Study of Deoxyribonucleic Acid Replication in Permeable Cells of *Bacillus subtilis* Using Mercurated Nucleotide Substrates†

Sudha Bhattacharya and Nilima Sarkar*

ABSTRACT: Using cells of *Bacillus subtilis* made permeable to small molecules by treatment with toluene, we have explored the utilization of 5-mercurideoxycytidine 5′-triphosphate as a substrate for DNA replication. The mercurated nucleotide could effectively substitute for dCTP in supporting ATP-dependent DNA synthesis. The initial rates of DNA synthesis were similar for the two substrates, and the apparent K_m's for dCTP and 5-mercurideoxycytidine triphosphate were 22 μM and 40 μM, respectively. The incorporation of the mercurated nucleotide into DNA could be demonstrated directly by the use of ^{203}Hg-labeled substrate, and the extent of incorporation agreed fairly well with the cytosine content of *B. subtilis* DNA. The reaction was inhibited by arabinofuranosylcytosine 5′-triphosphate, novobiocin, and 6-[(*p*-hydroxyphenyl)azo]uracil, suggesting that the incorporation of the mercurated nucleotide into DNA required DNA polymerase III and represented DNA replication rather than repair. The high density of mercurated nucleotides made possible their use as pycnographic probes. More than 85% of the DNA synthesized in the presence of 5-mercurideoxycytidine triphosphate banded in Cs_2SO_4 gradients at a higher density than parental DNA. This was consistent with the pattern expected for semiconservative DNA synthesis and provided further evidence that the incorporation of the mercurated nucleotide into DNA was primarily a manifestation of DNA replication. Sedimentation analysis in alkaline sucrose gradients showed that DNA synthesized in the presence of 5-mercuricytidine triphosphate had a smaller average size ($s_{20,w} \sim 30$ s) than DNA synthesized in the presence of the normal substrate and that the size of the DNA product was a function of the relative amount of mercurated substrate used. This could, in part, have been due to an increased sensitivity of mercurated DNA to limited endonucleolytic cleavage since the average size of DNA labeled for 30 min was slightly less than that seen after 5 or 10 min. On the other hand, pulse–chase experiments indicated that mercurated DNA was less efficiently processed into high molecular weight material than normal DNA. Experiments in which DNA ligase was inhibited by NMN indicated that mercurated DNA could be utilized by the DNA joining enzyme; however, it was not determined whether ligation of mercurated DNA occurred at a somewhat reduced rate. It is possible that the abnormally small size of mercurated DNA is due to a combination of a slightly enhanced rate of endonucleolytic cleavage and a somewhat reduced rate of ligation. Our results indicate that mercurated nucleotides constitute useful probes for the study of bacterial DNA replication, both as radioactive and as density markers. Since mercurated nucleotides are also potential affinity probes by virtue of their reactivity with thiol-substituted matrices, they show considerable promise for the study of replicative intermediates.

The synthesis of mercury-substituted nucleotides and their incorporation into polynucleotides by various DNA and RNA polymerases were first described by Dale and co-workers (1973, 1975). Subsequently, 5-mercuripyrimidine ribonucleoside triphosphates have been widely used in studies of transcription in eukaryotic systems (Smith & Huang, 1976; Crouse et al., 1976; Tsai et al., 1978; Nguyen-Huu et al., 1978). Their usefulness is based on the reactivity of mercurated nucleotides with thiols, which permits the selective isolation of mercury-labeled nucleic acids on thiol-substituted affinity matrices (Dale & Ward, 1975). It is thus possible to separate nucleic acids synthesized in the presence of mercurated substrates from preexisting polynucleotides.

In recent years, much attention has been focused on the early intermediates in bacterial DNA synthesis, especially in relation to the question whether they are covalently linked to ribonucleotide primers (Sugino et al., 1972; Kornberg, 1976), but this work has been hampered by the inadequacy of methods for the separation of newly synthesized DNA from other polynucleotide material. No studies described to date have made use of mercurated nucleotides for the study of DNA replication; however, the availability of selective isolation methods for mercurated polynucleotides suggested to us that such an approach might solve some of the technical problems encountered in earlier studies. In order to use mercurated nucleotides as substrates for DNA synthesis, it is necessary to employ a system for DNA replication which is accessible to nucleotide substrates. Such a system was first described for *Escherichia coli* by Moses & Richardson (1970) and later adapted to *Bacillus subtilis* by Matsushita et al. (1972) and consists of cells that have been made selectively permeable to small molecules by treatment with toluene. Such cells are known to carry out ATP-dependent semiconservative replication accompanied by little repair, normal advancement of preexisting replication forks occurs, and the newly synthesized DNA is biologically active (Moses & Richardson, 1970; Matsushita et al., 1972).

As a first step to the study of replicative intermediates in *B. subtilis*, we have studied the utilization of mercurated nucleotides as substrates for DNA replication in toluene-treated cells. The results described in this paper show that Hg-dCTP[1] can effectively substitute for the normal substrate

† From the Department of Metabolic Regulation, Boston Biomedical Research Institute, Boston, Massachusetts 02114 (S.B. and N.S.), and the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115 (N.S.). *Received September 2, 1980.* This investigation was supported by Grant GM25022 from the National Institute of General Medical Sciences. A preliminary report of some of this work was presented at the 71st Meeting of the American Society of Biological Chemists, New Orleans, LA, 1980, and at the 9th Annual ICN–UCLA Symposium, Keystone, CO, 1980.

