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Method for Restricting Incorporation of Radioactivity from ^{3}H-Thymidine into Deoxyribonucleic Acid Only During Outgrowth of Spores of *Bacillus cereus* T

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When heat-activated spores of *Bacillus cereus* T (*thy*$^-$) were germinated and grown in medium containing ^{3}H-thymidine, a significant amount of radioactivity was incorporated into ribonucleic acid and deoxyribonucleic acid (DNA). A method was developed to restrict the incorporation of radioactivity from ^{3}H-thymidine into DNA only. This was accomplished by labeling the cells with ^{3}H-thymidine in the presence of 2 mg of 2-deoxyadenosine per ml, 250 μg each of uracil, cytosine, and guanosine per ml, and 500 μg of adenosine per ml. Under these conditions, 97% of the radioactivity incorporated into cold trichloroacetic acid-insoluble material was associated with DNA only. In the absence of these compounds, DNA contained only 72% of the total radioactivity incorporated into cold acid-insoluble material.

The effect of chloramphenicol on deoxyribonucleic acid (DNA) synthesis during outgrowth of *Bacillus cereus* T (*thy*$^-$) was studied. The incorporation of ^{3}H-thymidine into cold trichloroacetic acid-insoluble material was used as a measure of DNA synthesis. When spores of *B. cereus* T (*thy*$^-$) were germinated and allowed to incorporate ^{3}H-thymidine for 30 min or longer, subsequent addition of chloramphenicol invariably resulted in the loss of significant amounts of radioactivity previously incorporated into cold acid-insoluble material. Because chloramphenicol does not interact with DNA directly, the loss of radioactivity could not be attributed to the degradation of DNA. These findings suggested that some radioactivity from ^{3}H-thymidine was probably incorporated into ribonucleic acid (RNA). This possibility could also explain the observed loss of radioactivity from cold acid-insoluble material, because a significant amount of RNA synthesized during early outgrowth is unstable. Thus, it was decided to examine the classes of nucleic acids which incorporated the radioactivity from ^{3}H-thymidine and to develop a method for selective labeling of DNA. The development of such a method was also necessary for investigating the relationship between protein and DNA synthesis during outgrowth of *B. cereus* T (*thy*$^-$).

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MATERIALS AND METHODS

Organism. A thymine-requiring auxotroph of *B. cereus* T isolated by Steinberg (15) was used in this study.

Growth and preparation of spores. Spores of the thymine-requiring strain were prepared in thymine-nutrient broth (Thy-NB) medium by procedures described previously (15). The final spore crops were lyophilized and stored at −20 C. All experiments with these spores were carried out at 30 C.

Media. The Thy-NB medium used for sporulation of the thymine-requiring auxotroph has been described previously (15). The synthetic medium used in this study was a modification of chemically defined growth and sporulation (CDGS) medium originally formulated by Nakata (11). The final composition in grams per liter is: L-glutamic acid, 1.8; L-leucine, 0.8; L-valine, 0.3; L-threonine, 0.170; L-methionine, 0.070; L-histidine, 0.050; L-serine, 0.6; L-aspartic acid, 0.4; L-arginine (free base), 0.28; L-isoleucine, 0.22; L-tryptophan, 0.1; L-glycine, 0.1; L-lysine, 0.08; L-phenylalanine, 0.05; adenosine, 0.5; L-alanine,

0.1; thymidine, 0.01; $FeSo_4 \cdot 7H_2O$, 0.005; $CuSO_4 \cdot 5H_2O$, 0.005; $ZnSO_4 \cdot 7H_2O$, 0.0005; $MnSO_4 \cdot 2H_2O$, 0.0305; $MgSO_4 \cdot 7H_2O$, 0.412; $(NH_4)_2SO_4$, 2.0; $CaCl_2 \cdot 2H_2O$, 0.007; glucose, 4.0; and potassium phosphate buffer, *p*H 7.0, 0.1 M final concentration. This medium will hereafter be termed as "supplemented CDGS medium." The modifications, where used, are described in the text.

