Alterations in the Structure of Deoxyribonucleic Acid on Chemical Methylation*

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ABSTRACT: The biological, chemical, and physicochemical properties of DNA from *Bacillus subtilis* and calf thymus were investigated both during and after methylation by dimethyl sulfate and methyl methanesulfonate. The transforming activity of *B. subtilis* DNA was destroyed as an immediate result of methylation under conditions where the viscosity, absorbance, molecular weight, and root-mean-square radius (R_G) changed only slightly. The only physical parameter which changed significantly as a direct consequence of methylation was the T_m, which decreased as a linear function of the extent of methylation. 7-Methylguanine and 3-methyladenine in a ratio of 6:1 were the only products of methylation.

The degree of methylation was a linear function of the dimethyl sulfate concentration. The viscosity of alkylated DNA solutions slowly decreased when incubated for extended periods at 37°, the exact rate of decrease depending on the extent of methylation. DNA methylated and ethylated to the same extent appeared to behave identically in this respect. The molecular weight and R_G determined by low-angle light scattering decreased at the same rate as the viscosity, suggesting that the changes taking place were due to double-strand scission and not to gradual denaturation or other conformational changes. Double-strand breaks could be the end result of depurination of the methylated purines, followed by single-strand cleavage at the apurinic sites. Where two single-strand breaks occurred close to each other on opposite strands, double-strand cleavage would take place. This mechanism was investigated by following the rate of release of 7-methylguanine and 3-methyladenine from methylated DNA. The loss of 300 methylated bases from DNA in which 30% of the guanine residues had been methylated led to one double-strand cleavage with a halving of the average molecular weight. DNA containing apurinic sites as well as single-strand breaks had hydrodynamic properties similar to that of native DNA.

Although the mutagenic, carcinogenic, cytotoxic, and antitumor properties of alkylating agents have been well documented, the mechanisms by which alkylating agents alter the genetic material and cellular processes are not yet fully understood (Ross, 1952; Alexander and Stacey, 1958; Lawley, 1966). The present work was undertaken to describe more completely the *in vitro* effects of methylation of DNA on its biological, chemical, and macromolecular properties.

The immediate biological and chemical results of DNA methylation have been characterized previously (Zamenhof *et al.*, 1956; Strauss, 1963; Strauss *et al.*, 1969), but the macromolecular changes have been studied less extensively. Lett *et al.* (1962), using viscometry, light-scattering, and sedimentation techniques, reported that alkylation produced a rapid decrease in viscosity with little change of the molecular weight or R_G. This result was obtained by previous workers (Chanutin and Gjessing, 1946; Butler *et al.*, 1952). Laurence (1963), on the other hand, reported that DNA alkylation produced little change in viscosity and found that the macromolecular properties changed only after prolonged incubation of the alkylated DNA. Leng *et al.* (1969) confirmed that the viscosity, sedimentation, and molecular weight of DNA changed very little as a direct result of alkylation. We have shown previously (Uhlenhopp and Krasna, 1969) that the reported decrease in viscosity is probably due to denaturation of the DNA by the alkali added to maintain the pH constant and not to alkylation *per se*. The results presented below show that there is very little alteration in the macromolecular structure of DNA during alkylation and immediately thereafter, while extensive changes occur subsequently, probably due to depurination of alkylated bases leading to single-strand scission and eventually to double-strand cleavage.

Materials

Reagent grade dimethyl sulfate and methyl methanesulfonate were obtained from Eastman Organic Chemical Co. [^{14}C]Dimethyl sulfate was obtained from Schwarz Bio-Research or New England Nuclear at a specific activity 1.5–9.1 mCi/mmole; 7-methylguanine, 3-methyladenine, and 1-methyladenine were purchased from Cyclo Chemical Corp.

