

Uptake and Incorporation of Thymine, Thymidine, Uracil, Uridine, and 5-Fluorouracil into the Nucleic Acids of *Bacillus subtilis*

WALTER F. BODMER AND SUSAN GREETHER

Department of Genetics, Stanford University School of Medicine, Palo Alto, California

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ABSTRACT

BODMER, WALTER F. (Stanford University School of Medicine, Palo Alto, Calif.), AND SUSAN GREETHER. Uptake and incorporation of thymine, thymidine, uracil, uridine, and 5-fluorouracil into the nucleic acids of *Bacillus subtilis*. *J. Bacteriol.* **89**:1011-1014. 1965.—From 55 to 95% of uracil, uridine, or 5-fluorouracil (FU) added to the culture medium is incorporated into the acid-insoluble fraction of cells of *Bacillus subtilis* strains SB 19 (prototroph) and SB 503 (FU-resistant). Thymine is poorly incorporated (less than 1%); thus, the incorporation of thymidine is limited (less than 12%) by the rapid degradation of the nucleoside to thymine, probably by the enzyme thymidine phosphorylase. Uracil, uridine, and FU were not incorporated into the deoxyribonucleic acid (DNA) of either strain, whereas all the incorporated thymidine was found in the DNA.

The preparation of H³-labeled deoxyribonucleic acid (DNA) from prototrophic *B. subtilis* was described by Bodmer and Schildkraut (1964). They showed that there is a limited uptake of thymidine-H³, as in *Escherichia coli* (Rachmeler, Gerhart, and Rosner, 1961). Rachmeler et al. (1961) explained the limited uptake in *E. coli* by the induction of the enzyme thymidine phosphorylase which decomposes thymidine into thymine plus deoxyribose-1-phosphate (Friedkin and Roberts, 1954). Free thymine does not enter the cell in appreciable quantities (Crawford, 1958).

In this paper, we describe the incorporation of radioactive thymine, thymidine, uracil, uridine, and 5-fluorouracil (FU) into the nucleic acids of a prototrophic strain of *B. subtilis*, and also into an FU-resistant strain sensitive to 5-fluorouridine (FUR) and 5-fluorodeoxyuridine (FUDR), which was studied in the hope of gaining some insight into the mechanism of the resistance. Thymidine phosphorylase activity of these strains is also described.

MATERIALS AND METHODS

The prototrophic *B. subtilis* strain, SB 19 (Nester and Lederberg, 1961), is completely inhibited by 1 μg/ml of FU, FUR, or FUDR. The FU-resistant strain chosen for this study, SB 503, was resistant to 50 μg/ml of FU, but was completely inhibited by 1 μg/ml of FUR or 1 μg/ml FUDR. It was selected on minimal agar

plates containing 3 μg/ml of FU. Thymidine-methyl-H³ and uridine-H³ were purchased from The New England Nuclear Corp., Boston, Mass. Thymine-2-C¹⁴, uracil-2-C¹⁴, and FU-2-C¹⁴ were purchased from Calbiochem. Pancreatic deoxyribonuclease and ribonuclease were obtained from Worthington Biochemical Corp., Freehold, N.J. The unlabeled FU, FUR, and FUDR were obtained from Ronald B. Ross and Hoffmann-La Roche, Inc., Nutley, N.J.

The uptake of labeled pyrimidine bases or nucleosides into cells growing in supplemented minimal medium was followed after addition of 0.1 μc/ml of labeled compounds during the log phase. Total counts were measured by spotting 10 μliter samples of the culture on Whatman GF/C glass filter papers. Uptake into whole cells was assayed by filtering 0.1 ml of the cultures through Millipore filters (HA; pore size, 0.45 μ), and washing the samples with 20 ml of dilute saline citrate (0.015 M NaCl and 0.0015 M trisodium citrate). Incorporation into the acid-insoluble fraction was assayed by adding 0.1 ml of the whole culture to 0.9 ml of 0.5 N HClO₄ followed by filtration through a Millipore filter and washing with 5.0 ml of 0.5 N HClO₄ and 15.0 ml of dilute saline citrate. The filters were dried and counted on a Tri-Carb liquid scintillation counter with the use of a toluene-2,5-diphenyloxazole (PPO)-1,4-bis[2-(5-phenyloxazolyl)]-benzene (POPOP) scintillator as described by Bodmer and Ganesan (1964). Counting efficiencies for H³ and C¹⁴ were approximately 10 and 40%, respectively.