Isolation of nucleic acids and analysis by MAK column chromatography. One gram of labeled spores or vegetative cells (in a wet pellet) was disrupted by grinding with acid-washed 120-μm glass beads in a prechilled (−20 C) mortar and pestle for 30 min in the presence of 0.1 g of sodium dodecyl sulfate (SDS) and 0.1 g of makaloid. Cold 0.05 M tris-(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (*p*H 7.8) containing 0.05 M ethylenediaminetetraacetic acid (EDTA) and 2% SDS was added. The crude extract was deproteinized three times with phenol saturated with the above-mentioned Tris buffer. Bulk nucleic acids were precipitated by the addition of three volumes of 95% ethyl alcohol at −20 C and storage overnight. Phenol was removed by three washings of precipitate in 95% ethyl alcohol at 4 C. Fifty optical density units (at 260 nm; OD_{260}) were loaded on a methylated albumin-kieselguhr (MAK) column. The column was washed with 250 ml of 0.3 M NaCl and then eluted with 400 ml of a 0.3 to 1.5 M NaCl linear gradient. Samples of 75 drops each were collected and checked for OD_{260}. A 1-ml sample from each fraction was used for measuring radioactivity in Bray's scintillation fluid. Refractive index of every fifth fraction was measured in a refractometer.

Hydrolysis of RNA and analysis of hydrolysate by paper chromatography. DNA from the bulk nucleic acid was removed by deoxyribonuclease treatment in 0.001 M magnesium acetate buffer, *p*H 5.0, containing 0.1 unit of deoxyribonuclease per ml and dialysis against 0.001 M magnesium acetate for 8 to 10 hr at room temperature. The mixture was deproteinized three times with phenol saturated with 0.01 M Tris buffer, *p*H 7.8. RNA was precipitated with three volumes of 95% ethyl alcohol at −20 C overnight. The phenol was removed by washing the precipitate three times in 95% ethyl alcohol. The precipitate was dissolved in 1.0 ml of 0.3 N KOH and incubated at 37 C for 18 to 20 hr. The digestion mixture was subsequently neutralized with Bio-Rad sulfonic acid resin AG-50W-X8 in pyridine form. The mixture was filtered through glass wool to remove the resin. The resulting filtrate was subjected to paper chromatography for the separation of adenosine monophosphate (Ap), guanosine monophosphate (Gp), cytidine monophosphate (Cp), and uridine monophosphate (Up).

A simple paper chromatographic technique developed by Yamazaki (*unpublished data*) and modified by Gay Samuelson (Ph.D. thesis, Univ. of Wisconsin, 1969) was used to separate the mononucleotides. Two OD_{260} units each of markers Cp, Ap, Gp, and Up were added to the sample. The sample was equally divided and allowed to flow under gravity through weighted syringes, luer-locked to perspex blocks, to a spot 2 cm from one end of strips (40 by 5 cm) of Whatman DE-81 paper. Each block was machined with a fine-bore drill allowing the sample to flow slowly from the syringe through the block to the paper. The sample absorption zone was small despite the large sample volume, since in the absence of salt the mononucleotides would be strongly attracted by the tertiary amino groups on the paper. For application of a 1.0-ml sample, approximately 15 min was required. Thereafter both papers were oven-dried. One paper was chromatographed in ascending manner to a line 20 cm from the origin in 1.0 M acetic acid to separate Ap from Cp (Up and Gp remained at the origin). At the same time, the second paper was chromatographed in similar manner in 3.0 M formic acid to separate Up from Gp (Ap and Cp moved with the solvent front). The chromatography time was approximately 1 hr. Papers were dried in an oven, and marker mononucleotide positions were recorded under ultraviolet light. Each paper was cut into 1-cm segments, and radioactivity was monitored in each segment by scintillation counting in 2,5-diphenyloxazole-1,4-bis-2-(5-phenyloxazolyl)-benzene scintillation fluid.