Buffers. Using nuclear magnetic resonance techniques, we have shown previously that dimethyl sulfate alkylates phosphate, citrate, and chloride ions which are commonly used for preparing buffer solutions for DNA studies (Uhlenhopp *et al.*, 1969). Cacodylate and perchlorate ions were inert toward dimethyl sulfate and were used to prepare the buffers in this investigation. The buffer used in this study contained 0.01 M cacodylic acid–0.07 M sodium cacodylate–0.001 M Na_2EDTA–0.128 M $NaClO_4$ (pH 7.0). This solution had sufficient buffer capacity to neutralize the acid released by the aqueous hydrolysis of a 50 mM concentration of alkylating agent without requiring any alkali addition to maintain the pH above 6.0 (Uhlenhopp and Krasna, 1969).

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Methods

DNA Preparation. Calf thymus DNA was prepared by the method of Kay *et al.* (1952), giving a product with an intrinsic viscosity of 94.1 dl/g and a calculated molecular weight (Crothers and Zimm, 1965) of 20×10^6. Transforming DNA from *Bacillus subtilis* 23 was prepared by the method of Saito and Miura (1963), and had an intrinsic viscosity of 122 dl/g and a calculated molecular weight of 30×10^6.

DNA concentrations were determined by measuring their absorbance at 259 mμ using an extinction coefficient of 0.0207 $cm^2/\mu g$ for calf thymus DNA and 0.0213 $cm^2/\mu g$ for *B. subtilis* DNA. For more accurate values, the Burton method (Burton, 1956) as modified by Giles and Myers (1965) was used.

Transformation. DNA from wild-type *B. subtilis* 23 was used to transform indole-requiring *B. subtilis* 168 i^- cells to prototrophy using the method of Anagnostopoulus and Spizizen (1961). Competent cells were prepared by inoculating overnight cultures of *B. subtilis* 168 i^- into Spizizen medium and growing for 4 hr at 37°. These competent cells were added to Spizizen medium II and incubated with DNA from wild type cells for 2 hr at 37° using DNA concentrations from 0.01 to 10 μg per ml. Viable counts were determined by plating on Penassay plates, and transformed cells were assayed with Spizizen minimal medium plates.

When methylated DNA was used for transformation, the wild-type DNA was incubated in cacodylate buffer at a concentration of 200 μg/ml with 42 mM dimethyl sulfate for 4 hr at 25°. Methylation by 42 mM methyl methanesulfonate required overnight incubation at 37°. The DNA was then used in the transformation assay.

Alkylation. Aqueous DNA solutions in cacodylate buffer were alkylated by the addition, with stirring, of small volumes of alkylating agent. DNA concentration, alkylating agent concentration, temperature, and time of reaction were carefully controlled. Concentrations of dimethyl sulfate above 10 mM required several minutes for complete dissolution, while methyl methanesulfonate dissolved more readily. Very low concentrations were achieved by diluting the alkylating agent with benzene and adding the diluted alkylating agent with a micropipet. The acid released by the aqueous hydrolysis of an alkylating agent was neutralized by a strong cacodylate buffer solution so that the pH never decreased below 6. In most cases the reaction was allowed to proceed until all the alkylating agent was consumed either by reaction with DNA or hydrolysis by H_2O. The reaction could be terminated earlier by the addition of sodium thiosulfate, a strong nucleophile. Thiosulfate addition alone had no effect on any of the properties of DNA.

Base Analysis. Methylated DNA was hydrolyzed with 90% formic acid at 175° for 45 min to release purines and pyrimidines or with 0.1 M H_2SO_4 at 100° for 35 min to release purines. The hydrolysate was spotted on Whatman No. 3MM paper and high-voltage electrophoresis conducted in a Savant water-cooled apparatus at 3000 V for 90 min using 0.05 M formate buffer (pH 3.25). The concentrations of the four bases and the two methylated bases thus separated were estimated by elution of the spots with 0.1 M HCl and determination of the absorbance at the λ_{max}.

When [^{14}C]dimethyl sulfate was used, the concentration of the methylated bases was determined by cutting out the radioactive areas of the electropherogram, placing the paper in scintillation vials containing scintillator solution (5.0 g of 2,5-diphenyloxazole plus 0.30 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene per l. of toluene), and counting in a liquid scintillation counter. The per cent methylation of a given DNA sample was defined as the per cent of total guanine converted to 7-methylguanine.

