UTILIZATION OF DEOXYRIBONUCLEOSIDE TRIPHOSPHATES IN THE CELLULAR SYNTHESIS OF DNA BY BACILLUS SUBTILIS *

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Summary. Deoxyribonucleoside triphosphates (dNTPs) are incorporated into DNA by Bacillus subtilis W23. Frozen cells show greater incorporating activity than toluene-treated cells. Incorporation is also observed in untreated cells. The incorporation of thymidine (Tdr) when substituted for dTTP has also been observed. The Tdr incorporating system differs from the dTTP incorporating system in being UV sensitive, exhibiting different kinetics of incorporation and in exhibiting less stringent requirements for magnesium and dNTPs.

Recently several studies on the utilization of deoxyribonucleoside triphosphates (dNTPs) in the synthesis of DNA by treated Escherichia coli have been reported. The treatments include exposure to EDTA (1) or toluene (2) as a means of altering cell permeability. B. subtilis treated with Brij 58 has recently been shown to utilize dNTPs in the synthesis of DNA (3). We have observed that B. subtilis will also incorporate dNTPs into DNA. Our initial results show that untreated or previously frozen B. subtilis cells were more proficient than toluene-treated cells in incorporating dTTP into DNA. A number of interesting observations relating to the utilization of B. subtilis of either labeled dTTP or thymidine (Tdr) in the synthesis of DNA have been made. The results indicate that the dTTP incorporating system may differ from that involved in Tdr incorporation into DNA.

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Materials and Methods. B. subtilis W23 and a thymine (T^-) requiring derivative were used for these studies. Cells were grown in the minimal medium described by Anagnostopoulos and Spizizen (4). The $W23T^-$ were grown in the presence of 10 µg/ml of thymine. Where indicated, the cellular DNA of $W23T^-$ was labeled for several generations by the addition of ^{14}C-thymine. The cells were harvested in exponential growth as determined by assay of colony forming units. Petroff-Hauser counts showed approximately 5×10^7 "cells" (very few single cells were observed, most cells exist as chains equal, on the average, to approximately four of the smallest single cells seen).

The harvested cells were washed once in SSC buffer (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and then resuspended in SSC in half of the original volume. Where applicable, the cells were frozen at this point. For the irradiation studies exposure took place before freezing. Freezing of the cells was accomplished by immersion in liquid nitrogen and the frozen preparation stored at -20°C. The cells were rapidly thawed at 37°C, harvested and resuspended in 0.05 M phosphate buffer (pH 7.4) at one tenth the original culture volume.

Toluene treatment was by the method of Moses and Richardson (2). A harvested and washed cell suspension in phosphate buffer was made 1% toluene and shaken at 37°C for 10 min prior to use.

The reaction mixture used for assay of DNA synthesis was similar to that described by Moses and Richardson (2) for their assay of toluene-treated cells. The reaction mixture contained the following: 25 µl of 260 mM $MgCl_2$; 50 µl of 13 mM ATP; 5 µl each of 1 mM of dATP, dCTP and dGTP; 100 µl of cells; and sufficient 0.05 M phosphate buffer (pH 7.4) to bring the total volume to 500 µl after the addition of radioactive label. The appropriate tritium label was added in the form of either ^3H-dTTP, ^3H-dTMP, ^3H-Tdr or ^3H-thymine (New England Nuclear Corporation) to give a final specific activity of 5 µcuries

in approximately 8 x 10^{-4} µmoles of substrate. The reaction mixture was incubated at 37°C without agitation. At appropriate times 50 µl samples were removed to 3 MM Whatman chromatography paper discs and acid insoluble counts determined. Selected batches of ^{3}H-dTTP were checked chromatographically and found to contain less than 1% of other radioactive products.

Crystalline pancreatic DNAase (DNAase I) was purchased from Worthington Biochemical Corporation. "Activated" thymus DNA was prepared by the method of Richardson (5).

CsCl isopycnic analysis of cell lysate was carried out as previously described (6).

Results and Discussion. B. subtilis, previously frozen for one hour, were at least 10 times more effective than toluene treated cells in incorporating ^{3}H-dTTP into acid insoluble material (Fig. 1). When untreated B. subtilis (cells harvested and held at room temperature) were used as the enzyme source both ^{3}H-dTTP and ^{3}H-Tdr were incorporated into DNA (Table I). The