Method for determining the fraction of radioactivity incorporated into RNA of vegetative cells labeled with ^{3}H-thymidine. Heat-shocked spores (20 min, 75 C, 2.5 mg of spores/ml) were germinated and grown up to 6 hr in medium containing ^{3}H-thymidine at 1 μCi per 100 μg per ml. Two samples of equal volume were taken and processed in the following manner. To one sample was added an equal volume of 10% trichloroacetic acid containing 100 μg of unlabeled thymidine per ml. The mixture was stored in ice for 1 to 2 hr and subsequently membrane-filtered (Millipore). The precipitate collected on the filter was washed three times with 5 ml of 5% trichloroacetic acid containing 50 μg of unlabeled thymidine per ml and three times with 5 ml of 95% ethyl alcohol. The filter paper was dried and monitored for radioactivity. Another sample was added to an equal volume of 2 N NaOH and incubated at 37 C for 18 to 20 hr. The mixture was neutralized with HCl, and 75 μg of carrier DNA per ml was added, followed by addition of cold 10% trichloroacetic acid containing 100 μg of unlabeled thymidine per ml; acid-insoluble material was collected, and radioactivity was monitored as described for the first sample. The fraction of radioactivity lost due to alkaline hydrolysis was calculated and assumed to represent the fraction of radioactivity from ^{3}H-thymidine being incorporated into RNA.

Materials. ^{3}H-thymidine and 2-deoxyadenosine were obtained from Schwarz BioResearch and Schwarz/Mann, respectively.

RESULTS

Effect of chloramphenicol on ^{3}H-thymidine incorporation during outgrowth. Heat-shocked spores were germinated in the medium containing 10 μg of ^{3}H-thymidine per ml (27 mCi/mmole). At various times, an appro-

priate volume was transferred to flasks containing chloramphenicol. The final concentration of chloramphenicol was 20 μg/ml. Incorporation of radioactivity into cold trichloroacetic acid-insoluble material was followed before and after the transfer of culture to chloramphenicol. As shown in Fig. 1, incorporation of radioactivity stopped immediately after transfer of the culture to chloramphenicol. At times later than 30 min, transfer of the culture to chloramphenicol led to a decrease in the cold acid-precipitable radioactivity. The rate of loss of radioactivity was greater immediately after transfer to chloramphenicol and decreased at later times.

MAK column chromatography of nucleic acids isolated from ^{3}H-thymidine-labeled spores and vegetative cells. The results presented in Fig. 1 made it necessary to examine the distribution of radioactivity from ^{3}H-thymidine into various nucleic acid fractions. Heat-shocked spores were inoculated into the medium containing ^{3}H-thymidine at 1 μCi per 100 μg per ml. The culture was divided into two equal volumes and incubated at 30 C. Vegetative cells were harvested after 8 hr of growth and washed extensively. The other culture was incubated until the completion of sporulation, and spores were harvested and washed repeatedly. Bulk nucleic acid was isolated from vegetative cells and spores by the procedure described above. Approximately 2.5 mg (50 OD_{260} units) of bulk nucleic acid was fractionated on MAK column, and radioactivity and OD_{260} were monitored in each fraction (Fig. 2). A very significant amount of radioactivity from ^{3}H-thymidine was found associated with various species of RNA.

Analysis of hydrolyzed RNA by paper chromatography. The ^{3}H label in thymidine used was in the methyl group. Thus, it was reasonable to assume that the component of RNA which received the radioactivity from ^{3}H-thymidine was probably a minor base component containing a methyl or hydroxymethyl group. The conversion of ^{3}H-thymidine to Cp, Up, Ap, or Gp would have resulted in the loss of radioactivity due to the removal of methyl group. Therefore it was very unlikely that the radioactivity in RNA was associated with these nucleotides. This possibility was confirmed by the analysis of hydrolyzed RNA by means of paper chromatography. RNA from ^{3}H-thymidine-labeled spores was isolated and hydrolyzed in 0.3 N KOH. Chromatography was carried out on Whatman DE-81 paper as described above. One sample was chromatographed in 1 M acetic acid, while the other was