Physicochemical Measurements. Viscosity measurements were made using a low-shear Zimm–Crothers Couette-type viscometer (Zimm and Crothers, 1962) with the temperature maintained constant to ±0.1°. The speed of rotation of the rotor was recorded automatically by directing a beam of light at a photoelectric cell mounted behind a small, well-defined slit on the opposite side of the stator. An opaque spot on the rotor interrupted this beam when it passed in front of the slit. The signal from the photocell was recorded on a Heath EUW-20A recorder with a variable chart speed.

Light-scattering measurements were made on the low-angle light-scattering apparatus described by Harpst *et al.* (1968a,b). DNA solutions at different concentrations were clarified by Millipore filtration as described by Krasna (1970), and the intensity of light scattered by each solution was measured over the angular range 10–90°. Light-scattering data were then processed using the reciprocal intensity method of Zimm (1948) with the aid of an IBM 360/91 computer. Final graphical output in the form of Zimm plots was obtained automatically using a Stromberg Carlson 4060 cathode ray plotter.

DNA melting curves were determined using a Gilford Model 2000 recording spectrophotometer equipped with a temperature monitoring channel and automatic zero adjustment. Temperature was controlled by a Tamson bath with an automatic temperature programming unit which increased the temperature continuously at a rate of 0.5°/min.

Results

Effect of Methylation on Transforming Activity. When *B. subtilis* DNA was methylated 30% (*i.e.*, when 30% of the guanine residues were converted into 7-methylguanine) by dimethyl sulfate, the transforming activity of the methylated DNA determined soon after methylation was 0.07% that of unmethylated DNA. When the same concentration of methyl methanesulfonate was used, the transforming activity decreased to the same extent. This result was the same as reported by Zamenhof *et al.* (1956).

Products of DNA Methylation. When calf thymus DNA was methylated with dimethyl sulfate and hydrolyzed with formic acid, the resulting hydrolysate was separated into its component bases quite easily by high-voltage electrophoresis at pH 3.25. By comparison with authentic 7-methylguanine, 3-methyladenine, and 1-methyladenine, the methylated bases in the DNA were identified as 7-methylguanine and 3-methyladenine. The order of the bases from anode to cathode on the pH 3.25 electropherogram were thymine (0.18), guanine (0.42), 7-methylguanine (0.55), adenine (0.75), 3-methyladenine (0.94), and cytosine (1.0). (The values in parentheses are the relative electrophoretic mobilities for each compound when cytosine is assigned a value of 1.) When [^{14}C]dimethyl sulfate was used, the areas corresponding to 7-methylguanine and 3-methyladenine were the only radioactive spots.

The effect of dimethyl sulfate concentration on the conversion of guanine and adenine to their respective methylated bases was next studied using [^{14}C]dimethyl sulfate. The results are given in Figure 1 as a log – log plot. The log of the per cent methylation was a linear function of the log of the dimethyl sulfate concentration up to about 10% methylation, at which point increases in dimethyl sulfate concentration produced progressively smaller increases in the per cent