[1] Abbreviations used: Hg-dCTP, 5-mercurideoxycytidine 5′-triphosphate; *ara*-CTP, 1-β-D-arabinofuranosylcytosine 5′-triphosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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in supporting DNA synthesis in a process which has the characteristics of DNA replication. Some of the properties of newly synthesized mercurated DNA are described.

Experimental Procedures

Materials. [*methyl*-^{14}C]Thymidine (53 mCi/mmol), [*methyl*-^{3}H]thymidine (20 Ci/mmol), [*methyl*-^{3}H]thymidine 5′-triphosphate (22 Ci/mmol), and [^{203}Hg]mercuric acetate were obtained from New England Nuclear, ATP, dATP, dGTP, dCTP, and dTTP were from P-L Biochemicals, NMN and CS_2SO_4 were from Sigma, Hg-dCTP, lysozyme, and proteinase K were from Boehringer, heparin was from Organon, and sucrose (ultrapure) was from Schwarz/Mann. ^{3}H-Labeled bacteriophage T7 DNA was the gift of Dr. C. C. Richardson. ^{203}Hg-dCTP was prepared by mercuration of dCTP with [^{203}Hg]mercuric acetate (100 Ci/mmol) by the procedure of Dale et al. (1973) and was purified by chromatography on DEAE-cellulose and Chelex-100 (Bio-Rad) (Dale et al., 1975).

Bacterial Growth. Bacillus subtilis ATCC 23857 was grown at 37 °C on a rotary shaker in Spizizen minimal salts medium (Anagnostopoulos & Spizizen, 1961) supplemented with glucose (5 g/L), Difco casamino acids (0.5 g/L), and indole (50 mg/L) (Winston & Matsushita, 1975). Cells with uniformly labeled DNA were prepared by labeling for three generations with [*methyl*-^{14}C]thymidine (0.16 μCi/mL) or with [*methyl*-^{3}H]thymidine (0.4 μCi/mL). Cell growth was monitored in a Klett–Summerson photoelectric colorimeter with a No. 42 filter. Cells were harvested in mid-exponential phase (40 Klett units) by centrifugation at 5000*g* for 7 min at room temperature.

Toluene Treatment of Cells. Freshly harvested mid-exponential cells were washed with 0.1 M potassium phosphate, pH 7.4, at room temperature and suspended in this buffer at a concentration of 3×10^9 cells/mL. The suspension was gently shaken for 10 min at 25 °C with 1% toluene. The cells were then collected by centrifugation at room temperature after dilution with 20% of the culture volume of phosphate buffer and resuspended in the same buffer at a concentration of 5×10^9 cells/mL. The suspension was divided into small aliquots, quickly frozen in liquid N_2, and stored at −80 °C.

Assay of DNA Synthesis. DNA synthesis in toluene-treated cells was measured by the incorporation of [^{3}H]dTTP or ^{203}Hg-dCTP or both into trichloroacetic acid insoluble material (Winston & Matsushita, 1975). The standard incubation mixture (0.1 mL) contained 70 mM potassium phosphate, pH 7.5, 1.5 mM ATP, 13 mM $MgSO_4$, 2 mM dithiothreitol, 40 μM each of dGTP, dCTP, and dATP, and 40 μM of [^{3}H]-dTTP (100 μCi/μmol). When ^{203}Hg-dCTP was used as the labeled substrate, it replaced dCTP at a concentration of 40 μM, and 5 mM 2-mercaptoethanol was added. After 30 min at 37 °C, the reaction was terminated by the addition of cold 0.3 M trichloroacetic acid containing 10 mM sodium pyrophosphate, and the precipitate was collected on Whatman GF/C glass fiber filters and washed with three 10-mL portions of the trichloroacetic acid reagent and then with ethanol. When ^{203}Hg-dCTP was used, the trichloroacetic acid washes were followed by further washing with five 10-mL portions of cold 1.2 M 2-mercaptoethanol to dissociate any mercurated substrates linked to thiol groups in the precipitated protein. The filters were then dried, and their radioactivity was determined in a liquid scintillation spectrometer with a toluene-based scintillation fluid.

Zone Sedimentation in Alkaline Sucrose Gradients. Linear gradients (12 mL) of 5–25% sucrose in 0.3 M NaOH, 0.7 M NaCl, and 1 mM EDTA were prepared in cellulose nitrate tubes with a cushion of 0.3 mL of 60% sucrose solution at the bottom. After the labeled cell lysate was layered onto the gradient, it was subjected to centrifugation for 15 h at 20 °C in a Beckman SW41 rotor at 22000 rpm. Fractions were collected from the bottom of the tubes, and trichloroacetic acid insoluble radioactivity was determined as described above after the addition of 25 μg of herring sperm DNA to each sample. Sedimentation coefficients were calculated by comparison with the sedimentation of bacteriophage T7 DNA, assuming $s^0_{20,w}$ = 37.2 S (Studier, 1965).