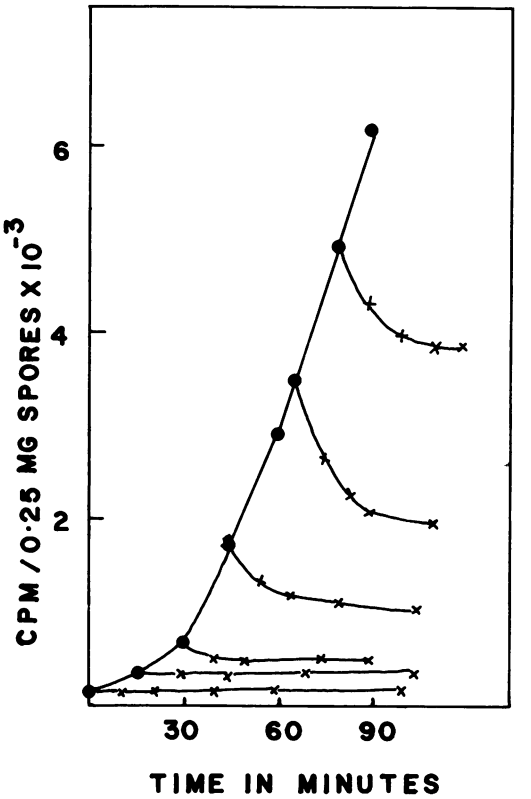

FIG. 1. *Effect of time of addition of chloramphenicol on ^{3}H-thymidine incorporation during outgrowth. Lyophilized spores were heat-shocked in distilled water and then centrifuged for 10 min at 5,000 × g, and the pellet was resuspended in water at 2.5 mg/ml. An appropriate volume of the spore suspension was inoculated (0.25 mg/ml) into supplemented CDGS medium (see text) containing ^{3}H-thymidine at specific activity of 1 μCi per 10 μg per ml. The culture was incubated at 30 C with mild shaking in a water bath. At indicated intervals, 5-ml samples were transferred to separate flasks containing chloramphenicol, and radioactivity incorporated into cold trichloroacetic acid-insoluble material was measured by the procedure described previously (15). Symbols: (●) control, no addition; (×) chloramphenicol added at times indicated.*

chromatographed in 3 M formic acid. The results of the experiment are shown in Fig. 3. The major peak of radioactivity was associated with the solvent front in 3 M formic acid (Fig. 3b). This peak, however, was not due to Ap or Cp, because it did not appear at expected positions for Ap and Cp in 1 M acetic acid. We have not attempted to identify this labeled component. We can only suggest that it is probably a minor base component. This appears to be supported by the high specific ac-

