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Evidence Suggestive of Compartmentalization of Deoxyribonucleic Acid-Synthesizing Systems in Freeze-Treated *Bacillus subtilis*

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Freezing of *Bacillus subtilis* in liquid nitrogen results, upon thawing of the cells, in an enhanced deoxyribonucleoside triphosphate and reduced thymidine (Tdr) incorporation into cellular deoxyribonucleic acid (DNA). The DNA synthesized from thymidine triphosphate (TTP) was made by a "repair"-type system as determined by density transfer experiments. The mono- and diphosphate precursors were also incorporated by a "repair"-type synthesis. When Tdr was used as the radioactive precursor in the assay mixture, the product was only that expected from a semiconservative synthesis. Superlethal ultraviolet light exposure of the freeze-treated cells stimulated incorporation of phosphorylated precursors into DNA. Tdr uptake was greatly reduced by ultraviolet exposure, and only repair synthesis was observed. TTP and Tdr do not compete with one another in this system. The possibility that two DNA synthesizing systems exist in separate, nonmixing cellular compartments is considered.

As part of an extensive effort to understand the biochemical nature of in vivo deoxyribonucleic acid (DNA) duplication, the ability of bacterial cells to incorporate exogenous phosphorylated precursors into their DNA has been the subject of several recent studies. A variety of treatments have been used to alter the permeability of the cells to allow penetration of the external precursors to the site of DNA synthesis. The treatments are usually highly toxic and include prior exposure to ethylenediaminetetraacetic acid (EDTA) (5), toluene (9), or detergents such as Brij 58 (6).

In a previous report a freeze treatment of *Bacillus subtilis* W23 was described which greatly enhanced the incorporation of exogenously supplied deoxyribonucleoside triphosphates into cellular DNA (3). These initial results indicated that the cellular thymidine triphosphate (TTP)-incorporating system differed in several ways from that involved in thymidine (Tdr) incorporation into DNA. In this communication we report that the DNA product containing labeled Tdr is synthesized semiconservatively, whereas the DNA synthesized with radioactive TTP, thymidine diphosphate (TDP), or thymidylic acid (TMP) results from a "repair"-type synthesis. The two synthesizing systems are noncompetitive with one another, raising the possibility that they exist in separate, nonmixing cellular compartments.

MATERIALS AND METHODS

Bacterial strains and cell growth. *B. subtilis* W23 and a thymine (T)-requiring derivative (W23T$^-$) were used for these studies. The bacteria were grown in supplemented, minimal medium similar to that described by Anagnostopoulos and Spizizen (1). The minimal medium contained the following salts per liter: K_2PO_4 (14 g); KH_2PO_4 (6 g); sodium citrate (1 g); NH_4Cl (0.8 g); $MgCl_2 \cdot 6\ H_2O$ (0.33 g); and Na_2SO_4 (0.014 g). The supplement consisted of glucose (1%) and Casamino Acids (0.5%; Difco). The W23T$^-$ cells were grown in the presence of 10 μg of thymine per ml. Where indicated, cellular DNA was labeled for several generations by the addition of ^{14}C-thymine (0.2 μCi/ml of [2-^{14}C]-thymine). The cells were harvested while in their exponential growth phase. Petroff-Hauser counts showed approximately 5×10^7 "cells"/ml. (Very few single cells were observed, most cells existing as chains whose average size was approximately four of the smallest single cells seen.) The plating efficiency of these cells was 50% or less.

"Freeze" treatment. The harvested cells were washed once in SSC buffer (0.15 M NaCl, 0.015 M sodium citrate, *p*H 7.0) and suspended in half the original volume of SSC. The cells were frozen at this stage. Freezing of the cells was accomplished by immersion in liquid nitrogen. The frozen preparation was stored at −76 C. For use in the DNA synthesizing reaction, the cells were rapidly thawed at 37 C, harvested by centrif-

ugation, and suspended in 0.05 M phosphate buffer (*p*H 7.4) at one-tenth the original culture volume. Colony-forming unit (CFU) assay after 1 hr of freezing showed that 50% or more of the CFU's survived. Some frozen preparations were held at −76 C for up to one month without apparent effect on their enzyme activity.