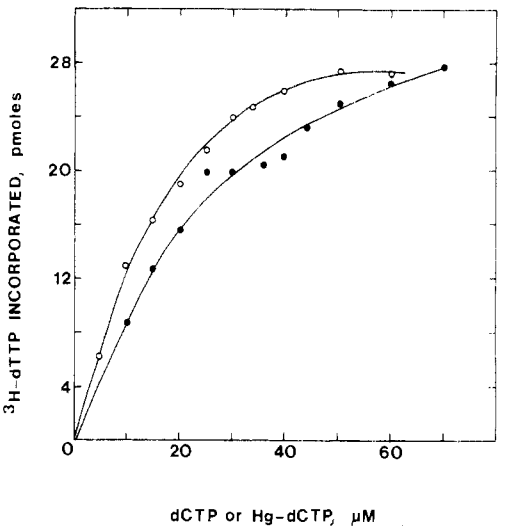

FIGURE 1: Comparison of dCTP and Hg-dCTP as substrates for DNA synthesis. DNA synthesis by 4×10^7 toluene-treated cells was measured as described under Experimental Procedures at the indicated concentrations of dCTP (○) or Hg-dCTP (●), with [^{3}H]dTTP as the labeled substrate.

Equilibrium Sedimentation in Cs_2SO_4 Gradients. DNA samples and 2.7 g of Cs_2SO_4 were brought to a final volume of 4.6 mL with 0.1 M Tris-HCl, pH 8.0, and 10 mM EDTA in a siliconized nitrocellulose tube (Sugino et al., 1972). The mixture was overlayed with light paraffin oil and centrifuged in a Beckman 50 Ti rotor for 42 h at 36000 rpm and 15 °C. Fractions (0.11 mL) were collected from the bottom of the tube, and acid-insoluble radioactivity was determined as described above.

Results

Hg-dCTP as Substrate for DNA Synthesis. The incorporation of [^{3}H]dTTP into DNA by toluene-treated cells of *B. subtilis* depends on the presence of all four deoxyribonucleoside triphosphates. As shown in Figure 1, the requirement for deoxycytidine nucleotides could be met either by dCTP or by Hg-dCTP. Both nucleotides exhibited hyperbolic saturation curves with similar maximum velocities. Double-reciprocal plots of the data indicated apparent K_m's for dCTP and Hg-dCTP of 24 and 40 μM, respectively. When the cytidine nucleotide concentration was held constant at 40 μM and dCTP was progressively replaced by Hg-dCTP, only a slight reduction in the rate of DNA synthesis was seen as the proportion of the mercurinucleotide increased (Figure 2).

For determination of whether the stimulation of DNA synthesis by Hg-dCTP actually involved the incorporation of the mercurinucleotide into DNA, ^{203}Hg-dCTP was prepared by the method of Dale et al. (1973, 1975). The incorporation of ^{203}Hg-dCTP and [^{3}H]dTTP into DNA showed a similar dependence on Hg-dCTP concentration (Figure 3). The molar ratio of the two nucleotides incorporated was about 0.70, which agreed fairly well with the cytosine/thymine ratio of 0.78 in *B. subtilis* DNA (Schildkraut et al., 1962).

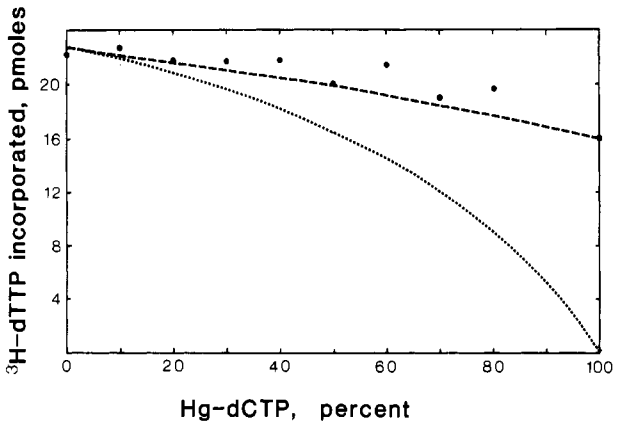

FIGURE 2: Effect of various proportions of dCTP and Hg-dCTP on DNA synthesis. DNA synthesis by 4×10^7 toluene-treated cells was measured as described under Experimental Procedures with [^{3}H]dTTP as the labeled substrate and at a total deoxycytidine nucleotide concentration of 40 μM, with dCTP and Hg-dCTP in the proportions indicated. The experimental points (●) are compared with the relationship expected if only dCTP were a substrate (---), given by the expression $v = V_{max}[dCTP](K_m^{dCTP} + [dCTP])^{-1}$, and with that expected if dCTP and Hg-dCTP were competing substrates (– –), given by the expression $v = V_{max}^{dCTP}[dCTP]\{K_m^{dCTP}(1 + [Hg\text{-}dCTP]/K_m^{Hg\text{-}dCTP}) + [dCTP]\}^{-1} + V_{max}^{Hg\text{-}dCTP}[Hg\text{-}dCTP]\{K_m^{Hg\text{-}dCTP}(1 + [dCTP]/K_m^{dCTP}) + [Hg\text{-}dCTP]\}^{-1}$. The values used for K_m^{dCTP} and $K_m^{Hg\text{-}dCTP}$ were 24 μM and 40 μM, respectively.

