Purification and Properties of Deoxyribonucleic Acid Methylase from *Bacillus subtilis**

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ABSTRACT: DNA methylase was purified about 100-fold from *Bacillus subtilis* strain 6633. The novel features of this enzyme are as follows: (1) during growth of bacteria, enzyme activity was detected principally in the early exponential phase. (2) The enzyme catalyzes the methylation of both native and heat-denatured DNA. (3) The product of the enzymatic methylation of DNA was 5-methylcytosine (5MC) only. (4) The extent of the methylation of both native and heat-denatured bacterial DNA is approximately proportional to its GC (guanine plus cytosine) content. The higher the per cent GC in DNA, the greater the extent of methylation that takes place, even if the ratio of 5MC to total cytosine residues in DNA is considered. The possibility of the selective methylation of one strand of DNA was also examined by using *B. subtilis* bacteriophage 2C DNA, whose strands, after denaturation, can be fractionated because of a bias in base composition. The H and L strands of phage 2C DNA were separated by MAK column chromatography or by centrifugation in alkaline CsCl density gradients, and it was found that both strands were equally methylated *in vivo* and *in vitro*.

The discovery of trace amounts of methylated bases in nucleic acids (Dunn, 1959; Dunn *et al.*, 1960) stimulated studies on nucleic acid methylation and a search for the role of methylated bases in cellular metabolism. Recent knowledge about the specificity of nucleic acid methylation can be summarized as follows: (1) methionine is the donor of methyl groups to nucleic acid *in vivo* (Biswass *et al.*, 1961; Fleissner and Borek, 1962). (2) The actual methyl donor in the *in vitro* methylation of nucleic acids is SAM[1] and this reaction is carried out by several specific enzymes (Hurwitz *et al.*, 1964a,b; Gold and Hurwitz, 1964a,b). (3) The methylation of nucleic acids occurs at the macromolecular level (Fleissner and Borek, 1962; Gold *et al.*, 1963a,b). (4) There exists a species specificity of nucleic acid methylation (Srinivasan and Borek, 1963; Gold *et al.*, 1963a), and, in general, nucleic acids cannot be methylated further by enzyme preparations from homologous bacterial strains. These features suggest the possibility that the methylation of nucleic acids might play some biological role in the maintenance and recognition of species specificity of organisms or on the control mechanisms of macromolecular syntheses primed by nucleic acids. In this paper, studies have been carried out on the characterization of methylating enzymes of several *Bacillus* species and on the methylation of DNA isolated from various sources. The possible role of methyl groups of DNA as a recognition site for strand selection by DNA-dependent RNA polymerase prompted an examination of the distribution of methyl groups on each strand of *B. subtilis* bacteriophage 2C DNA. The latter system was selected for this purpose since the individual strands can be separated, fractionated, and characterized (Pène and Marmur, 1964).

Materials and Methods

Bacterial Strains and Bacteriophages. The following strains, which are from the stock collection of this laboratory, were used in this study: *Clostridium perfringens* 876, *B. cereus* MB19, *B. megaterium*, *Proteus mirabilis* 35, *B. subtilis* ATCC 6633, *B. subtilis* 168, *B. subtilis* W23, *B. subtilis* var *aterrimus* ATCC 6460, *B. subtilis* Marburg strain, *B. natto* MB 275, *B. niger* ATCC 6554, *Escherichia coli* B, *Aerobacter aerogenes* 1088, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Micrococcus lysodeikitcus*, NRRL B287, and *Sarcina lutea* 26C. The mutant strain of *B. subtilis* 6633 (thy⁻met⁻cys⁻) was supplied by Dr. W. J. Brabander. The bacteriophages used here are 2C, SP8* (Brodetsky and Romig, 1965), and SP82. SP8* and, SP82 were gifts, respectively,

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[1] Abbreviations used in this work: 5MC, 5-methylcytosine; 6MAP, 6-methylaminopurine; SAM, *S*-adenosyl-L-methionine; SAE, *S*-adenosyl-L-ethionine; MA, methylated albumin; MAK, methylated albumin kieselguhr; BSA, bovine serum albumin; SSC, standard saline citrate (0.15 M NaCl, 0.015 M Na citrate, pH 7.0); MM, Spizizen's glucose–mineral salts medium; MAM, Difco methionine assay medium; RI, refractive index; H, heavy phage strand; L, light phage strand; PCA, perchloric acid; TCA, trichloroacetic acid; moi, multiplicity of infection; Thy, thymine; Met, methionine; Cys, cystine; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; GC, guanine plus cytosine.

from Drs. W. R. Romig, University of California at Los Angeles, and D. M. Green, University of Pittsburgh.

Enzymes and Other Materials. Beef pancreas DNAase I (1× crystallized), RNAase (1× crystallized), calf thymus DNA and salmon sperm DNA were purchased from Worthington Biochemical Corporation. Pronase was purchased from the Enzyme Development Corporation. *E. coli* K12 DNA was a commercial preparation from General Biochemicals. DEAE-cellulose and phosphocellulose were purchased from Carl Schleicher and Schuell Company and W. & R. Balston Ltd., respectively. Alumina C_γ gel was a commercial product from Sigma Chemical Co. [methyl-^{14}C]SAM (1.4–2.0 × 10^7 cpm/μmole) and [1-ethyl-^{14}C]SAE (1.3 × 10^7 cpm/μmole) were obtained from New England Nuclear Corporation. L[methyl-^{14}C]Methionine (10 mc/μmole) was purchased from Calbiochem. CsCl was purchased from the Harshaw Chemical Company or American Fluoride. Species of RNA from *B. subtilis* 6633 or *B. subtilis* 168 (4S, 16S, and 23S) were the gift of Dr. David Dubnau of this laboratory. Single-stranded φX-174 DNA was the generous gift of Dr. R. L. Sinsheimer. T2 and T4 bacteriophage DNA were kindly supplied by Dr. M. Gold.

Preparation of DNA from Bacteria. Bacterial DNA was prepared by the method of Marmur (1961). For the preparation of [^{14}C]methyl-labeled bacterial DNA, *B. subtilis* 6633 (thy$^-$cys$^-$met$^-$) and *B. subtilis* 168 N5 (met$^-$)were cultivated in 200 ml of MM (Anagnostopoulos and Spizizen, 1961) supplemented with 0.05% of MAM purchased from Difco laboratories, 100 μc of L-[methyl-^{14}C]methionine, and, in the case of *B. subtilis* strain 6633 (thy$^-$cys$^-$met$^-$), with 5 μg/ml of thymidine and 20 μg/ml of cysteine. The medium was inoculated with 1 ml of overnight culture, grown at 37° on a rotary shaker, and the cells were harvested when they reached the stationary phase.

Preparation of Bacteriophage DNA. Phage lysates were prepared by adding a stock phage suspension (moi was 0.5–3) to a fresh bacterial culture (*ca.* 10^8 cells/ml) grown in MM supplemented with a 0.1% Difco yeast extract. After lysis, the lysate was centrifuged at 10,000*g* for 10 min. To the supernatant fluid 2 μg/ml of DNAase I and 5 μg/ml of RNAase were added and the solution made 0.01 M with regard to $MgCl_2$. After incubation at 37° for 30 min, the lysate was again centrifuged at 10,000*g* for 10 min. The supernatant solution was adjusted to 0.02 M in EDTA and chilled in ice. Phage pellets were collected by centrifugation at 20,000 rpm for 45 min in the 21 rotor of the Spinco L2 preparative ultracentrifuge, and suspended in saline–Versene (0.15 M NaCl, 0.1 M EDTA, pH 8.0). The phage suspension was gently mixed with a tissue homogenizer and deproteinized 3–4 times with water-saturated phenol, previously adjusted to pH 8–9 with 1 N NaOH. The phage DNA was precipitated by the addition of 2 volumes of 95% ethanol and then dissolved in $^1/_{10}$ SSC. The ratio of absorbance at 280 mμ:260 mμ was 0.49, indicating that almost all protein was eliminated during successive deproteinization procedures. No bacterial DNA contamination was observed by CsCl density gradient centrifugation and the $s_{20,w}$ value of the phage DNA was about 30, corresponding to a MW of approximately 20 × 10^6. This would indicate that the phage 2C DNA isolated by this method has been sheared to approximately $^1/_4$–$^1/_5$ of its original size.[2]

For the isolation of [^{14}C]methyl-labeled phage 2C DNA, the following procedure was used. An auxotroph of either *B. subtilis* 6633 (thy$^-$met$^-$cys$^-$) or 168 (met$^-$) was grown in 1 l. of MM supplemented with 0.05% of MAM, 20 μg/ml of L-methionine, 5 μg/ml of thymidine, and 20 μg/ml of L-cysteine. The cells were harvested at the early exponential phase (*ca.* 10^8 cells/ml), washed once with the same medium but containing no methionine, and suspended in 400 ml of the same prewarmed medium. Methionine starvation was carried out by incubating the bacterial suspension at 37° for about 1 hr, after which time 200 μc of L[methyl-^{14}C]-methionine and the stock phage suspension (moi was about 0.5) were added to the culture (2 × 10^8 cells/ml) simultaneously. After lysis, the procedure described above for the isolation of phage DNA was carried out.