Assay for DNA synthesizing ability. The reaction mixture used for assay of DNA synthesis was similar to that described by Moses and Richardson (9) for their assay of toluene-treated cells. The reaction mixture contained the following: 25 μliters of 260 mM $MgCl_2$; 50 μliters of 13 mM adenosine-5-triphosphate; 5 μliters each of 1 mM deoxyguanosine-5-triphosphate, deoxyadenosine-5-triphosphate, and deoxycytidine-5-triphosphate; 100 μliters of cells; and sufficient 0.05 M phosphate buffer (*p*H 7.4) to bring the total volume to 500 μliters after the addition of radioactive label. The appropriate thymine-*methyl*-^{3}H-containing precursor was added in the form of either ^{3}H-TTP, ^{3}H-TMP, ^{3}H-Tdr, or ^{3}H-TDP (New England Nuclear Corp.) to give a final specific activity of 5 μCi in approximately 10^{-3} μmoles of substrate. The reaction mixture was incubated at 37 C without agitation. At appropriate times, 50-μliter samples were removed to 3-mm chromatography paper discs (Whatman), and acid-insoluble counts were determined as previously described (13).

Density transfer. To characterize the products of synthesis, cells were grown in a minimal medium containing $^{15}NH_4Cl$ and ^2H_2O in place of $^{14}NH_4Cl$ and H_2O, and supplemented with 0.05% deuterated amino acids (Merck, Sharp, and Dohme of Canada, Ltd.), plus 4 mg of deuterated sugars per ml to provide a cellular DNA with a buoyant density of 1.738 instead of 1.703 gm/ml in cesium chloride. To reduce pools of "heavy" precursors, the cells were incubated in light medium for 15 min before freezing. These cells were freeze-treated as described and used as the "enzyme" preparation in the assay mixture. Therefore, in the density transfer experiments, the cellular DNA was ^{15}N-, ^{2}H-, and ^{14}C-labeled.

After 90 min of incubation, the remaining reaction mixture (usually 400 μliters, since two 50-μliter samples were removed for 0- and 90-min uptake analysis) was added to approximately 10^9 fresh cells and lysed by incubation for 5 min at 37 C in the presence of 1 mg of lysozyme per ml. Then 0.01 ml of 10% sodium dodecyl sulfate in 1× SSC was added to clear the lysate. Cesium chloride (CsCl) density gradient centrifugation of the whole lysate was carried out in a Spinco 50Ti fixed-angle rotor at 33,000 rev/min for 65 hr at 20 C. Density calculations were based on refractive index and calibrated by *B. subtilis* DNA of normal (LL), hybrid (LH), and heavy (HH) density. The density positions of LL, LH, and HH DNA at equilibrium were 1.703, 1.722, and 1.738 gm/ml, respectively. Details of the procedure for analysis of the distribution of radioactivity in the gradient were as previously described (2).

Crystalline pancreatic deoxyribonuclease I (EC 3.1.4.5) was purchased from Worthington Biochemical Corp. "Activated" thymus DNA was prepared by the method of Richardson (10).

Ultraviolet light irradiation. Exposure to ultraviolet light (UV) was usually carried out just before addition of the freeze-treated cells to the assay mixture. Details of UV exposure were as described elsewhere (4).

RESULTS

Incorporation of thymine precursors into DNA. Freeze-treated *B. subtilis* W23T$^-$, when incubated with ^{3}H-thymine derivatives and the other three deoxyribonucleotides, exhibits a variety of DNA incorporation patterns which depend on the type of precursor used (Fig. 1). In this system ^{3}H-thymine uptake is minimal. ^{3}H-Tdr uptake is initially rapid and then levels off. In the experiment from which the data presented in Fig. 1 were obtained, approximately 20 pmoles of Tdr was incorporated per 10^7 cells in the initial 10 min of synthesis. This represents about a 10% increment in DNA [10^7 cells possess 0.36 μg of DNA as determined by colorimetric analysis (8)].