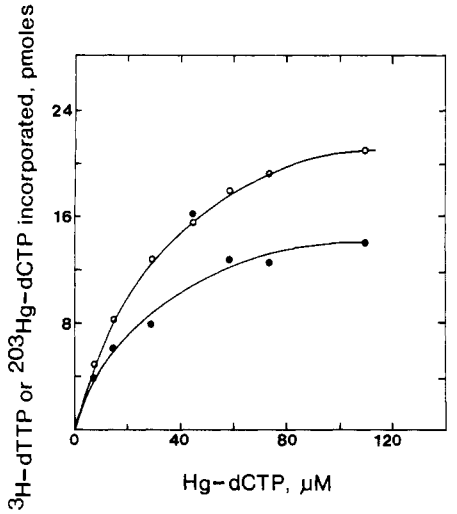

FIGURE 3: Comparison of the incorporation of [^{3}H]dTTP and ^{203}Hg-dCTP into DNA at different concentrations of Hg-dCTP. DNA synthesis by 4×10^7 toluene-treated cells was measured as described under Experimental Procedures with both [^{3}H]dTTP and ^{203}Hg-dCTP as the labeled substrates, at the concentrations of ^{203}Hg-dCTP indicated. (○) Incorporation of ^{3}H; (●) incorporation of ^{203}Hg.

The rates of DNA synthesis with 40 μM Hg-dCTP, measured by the incorporation of ^{203}Hg, and that seen in the presence of 40 μM dCTP, measured by [^{3}H]dTTP incorporation, were constant for more than 1 h (results not shown). The rates of incorporation of ^{203}Hg-dCTP and [^{3}H]dTTP were 0.35 pmol min^{-1} per 10^7 cells and 0.72 p mol min^{-1} per 10^7 cells, respectively. The lower rate seen with the mercurated substrate is consistent with the higher apparent K_m for Hg-dCTP.

Characterization of Hg-dCTP Incorporation into DNA. The incorporation of mercurinucleotide into DNA required the presence of a mercaptan as the mercury ligand, the chloride and acetate salts being ineffective as substrates. The rate of DNA synthesis was greatest in the presence of 5 mM 2-mercaptoethanol whereas dithiothreitol, thioacetic acid, and ethyl mercaptan were less effective. In the presence of 5 mM 2-mercaptoethanol, 2 mM dithiothreitol had no inhibitory effect on the incorporation of ^{203}Hg-dCTP into DNA. The effect of other substances on DNA synthesis in an experiment using both ^{203}Hg-dCTP and [^{3}H]dTTP as labeled precursors is shown in Table I. DNA synthesis required the presence of all four deoxyribonucleoside triphosphates and of ATP and was inhibited by arabinosylcytosine triphosphate, novobiocin, and 6-[(*p*-hydroxyphenyl)azo]uracil, and the incorporation of both precursors was affected in parallel by these agents. Since the incorporation of ^{203}Hg-dCTP required the presence of mercaptans, it was not possible to study the effect of sulfhydryl reagents under the conditions of this experiment. However, in a parallel experiment in which the incorporation of [^{3}H]-dTTP into DNA by toluene-treated cells was studied in the presence of dCTP, complete inhibition of DNA synthesis was produced by 12 mM *N*-ethylmaleimide or by 0.5 mM *p*-(hydroxymercuri)phenylsulfonate (data not shown).

Table I: Requirements and Effects of Inhibitors on the Incorporation of [^{3}H]dTTP and ^{203}Hg-dCTP into DNA[a]

addition or deletion	relative incorporation of [^{3}H]dTTP	relative incorporation of ^{203}Hg-dCTP
none	[100][b]	[100][c]
−dATP and dGTP	4.9	1.5
−ATP	5.4	2.1
−2-mercaptoethanol and dithiothreitol	0	0
+*ara*-CTP (0.1 mM)	15	14
+novobiocin (50 μg/mL)	14	16
+6-[(*p*-hydroxyphenyl)azo]uracil (0.2 mM)	1.7	2.7

[a] DNA synthesis by 4×10^7 toluene-treated cells was measured as described under Experimental Procedures, with both [^{3}H]dTTP and ^{203}Hg-dCTP as labeled substrates and with additions or omissions as indicated. [b] 20 pmol incorporated. [c] 12.8 pmol incorporated.

Pycnographic Analysis of DNA Synthesized with Hg-dCTP. Because mercury is a heavy metal, mercurinucleotides can also be used as a density label for pycnographic analysis of DNA synthesized in toluene-treated cells. *Bacillus subtilis* DNA was uniformly labeled with [^{3}H]thymidine for several generations, and the ^{3}H-labeled cells were then permeabilized with toluene and allowed to engage in DNA synthesis with ^{203}Hg-dCTP as the labeled substrate. Upon pycnographic analysis of cellular DNA, more than 85% of the ^{203}Hg-labeled DNA was found to band at a higher density in Cs_2SO_4 gradients than the ^{3}H-labeled parental DNA (Figure 4A). In a parallel experiment, unlabeled permeabilized cells were incubated with both ^{203}Hg-dCTP and [^{3}H]dTTP, and the resulting labeled DNA was analyzed pycnographically. As shown in Figure 4B, ^{3}H and ^{203}Hg were distributed in a similar manner, indicating that the two labeled precursors were incorporated into the same species of DNA.