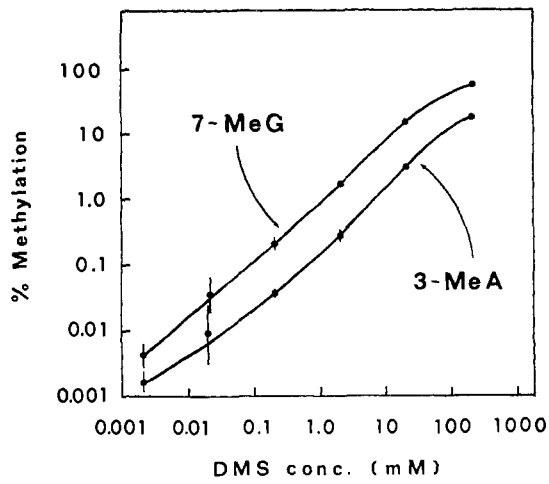

FIGURE 1: Extent of methylation as a function of dimethyl sulfate concentration. To 5 ml of calf thymus DNA (400 μg/ml) in cacodylate buffer, various amounts of [^{14}C]dimethyl sulfate were added. The highest dimethyl sulfate concentration was conducted on 0.2 M cacodylate buffer (pH 7.0) in order to neutralize the acid produced. Samples were incubated for 1 hr at 37°, precipitated with two volumes of cold ethanol, and hydrolyzed with 0.05 ml of 90% formic acid. The hydrolysates were spotted on Whatman No. 3MM paper along with carrier 7-methylguanine and 3-methyladenine, and the bases were separated by electrophoresis at 3000 V for 75 min using 0.05 M citrate buffer (pH 3.25). The areas containing the methylated bases were cut out and their radioactivity measured. The unmethylated bases guanine and adenine were eluted from the paper with 0.1 M HCl and their concentrations determined by measuring absorbances at their λ_{max}. Vertical bars represent the range of per cent methylation values for the four separate electrophoresis runs made on each sample.

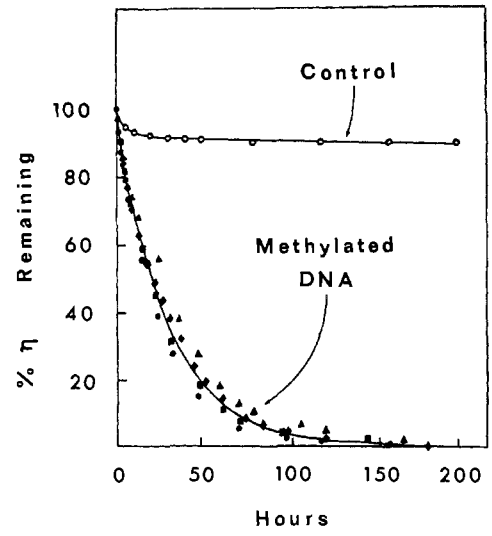

FIGURE 3: Change in viscosity of methylated DNA. Four calf thymus DNA samples in cacodylate buffer were methylated with 42 mM dimethyl sulfate for 1 hr at 37°, resulting in 30% methylation. Solutions of this methylated DNA were incubated at 37° for 200 hr and portions were removed periodically for viscosity measurements. "% η remaining" is the ratio of the specific viscosity at each time interval to the specific viscosity at $t = 0$, multiplied by 100. The different symbols represent the four different experiments, and the upper curve represents control, unmethylated DNA, incubated under identical conditions.

methylation. Guanine methylation ranged from 0.003% at 2.11 μM dimethyl sulfate (representing less than one 7-methylguanine molecule per DNA molecule of 20×10^6 mol wt) up to 85% at 0.211 M. Similar curves were obtained using denatured DNA as the substrate. Diethyl sulfate was much less effective in alkylating the purine bases when compared to dimethyl sulfate. For example, 50 mM dimethyl sulfate gave 30% methylation, whereas 50 mM diethyl sulfate gave only about 3% ethylation; 30% ethylation could only be achieved by increasing the diethyl sulfate concentration to 1.3 M.

Changes in DNA Structure During Methylation and Immediately Thereafter. Both calf thymus and *B. subtilis* DNA methylated 30% retained 97% of their initial viscosity. Neither the molecular weight nor the R_G was changed by methylation. The spectrum of DNA exhibited a slight hypochromic shift around 259 mμ on methylation. The only property of DNA besides transforming activity which was found to be significantly altered as a direct result of methylation was the T_m. The melting curves of methylated DNA were as sharp as for unmethylated DNA, but were shifted to lower temperatures. The T_m decrease was a function of the degree of methylation, as shown in Figure 2.