TDP and TTP incorporation was continuous for the 90-min period of study, whereas TMP uptake was initially rapid and then slowed.

Exposure of the freeze-treated cells to 2,000 ergs/mm^2 of UV (resulting in less than 0.01%

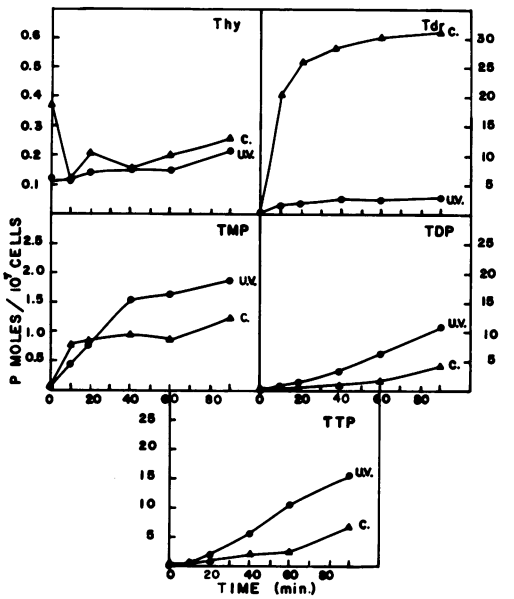

FIG. 1. *Incorporation of labeled thymine precursors into DNA. Polymerase assay mixture was as described in the text. A 100-μliter amount of freeze-treated log phase Bacillus subtilis W23T$^-$ (about 5 × 10^7 cells) was added as the enzyme preparation. Each ^{3}H-labeled precursor (shown in upper right hand corner of each frame) was present at a final specific activity of 986 pmoles/5 μCi. Samples (50 μliters) were removed for analysis of cold acid-insoluble radioactivity. The ultraviolet light dose was 2,000 ergs/mm^2.*

survivors) resulted in marked inhibition of Tdr uptake but stimulated the incorporation of TMP, TDP, and TTP (Fig. 1).

^{3}H-TTP product. A density shift analysis of product DNA, synthesized from the labeled precursors, provided the results illustrated in Fig. 2, 3, and 4. When freeze-treated cells are grown in heavy medium containing ^{14}C-thymine (cell DNA would be ^{15}N-, ^{2}H-, ^{14}C-labeled), any DNA product made *semiconservatively* in an assay mixture containing ^{3}H-labeled precursor should result in hybrid density (HL) banding of ^{3}H-labeled DNA in a CsCl gradient. On the other hand, if a "repair"-type synthesis occurred (limited incorporation of ^{3}H-label into parental DNA), the ^{3}H-label should band with the heavy parental DNA.

The product DNA in a ^{3}H-TTP assay was observed banding at the same density region as the heavy (HH) parental DNA (Fig. 2). UV-exposed cells produced a similar product pattern in the CsCl gradients. These results show that TTP incorporation in this system occurs by a "repair"-type synthesis which is stimulated by UV exposure of the cells.

^{3}H-Tdr product. A similar experiment was carried out with ^{3}H-Tdr used in place of ^{3}H-TTP during the polymerase assay. The product DNA banded in the hybrid density region (HL) only (Fig. 3). Therefore, Tdr incorporation was by a semiconservative mode of synthesis. This is substantiated by the appearance of about 20% of the parental ^{14}C-labeled DNA in the hybrid position. Little, if any, ^{3}H-Tdr was incorporated by a "repair"-type synthesis; therefore, Tdr is not available to the "repair"-type system under these conditions. However, UV exposure of the cell preparation before assay resulted in an inhibition of semiconservative synthesis and induced ^{3}H-Tdr utilization via repair synthesis (Fig. 3). When unlabeled deoxyribonucleosides were substituted for the unlabeled deoxyribonucleoside triphosphates in the assay mixture, the incorporation of ^{3}H-Tdr was reduced 70%.