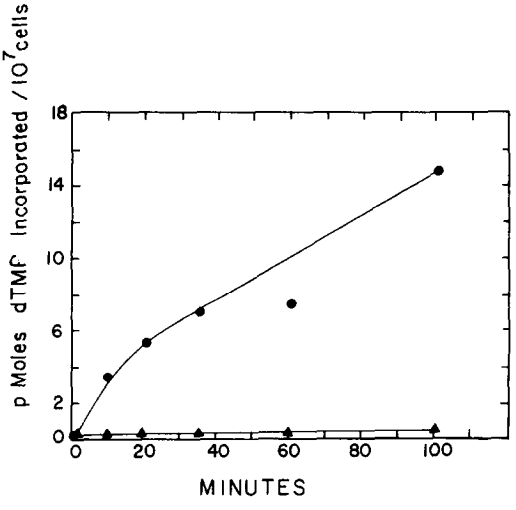

Figure 1 Appearance of ^{3}H-label from ^{3}H-dTTP into the acid insoluble fraction of frozen or toluene-treated B. subtilis. Cell preparation and enzyme assay were as described in text. Frozen cells (●); Toluene treated cells (▲).

TABLE I

EFFECT OF FREEZING ON INCORPORATION OF RADIOACTIVE PRECURSOR INTO DNA BY B. SUBTILIS

PreTreatment of Cells	pMoles incorporated/10^7 cells [a] ^{3}H-dTTP	^{3}H-Tdr	^{3}H-dTMP
None	0.4	24.0	-
-20°C, 1 hr	4.5	8.4	-
-20°C, 14 hrs	11.3	7.3	6.2

a) Complete assay mixture except thymine derivatives substituted for dTTP as indicated. Incubated for 90 min at 37°C.

1 hour freezing treatment enhanced ^{3}H-dTTP incorporation but reduced ^{3}H-Tdr utilization. In this experiment a 14 hour period at -20°C enhanced ^{3}H-dTTP incorporation. However in other studies, freezing for 24 hours or longer was observed to reduce ^{3}H-dTTP incorporation. ^{3}H-dTMP was incorporated to the same extent at ^{3}H- Tdr (Table I). [α-^{32}P]-dATP was incorporated into DNA at a rate similar to that observed for dTTP (data not shown). Since a one hour freeze treatment stimulated dNTP incorporation, it was routinely employed in the remaining studies.

The acid insoluble ^{3}H-labeled product made by B. subtilis was confirmed to be DNA as follows: a) B. subtilis DNA was ^{14}C-labeled by cell growth for many generations in the presence of ^{14}C-thymine. These cells were then used as the enzyme source for DNA synthesis in a reaction mixture containing ^{3}H-dTTP. At the end of a 90 min incubation period the cells were lysed with lysozyme (1 mg/ml). In the presence of 40 µg/ml of DNAase I, 90% of both the ^{14}C- and ^{3}H-label was solubilized in 90 min at 37°C. b) Cells, ^{14}C- and ^{3}H-labeled as described above (a), were lysed and the lysate subjected to CsCl gradient analysis. The banding patterns observed for both ^{14}C-DNA and

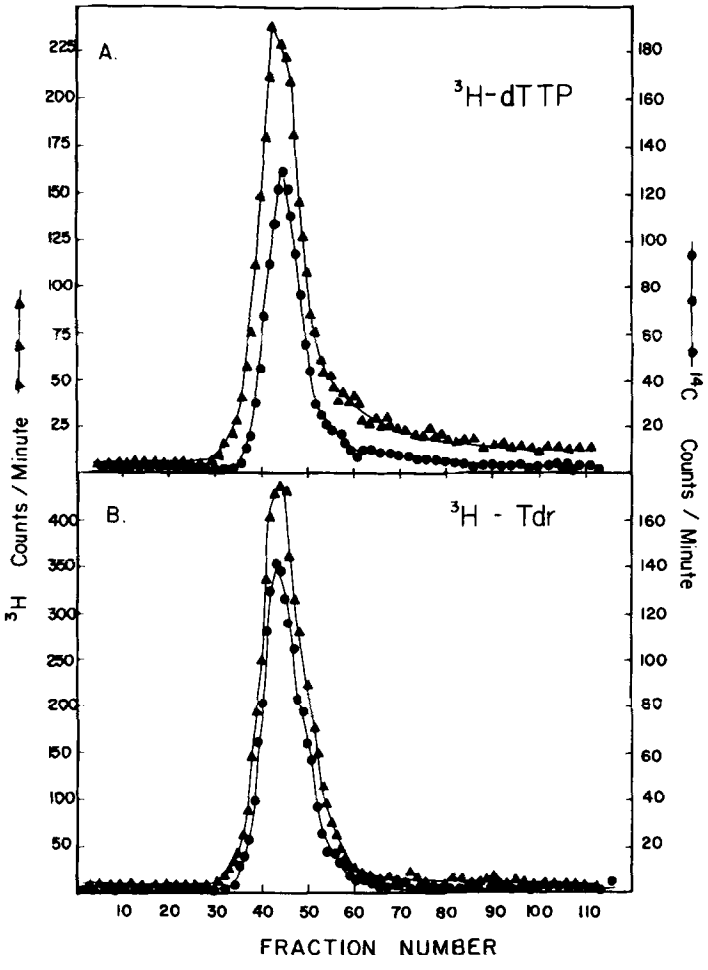

Figure 2 CsCl isopycnic banding pattern for ^{14}C- and ^{3}H-labeled DNA. The ^{14}C-label is in DNA synthesized during cellular growth. ^{3}H-label represents incorporation by cells held in a reaction mixture containing either ^{3}H-dTTP (A) or ^{3}H-Tdr (B). Details are given in text . Increasing density from right to left.