Sedimentation Analysis of DNA Synthesized with Hg-dCTP. DNA synthesized in the presence of Hg-dCTP was analyzed by zone sedimentation in alkaline sucrose gradients. Cells were first labeled for three generations with [^{14}C]thymidine and then permeabilized with toluene and permitted to engage in DNA synthesis in the presence of either dCTP or Hg-dCTP, with [^{3}H]dTTP as the labeled substrate. The sedimentation profile of DNA synthesized in the presence of dCTP was similar to that of parental DNA that had been uniformly labeled in vivo and consisted of a broad rapidly sedimenting peak (Figure 5). In contrast, DNA synthesized in the presence of Hg-dCTP sedimented more slowly than parental DNA, with an average sedimentation coefficient of

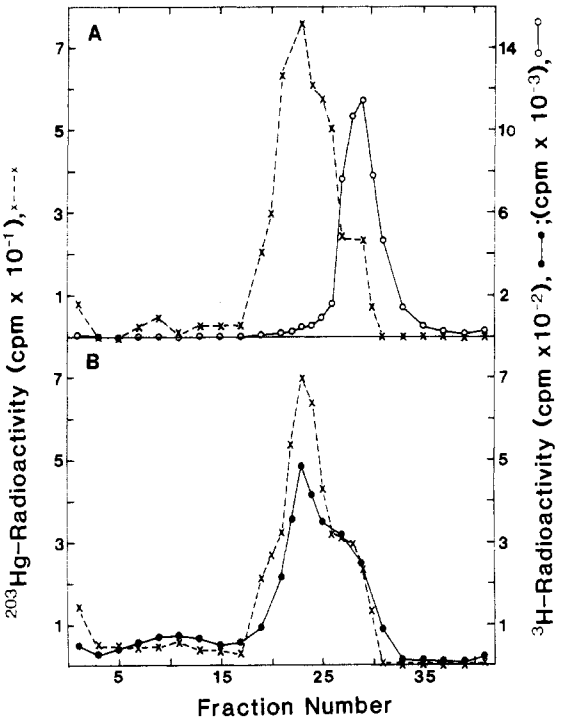

FIGURE 4: Pycnographic analysis of DNA synthesized with Hg-dCTP. (A) Toluene-treated cells (2×10^8) from a culture that had been labeled with [^{3}H]thymidine for three generations during early exponential growth were incubated with 60 μM ^{203}Hg-dCTP as described under Experimental Procedures, except that the reaction volume was scaled up to 0.4 mL. (B) Unlabeled toluene-treated cells (2×10^8) were incubated with both ^{203}Hg-dCTP and [^{3}H]dTTP in a final volume of 0.4 mL. After 30 min at 37 °C, DNA synthesis was terminated by the addition of 20 mM EDTA, pH 8.0, and the mixtures were incubated for 30 min at 37 °C in the presence of 0.1 M NaCl and lysozyme (1 mg/mL) and then for a further 40-min after supplementation with Sarkosyl (10 mg/mL), heparin (0.1 mg/mL), and proteinase K (0.5 mg/mL). After dispersal on a vortex mixer, the samples were passed through 15-mL columns of Sephadex G-25 equilibrated with 10 mM Tris-HCl, pH 7.5, and 0.1 M NaCl. The material excluded from the gel was pooled, mixed with 2.7 g of Cs_2SO_4, adjusted to a final volume of 4.6 mL, and subjected to pycnographic analysis as described under Experimental Procedures. (×) ^{203}Hg incorporated in vitro; (●) ^{3}H incorporated in vitro; (○) [^{3}H]thymidine-labeled parental DNA.

about 30 S (Figure 5). The sedimentation pattern of parental DNA was not affected by subsequent incubation with Hg-dCTP.

One possible cause of the smaller size of DNA synthesized in the presence of Hg-dCTP is the preferential degradation of mercurated DNA. This possibility was investigated by examining the size of mercurated DNA as a function of time. The average sedimentation coefficients of DNA synthesized in the presence of Hg-dCTP in 5-, 10-, and 30-min periods were 31, 33, and 25 S, respectively. This suggested that some degradation of mercurated DNA may be occurring over a 30-min period. However, it should be noted that mercurated DNA sedimented much more slowly than parental DNA even after 5 and 10 min of incubation, a time period over which no degradation was seen. It is therefore not likely that degradation is a primary cause for the decrease in DNA size seen in the presence of Hg-dCTP.

Another possible explanation for the accumulation of relatively low molecular weight mercurated DNA is that it represents an intermediate in DNA replication whose processing to high molecular weight DNA is somehow inhibited. Such a possibility is supported by the observation that the size of labeled DNA produced during very short periods of synthesis (90 s) in the presence of either dCTP or Hg-dCTP was