Assay of DNA Methylase. Optimal conditions for measuring DNA methylase activity from *B. subtilis* were found to be the same as for the *E. coli* enzyme. Thus, the following method (Gold and Hurwitz, 1964a) was used. The reaction mixture (0.25 ml) contained 10 μmoles of Tris buffer, pH 8.0, 1 μmole of $MgCl_2$, 4 μmoles of 2-mercaptoethanol, 10 mμmoles of [methyl-^{14}C]SAM, 100 mμmoles of DNA expressed as nucleotide residues, and 0.01–0.3 unit of enzyme protein. After incubation at 37° for 30 min, the mixture was chilled in ice and 0.1 ml of 0.1 M sodium pyrophosphate, 0.05 ml of BSA (0.5%), and 0.4 ml of cold 7% PCA were added. The suspension was vigorously mixed on a vortex mixer and centrifuged at 3000*g* for 5 min. The supernatant fluid was removed, and the precipitate was washed twice with 3 ml of cold 2% PCA containing 2 μmoles/ml of sodium pyrophosphate. The resulting precipitate was dissolved in 1.5 ml of 0.2 N NH_4OH, transferred to a metal planchet, dried, and counted in a window gas flow counter. One unit of enzyme was defined as the amount catalyzing the incorporation of 1 mμmole of [^{14}C]methyl groups into the acid-precipitable fraction in 30 min. Specific activity was expressed as enzyme units for 1 mg of protein.

Measurement of the Amount of DNA and Protein. DNA was determined by the Burton modification of the diphenylamine reaction (Burton, 1956) or by measuring its absorbance at 260 mμ (OD 1.0 corresponds to 50 μg/ml DNA). Protein was determined by the method of Lowry *et al.* (1951) after precipitation by cold TCA. Absorbance measurements in the ultraviolet region were made with the Zeiss-PMQ II spectrophotometer.

Separation of Methylated Bases by Paper Chromatography. To degrade any RNA that might be present, KOH was added to a final concentration of 0.3 N to

[2] The MW of phage 2C DNA was estimated to be approximately 1 × 10^8 (P. May, 1965, unpublished data).

50–100 μg of DNA methylated either *in vivo* or *in vitro* with [^{14}C]methyl groups and incubated overnight at 37°. The pH of the solution was then adjusted to 4–5 with 1 N acetic acid and DNA was precipitated by addition (2 volumes) of 95% ethanol. The mixture was kept in ice for at least 1 hr. After centrifugation, the precipitate was washed twice with 70% ethanol. The washed DNA was dried, and hydrolyzed with 0.2 ml of 90% formic acid in a sealed tube at 175° for 1 hr. After hydrolysis, the formic acid was evaporated at reduced pressure over KOH and the resulting precipitate was dried and dissolved in 0.05 ml of 0.5 N HCl. A 0.005-ml aliquot was mixed with 10 ml of Bray's solution and counted in a liquid scintillation counter (Nuclear Chicago, Model 6860). The sample (30–100 μg) with about 0.2 μmole of 5MC and 6MAP as standards was spotted on a 64 × 57 cm sheet of Whatman No. 3MM filter paper and developed by descending chromatography using butanol–H_2O–NH_4OH (86:13:1) for about 18 hr. The ultraviolet-absorbing spots were examined with a short-wave ultraviolet lamp and the paper was cut into appropriate strips and counted for [^{14}C] in a Vanguard automatic chromatogram scanner.

MAK Column Separation of H and L Strands of Bacteriophage 2C DNA. MA was prepared according to Mandel and Hershey (1960). A MAK column was prepared by the modified method of Sueoka (Sueoka and Cheng, 1961): 10 g of kieselguhr was suspended in 30 ml of 0.05 M potassium phosphate buffer, pH 6.7–6.8, containing 0.1 M sodium chloride, and twice boiled and cooled gradually to room temperature. Three milliliters of a 1% MA soln were then added dropwise, stirring at least 10 min, and 40 ml of the same buffer was added. Phage 2C DNA was denatured at a concentration of 30 μg/ml in $^1/_{10}$ SSC by heating for 10 min at 100°, followed by rapid cooling to 0°. The column was prepared by pouring 15 ml of MAK solution into a 2 cm diameter column which was then packed under pressure (*ca.* 1 psi). The column was washed with 30 ml of 0.05 M potassium phosphate buffer containing 0.1 M NaCl, and with the same buffer containing 0.5 M NaCl. The concentration of NaCl in the heat-denatured DNA solution was adjusted to 0.5 M and added to the column. After passing the solution through the column, the L phage DNA strand was eluted with 18 ml of the same buffer containing 0.665 M NaCl, followed by 14 ml of the buffer containing 1.5 M NaCl for the elution of the H phage DNA strand. Fractions of 2 ml were collected.

CsCl Density Gradient Centrifugation. Analytical CsCl density gradient centrifugation was carried out essentially according to Meselson *et al.* (1957). Samples were centrifuged at 44,770 rpm at 25° in a Spinco Model E analytical ultracentrifuge. After about 18 hr, ultraviolet-absorption photographs were taken, and tracings were made with a Joyce-Loebl recording microdensitometer. For the examination of the purity of phage 2C native and denatured DNA, PBS2 (Takahashi and Marmur, 1963) phage DNA (ρ = 1.722 g/cm^3) was used as the reference density marker. All densities are related to that of *E. coli* DNA which is taken as 1.710 g/cm^3.

For preparative CsCl density gradient centrifugation at alkaline pH, 3.3 g of CsCl was dissolved in 0.4 ml of Na_2CO_3–NaOH buffer (0.5 M Na_2CO_3 + 0.044 M NaOH, pH 12.0) (Schildkraut *et al.*, 1964), and 0.05 ml of 0.1 M EDTA and 1.8 ml of phage DNA solution containing about 100 μg were added. The RI was *ca.* 1.4050. The final volume of the solution was about 3 ml, which was transferred to a polyallomer tube, layered with heavy mineral oil to fill the volume, and run at 35,000 rpm for about 90 hr at 25° in the SW 39 rotor of the Spinco Model L2 ultracentrifuge. At equilibrium, two-drop fractions were collected through a hole punctured at the bottom of the tube with an insect pin (size 00, Clay–Adams Inc., N. Y.).

Liquid Scintillation Counting of [^{14}C]Methyl-Labeled DNA. After fractionation of [^{14}C]methyl-labeled phage 2C DNA, either by MAK column chromatography or by alkaline CsCl density gradient centrifugation, 200 μg of BSA and 5% TCA at final concentrations were added to an aliquot of the fractions. The mixture was chilled in ice for at least 10 min and centrifuged at 2000*g* for 5 min in the cold. The precipitate was washed twice with cold 5% TCA by centrifugation, and finally suspended in about 3 ml of cold 5% TCA. The suspension was then filtered through a nitrocellulose membrane filter (Carl Schleicher and Schuell Co., B-6, 24MM) previously soaked in 0.5 M KCl containing 0.01 M Tris, pH 7.5. The membrane filter was dried, placed in a vial with 5 ml of toluene scintillator (4 g of PPO and 0.1 g of POPOP/l toluene), and counted.