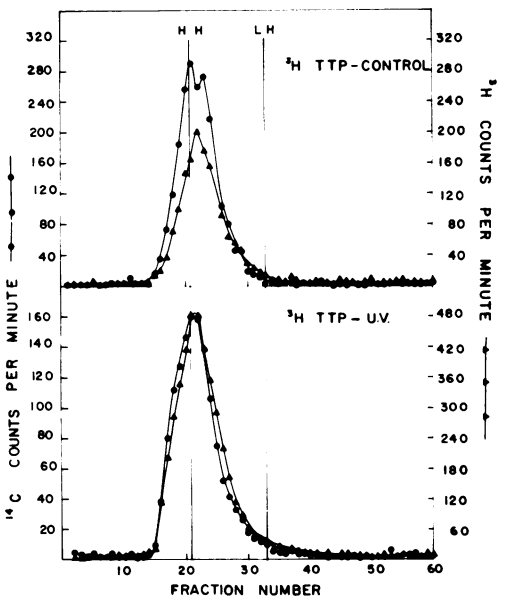

FIG. 2. *Pycnographic analysis of the DNA product synthesized from ^{3}H-TTP. Freeze-treated cells, grown in the heavy, ^{15}N-, ^{2}H-, ^{14}C-containing medium, were used as the enzyme preparation. Details of cell growth, DNA synthesis assay, and pycnographic analysis were as described in the text. The ultraviolet light dose was 2,000 ergs/mm^{2}. Incubation time in the enzyme assay mixture was 90 min. The specific activity of the ^{3}H precursor was 5 μCi in 782 pmoles.*

^{3}H-TMP and ^{3}H-TDP products. The products of ^{3}H-TMP and ^{3}H-TDP incorporation by the freeze-treated cells reflected DNA synthesis by a "repair"-type system (Fig. 4).

Association of the product DNA with the cell. The possibility that product DNA was synthesized externally to the cells and represented the action of enzymes released from the cell preparation by lysis was considered. If this were so, then the product DNA should be found in the supernatant fluid and not with the cells after centrifugation of the assay mixture. The results given in Table 1 were obtained with ^{15}N-, ^{2}H-grown cells. Similar results were obtained with cells grown in the light medium. The ^{3}H- and ^{14}C-labels represent distribution of product and parental DNA, respectively, at the end of a 90-min assay. The cells retained from 79 to 97% of the product DNA made using ^{3}H-TTP and an approximately equal amount of parental DNA. Therefore, most of the product DNA made from TTP is cell-associated. The results were similar when ^{3}H-Tdr was the labeled precursor.

Release of TTP- and Tdr-incorporating activity. We preincubated the freeze-treated cells in phosphate buffer only at 37 C for 90 min to determine whether precursor-incorporating enzymes were released under these conditions. When the preincubated cells were separated by centrifugation from the buffer solution after 90 min, only the cells showed ^{3}H-TTP- or ^{3}H-Tdr-incorporating activity (compare Fig. 5B and C). However, when calf thymus DNA (10 μg) was added (either "activated" or untreated DNA will suffice), the supernatant fraction showed both ^{3}H-TTP- and ^{3}H-Tdr-incorporating activities (Fig. 5B). ^{3}H-Tdr is probably not used as such but is converted to TTP. This may account for the delay observed (Fig. 5B, Tdr). However, we have no evidence to support this supposition.

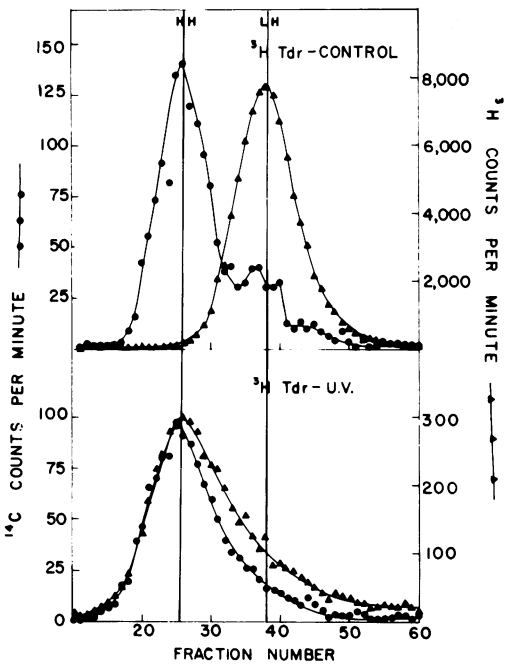

FIG. 3. *Pycnographic analysis of the DNA product synthesized from ^{3}H-Tdr. Details as given for Fig. 2. The specific activity of ^{3}H-Tdr was 5 μCi in 782 pmoles.*