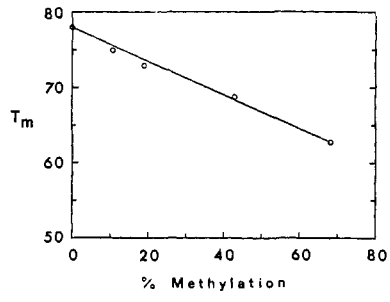

FIGURE 2: Dependence of T_m on extent of methylation. The T_m values of the methylated DNA samples were determined in cacodylate buffer diluted 20-fold to give a final sodium concentration of 0.01 M. This dilution was necessary in order to lower the T_m to minimize depurination at the elevated temperature.

Changes in DNA Structure Following Alkylation. Although methylation *per se* produced few alterations in the secondary structure of DNA, prolonged incubation of the methylated product caused much greater structural changes. Figure 3 shows the decrease in viscosity for 30% methylated DNA incubated for 200 hr at 37°. (Methylation by dimethyl sulfate was essentially complete after 1 hr at 37°, during which time the viscosity decreased very little, as mentioned above.) The exact rate at which the viscosity of methylated DNA decreased depended on the extent of methylation. Figure 4 shows that the rate of viscosity loss increased as the extent of methylation increased.

When the viscosity changes for 1% methylated DNA were compared to DNA ethylated with diethyl sulfate to approximately the same extent, very similar behavior was observed, suggesting that both types of DNA undergo similar structural modifications.

The loss of viscosity on prolonged incubation of methylated DNA could be the end result of diverse structural changes in the native molecule. Gradual denaturation or any other similar process which would increase the molecular flexibility would lead to a loss in viscosity. Alternately, double-strand scission could be taking place leading to the loss of viscosity. Krasna has shown (Krasna, 1970; Krasna *et al.*, 1970) that the two processes, denaturation *vs.* double-strand scission, can easily be differentiated by low-angle light scattering. Denaturation results in a complete loss of viscosity, a

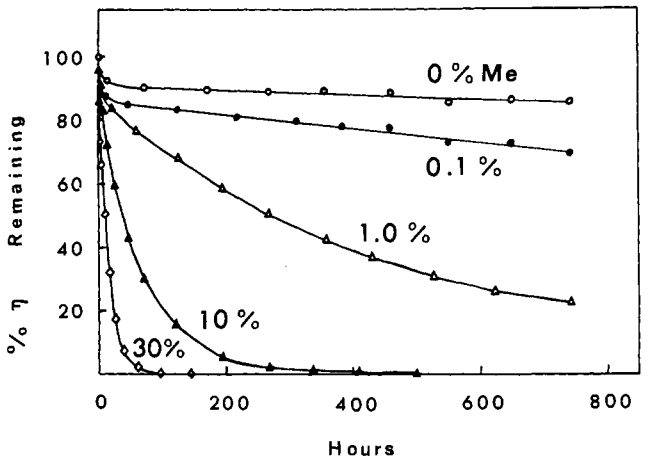

FIGURE 4: Viscosity loss for DNA methylated to different extents. DNA solutions (91 μg/ml) were methylated to 0, 0.1, 1.0, 10, and 30% using dimethyl sulfate concentrations obtained from the upper curve in Figure 1. The solutions were incubated at 37°, and aliquots were removed directly from the incubation flasks for viscosity determinations.