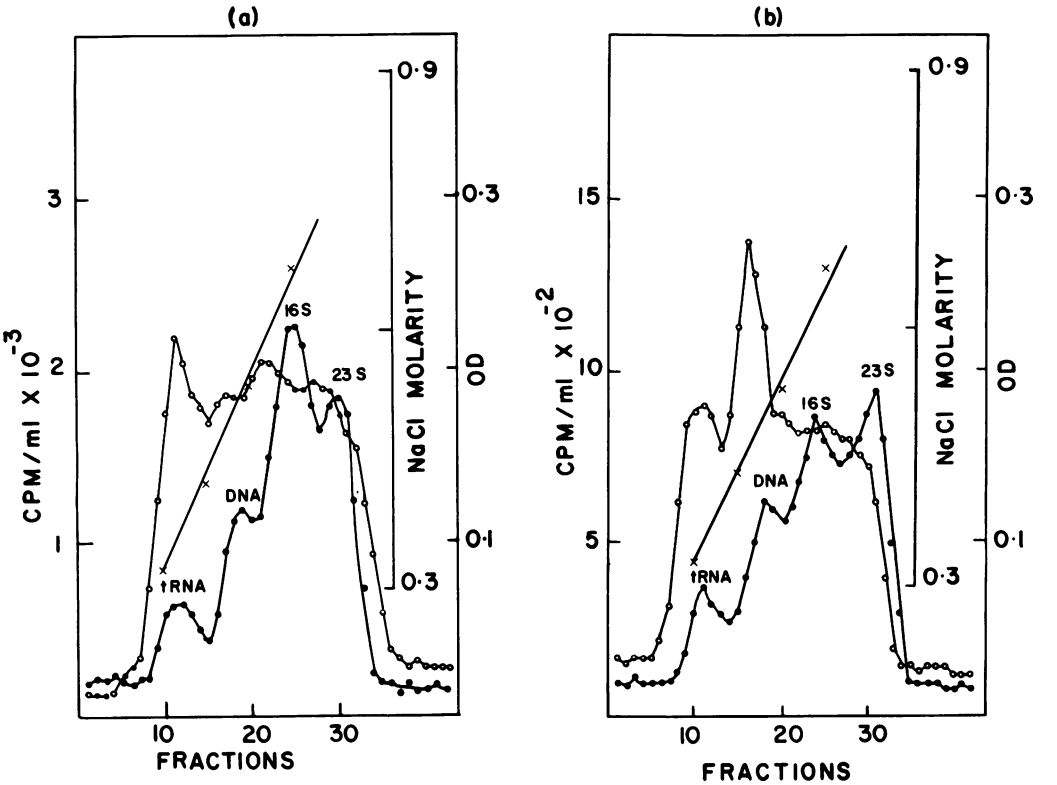

FIG. 2. *MAK column chromatography of bulk nucleic acid from ^{3}H-thymidine-labeled spores and vegetative cells. Nucleic acid was isolated and fractionated on MAK column by elution with a linear 0.3 to 1.5 M NaCl gradient. Radioactivity and optical density at 260 nm were recorded for each fraction. (a) Nucleic acid from spores; (b) nucleic acid from vegetative cells; (○) counts/min; (●) optical density; (×) NaCl molarity.*

tivity of counts in transfer RNA (tRNA) region in MAK column chromatography results (Fig. 2a).

Development of conditions for restricting the incorporation of ^{3}H-thymidine into DNA only. Budman and Pardee (4) have reported that deoxyadenosine competitively inhibits the activity of thymidine phosphorylase, thus preventing the degradation of thymidine to thymine in *Escherichia coli*. We expected that conversion of thymidine to other nucleotides might be occuring via its initial degradation to thymine. For this reason we decided to study the incorporation of radioactivity from ^{3}H-thymidine into DNA and RNA in the presence of 2 mg of 2-deoxyadenosine per ml. The effect of addition of adenosine (A), uracil (U), cytosine (C), and guanosine (G) together with 2-deoxyadenosine was also studied. In these experiments alkaline hydrolysis was used to study the distribution of radioactivity into RNA and DNA. Heat-shocked spores were germinated in medium containing 100 μg of ^{3}H-thymidine per ml (2.7 μCi/mmole) and grown for 6 hr under various conditions described in Table 1, and the distribution of counts in alkali stable and alkali hydrolyzable fractions under various conditions was determined. In the presence of 2-deoxyadenosine alone, 94% of the radioactivity incorporated was restricted to DNA only. This was further improved to 97% by addition of cold U, G, and C (250 μg/ml each) and A (500 μg/ml). Comparatively, when no additions were made, only 72% of the radioactivity incorporated was recovered in DNA.

MAK column chromatography of nucleic acids from spores labeled with ^{3}H-thymidine in the presence of 2-deoxyadenosine and A, U, G, and C. Because addition of deoxyadenosine and A, U, G, and C restricts the incorporation of radioactivity from ^{3}H-thymidine into DNA of vegetative cells, parallel experiments were conducted to determine the behavior of ^{3}H-thymidine-labeled spores under similar conditions. Bulk nucleic acid was isolated from ^{3}H-thymidine-labeled spores obtained in the presence of deoxyadenosine

and A, U, G, and C, respectively. Approximately 2.5 mg was fractionated on MAK column by using a 0.3 to 1.5 M linear NaCl gradient, and OD_{260} and radioactivity were recorded for each fraction. As seen in Fig. 4, only one peak of radioactivity appeared which coincided with the elution peak of DNA.