Determination of $s_{20,w}$ and Molecular Weight of DNA. For the determination of $s_{20,w}$ of DNA preparations used, the DNA was dissolved in SSC at a final concentration of 20 μg/ml and introduced into the 30-mm cell from the window end while partially assembled. The centrifugation was carried out in a Spinco Model E analytical ultracentrifuge at 35,600 rpm, and the tracings of the ultraviolet absorption photographs were used to calculate the $s_{20,w}$ value. The Ws of native and heat-denatured DNA were estimated according to Eigner and Doty (1965). The equation used to convert the $s_{20,w}$ value of native DNA to MW was $s_{20,w} = 0.034M^{0.405}$.

Results

Production of DNA Methylase and Species Specificity of DNA Methylation in the Genus Bacillus. Among the *B. subtilis* strains examined, only *B. subtilis* 6633 had a highly active DNA methylase activity, whereas the other strains contained little enzyme activity (Table I). It has been demonstrated that the methylation of *s*-RNA and DNA is species specific (Srinivasan and Borek, 1963; Gold *et al.*, 1963). A similar type of specificity of *in vitro* methylation of DNA was also observed in the genus *Bacillus* as shown in Table I.

Production of DNA Methylase during Growth of Bacteria. The production of DNA methylase by *B. subtilis* 6633 was examined in several complex media, and MM supplemented with 0.1% yeast extract yielded cells containing the highest amount of methylase. As

TABLE I: Production of DNA Methylase and Species Specificity of DNA Methylation in *Bacillus*.[a]

Crude Extract	DNA						
	E. coli B	*E. subtilis* 6633	*B. subtilis* var *aterrimus*	*B. natto*	*B. subtilis* 168	*B. subtilis* W23	*B. niger*
B. subtilis 6633	0.100	0.002	0.065	0.066	0.068	0.078	0.090
B. subtilis var *aterrimus*	0.006	0.003	0.001	0.003	0.003	0.004	0.001
B. natto	0.007	0.004	0.004	0.001	0.005	0.004	0.003
B. subtilis 168	0.005	0.008	0.003	0.003	0.002	0.003	0.006
B. subtilis W23	0.005	0.007	0.003	0.003	0.003	0.002	0.004
B. subtilis Marburg	0.003	0.004	0.003	0.002	0.003	0.003	0.005
B. niger	0.002	0.002	0.002	0.002	0.003	0.002	0.001

[a] Crude extracts were prepared from cells indicated in the table, harvested in the early exponential phase (*ca.* 10^8 cells/ml). Protein (100 μg) from the crude extract and 100 mμmoles of DNA as nucleotide residues were added to the reaction mixture. The values (in mμmoles) indicate [^{14}C]methyl groups incorporated in 30 min at 37°.

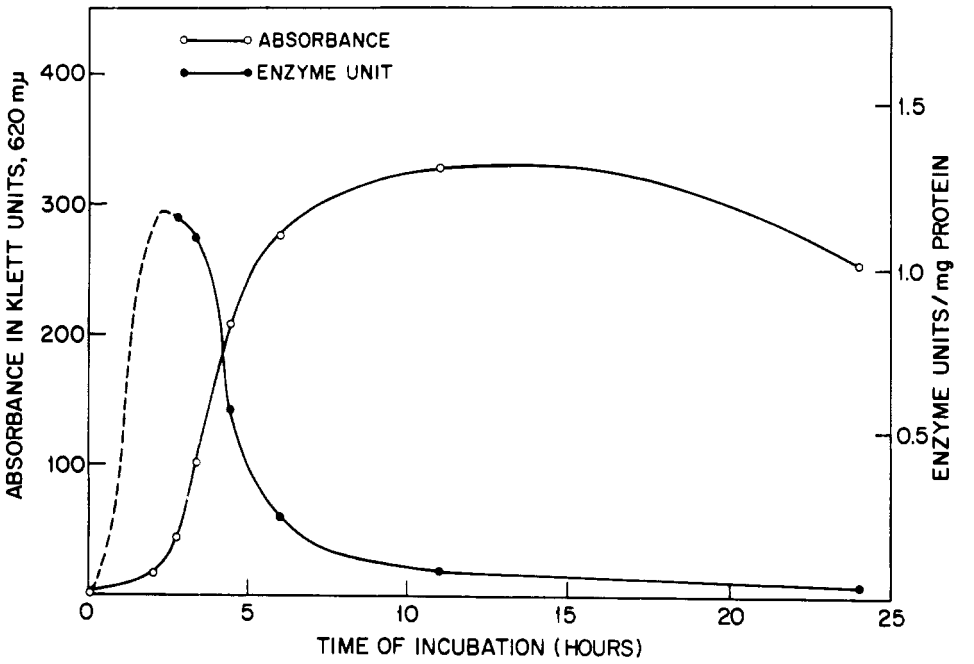

FIGURE 1: Production of DNA methylase during growth of *B. subtilis* 6633. *B. subtilis* 6633 was grown in 2 l. of MM supplemented with 0.1% yeast extract. At the times indicated, 200–300 ml aliquots of the culture were withdrawn and rapidly cooled in an ice bath. After washing the cells, the crude extracts were prepared as described in Materials and Methods. Protein (100 μg) in the crude extract and 100 mμmoles of *E. coli* B DNA as nucleotide residues were used as the enzyme and the methyl group acceptor. The reaction mixture has been described in Materials and Methods.

shown in Figure 1, enzyme activity expressed as units per mg of crude extract was the highest at the early exponential phase of growth but markedly decreased thereafter, so that after 24 hr almost no activity was observed in crude cell extract. The reason for this phenomenon is unknown. The enzyme might be excreted into the medium or inactivated in the cells. No enzyme activity responsible for the cleavage of SAM (Gold *et al.*, 1964) was observed when SAM was incubated with crude extracts prepared from cells harvested in the stationary phase. The decrease in methylase activity in the later stage of growth was also observed with other *Bacillus* strains. Addition of the crude extract prepared from either *B. subtilis* 168 cells or *B. subtilis* 6633 cells harvested in the stationary phase to a crude extract prepared from *B. subtilis* 6633 in the early exponential phase did not alter the activity of the latter.

Effects of Phage Infection on DNA Methylase Activity

in B. subtilis. Phage infection of *E. coli* may result in either an increase or decrease in the methylase activity of the infected cell extracts. T2 infection results in an 100-fold increase of phage-specific DNA methylase, whereas infection of *E. coli* with T3 induces an enzyme which splits the methyl donor SAM (Gold *et al.*, 1964). The effect on DNA methylase activity of phage infection of *B. subtilis* with several virulent bacteriophages was also examined. As shown in Table II, there was no effect of bacteriophage infection on enzyme activity.

TABLE II: Effect of Phage Infection on DNA Methylase Activity in *B. subtilis*.[a]

Source of Crude Extract *B. subtilis* strain	Phage	moi	Time after Infection (min)	Enzyme Activity [^{14}C]Methyl Group Inc $\mu\mu$moles
6633	...		0	185.0
			15	129.0
			30	93.5
6633	2C	1.3	15	118.0
			30	91.5
168	...		0	7.2
			15	7.0
			30	9.9
168	2C	4	15	9.8
			30	7.2
168	SP8*	4	15	8.0
			30	7.2
168	SP82	4	15	6.3
			30	7.8

[a] One liter of *B. subtilis* 6633 and 1.6 l. of *B. subtilis* 168 cultures were grown at 37° with vigorous shaking until a cell density corresponding to a Klett unit (No. 62 filter) of 80 (*ca.* 10^8 cells/ml) was reached. Cultures (150 ml) were harvested at zero time and the remaining cultures were divided into 350-ml portions. The phage suspension was added at the multiplicity indicated in the table. After 15- and 30-min infection, 150 ml of the culture was harvested, and immediately chilled in an ice bath. The crude extracts were prepared as described in Materials and Methods. Protein (150 μg) from the crude extracts were used as enzyme, and 100 mμmoles of *E. coli* B DNA as methyl group acceptor. The reaction was carried out at 37° for 30 min. Infected cultures were lysed completely after 3-hr infection.

Purification of Enzyme. All operations carried out during the purification of the *B. subtilis* DNA methylase were conducted at 0–5° and all solutions contained 0.01 M 2-mercaptoethanol and 0.002 M EDTA. The enzyme activity was assayed using 100 mμmoles of *E. coli* B DNA as nucleotide residues as the methyl acceptor.