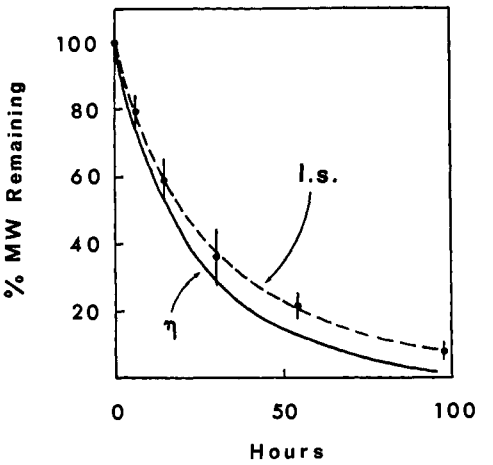

FIGURE 5: Long-term changes in molecular weight of methylated DNA. A stock solution of calf thymus DNA (70 μg/ml) in cacodylate buffer was prefiltered through 1.2, 0.8, and 0.65 μ Millipore filters and then through a single 0.45 μ filter as described by Krasna (1970). The solution was made 42 mM in dimethyl sulfate and incubated for several days at 37°. At t = 0, 5, 14, 30, 55, and 98 hr, aliquots were removed for dilution to 10, 20, 30, and 40 μg per ml. Each concentration of methylated DNA was filtered through a 0.45 μ filter directly into the light-scattering cell for measurement of scattered intensities from 90° down to 10°. Results were calculated as described in Methods. Vertical bars represent the range of data obtained from several experiments. The "l.s." curve is the data obtained from these light-scattering experiments, and the "η" curve is the molecular weight calculated from the viscosity curve in figure 3 using the equation of Crothers and Zimm (1965).

drastic decrease in R_G, and only a halving of the molecular weight. Double-strand scission, on the other hand, leads to a gradual decrease in all these parameters. Therefore, by measurement of the light-scattering molecular weight and R_G, it should be possible to assess the nature of the viscosity loss for methylated DNA. Figure 5 compares the light-scattering molecular weight to the molecular weight calculated from the intrinsic viscosity using the equation of Crothers and Zimm (1965) on the assumption that the methylated DNA undergoing change has a double-stranded helical structure. Clearly the molecular weight estimated by both methods is approximately the same, suggesting that the process being observed is a double-strand cleavage rather than conformational changes which would affect macromolecular flexibility and viscosity without affecting molecular weight.

Denaturation would not account for the result obtained in Figure 5. Conformational changes without double-strand cleavage would be expected to alter the R_G much more rapidly than the molecular weight (Krasna, 1970), while double-strand cleavage would lead to only small changes in R_G. Figure 6 shows that the decrease in R_G follows that observed in viscosity and light-scattering molecular weight and again suggests that the only event taking place is double-strand cleavage.

The most satisfactory mechanism for explaining the production of double-strand breaks in methylated DNA is a process involving depurination of methylated bases followed by the introduction of single-strand breaks at apurinic sites (Lawley, 1966; Strauss and Hill, 1970). Such a mechanism would be consistent with the viscometry and light-scattering results presented above. The decrease in viscosity, molecular weight, and R_G could be accounted for by assuming that double-strand cleavage took place where single-strand breaks occurred close enough to each other on opposite strands. The feasibility of this mechanism was investigated by studying the rate of depurination of methylated bases.

In order to observe the release of methylated bases from DNA by depurination, [^{14}C]dimethyl sulfate was used as the alkylating agent and the free methylated bases separated from the intact DNA by high-voltage electrophoresis. Figure 7 shows the rate of depurination of 7-methylguanine and 3-methyladenine from 30% methylated DNA at 70° and at 37°. The first-order rate constant for 7-methylguanine depurination at 70° was 1.33 × 10^{-2} min^{-1}, which is slightly greater than the value obtained by Strauss and Hill (1970) at pH 7.8. When the rate of depurination at 37° was compared to the rate of decrease in molecular weight, it was estimated that approximately 300 depurinations are necessary before the equivalent of one double-strand break (to halve the average molecular weight) has been introduced into calf thymus DNA methylated 30%.

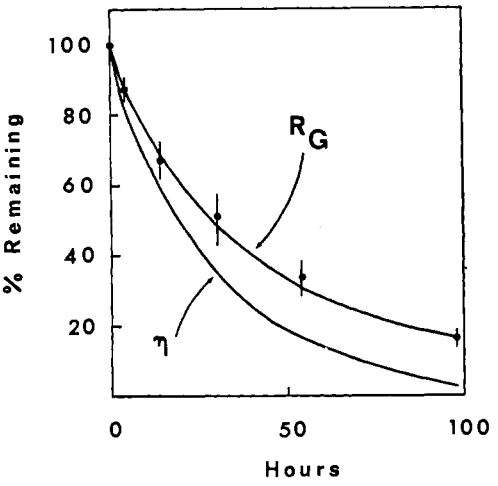

FIGURE 6: Change in R_G for methylated DNA. The R_G values were obtained from the Zimm plot light-scattering data of methylated DNA as described in Figure 5. The viscosity curve is provided for comparison.