DISCUSSION

Exogeneous thymidine is very poorly incorporated into DNA of wild-type *E. coli*. Addition of thymidine to the medium leads to the induction of thymidine phosphorylase in *E. coli* (12) which cleaves thymidine to thymine. Thus, a large proportion of exogenous thymidine is degraded rather than incorporated into DNA (12). In *Bacillus subtilis*, thymidine incorporation is limited by the rapid degradation of thymidine to thymine, probably by the same enzyme (1). Thymidine itself, however, may not be the actual inducer of this enzyme. Razzel and Cashyap (13) have suggested that the inducer could be deoxyribose phosphate which accumulates as a result of deoxynucleoside phosphorylase activity.

Thymine, whether produced by the degradation of thymidine or exogenously added, is poorly incorporated into DNA. The poor utilization of exogenous thymine has been related to the limited availability of deoxyribosyl donors (9). This is supported by the observation that addition of deoxyadenosine to the medium leads to a more efficient incorporation of thymine (4). Deoxyadenosine can act as a source of deoxyribosyl for converting thymine to thymidine. This can occur via a transdeoxy-

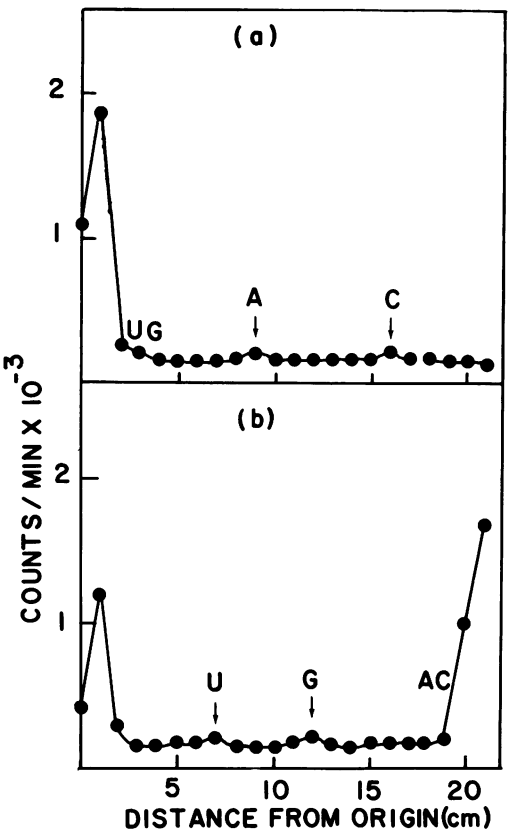

FIG. 3. *Paper chromatography of nucleotides obtained by alkaline hydrolysis of RNA. RNA from vegetative cells labeled with ^{3}H-thymidine was isolated and hydrolyzed as described in the text. Chromatography on Whatman DE-81 paper was done in two different solvent systems. In each case, unlabeled nucleotides were added as markers and their positions were identified under ultraviolet light. Each paper was cut into 1-cm segments, and radioactivity was monitored for each segment (for details, see text). (a) Chromatography in 1 M acetate; (b) chromatography in 3 N formic acid. UG and UC represent mixtures of uracil and guanosine, and uracil and cytosine, respectively.*

TABLE 1. *Effect of nucleosides on the distribution of radioactivity into DNA and RNA after ^{3}H-thymidine incorporation*[a]

Treatment	Counts/min[b] (DNA + RNA)	Counts/min[c] (DNA)	Radioactivity in DNA (%)
No treatment (control)	16,302	11,791	72.33
2-Deoxyadenosine (2 mg/ml)	25,020	23,520	94.00
2-Deoxyadenosine (2 mg/ml); uracil, cytosine, and guanosine (250 μg/ml; adenosine (500 μg/ml)	18,027	17,405	96.55