Crude lysates were generally prepared after 30 min of contact with a given isotope; 10-ml cul-

tures were resuspended in 0.2 ml of saline-ethylenediaminetetraacetate (EDTA; Marmur, 1961), and incubated at 37 C with 5.0 mg of lysozyme for 15 min. Next, 10 μ liters of 25% sodium lauryl sulfate were added to complete lysis, followed by the addition of 0.1 ml of saline-EDTA, 0.1 ml of 5 M NaClO₄, and 0.5 ml of chloroform-ethanol (5:1). The preparation was shaken for 30 min and then centrifuged for 20 min at 13,000 \times *g*. The nucleic acids were precipitated from the supernatant with 0.5 ml of cold 95% ethyl alcohol, and then redissolved in 0.1 M phosphate buffer (pH 7.5). The amount of label incorporated into DNA and RNA was determined from the release of acid-soluble counts after treatment of the lysate with deoxyribonuclease and ribonuclease and also from the distribution of label on pycnographic fractionation in a CsCl density gradient. Samples of the lysate were incubated for 60 min at 37 C with 50 μ g/ml of deoxyribonuclease or ribonuclease, or both, or with no enzyme. Carrier DNA (3 μ g) was then added to each, and the samples were precipitated in cold 10% trichloroacetic acid. (Subsequently it has been found that the addition of carrier DNA is unnecessary.) Both the filter and the filtrate were counted. Preparative pycnography of the lysates in a CsCl density gradient was carried out as described by Ganesan and Lederberg (1964). Samples of 10 μ liters were counted on filter paper as described above. Location of the DNA in the fractions was established by transformation with SB 168 (*try₂⁻*; Nester and Lederberg, 1961) as the recipient strain.

Thymidine phosphorylase activity was assayed by following the conversion of thymidine to thymine chromatographically. (The activity measured was the conversion of thymidine into thymine. Conclusive evidence that the enzymatic activity is thymidine phosphorylase would require the identification of deoxyribose-1-phosphate as a product of the reaction.) Thymidine-H³ (5 μ c/ml) was added to the cultures after 3.5 hr of growth in a supplemented minimal medium. Portions of 0.5 ml were withdrawn at intervals, precipitated in 0.5 ml of cold 10% trichloroacetic acid, passed through a Millipore filter, and the filtrate was collected. A sample (20 μ liters) of each filtrate was run on a descending chromatogram for 6 hr with the use of Whatman no. 1 filter paper and a solvent system consisting of ethyl acetate-formic acid-water (60:5:35, v/v/v; Heidelberger and Kaldor, 1959). Excess cold thymidine and thymine were run with each sample for visual detection of the base and nucleoside under ultraviolet light. After drying the chromatograms, the thymine and thymidine spots were cut out, placed in scintillation vials, and counted as described above with the toluene-PPO-POPOP scintillator solution.

To identify FU, the nucleic acids were first hydrolyzed in formic acid at 175 C for 30 min. The hydrolysate was then run on a descending chromatogram with the use of Whatman no. 1

filter paper and a solvent system of isopropanol-hydrochloric acid-water (85:22:8, v/v/v; Heidelberger and Kaldor, 1959).