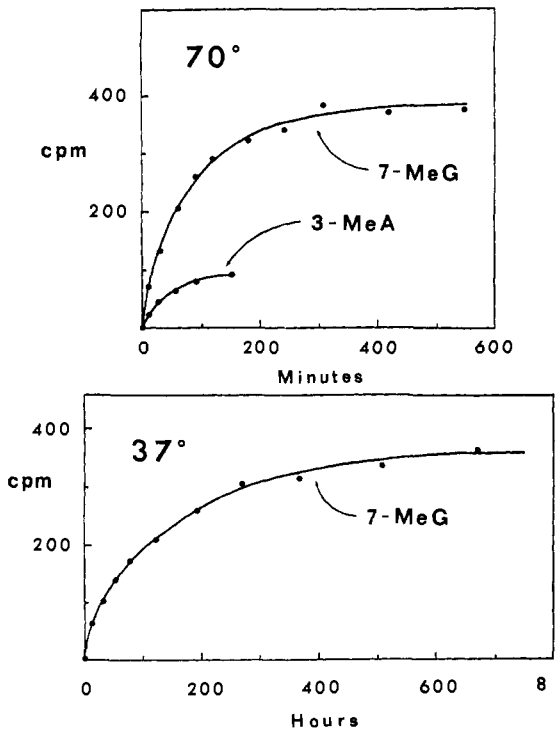

FIGURE 7: Depurination of methylated bases from methylated DNA. Two 1.25-ml reaction mixtures containing 254 μg/ml of calf thymus DNA in cacodylate buffer and 42 mM [^{14}C]dimethyl sulfate (9.1 mCi/mmole) were incubated in 2.0-ml glass-stoppered volumetric flasks for 1 hr at 37° to fully methylate the DNA. One flask was then incubated at 70° (upper graph) and the other at 37° (lower graph). From each reaction mixture, aliquots were removed (10 μl) and spotted on two sheets of Whatman No. 3MM paper over carrier 7-methylguanine and 3-methyladenine. Electrophoresis of the first sheet was conducted at 5000 V for 55 min in 0.05 M formate buffer (pH 3.0) and of the second sheet at 5000 V for 75 min in 0.05 M cacodylate buffer (pH 5.9). The pH 5.9 electrophoresis was run for the estimation of 3-methyladenine and the pH 3.0 electrophoresis for the estimation of 7-methylguanine. Areas containing the 7-methylguanine and 3-methyladenine spots were cut out from the appropriate electropherograms and counted.

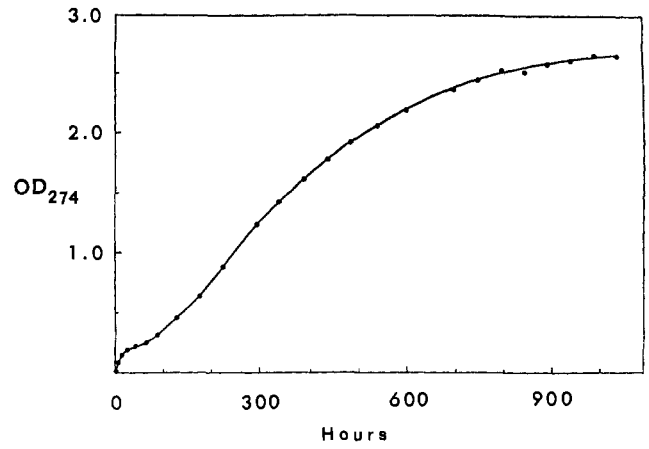

FIGURE 8: Appearance of dialyzable material from methylated DNA. A DNA solution (30 ml containing 320 μg/ml) in 0.25 M cacodylate buffer (pH 7.0) was methylated with 105 mM dimethyl sulfate for 2 hr at 37°. The reaction mixture was transferred to 1 m of 7-mm i.d. dialysis tubing and the tubing placed in a specially constructed flask with quartz cuvet attached. Cacodylate buffer (25 ml) was added, filling the flask to capacity. The entire apparatus was incubated at 37° and the OD_{274} of the dialysate monitored (274 mμ is the λ_{max} for 7-methylguanine).