As expected from the product DNA distribution study, insufficient DNA is released under these conditions (at least in suitable form) to serve as primer-template for the solubilized enzymes. Note the change in Tdr uptake kinetics when cells were first preincubated (Fig. 5C, Tdr). This was routinely observed. Cells which were not preincubated were also assayed as a control (Fig. 5A).

If, instead of phosphate buffer, the standard assay mixture was used for the preincubation period, the activity of the supernatant fraction was about 50% of that observed for incubation in phosphate buffer.

Sensitivity to deoxyribonuclease I. The addition of 25 μg of deoxyribonuclease I to the assay mixture did not block the incorporation of ^{3}H-TTP initially (Fig. 6). However, by 30 min a loss of both product and parental DNA was observed. The presence of deoxyribonuclease I did not grossly effect ^{3}H-Tdr incorporation; but loss of parental DNA was observed.

Precursor competition experiments. TTP and Tdr were not competitive precursors. The presence of Tdr at 100 times the concentration of ^{3}H-TTP in the same assay did not reduce the incorporation of ^{3}H-TPP label (Fig. 7). Similarly, the presence of TTP in 100-fold concentration over that of Tdr did not interfere with ^{3}H-Tdr uptake into DNA.

DISCUSSION

Freeze-treated *B. subtilis* W23 exhibits enhanced incorporation of TTP into cellular DNA. Product analysis by density shift techniques shows that ^{3}H-TTP, ^{3}H-TDP, or ^{3}H-TMP was incorporated into DNA by a "repair"-type synthesis. However, these cells incorporated ^{3}H-Tdr into their DNA by a semiconservative synthesizing mechanism. To explain the two seemingly independent DNA-incorporating systems, we currently favor an interpretation which invokes the existence of a semiconservative DNA-synthesizing system either functionally (e.g., unique substrates) or physically compartmentalized from a "repair"-type system. Evidence supporting this interpretation includes (i) the observation that exogenous TTP does not compete with Tdr nor Tdr with TTP for incorporation into DNA when both synthesizing systems are functioning simultaneously (Fig. 7), and (ii) while TTP is incorporated essentially only by the "repair"-type synthesis, there is little "repair"-

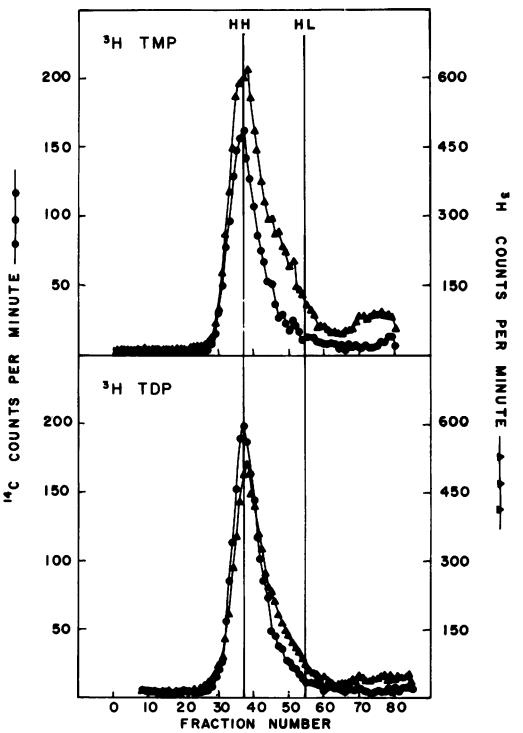

FIG. 4. *Pycnographic analysis of the DNA product synthesized from ^{3}H-TMP or ^{3}H-TDP. Details as given for Fig. 2. Specific activity of both ^{3}H-TMP and ^{3}H-TDP was 5 μCi in 986 pmoles.*