(i) CRUDE EXTRACT. *B. subtilis* 6633 was grown in 12 l. of MM supplemented with 0.1% yeast extract at 37° on a rotory shaker. The cells were harvested during the early exponential phase (*ca.* 10^8 cells/ml) and washed once with 0.5% NaCl–0.5% KCl. The yield of packed cells was 15 g. The washed cells were suspended in 65 ml of 0.05 M Tris, pH 7.4 and disrupted with a French press. The disrupted cell suspension was centrifuged at 12,000*g* for 10 min and the supernatant solution was collected. The pellet was again suspended in 30 ml of the same buffer and similarly disrupted. After centrifugation, the combined supernatant solutions were centrifuged at 40,000 rpm for 90 min in the R40 rotor of the Spinco Model L ultracentrifuge. The resulting supernatant solution (87 ml) was kept in an ice bucket overnight, and used as a crude extract (Fraction I, Table III).

TABLE III: Purification of DNA Methylase from *B. subtilis* 6633.

Fraction	Protein (mg/ml)	Total Enzyme Units	Specific Activity
I Crude extract	13.0	922	0.82
II Alumina C_γ gel	1.08	482	2.36
III Ammonium sulfate	7.6	341	3.83
IV Phosphocellulose	0.83	218	13.30
V DEAE-cellulose	0.018	75	138.00

(ii) ALUMINA C_γ GEL. The crude extract was diluted twofold with cold water containing 0.01 M 2-mercaptoethanol and 0.002 M EDTA, and the pH was adjusted to 6.7 by the dropwise addition of 0.2 M acetic acid. Alumina C_γ gel (1.7 g dry wt) was added to the solution (gel:protein ratio = 1.5) and stirred for 30 min. The suspension was then centrifuged at 10,000*g* for 10 min and the supernatant fluid was discarded. The gel was washed by homogenization with 100 ml of 0.05 M potassium phosphate buffer, pH 7.5. After 10 min the suspension was centrifuged at 10,000*g* for 10 min, and the supernatant solution was discarded. This procedure was repeated twice more and the DNA methylase was then eluted from the gel by homogenization with 100 ml of 0.4 M potassium phosphate buffer, pH 7.5, and centrifuged at 10,000*g* for 10 min. The gel was reextracted with 80 ml of the same buffer, and the two eluates were combined (191 ml, Fraction 2, Table III).

(iii) AMMONIUM SULFATE FRACTIONATION. To fraction 2, 40 g of solid ammonium sulfate was gradually added. After standing for 30 min, the suspension was centrifuged at 10,000*g* for 10 min. To the resulting supernatant solution 33.5 g of solid ammonium sulfate was added gradually, and stirred for 30 min. After centrifugation, the supernatant solution was discarded, and the pellet was dissolved in 10 ml of 0.05 M potas-

sium phosphate buffer, pH 7.4. The solution was dialyzed against 2 l. of the same buffer for 1 hr, then against 2 l. of 0.05 M potassium phosphate buffer, pH 8.0, for 1 hr. The volume of the solution increased to 11.7 ml (Fraction 3, Table III).

(IV) PHOSPHOCELLULOSE CHROMATOGRAPHY. Phosphocellulose was suspended in water and the fine particles were removed by decantation. The washed phosphocellulose was then equilibrated with 0.05 M potassium phosphate buffer, pH 8.0, and poured into the column. The bed volume was 50 cm^3 (2.4 cm × 11.5 cm). After washing the column with the same buffer, the dialyzed sample, diluted twice with the same buffer, was gently added to the top of the column. The column was then washed with 50 ml of 0.05 M potassium phosphate buffer, pH 7.4. The enzyme activity was eluted from the column with 0.4 M potassium phosphate buffer, pH 7.4, at a flow rate of *ca.* 1 ml/min, and 10-ml fractions were collected. Usually, almost all enzyme activity was found in fractions 4–6. Two fractions were combined and stored in an ice bucket (Fraction 4, Table III).

(V) DEAE-CELLULOSE CHROMATOGRAPHY. DEAE-cellulose, previously washed successively with water, 0.5 N NaOH, 0.5 N HCl, 0.5 N NaOH, and water, was equilibrated with 0.02 M potassium phosphate buffer, pH 8.0. After pouring the DEAE-cellulose suspension onto the column (2.0 × 9.5 cm), it was washed with the same buffer. Fraction 4 was dialyzed against 2 l. of 0.02 M potassium phosphate buffer, pH 7.4, and 2 l. of 0.02 M potassium phosphate buffer, pH 8.0, each for 1 hr. The dialyzed solution was diluted twofold with water containing 0.01 M 2-mercaptoethanol and 0.002 M EDTA, and added to the column. After washing with 30 ml of 0.02 M potassium phosphate buffer, pH 7.5, the enzyme activity was eluted with 0.05 M potassium phosphate buffer at a flow rate of 1 ml/min, and 10-ml fractions were collected. Most of the enzyme activity was found in Fractions 4, 5, and 6. (30 ml, Fraction 5, Table III).

(VI) CONCENTRATION OF ENZYME.[3] Fraction 5 was transferred to dialysis tubing and the bag covered with cold polyethylene glycol powder for about 1 hr to reduce the volume. The enzyme was then dialyzed against 0.05 M Tris buffer, pH 7.4, for 30 min. The final concentration of protein was about 150 μg/ml. The purified enzyme was free of nuclease when assayed on transforming DNA in the presence of EDTA.

Properties of the Enzyme. (I) REQUIREMENT FOR THE REACTION AND OPTIMUM pH. The requirements for the methylation of DNA are summarized in Table IV. No detectable methylation occurred in the absence of added DNA. The latter requirement was partially satisfied with heat-denatured DNA. The ability of denatured DNA to act as an acceptor was not limited to DNA from *B. subtilis* 168 but was a general property of all DNA acceptors (see below, Table V). The ability of denatured DNA to act as a methyl group acceptor with the enzyme from *B. subtilis* was different from that of the DNA methylase of *E. coli*, which did not catalyze the methylation of heat denatured DNA (Gold and Hurwitz, 1964b). The *Ks* value for native and heat-denatured *B. natto* DNA was 1.8×10^{-4} M. Very little methylation occurred when s-RNA and r-RNA from *B. subtilis* 6633 and 168 were used as substrates. The addition of divalent metals or EDTA were without effect while the omission of 2-mercaptoethanol resulted in a slight decrease in the rate of methylation. The enzyme appears to be specific for SAM as the alkylating agent since [1-ethyl-^{14}C]SAE was inactive in the reaction. The effect of pH and different buffers on the activity of DNA methylase was studied (Figure 2). The maximum rate of methylation occurred at pH 8.0 in Tris buffer, while Tris–maleate buffer was markedly inhibitory.

(II) IDENTIFICATION OF THE METHYLATED BASES

TABLE IV: Requirement for DNA Methylation.[a]

Reaction Mixture	[^{14}C] Incorporated (μμmoles)
Complete system	170
−DNA	1
−DNA, + heat denatured DNA	70
−DNA, + 4S RNA[b]	1.4
−DNA, + 16S RNA[c]	1.8
−DNA, + 23S RNA[c]	1.9
−2-Mercaptoethanol	143
−Mg (no metal)	178
−Mg, + Mn	173
−Mg, + Ca	184
−Mg, + EDTA	160
−SAM, +SAE	4

[a] The complete system has been described in Materials and Methods. In this experiment, 100 mμmoles of *B. subtilis* 168 DNA, and for RNA 50 mμmoles, as nucleotide residues were used as the methyl group acceptor. Where indicated $MnCl_2$, $CaCl_2$, and EDTA were added to final concentrations of 4×10^{-3} M, 4×10^{-3} M, and 10^{-2} M, respectively. Ten millimicromoles of [1-ethyl-^{14}C]SAE were present where used. The specific activity of [methyl-^{14}C]SAM and [1-ethyl-^{14}C]SAE were 1.7×10^4 cpm/mμmole and 1.3×10^4 cpm/mμmole, respectively. All reaction mixtures contained 1.86 μg of protein of DEAE-cellulose preparation (105 enzyme units/mg protein) and were incubated at 37° for 30 min. Under this condition the incorporation of [^{14}C]methyl groups into DNA was linear for at least 30 min. [b] Prepared from *B. subtilis* 6633. [c] Prepared from *B. subtilis* 168.