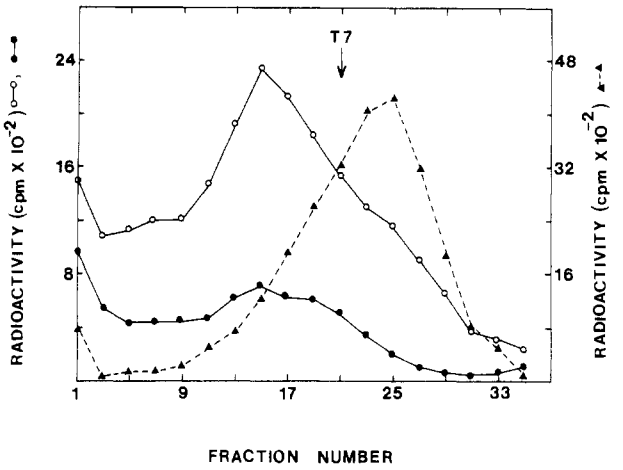

FIGURE 5: Sedimentation analysis in alkaline sucrose gradients of DNA synthesized with dCTP or with Hg-dCTP. Toluene-treated cells (5×10^8) from a culture that had been labeled with [^{14}C]thymidine for three generations during early exponential growth were incubated with [^{3}H]dTTP and either dCTP or Hg-dCTP as described under Experimental Procedures, except that the reaction volume was scaled up to 1 mL. After 30 min at 37 °C, the reaction mixtures were rapidly chilled to 4 °C and subjected to centrifugation at 12000*g* for 5 min. The pellets were resuspended in 20 mM EDTA and 0.1 M NaCl and digested with lysozyme and then with proteinase K as described in the legend to Figure 4. After proteinase K digestion, NaOH was added to the lysate to a final concentration of 0.2 M, and the solution (0.24 mL) was clarified by centrifugation at 12000*g* for 3 min. Samples (0.16 mL) were layered onto a linear sucrose gradient (5–20%) in 4.8 mL of 0.7 M NaCl, 0.3 N NaOH, and 1 mM EDTA and centrifuged at 4 °C in a Beckman SW50.1 rotor for 13 h at 19000 rpm. (○) [^{3}H]dTTP incorporated in the presence of dCTP; (▲) [^{3}H]dTTP incorporated in the presence of Hg-dCTP; (●) ^{14}C-labeled parental DNA. The arrow indicates the position of ^{3}H-labeled bacteriophage T7 DNA sedimented under identical conditions.

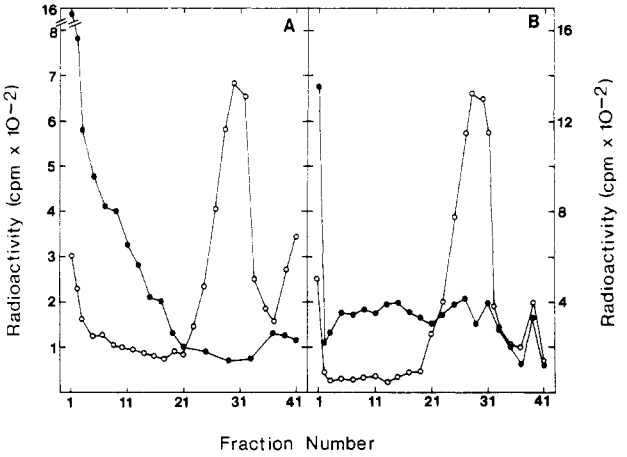

FIGURE 6: Sedimentation analysis of DNA pulse labeled with [^{3}H]dTTP in the presence of dCTP or Hg-dCTP and then incubated further with unlabeled substrates and an excess of dCTP. Duplicate samples containing 8×10^7 toluene-treated cells were incubated with 8 μM [^{3}H]dTTP (18.5 Ci/mmol) and either dCTP (A) or Hg-dCTP (B) as described under Experimental Procedures. After 90 s at 37 °C, the reaction in one set of mixtures was terminated by the addition of EDTA as described in the legend to Figure 5 whereas the duplicate mixtures were supplemented with a 124-fold excess of unlabeled dTTP (1 mM) and, in the case of the mixture containing Hg-dCTP, with a 50-fold excess of dCTP (2 mM) and incubated for an additional 25 min at 37 °C before termination by the addition of EDTA. Digestion with lysozyme and proteinase K and sedimentation analysis were carried out as described in the legend to Figure 5. (○) ^{3}H-Labeled DNA after 90-s pulse; (●) ^{3}H-labeled DNA after 90-s pulse followed by 25-min chase.

quite small ($s_{20,w} \approx 25$ S). However, whereas further incubation with an excess of unlabeled dTTP caused the conversion of most of the DNA synthesized with dCTP to high molecular

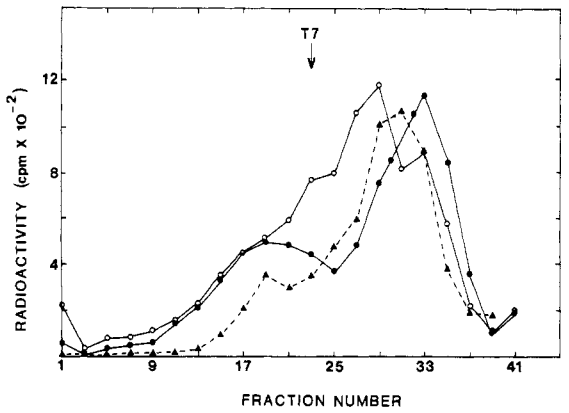

FIGURE 7: Effect of inhibition of polynucleotide ligase by NMN on the sedimentation profiles of DNA synthesized in the presence of dCTP or Hg-dCTP. Toluene-treated cells (8×10^7) were incubated with [^{3}H]dTTP and either 40 μM dCTP or 60 μM Hg-dCTP as described under Experimental Procedures, except that the final incubation volume was 0.2 mL and NMN (5 mM) was added as indicated. The DNA was isolated and analyzed by sucrose gradient sedimentation as described in the legend to Figure 5. ^{3}H-Labeled DNA synthesized (○) in the presence of Hg-dCTP, (▲) in the presence of Hg-dCTP plus NMN, and (●) in the presence of dCTP plus NMN.

weight material (Figure 6A), mercurated DNA was converted only partially to high molecular weight DNA, even when the subsequent incubation contained a 50-fold excess of dCTP (Figure 6B).