^{3}H-DNA coincided (Fig. 2A). These results showed that ^{3}H-label was in DNA and the tritium-containing DNA existed as a double stranded duplex. A similar observation was made using ^{3}H-Tdr in place of dTTP (Fig. 2B).

The requirements for DNA synthesis in this system was determined. ^{3}H-dTTP incorporation was completely dependent on the presence of Mg++, almost totally dependent on the presence of the dNTPs,

TABLE II

REQUIREMENTS FOR DNA SYNTHESIS
IN B. SUBTILIS

Condition	Activity (%) [a] Radioactive Precursor ^{3}H-Tdr	^{3}H-dTTP
Complete	100	100
+DNA	192	236
-ATP	48	88
-Mg++	24	1
-dGTP,dATP,dCTP	32	6

a) Cells held at -20°C for 14 hours prior to use. Reaction mixture incubated 90 min at 37°C.

but not greatly stimulated by ATP (Table II). The requirement for Mg++ or dNTPs was not as stringent for ^{3}H-Tdr incorporation into DNA (Table II). ATP appeared to be stimulatory for Tdr incorporation (Table II).

The addition of "activated" calf thymus DNA enhanced radioactive precursor incorporation (Table II). We are currently investigating the cause of this stimulation.

UV irradiation is known to block DNA replication in bacterial cells including B. subtilis (7). The effects of UV exposure on the incorporation of ^{3}H-dTTP and ^{3}H-Tdr is shown in Fig. 3. In this experiment the cells received 2000 ergs/mm^2, resulting in less than 0.01% survivors when assayed by the colony forming unit method. The incorporation of ^{3}H-dTTP into DNA was not reduced (Fig. 3A and 3B), whereas ^{3}H-Tdr incorporation was markedly inhibited (Fig. 3C and 3D). These data clearly show that UV irradiation selectively effects two DNA replicating systems: the one utilizing dTTP as substrate being insensitive, while the Tdr incorporating system is markedly inhibited.

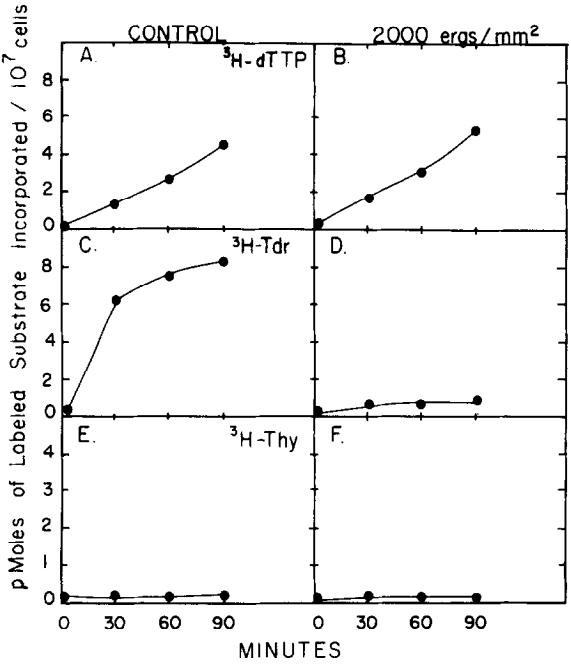

Figure 3 A comparison of ^{3}H-dTTP, ^{3}H-Tdr or ^{3}H-thymine incorporation into DNA in UV exposed or control cells.

^{3}H-thymine incorporation is minimal under the conditions used for assay (Fig. 3E). UV was without apparent effect on ^{3}H-thymine incorporation (Fig. 3F).

In addition to showing that B. subtilis can utilize dNTPs, in DNA synthesis, these results provide preliminary evidence for the existence of two DNA synthesizing systems in B. subtilis. One polymerizing system utilizes dNTPs, requires magnesium and all four dNTPs, and is UV resistant. The other system incorporates Tdr into its DNA, is extremely sensitive to UV, is not as completely dependent on magnesium or the presence of dNTPs, and differs in its kinetics of incorporation. We are now in the process of determining whether or not the DNA product of each system reflects a semiconservative or repair-type synthesis.

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