[3] DNA methylase from *B. subtilis* is very unstable; about 50% of its activity was lost in 3 days' storage in ice, and about 90% in 1 week. None of the methods thus far used has protected the enzyme from inactivation. Freezing and thawing the cells or the enzyme extract at any stage in the purification procedure should be avoided.

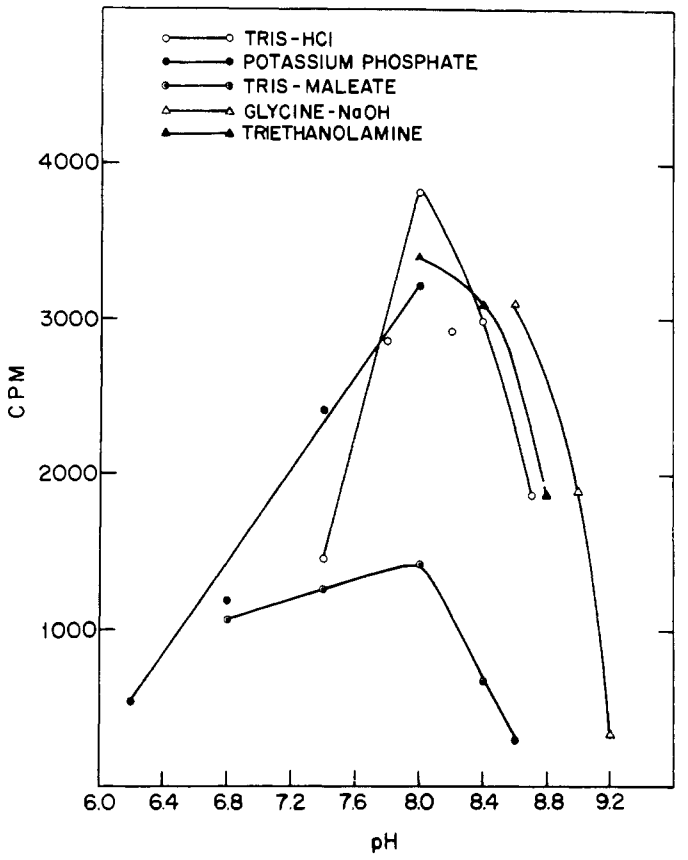

FIGURE 2: Optimum pH for DNA methylation. The reaction mixture described in Materials and Methods was used, except for the buffer indicated which replaced the usual one. To each assay 100 mμmoles of *B. subtilis* 168 DNA as nucleotide residues and 1.65 μg of DEAE-cellulose preparation (86 enzyme units/mg of protein) were added. Specific activity of the [methyl-^{14}C]SAM was 2.1 × 10^4 cpm/mμmole. The reaction mixture was incubated at 37° for 30 min.

FORMED IN DNA BY PAPER CHROMATOGRAPHY. The qualitative assay of the methylated bases in DNA is shown in Figure 3. In the case of the *in vitro* methylation with purified *B. subtilis* 6633 enzyme, only 5MC was found in all the methylated DNA samples listed in Table V. The amount of labeled 5MC found on the paper was proportional to the extent of the DNA methylation. No other methylated bases were detected when crude extract from the same strain was used as the source of enzyme.

For the study of *in vivo* methylation, DNA samples were extracted from *B. subtilis* 6633 and 168 after growth in a medium containing [methyl-^{14}C]methionine (see Materials and Methods). As shown in Figure 3 (a) and (b), the amount of 5MC in *B. subtilis* 6633 DNA was almost ten times greater than that in *B. subtilis* 168. This result, together with the previous observation, indicates that the extent of the DNA methylation *in vivo* is related to the amount of the enzyme which can be isolated from the strain *in vitro*. Doskočil and Šormová (1965a) have reported the presence of 6MAP in addition to 5MC in *B. subtilis* 168 DNA. However, no 6MAP was detected under the conditions used in this experiment. The other radioactive peak shown in Figure 3 (a) was thymine; this was confirmed by the rechromatography of the sample with another solvent (170 ml of 2-propanol, 44 ml of HCl, 36 ml of H_2O).

The extent of the methylation of phage 2C DNA depended on the host strain used to prepare the lysates. As can be seen in Figure 3 (c) and (d), DNA from phage 2C grown on *B. subtilis* 6633 was methylated to a greater extent than DNA of 2C grown on *B. subtilis* 168. Employing liquid scintillation counting, the specific activity of the former and the latter ranged from 8 to 20 cpm and from 2 to 5 cpm/μg of DNA, respectively. Phage 2C DNA isolated from lysates prepared from *B. subtilis* 168 was methylated to a greater extent by the purified *B. subtilis* 6633 enzyme *in vitro* than 2C phage DNA isolated from lysates of *B. subtilis* 6633 was methylated *in vivo*. The *in vitro* methylated phage 2C DNA had a specific activity of approximately 100 cpm/μg DNA.

(III) METHYL GROUP ACCEPTOR ACTIVITY OF DNA ISOLATED FROM VARIOUS SOURCES. DNA's isolated from various sources were examined and compared for their rate and extent of methylation with the enzyme purified from *B. subtilis* 6633. Reaction mixtures were incubated

TABLE V: Methyl Group Acceptor Activity of DNA Isolated from Various Sources.[a]

DNA Source	GC (%)	$s_{20,w}$	[^{14}C]Methyl Incorporated[b] (mμmoles) Native DNA (0.5 hr)	(5 hr)	Denatured DNA (5 hr)	5MC/Total Cytosine (%) Native DNA (5 hr)	Denatured DNA (5 hr)
Bacteria							
Cl. perfringens	26.5	21.0	0.016	0.021	0.007	0.16	0.05
B. cereus	33	23.1	0.060	0.093	0.042	0.56	0.26
B. megaterium	37	33.7	0.117	0.125	0.044	0.68	0.24
Pr. mirabilis	38	26.8	0.104	0.123	0.074	0.65	0.39
B. subtilis 6633	43	18.5	0.002	0.003	0.007	0.02	0.04
B. subtilis 168	43	19.2	0.131	0.214	0.081	0.10	0.38
B. subtilis W23	43		0.154	0.197	0.081	0.92	0.38
B. natto	43	21.2	0.127	0.210	0.074	0.98	0.34
B. niger	43	23.4	0.177	0.221	0.081	0.98	0.38
E. coli B	50	26.8	0.204	0.478	0.221	1.91	0.89
E. coli K12	50	28.9	0.205	0.459	0.266	1.87	1.06
A. aerogenes	57.5	20.0	0.274	0.665	0.229	2.31	0.80
S. marcescens	57.5	22.5	0.213	0.455	0.236	1.58	0.82
Ps. aeruginosa	66		0.295	0.894	0.484	2.63	1.47
M. lysodeikticus	72	27.6	0.211	0.955	0.862	2.65	2.49
S. lutea	72	25.1	0.211	0.260	0.100	0.72	0.28
Bacteriophages							
SP8	43	54.6	0.009	0.013	0.006	0.06	0.03
2C (6633)	43	31.4	0.009	0.012	0.014	0.06	0.07
2C (168)	43	30.9	0.028	0.033	0.014	0.15	0.07
T2[c]	34		0	0			
T4[c]	34		0	0			
φX-174[c]			0	0			
Higher Organisms							
Calf thymus	42	25.0	0.021	0.032	0.016	0.15	0.08
Salmon sperm	42	20.6	0.008	0.034	0.012	0.16	0.06

[a] DNA (100 mμmoles) as nucleotide residues and 3.3 μg (0.21 unit) of protein of DEAE-cellulose preparation from *B. subtilis* 6633 were added to the reaction mixtures. The same reaction mixture described in Materials and Methods was used except 1 μmole of $MgCl_2$ was replaced by 0.25 μmole of EDTA. [b] Since 100 mμmoles of DNA as nucleotide residues were used in each methylation assay, this figure also expresses directly the per cent of the total bases methylated. [c] DNA (50 mμmoles) as nucleotide residues were used.

