interesting, however, since this result initially obtained by Lawley and Brookes (11) does not appear to have been confirmed previously.

The relative reactivity of bases in the

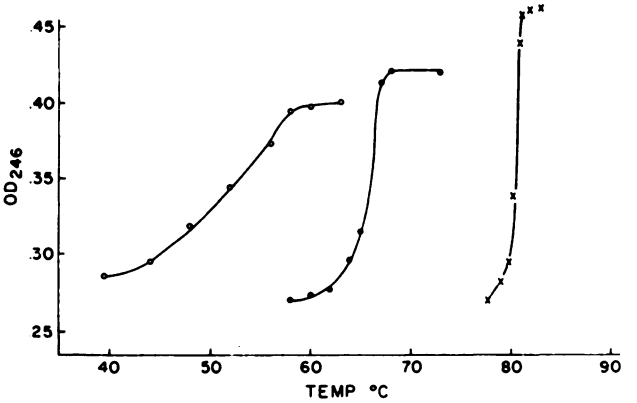

FIG. 4. *Melting curves for poly I–poly C complexes*

Optical density at 246 mμ for equimolar mixtures of poly I and poly C in 0.15 M NaCl–0.015 M sodium citrate, pH 7, *versus* temperature, ○, 8% Methylated poly C–poly I; ●, Poly C–Poly I; X, Poly 5-methyl C–Poly I. Data of Szer and Shugar (15).

polymers in Fig. 1 is different from that reported at 37° for the naturally occurring nucleic acids (11), among which the order is guanine > adenine > cytosine > uracil. This difference in reactivity might be caused by the difference in temperature, but it is probably related to the effects of secondary structure on reaction rates. Of the polynucleotides studied here, it is likely that poly A has the most highly ordered secondary structure, and this could decrease the reactivity of adenine relative to cytosine in poly C. This result emphasizes the fact that the site and extent of alkylation in nucleic acids may be quite dependent on the local structure of the polymer.

Physical studies provide strong evidence that methyl methanesulfonate does not attack the phosphodiester bond in polyribonucleotides. Such methylation would decrease the negative charge on the polymer and would certainly result in a decreased viscosity and radius of gyration. Results obtained with poly U show that this does not occur. Since the phosphate group is presumably as available for methylation in this polymer as in the other polynucleotides, this is strong evidence against methylation of phosphate groups in any polynucleotide. Such a conclusion is in agreement with the results of Brimacombe *et al.* (2), who showed that there was no methylation of the phosphate group in the model compound, adenylyl (3′–5′) uridine by dimethyl sulfate.

The changes in secondary structure which accompany methylation of poly A and poly C are particularly interesting in view of the unknown function of methylated bases in naturally occurring nucleic acids. Borek and Christman (16) have reported spectral changes similar to those seen during methylation of poly A when methyl-deficient transfer RNA is methylated. These results suggest that biological methylation may also affect secondary structure, and since such changes affect, for example, the ability of synthetic polynucleotides to code for polypeptide synthesis, methylation might have a regulatory role in nature mediated by changes in secondary structure.

The effects produced by pharmacologically useful alkylating agents may be quite different from those seen with methyl methanesulfonate. The approach outlined here is extremely useful in elucidating the chemistry and physical chemistry of such alkylation reactions. After alkylation, the substituted polymers may be used to determine the effects that these reactions have on the ability of the polynucleotides to function in model biological systems.

ACKNOWLEDGMENT

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