The failure of mercurated DNA to be efficiently processed to parental size DNA could be due to its inability to be joined by polynucleotide ligase. It was thus of interest to examine the effect of inhibition of DNA ligase by NMN on the size of DNA synthesized in the presence of dCTP or Hg-dCTP. As shown in Figure 7, the presence of NMN caused a major portion of DNA synthesized in the presence of dCTP to sediment as a peak of 17 S, while the peak of DNA synthesized in the presence of Hg-dCTP shifted from 25 S to about 21 S. The reduced size of mercurated DNA seen in the presence of the ligase inhibitor indicated that mercurated DNA is indeed a substrate for DNA ligase. Nevertheless, our results do not rule out the possibility that mercurated DNA is ligated less efficiently than normal DNA, causing it to sediment at a position intermediate between normal DNA (greater than 60 S) and unligated DNA (about 17 S).

Discussion

The primary aim of the experiments described in this paper was to evaluate the potential of mercurinucleotides as probes for the study of DNA replication. It was thus important to determine whether Hg-dCTP could effectively substitute for dCTP as substrate for DNA synthesis. The experimental systems used for this purpose were cells of *B. subtilis* that had been made permeable to small molecules by treatment with toluene. Other workers have shown that such cells can utilize deoxyribonucleoside triphosphates as substrates for DNA synthesis which represents predominantly semiconservative replication and yields biologically active DNA (Matsushita et al., 1972). Our results showed that Hg-dCTP could replace dCTP in supporting DNA synthesis in this system. The maximal rates of DNA synthesis observed with dCTP and with the mercurated analogue were nearly the same, and the apparent K_m for Hg-dCTP was less than twice that for the normal substrate. The rate of DNA synthesis observed with mixtures of dCTP and Hg-dCTP agreed well with that expected if the two nucleotides acted as alternate substrates for a single enzyme with apparent K_m's of 24 μM and 40 μM and apparent V_{max}'s of 36 and 32 pmol (30 min)$^{-1}$ per 5×10^7 cells, respectively (Figure 2). The slight deviation seen from the predicted relationship is probably due to the fact that the calculations assumed the K_m's and inhibition constants to be identical.

The incorporation of Hg-dCTP into DNA could be confirmed directly by studying the incorporation of the ^{203}Hg-labeled nucleotide. The incorporation of ^{203}Hg-dCTP and [^{3}H]dTTP into DNA at different Hg-dCTP concentrations occurred at a constant ratio of 0.70 (Figure 3). The slightly lower value of this ratio than the cytosine/thymine ratio of 0.78 in *B. subtilis* DNA may be due to some demercuration (Dale et al., 1975) that occurs when the trichloroacetic acid precipitated material is extensively washed with 2-mercaptoethanol to remove mercurated nucleotides unspecifically bound to protein.

The rate of DNA synthesis in toluene-treated cells with either dCTP or Hg-dCTP as substrate was constant for prolonged time periods. Hg-dCTP-dependent DNA synthesis required the presence of a thiol, 2-mercaptoethanol being the most effective. The function of the thiol is probably to act as a mercury ligand, as has been observed with purified DNA polymerases (Dale et al., 1973). A nearly absolute requirement for ATP suggested that the incorporation of Hg-dCTP into DNA represented replicative DNA synthesis, and by the use of specific inhibitors it was possible to obtain some information on the enzymes involved. Hg-dCTP-dependent DNA synthesis was strongly inhibited by low concentrations of 6-[(*p*-hydroxyphenyl)azo]uracil, *ara*-CTP, and novobiocin (Table I). 6-[(*p*-Hydroxyphenyl)azo]uracil is a known inhibitor of DNA replication in *B. subtilis*, affecting directly DNA polymerase III (Mackenzie et al., 1973; Gass et al., 1973), and the targets of *ara*-CTP in *B. subtilis* are DNA polymerases II and III (Gass & Cozzarelli, 1973). Novobiocin is an inhibitor of DNA gyrase (Gellert et al., 1976) and inhibits DNA replication in vivo (Smith & Davis, 1967). Taken together, these characteristics of the incorporation of Hg-dCTP into DNA constitute strong evidence that this reaction represents DNA replication rather than repair.

Since the incorporation of Hg-dCTP significantly increases the buoyant density of DNA, it was possible to study DNA synthesized in the presence of Hg-dCTP pycnographically. If the mercurated DNA were synthesized in a semiconservative manner, one would expect it to band in a Cs_2SO_4 gradient at a density higher than that of parental DNA; in the case of repair synthesis, the labeled DNA would be dispersed throughout parental DNA and band at a corresponding density. We found that 85% of DNA labeled with ^{203}Hg-dCTP banded at a higher buoyant density than parental DNA, indicating that it is a product of replication rather than repair.

Sedimentation analysis in alkaline sucrose gradients revealed an unusual feature of DNA synthesized in the presence of mercurated nucleotides. Consistent with a discontinuous mode of replication (Okazaki et al., 1968), DNA labeled for short time periods (90 s) in the presence of either dCTP or Hg-dCTP was of relatively small size (about 25 S). Further incubation in the presence of unlabeled substrates led to the conversion of normal pulse-labeled DNA to higher molecular weight material whereas DNA synthesized in the presence of Hg-dCTP showed a much smaller increase in molecular weight (Figure 6). Longer labeling periods produced mercurated DNA of a slightly higher sedimentation coefficient (≈30 S), while DNA synthesized in the presence of the normal substrates attained the size of parental DNA (Figure 5).