TABLE 1. Comparative uptake of labeled bases and nucleosides by SB 19 and SB 503*

Isotope	Per cent of added label in washed cells		Per cent of added label in acid-insoluble fraction	
	SB 19	SB 503	SB 19	SB 503
Thymine-2-C ¹⁴ ..	0.56	0.004	0.63	0.003
Thymidine-methyl-H ³	11.73	12.08	11.15	11.70
Uracil-2-C ¹⁴	93.54	77.86	89.00	63.20
Uridine-H ³	95.27	45.68	94.61	36.40
5-Fluorouracil-2-C ¹⁴	63.21	78.06	58.42	54.46

* The uptake of labeled compounds was determined as described in Materials and Methods; 0.1 μ c of label was added to a 10.0-ml log-phase culture growing in minimal medium. Maximal incorporation of the isotopes occurred by 30 min, at which time the above determinations were made.

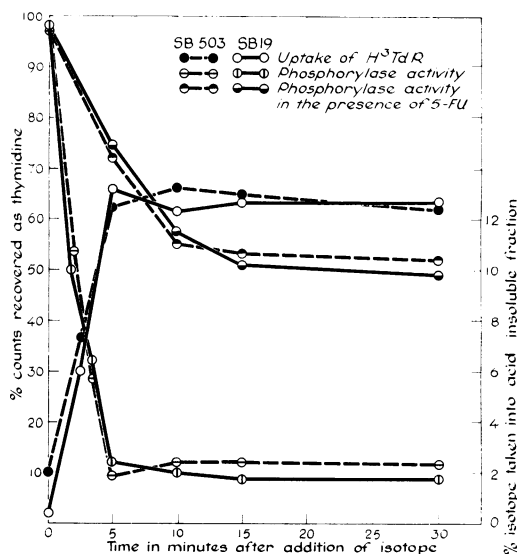


FIG. 1. Thymidine uptake and concomitant thymidine phosphorylase activity of SB 19 and SB 503. The simultaneous uptake of thymidine-H³ into the acid-insoluble fraction of the cells and its conversion to thymine + deoxyribose-1-phosphate by thymidine phosphorylase was followed as described in Materials and Methods. Where indicated, 10 μ g/ml of 5-fluorouracil were added with the isotope. Approximately 90% of the counts chromatographed were recovered in the thymidine and thymine spots. The percentage of counts in thymidine are based on this total.

TABLE 2. Percentage of incorporated counts made acid-soluble after nuclease treatment of extracted lysates*

Isotope	Control		Deoxyribonuclease		Ribonuclease		Deoxyribonuclease + ribonuclease	
	SB 19	SB 503	SB 19	SB 503	SB 19	SB 503	SB 19	SB 503
Thymidine-methyl- H^3	0.66	0.54	99.50	98.70	1.83	0.98	97.77	99.13
Uridine- H^3	3.0	1.72	13.33	15.76	98.23	97.43	98.56	98.00
Uracil- $2-C^{14}$	2.76	4.71	11.50	9.78	96.42	84.50	95.01	96.02
5-Fluorouracil- $2-C^{14}$	5.33	2.97	24.92	15.41	99.31	91.84	98.73	99.63

* Nuclease treatment of the extracted lysates was carried out as described in Materials and Methods. Each determination was based on a total of approximately 10^5 count/min.

RESULTS

In both SB 19 and 503, maximal incorporation of the isotopes occurred within 30 min after addition of the label. The per cent incorporation of each isotope into whole cells and into the acid-insoluble fractions is given in Table 1. Both strains showed a limited incorporation of thymidine to a level of about 12%. There was a low but significant incorporation of thymine into the prototroph SB 19, whereas uptake of thymine by the FU-resistant strain SB 503 was completely blocked. Uracil and uridine were almost completely incorporated by SB 19 within 30 min, but were incorporated to a lesser extent by SB 503. However, the uptake of FU was somewhat higher in SB 503 than in SB 19, although the percentage incorporated into the acid-insoluble fraction was the same for both strains.

The incorporation of thymidine was rapid initially, but leveled off within 5 min (Fig. 1), by which time 88% of the thymidine was converted to thymine. The simultaneous addition of $10 \mu\text{g}/\text{ml}$ of cold thymine with the thymidine H^3 did not alter the kinetics of conversion, whereas $10 \mu\text{g}/\text{ml}$ of FU resulted in a 50% inhibition of enzyme activity. Cell-free culture filtrates showed no detectable thymidine phosphorylase activity.