Once depurination of a methylated base has occurred, single-strand cleavage can take place at the apurinic site, presumably *via* β elimination of the 3′-phosphate (Brown and Todd, 1955). When a sufficient number of single-strand breaks has been introduced, two events can take place. (1) If two single-strand breaks are located on *opposite* strands of the double helix and separated by only a few base pairs (Thomas, 1956; Freifelder and Trumbo, 1967), a double-strand break results and the viscosity, molecular weight, and R_G fall as described above. (2) If two single-strand breaks occur near each other on the *same* DNA strand, however, another process could occur. A small oligonucleotide fragment could be released if the number of base pairs holding the fragment to the opposite strand is small. In this case, the small oligonucleotide fragment would diffuse away from its complementary strand and be detectable. This possibility was confirmed experimentally by observing the release of small ultraviolet-absorbing fragments in the dialysate when the methylated DNA was held in dialysis tubing (Figure 8). The ultraviolet-absorbing material appearing in the dialysate resulted in an absorbance much higher than could be accounted for solely by depurination of methylated bases. The rate of release of ultraviolet-absorbing material was also much slower than the rate of depurination (*cf.* Figure 7), which would be expected if release of dialyzable fragments depended on depurination and single-strand cleavage. Moreover, the material in the dialysate contained deoxyribose in addition to free methylated purines.

Discussion

Methylation of native DNA produced 7-methylguanine as the predominant product with smaller amounts of 3-methyladenine. This result was previously found by Brookes and Lawley (1961a), who also reported that methylation of RNA or denatured DNA produced 1-methyladenine in addition to 3-methyladenine. The difference in products was presumably due to the fact that the N-1 position of adenine in native DNA was involved in hydrogen bonding and was not available for alkylation. It should be noted that enzymatic methylation of DNA leads predominantly to 6-methylaminopurine and 5-methylcytosine (Srinivasan and Borek, 1966).

The very small degree of methylation detectable using radioactive alkylating agents and high-voltage electrophoresis is particularly significant in attempting to duplicate the degree of alkylation encountered in *in vivo* studies. Cancer chemotherapy and cytotoxicity studies can involve as little as 0.001–0.002% alkylation (Rutman *et al.*, 1969).

In contrast to the lack of transforming activity of methylated DNA, its physicochemical stability is rather remarkable, as shown here and as has been noted previously (Leng *et al.*, 1969). This stability was only observed when the alkylation was carried out under conditions which did not lead to denaturation (Uhlenhopp and Krasna, 1969).

The decrease in T_m which results from DNA alkylation has been reported by other workers (Leng *et al.*, 1969; Strauss, 1962; Pochon and Michelson, 1967; Walles *et al.*, 1967) and can be explained either in terms of a weakening of guanine–cytosine hydrogen-bonded base pairs due to ionization of the N-1 proton of 7-methylguanine (Brookes and Lawley, 1961b) or to a disruption of stacking interactions due to introduction of a positive charge by quaternization of the N-7 position of guanine (Hendler *et al.*, 1970).

Incubation of methylated DNA for extended periods at 37° leads to the eventual destruction of the secondary structure. DNA undergoing depurination and single-strand cleavage showed no great increase in flexibility, as witnessed by the fact that the viscosity and R_G did not decrease more rapidly than the light-scattering molecular weight. Similar conclusions have been reached by other workers in connection with single-strand breaks caused by DNase (Thomas, 1956; Hays and Zimm, 1970). If the DNA was methylated to a very small extent, it was relatively stable for long periods of time, as is seen by the retention of viscosity for DNA methylated 0.1% (Figure 4).

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