for 30 min and 5 hr to determine the rate and yield, respectively. Under these conditions, the incorporation of [^{14}C]methyl groups into DNA was linear for at least 30 min. The $s_{20,w}$ of the DNA samples used ranged from about 20 S to 30 S, corresponding to MW 6–20 × 10^6. As shown in Table V, the extent of methylation of DNA was approximately proportional to its GC content, with few exceptions. For example, *Cl. perfringens* DNA (GC 26.5%) accepted about 20 μμmoles of [^{14}C]methyl groups. This corresponds to 1 methylated nucleotide base in 5000 nucleotides, or about 0.02% methylation in *Cl. perfringens* DNA; with DNA from *M. lysodeikticus* (GC 72%) approximately 1 mμmole of methyl group was incorporated, which corresponds to 1% methylation. As described in the previous section, the enzyme from *B. subtilis* 6633 catalyzes the methylation of only cytosine residues in DNA; thus, the ratio of the number of 5MC to total number of cytosine residues in DNA was 0.16% in *Cl. perfringens* DNA and 2.7% in *M. lysodeikticus* DNA. When the methylation of DNA from *B. subtilis* 168 and *E. coli* B were studied, the ratio of 5MC to total bases was about 0.2% and 0.5%, and the ratio of 5MC to total cytosine residues was about 1 and 2%, respectively. This indicates that the higher the per cent GC in DNA, the more methylation that takes place, even if the ratio of 5MC to total cytosine residues in DNA is considered. However, there are a few exceptions; *e.g.*, *Sarcina lutea* DNA (GC 72%) was methylated to a lower extent than DNA of *M. lysodeikticus* although both have similar GC contents. This might be due to either the fact that the *S. lutea* DNA used as the methyl acceptor *in vitro* has already been extensively methylated *in vivo* or that the specific base sequences of the DNA of the latter two

organisms are different. That methylated bases appear only in specific base sequences has been demonstrated in the DNA extracted from *E. coli* and *B. subtilis* by Doskočil and Šormová (1965b).

The rate of enzymatic incorporation of methyl groups into DNA during the first 30 min did not vary greatly among different DNA samples, even though the extent of methylation showed a greater variation related to the GC content of the DNA. This would imply that the methylation enzyme possesses a high degree of affinity for particular sites in DNA.

The extent of methylation of heat denatured DNA is also approximately proportional to its GC content; however, it appears that the ratio of the yield of methylation for denatured to native DNA samples is higher with DNA of high GC content than with DNA having a low GC concentration. For example, denatured *M. lysodeikticus* DNA was methylated to the extent of 90% of the value obtained for the native DNA preparation, while denatured *Cl. perfringens* DNA was methylated to an extent of only about 30% of that obtained with native DNA. The reason native DNA is a better substrate than denatured DNA for methylation is presently unknown. However, after heat denaturation of *B. subtilis* 6633, as well as of 2C DNA isolated from phage grown on *B. subtilis* 6633, the extent of methylation by *B. subtilis* 6633 enzyme was increased by a factor of two compared to native DNA, although the extent of DNA methylation in this homologous system was very low. This doubling in the extent of methylation was also induced when the homologous DNA was irradiated with ultraviolet light for 10 min at a distance of 20 cm using a Westinghouse sterile lamp (G15T8, 15 W) (Ryan and Borek, 1964). Ultraviolet irradiation of heterologous DNA did not change its ability to accept methyl groups.

B. subtilis phage SP8* and 2C DNA's were methylated poorly. However, DNA isolated from phage 2C grown on *B. subtilis* 168 was methylated to a greater extent than DNA from phage 2C grown on *B. subtilis* 6633 by the *B. subtilis* 6633 enzyme. T2 and T4 DNA's, which contain 5-hydroxymethylcytosine in place of cytosine, were not methylated by the enzyme. This observation is in keeping with other results which indicate that the *B. subtilis* 6633 enzyme does not methylate the adenine residues of DNA. DNA from the single-stranded *E. coli* phage ϕX174 also was not methylated. Calf thymus and salmon sperm DNA's were poor methyl group acceptors.

Methylation of the H and L Strands of Phage 2C DNA. In order to examine the possible role of DNA methylation on strand selection mechanisms involved in the DNA-dependent RNA polymerase, phage 2C DNA, whose strands are readily identified by their density difference, was methylated *in vivo* and *in vitro* with [^{14}C]methyl groups. The separation of these two strands was carried out by MAK column chromatography or by alkaline CsCl density gradient centrifugation. As shown in Figure 5 (a), the H and L strands of 2C DNA were methylated to approximately the same extent *in vivo*. The acid-precipitable radioactivity was rendered

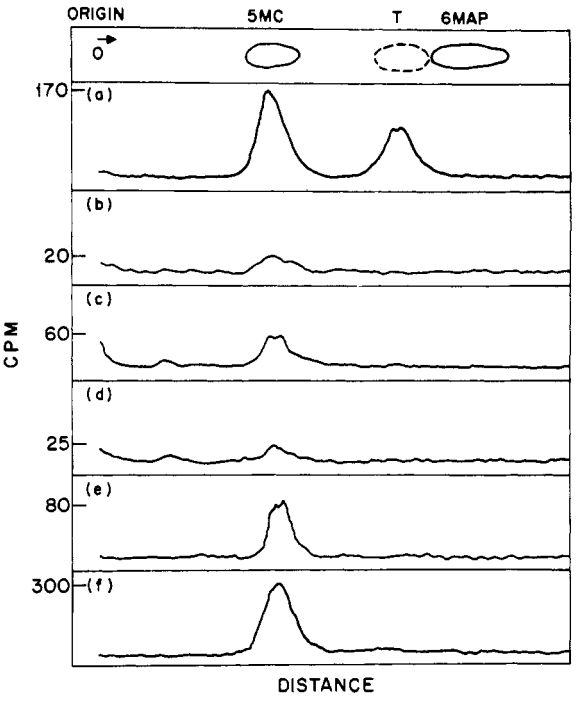

FIGURE 3: Separation of methylated bases by paper chromatography. The methylated DNA was hydrolyzed as described in Materials and Methods. Hydrolyzed DNA samples (from 30 to 100 μg) were spotted on the paper. The specific activities of L[methyl-^{14}C]-methionine and [methyl-^{14}C]SAM were 4×10^6 cpm/μmole and 1.5×10^7 cpm/μmole, respectively. (a) *B. subtilis* 6633 DNA, methylated *in vivo*. (b) *B. subtilis* 168 DNA, methylated *in vivo*. (c) 2C DNA isolated from phages grown on *B. subtilis* 6633 (thy$^-$met$^-$cys$^-$), methylated *in vivo*. (d) 2C DNA isolated from phages grown on *B. subtilis* 168 (met$^-$), methylated *in vivo*. (e) 2C DNA isolated from phages grown on *B. subtilis* 168 (met$^-$), methylated *in vitro*. (f) *B. subtilis* 168 DNA methylated *in vitro*.

acid soluble after DNAase treatment. The purity of materials from the two peaks obtained from MAK column chromatography was examined by analytical CsCl density gradient centrifugation. As observed in Figure 4 (d), the first peak consisted of pure L strand (ρ = 1.752 g/cm^3) and the second one consisted of pure H strand (ρ = 1.762 g/cm^3). Samples were also treated with 50 μg/ml of pronase, as shown in Figure 5 (b), and almost all of the radioactivity (80%) remained acid precipitable. The decrease in acid-insoluble radioactivity may be due to a trace contamination of DNAase in the pronase preparation. The $s_{20,w}$ value of [^{14}C]-methyl-labeled phage 2C DNA, isolated as described in Materials and Methods, was 32.4, corresponding to MW of approximately 22×10^6. This would indicate that it was degraded during the isolation to a MW approximately one-quarter that of the native phage 2C DNA. After MAK column separation, the MW of