TABLE 1. *Distribution of parental and product DNA at end of in vitro synthesis*

Preparation	Counts/minute[a] in DNA when precursor was			
	^{3}H-TTP		^{3}H-Tdr	
	^{3}H	^{14}C	^{3}H	^{14}C
Expt. 1				
Cells	22,880	2,900	45,670	3,150
supernatant fraction	6,260	930	13,680	950
Expt. 2				
Cells	31,980	3,760	56,850	3,590
Supernatant fraction	930	150	4,730	40

[a] The total counts were measured at the end of the 90-min assay. The assay mixture was centrifuged at 5,000 rev/min for 15 min, and the supernatant fraction was sampled for acid-insoluble radioactivity. Parental DNA was prelabeled with ^{14}C-thymine.

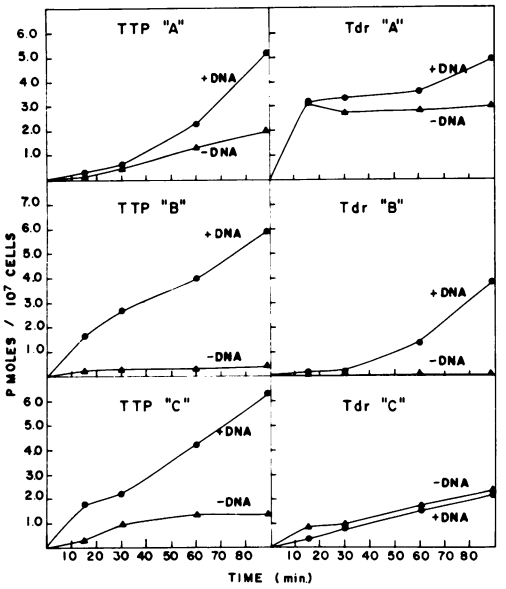

FIG. 5. *Release of TTP- and Tdr-incorporating activity during incubation in buffer. "A," Uptake by the cells of the indicated labeled precursor into DNA without preincubation. "B," Incorporating activity of the supernatant fluid from cells which had been preincubated for 90 min in phosphate buffer at 37 C. "C," Residual activity of the preincubated cells. "Activated" calf thymus DNA was present as indicated.*

type synthesis observed during Tdr incorporation into DNA (Fig. 2 and 3).

In this regard, it is of interest that *Escherichia coli* has recently been reported to be able to distinguish between thymine and Tdr in DNA synthesis (14).

A trivial explanation for our results would be that intact cells are impermeable to the phospho-

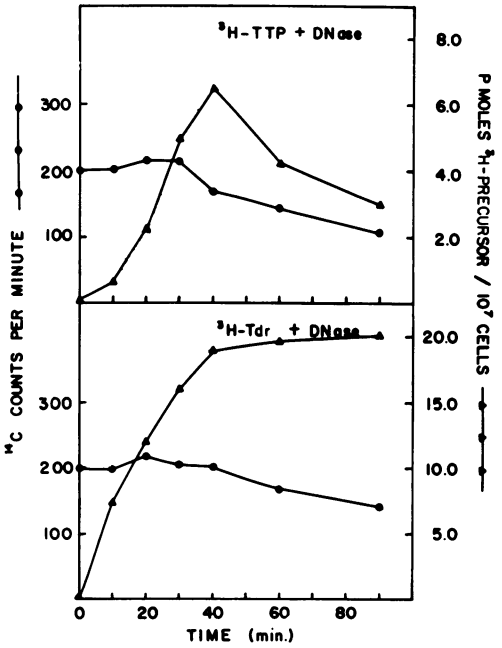

FIG. 6. *Effect on deoxyribonuclease (DNase) I on parental and product DNA. Immediately after addition of the freeze-treated cells to the assay mixture, 25 μg of deoxyribonuclease I was added. Assay of synthesis was as described in the text. The specific activity of ^{3}H-TTP or ^{3}H-Tdr is 5 μCi in 782 pmoles.*