The percentage of the incorporated counts made acid-soluble by deoxyribonuclease and ribonuclease treatments of the extracted lysates is given in Table 2. After deoxyribonuclease digestion, 98 to 99% of the thymidine- H^3 counts in each strain were acid-soluble; 95 to 100% of H^3 or C^{14} counts from uridine, uracil, or FU were acid-soluble after ribonuclease digestion. The relatively high percentage of counts from uridine, uracil, and FU made acid-soluble by deoxyribonuclease treatment is probably due to residual ribonuclease activity in the deoxyribonuclease which was used. An example of a CsCl pycnogram of a uridine- H^3 -labeled lysate is shown in Fig. 2. Almost all the counts are at the bot-

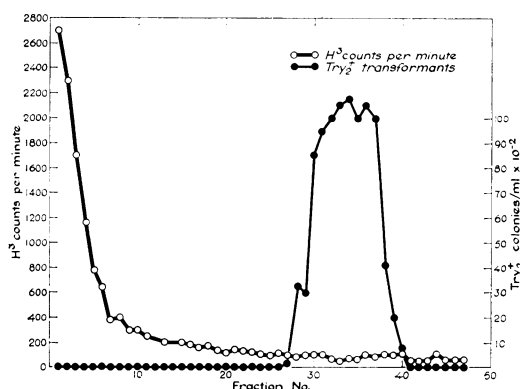


FIG. 2. Preparative pycnography of a uridine- H^3 -labeled SB 19 lysate. Preparative pycnographic fractionation was performed as described by Ganesan and Lederberg (1964). Isotopic content of the fractions was determined by counting 10 μliter samples as described in Materials and Methods. Biological activity was tested by adding 10 μliters of each fraction to 0.05 ml of 1 M MgCl_2 , to which was then added 1.0 ml of competent SB 168 (try_2^-) at an approximate titer of 5×10^8 cells per milliliter. Transformation was carried out according to Nester and Lederberg (1961).

tom of the tube, corresponding to the expected position of the ribonucleic acid (RNA). Less than 1.1% are in the DNA region, as located by the distribution of try_2^+ transforming activity. Moreover, most of these counts are probably due to trailing of the RNA peak. Results from similar experiments with uracil and FU, as well as uridine, showed less than 3% incorporation into the DNA of either strain. Chromatography of a hydrolysate of the combined 10 heaviest fractions from a preparative gradient with SB 19 labeled with FU- $2-C^{14}$ showed that more than 94% of the counts were still in FU.

DISCUSSION

The low efficiency of incorporation of thymine compared with that of thymidine in *B. subtilis*

parallels the results reported by other workers with *E. coli* (Crawford, 1958; Brockman, Davis, and Stutts, 1960; White and Nichol, 1963). The limited incorporation of thymidine in *B. subtilis* can be explained by its rapid conversion to thymine by thymidine phosphorylase. The FU-resistant strain shows the same level of thymidine phosphorylase activity as the control (SB 19) and has a similar uptake pattern. There is, thus, no obvious indication of the biochemical change which is responsible for the FU resistance of SB 503.

The lack of incorporation of FU into DNA and its incorporation in RNA is consistent with results reported by other workers (Gordon and Staehelin, 1959; Horowitz, Saukkonen, and Chargaff, 1960).

Uracil is incorporated into the DNA (as well as the RNA) of Ehrlich ascites cells (Harbers, Chandhuri, and Heidelberger, 1958). Uridine-H³ has also been used in the preparation of H³-labeled DNA from *E. coli* phage λ (Burgi, 1963). Labeling of the DNA by uracil and uridine presumably follows the methylation pathway described by Friedkin (1957). The failure of incorporation of exogenous uracil and uridine into the DNA of prototrophic *B. subtilis* deserves further study.

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