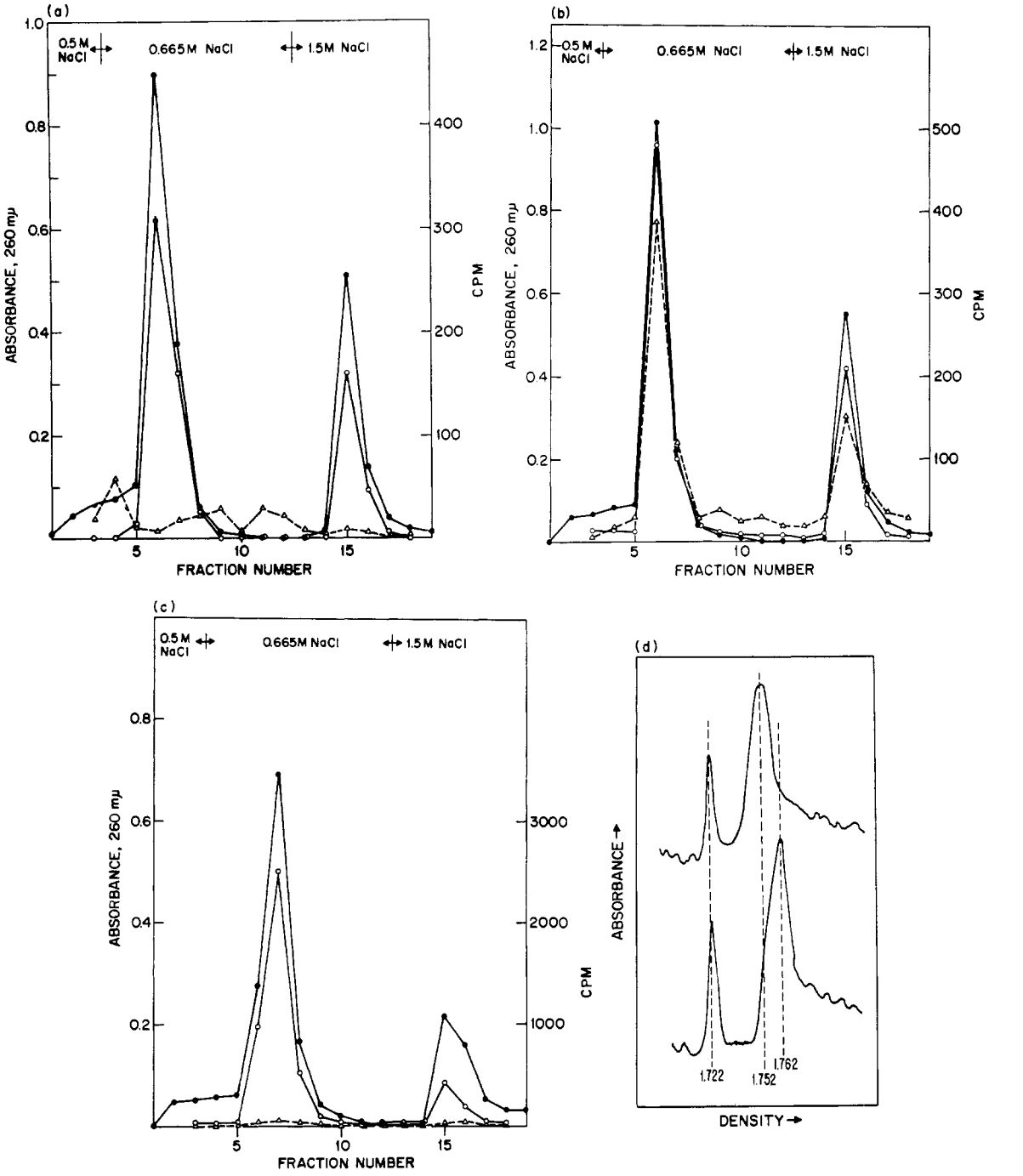

FIGURE 4: MAK column separation of the H and L strands of phage 2C DNA. (a) [^{14}C]Methyl-labeled DNA (240 μg) from phage 2C grown on *B. subtilis* 6633 was heat denatured, rapidly cooled, then charged onto the column. Specific activity of the DNA was 18 cpm/μg. Two-ml fractions were collected and dialyzed against 0.05 M Tris buffer, pH 7.5, for several hours and the dialyzed fractions were divided into 2 equal volumes. One of the divided fractions was treated with pancreatic DNAase I at 37° for 1 hr. The reaction mixture contained 0.05 M Tris, pH 7.5, 2 μg/ml of DNAase I, and 0.01 M $MgCl_2$. [^{14}C]Methyl DNA samples in both fractions were counted in a liquid scintillation counter as described in Materials and Methods. (b) The same experiment as described in (a) was carried out, except that pronase (50 μg/ml) was substituted for DNAase I. (c) DNA (180 μg) from phage 2C grown on *B. subtilis* 168, methylated *in vitro* with purified *B. subtilis* 6633 enzyme, was heat denatured and charged onto the column. Specific activity of the DNA was 80 cpm/μg. One of the divided fractions was treated with DNAase I, as described in (a). (●), absorbance at 260 mμ; (○), cpm; (Δ), cpm after DNAase or pronase treatment. (d) Samples (0.2 ml) were taken from fractions 6 and 15 in Figure 4 (a) and their density was analyzed by CsCl density gradient centrifugation. In both cases, PBS2 phage DNA was used as the reference density.

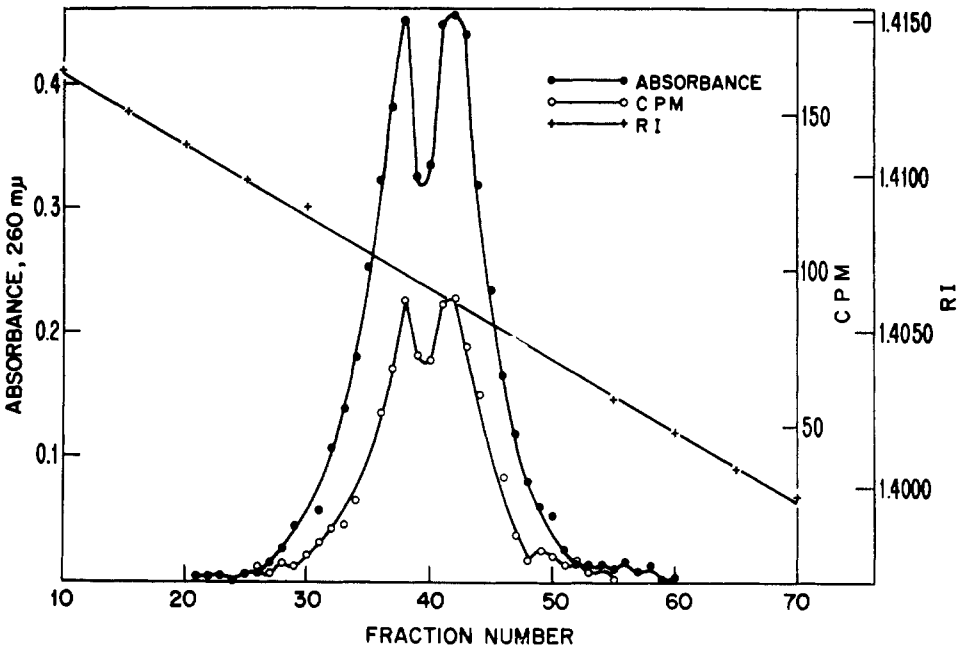

FIGURE 5: Alkaline CsCl density gradient centrifugation of phage 2C DNA. DNA (110 μg) isolated from phage 2C grown on *B. subtilis* 6633 was added to an alkaline CsCl solution (pH 12.0). Specific activity of the DNA was 8.5 cpm/μg. Tris buffer (0.5 ml), pH 7.5, was added to each fraction and the acid-insoluble radioactivity was analyzed as described in Materials and Methods.

the H and L strands was estimated according to Eigner and Doty (1965). The $s_{20,w}$ values of the H and L strands was 14.1 and 9.6 corresponding to MW values of 4–5 × 10^6 and 1.5 × 10^6, respectively.

DNA isolated from phage 2C grown on *B. subtilis* 168, and methylated *in vitro* with the enzyme from *B. subtilis* 6633, was also examined for the extent of methylation of the complementary strands by the method described above. The result was identical with that observed with *in vivo* methylated DNA and is shown in Figure 4 (c). The recovery from the MAK column of the L strand was almost 100% while that of the H strand was at most 50%. The reason for the poor recovery of the H strand is unknown. The separation of the two strands was also carried out by alkaline CsCl density gradient centrifugation. As shown in Figure 5, the separation was incomplete because of the small density difference between the strands ($\Delta\rho$ = 0.01 g/cm^3); however, the location of the H strand in the preparative CsCl density gradient can be distinguished from that of the L strand. Under these conditions, the densities of the H and L strands were 1.780 g/cm^3 and 1.770 g/cm^3, respectively. Based on these results, it was concluded that both H and L strands of phage 2C DNA were methylated *in vivo* and *in vitro*.