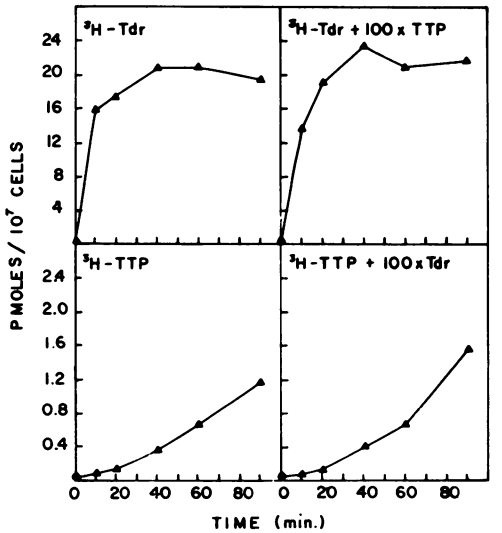

FIG. 7. *Precursor independence. Duplicate assays were run by using either ^{3}H-TTP or ^{3}H-Tdr (specific activity of 5 μCi in 445 pmoles). To one of each pair, cold Tdr or TTP, respectively, at 100 times the concentration of the labeled precursor, was added. DNA synthesis was determined as described in the text.*

rylated precursors while permitting the penetration of Tdr and that uptake of the phosphorylated precursors would result from cell rupture. Cell rupture would lead to the availability of polymerase activity and DNA in the supernatant fluid. However, several observations make this an unlikely explanation. These include: (i) the cellular association of the product DNA; (ii) the failure of deoxyribonuclease I to initially prevent TTP incorporation; and (iii) the lack of TTP uptake by the activity of the supernatnat fraction unless exogenous DNA is added. Because we have not yet differentiated between incorporation by the viable and nonviable intact cells in the system, we cannot rule out the possibility that compartmentalization represents differential incorporation by the two populations.

Our data tempt us to suggest that the TTP-incorporating system is located at, or within, the periplasmic region and the system required for semiconservative synthesis is located elsewhere in the cell. [See Ryter (11) for a review of the morphological evidence for DNA association with mesosomes and membrane.] Based on this reasoning, our data would be compatible with the following interpretation. Added phosphorylated precursors are accessible to the "repair"-type synthesizing system only. The freeze-treatment enhances this accessibility by inducing additional changes in the cell surface. However, the permeability barrier to penetration of phosphorylated DNA precursors does not allow access of these precursors to the replicase system responsible for semiconservative synthesis. The "barrier" hypothesis is supported by the data in Fig. 6, which show that the DNA product of Tdr incorporation is inaccessible to added deoxyribonuclease activity. Deoxyribonucleosides can move through the barrier and are utilized in semiconservative synthesis after being converted (perhaps only inside the cell) to the proper phosphorylated precursor form. The introduction by UV of dimers in the DNA would set in motion the enzyme sequence involved in excision-repair. Excision-repair may or may not involve the same periplasmic-associated enzyme system responsible for the "repair"-like action utilizing exogenously supplied phosphorylated precursors. However, UV-induced repair synthesis occurs with *either* TTP or Tdr as the radioactive precursor.

The nature of the "compartmentalization" of precursors for DNA synthesis in *B. subtilis* can only be conjectured at this time. However, several attributes of *B. subtilis* are worth bearing in mind in considering the possibilities. Certain strains of *B. subtilis* can be transformed. This process involves the passage, processing, and integration of DNA fragments. Vesicles of unknown function in the periplasmic region (7) and "membranous bodies" (15) may be involved in transformation (15). DNA in *B. subtilis* is released extracellularly into the medium during cell growth and especially during development of competence (12). It appears reasonable to conclude that in *B. subtilis* the surface is active in DNA metabolism.

A superlethal dose of UV stops Tdr incorporation by the semiconservative mode and induces repair synthesis. The relationship between UV-induced repair synthesis (4) and "repair"-type synthesis in these freeze-treated cells is not currently known. They may be identical, or two independent systems. A detailed analysis of the effects of UV on DNA synthesis in freeze-treated cells will be given in a subsequent report.

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