The smaller size of DNA synthesized in the presence of Hg-dCTP could be due either to an increased susceptibility

to degradation or to a slower rate of processing of mercurated DNA. The observations that the rate of synthesis of both normal and mercurated DNA were similar and constant for more than 60 min and that the amount of DNA synthesized was nearly independent of the proportion of mercurated nucleotide substrate (Figure 2) speak against massive DNA degradation. On the other hand, the small decrease in the average size of mercurated DNA seen during prolonged labeling periods indicates the occurrence of some degradation. However, it is also possible that substitution with mercury could interfere with the processing of nascent DNA chains. Since DNA ligase plays an important role in the formation of high molecular weight DNA (Olivera et al., 1968), it was of interest to examine the effect of NMN, an inhibitor of DNA ligation (Olivera & Bonhoeffer, 1972), on the size of DNA synthesized with either Hg-dCTP or the normal substrate. The presence of NMN led to a reduction in the size of the mercurated DNA product, suggesting that even the formation of the relatively small mercurated DNA had involved DNA ligase action. The relatively small size of mercurated DNA appears thus to be due neither to extensive degradation nor to the inability to be ligated. However, this does not preclude the possibility that mercurated DNA may be processed more slowly than normal DNA and that a reduced rate of ligation and perhaps a slightly enhanced rate of endonucleolytic cleavage combine to yield a product of lower average size.

It is clear from the results described here that Hg-dCTP can effectively substitute for the normal substrate in DNA replication in cells of *B. subtilis* made permeable to small molecules by treatment with toluene. In our experiments, we have made use of the mercurated nucleotide both as a radioactive tracer and a density label to characterize newly synthesized DNA. However, an even greater advantage can accrue from the use of mercurated substrates by virtue of their reactivity with thiols, which makes them a chemical probe for the study of intermediates in DNA replication. Much effort has been devoted to the characterization of early intermediates in DNA replication, especially in regard to their ribonucleotide content, but this work has been hampered by the lack of unambiguous methods for the separation of newly synthesized DNA from preexisting DNA. Mercurated polynucleotides can be selectively absorbed to thiol-substituted affinity matrices (Dale & Ward, 1975), a property which has been widely used in the analysis of transcription (Smith & Huang, 1976; Crouse et al., 1976; Tsai et al., 1978; Nguyen-Huu et al., 1978). A similar approach should be applicable to the analysis of replicative intermediates. Preliminary experiments have shown that DNA synthesized in the presence of Hg-dCTP can be selectively isolated in good yield by absorption to columns of agarose substituted with *N*-acetylcysteine and elution with mercaptoethanol. The mercurated DNA isolated by this procedure can transform *B. subtilis* with respect to several different auxotrophic markers (S. Bhattacharya and N. Sarkar, unpublished observations) and must therefore represent a biologically functional replication product.

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References

Anagnostopoulos, C., & Spizizen, J. (1961) *J. Bacteriol. 81*, 741–746.

Crouse, G. F., Fodor, E. J., & Doty, P. (1976) *Proc. Natl. Acad. Sci. U.S.A. 70*, 1564–1567.

Dale, R. M. K., & Ward, D. C. (1975) *Biochemistry 14*, 2458–2469.

Dale, R. M. K., Livingston, D. C., & Ward, D. C. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2238–2242.

Dale, R. M. K., Martin, E., Livingston, D. C., & Ward, D. C. (1975) *Biochemistry 14*, 2447–2457.

Gass, K. B., & Cozzarelli, N. R. (1973) *J. Biol. Chem. 248*, 7688–7700.

Gass, K. B., Low, R. L., & Cozzarelli, N. R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 103–107.

Gellert, M., O'Dea, M. H., Itoh, T., & Tomizawa, J. I. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4474–4478.

Kornberg, A. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M., Eds.) pp 331–352, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Mackenzie, J. M., Neville, M. M., Wright, G. E., & Brown, N. C. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 512–516.

Matsushita, T., White, K., & Sueoka, N. (1972) *Nature (London) 232*, 111–114.

Moses, R. E., & Richardson, C. C. (1970) *Proc. Natl. Acad. Sci. U.S.A. 67*, 674–681.

Nguyen-Huu, M. C., Sippel, A. A., Hynes, N. E., Groner, B., & Schutz, G. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 686–690.

Okazaki, R., Okazaki, T., Sakabe, K., Sugimoto, K., Kainuma, R., Subino, A., & Iwatzuki, N. (1968) *Cold Spring Harbor Symp. Quant. Biol. 33*, 129–142.

Olivera, B. M., & Bonhoeffer, F. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 25–29.

Olivera, B. M., Hall, Z., Anraku, Y., Chien, J., & Lehman, I. R. (1968) *Cold Spring Harbor Symp. Quant. Biol. 33*, 27–34.

Schildkraut, C. L., Marmur, J., & Doty, P. (1962) *J. Mol. Biol. 4*, 430–443.

Smith, D. H., & Davis, B. D. (1967) *J. Bacteriol. 93*, 71–79.

Smith, M. M., & Huang, R. C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 775–779.

Studier, F. W. (1965) *J. Mol. Biol. 11*, 373–390.

Sugino, A., Hirose, S., & Okazaki, R. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1863–1867.

Tsai, M., Tsai, S. Y., Chang, C. W., & O'Malley, B. W. (1978) *Biochim. Biophys. Acta 521*, 689–707.

Winston, S., & Matsushita, T. (1975) *J. Bacteriol. 123*, 921–927.