Discussion

In this paper, the presence of DNA methylase in *B. subtilis* has been demonstrated, and its properties and specificity have been studied. DNA methylase in *B. subtilis* was produced only in the early exponential phase, and disappeared rapidly upon further cellular growth. The enzyme might be excreted into the medium together with other known exoenzymes in *B. subtilis*, *e.g.*, RNAase (Nishimura, 1960) and DNAase (Kerr *et al.*, 1965), or it might be inactivated during the latter period of the growth curve.

It has been demonstrated that besides 6MAP, 5MC also exists in bacterial DNA (Doskočil and Šormová, 1965a) as well as in phage λ DNA (Ledinko, 1964) and that the DNA methylase isolated from *E. coli* W could methylate both the adenine and cytosine residues of macromolecular DNA (Gold and Hurwitz, 1964a). The specificity of the methylating enzyme isolated from *B. subtilis* 6633 is quite different from that of *E. coli* and from the T2 phage-induced enzyme which specifically methylates the adenine residues of DNA (Hausmann and Gold, 1966). The enzyme isolated from *B. subtilis* 6633 methylates only the cytosine residues of DNA. Another interesting property of the *B. subtilis* enzyme, not shared by the *E. coli* methylase, is its ability to methylate heat-denatured DNA. The extent of the methylation of native bacterial DNA by the *B. subtilis* 6633 enzyme appears to be proportional to its GC content, with few exceptions. The proportion of the cytosine residues methylated *in vitro* increases with the GC content of DNA. The extent of the methylation of heat denatured bacterial DNA is also dependent on its GC content. If the DNA has a GC content greater than 50%, after denaturation it was methylated to an extent 50–100% that of native DNA; if the GC content of the DNA

substrate is lower than 50%, denatured DNA was methylated less than 50% that of native DNA.

In the case of the methylation of *B. subtilis* DNA *in vivo*, Doskočil and Šormová (1965a) observed the existence of both 6MAP and 5MC; however, no 6MAP was detected in the DNA extracted from methionine-requiring strains of *B. subtilis* 6633 and 168 incubated in the presence of L-[methyl-^{14}C]methionine. Therefore, if 6MAP is present in *B. subtilis* DNA, it is present to a considerably lesser extent than 5MC, so that it might have escaped detection by the methods described in Materials and Methods. In the studies on the methylation of *B. subtilis* 6633 DNA *in vivo*, a thy$^-$cys$^-$met$^-$ auxotroph was inoculated with L-[methyl-^{14}C]methionine in the presence of thymine and cysteine. It seems that under these conditions, a trace amount of thymine would be produced (see Figure 3 (a)) from uracil by transferring a methyl group from methionine. However, no such enzyme has been described in *B. subtilis*.

It has been inferred from hybridization experiments that only one of the phage DNA strands acts as the template *in vivo* for the synthesis of phage-specific RNA in infected cells (Marmur and Greenspan, 1963; Tocchini-Valentini *et al.*, 1963). Phage 2C behaves like SP8 in having the H phage DNA strand preferentially transcribed *in vivo* (J. Pène, unpublished results). In order to determine whether or not DNA methylation plays some role in the strand selection mechanism, the methylation of the H and L strands of virulent *B. subtilis* phage 2C DNA both *in vivo* and *in vitro* was examined. The results obtained when the two strands were separated either by MAK column chromatography or by alkaline CsCl density gradient centrifugation showed the same extent of methylation in both the H and L complementary DNA strands.

In attempting to elucidate the role of the methylated bases in DNA, the enzymological approach has yielded some information; however, the biological significance of the presence of 5MC and/or 6MAP in macromolecular DNA still remains obscure. *In vitro* studies have revealed the high degree of specificity of nucleic acid methylation; in fact, the methylating enzymes can be compared to bacterial transformation in distinguishing the heterologous nature of DNA from different sources (Marmur *et al.*, 1963). Coupled with the unique ability of the methylating enzyme to recognize specific DNA base sequences is the fact that cytosine is preferentially methylated by the enzyme isolated from *B. subtilis* and 6MAP by the enzyme extracted from T2 infected *E. coli* cells (Hausmann and Gold, 1966). In yet another system, when *E. coli* is infected with phage T3, the induction of an enzyme which cleaves SAM results in phage lysates with methyl-free DNA (Gold *et al.*, 1964). It is therefore difficult at the present time to assign a biological role to the methylated bases of DNA which is consistent with the above facts. If one is to assign the same general role to methylated bases in DNA as in RNA, the question becomes still more complicated. Quantitative analyses have shown no differences in the amount of methyl groups in the complementary strands of phage 2C DNA. It has also been reported that the DNA of phage λ grown in strains of *E. coli*, which differ genetically only in their ability to modify this bacteriophage, do not differ in their content of methylated bases (Wood, 1965, quoted in Stacey, 1965). Furthermore, supermethylation of transforming DNA by enzymes from heterologous strains did not alter its efficiency or rate of expression (K. Oda, unpublished results). These results tend to rule out the role of methylation in selective DNA strand transcription as well as in the host controlled modifications of phage λ (Arber and Dussoix, 1962). The sequence pattern of the methylated bases in 2C or λ phage DNA, however, has not yet been examined for its possible involvement in strand selection and modification. The role played by the methylated DNA bases would appear to be subtle and remains elusive at the present time.

Acknowledgment

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The Chemistry of Pseudouridine. VII. Selective Cleavage of Polynucleotides Containing Pseudouridylic Acid Residues by a Unique Photochemical Reaction*

Maria Tomasz and Robert Warner Chambers†

ABSTRACT: Specific cleavage of the oligonucleotide, TpΨpCpGp, at its pseudouridylic acid residue was achieved by irradiation with ultraviolet light

$$\text{TpΨpCpGp} \xrightarrow{h\nu} \text{TpY} + \text{5-formyluracil} + \text{pCpGp}$$

where Y is an unknown fragment derived from the pseudouridylic acid moiety. Examination of the stoichiometry of the reaction indicated that other, nonspecific, photochemical reactions were also occurring. These reactions caused a 15–20% loss in ultraviolet absorbing material, but did not interfere with the detection of the predicted products. Irradiation of purified alanine-soluble ribonucleic acid (s-RNA) followed by gel filtration on a Sephadex G-100 column at 56° gave four partially resolved peaks. According to the nucleotide sequence of yeast alanine-s-RNA, five fragments (55, 39, 37, 21, and 15 nucleotides) could be produced by specific photolysis of the pseudouridylic acid residues. The positions of the peaks emerging from the Sephadex column are consistent with this prediction and the peaks may represent polynucleotides containing 77 (starting material), 55, (39 + 37), and (21 + 15) nucleotides.

During our recent studies on the chemistry of pseudouridine, we discovered a unique photochemical reaction in which pseudouridine 3′-monophosphate (Ψ3′P)[1] (I, R = H) is converted to 5-formyluracil (II), inorganic phosphate, and an unknown fragment, RY (Tomasz and Chambers, 1964). A similar reaction occurs with pseudouridine 3′,5′-diphosphate (Ψ3′,5′DP) (I, R = PO_3^{2-}), while Ψ2′P, pseudouridine 2′,3′-cyclic phosphate, or pseudouridine itself produces only traces of 5-formyluracil under identical conditions. This unusual behavior of Ψ3′P and Ψ3′,5′DP is in sharp contrast to the well-known photochemical reactions of pyrimidine nucleotides which usually involve a reversible

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[1] The following abbreviations will be used: Ψ, pseudouridine (*C* isomer); Ψ3′P, pseudouridine 3′-monophosphate; Ψ3′,5′DP, pseudouridine 3′,5′-diphosphate; C2′(3′)P, cytidine 2′- and 3′-monophosphate mixture; C2′(3′)5′DP, cytidine 2′,5′-diphosphate and cytidine 3′,5′-diphosphate mixture; dT5′P, deoxyribothymidine 5′-monophosphate; T3′P, ribothymidine 3′-monophosphate; ATP, adenosine triphosphate; TpψpCpGp, thymidylylpseudouridylylcytidylylguanylic 3′-acid. Abbreviations according to this system will be used for all other nucleotides.