[a] Heat-activated spores were germinated at 0.25 mg/ml in three different flasks containing supplemented DCGS medium plus ^{3}H-thymidine at the specific activity of 1 μCi per 10 μg per ml. Adenosine in the medium was replaced by 15 μg of inosine per ml to initiate germination. Flask 2 also contained 2-deoxyadenosine, while flask 3 contained 2-deoxyadenosine together with uracil, cytosine, guanosine, and adenosine at concentrations described above. All three cultures were incubated at 30 C for 6 hr. At the end of 6 hr, eight samples of 1 ml each were taken from each flask. Four samples were immediately processed for measuring the radioactivity incorporated into cold trichloroacetic acid-insoluble material; the remaining four samples were subjected to alkaline hydrolysis before the radioactivity in cold acid-insoluble material was measured.

[b] Average of four samples of acid-insoluble material without alkaline hydrolysis.

[c] Average of four samples of acid-insoluble material after alkaline hydrolysis.

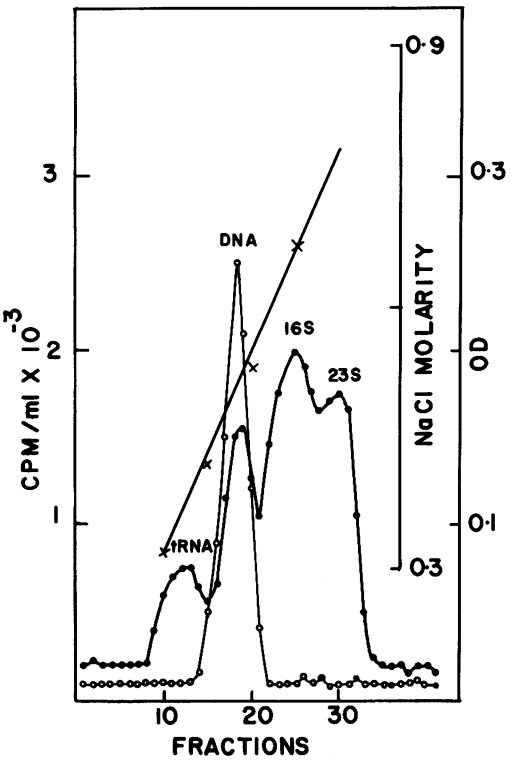

FIG. 4. *MAK column chromatography of bulk nucleic acid from spores labeled with ^{3}H-thymidine, in the presence of 2-deoxyadenosine (2 mg/ml), adenosine (500 μg/ml), guanosine (250 μg/ml), cytosine (250 μg/ml), and uracil (250 μg/ml). The procedure used was the same as described in legend to Fig. 2. Symbols:* (○) *counts/min;* (●) *optical density;* (×) *NaCl molarity.*

ribosylation or coupled deoxynucleoside phosphorylation reactions, or both (3). Deoxyadenosine increases the uptake of thymidine also (2). Recently Budman and Pardee (4) showed that deoxyadenosine stimulates thymidine incorporation in two ways. First, it prevents the conversion of thymidine to thymine by competitively inhibiting the activity of thymidine phosphorylase; and second, it can provide deoxyribose for the reformation of thymidine from thymine (4). Uridine also is effective in stimulating thymidine incorporation; this, however, does not provide deoxyribose and acts primarily by competitively inhibiting thymidine phosphorylase and also by preventing the formation of inducer(s) required for the induction of this enzyme (4). Uridine stimulates the incorporation of thymidine but inhibits the incorporation of thymine in thymine-requiring mutants (5 through 8), because thymine cannot be converted to thymidine due to the inhibition of thymidine phosphorylase (4). Thus, deoxyadenosine appears to be the more useful compound because it stimulates the incorporation of both thymine and thymidine.

Aminopterin has been successfully used to obtain thymineless mutants in several bacteria. In most cases, however, the mutants obtained by this method retain the poor thymine incorporation characteristic of wild type and require high concentrations of thymine to grow (7). It has been observed that these mutants grow very well on thymidylate but poorly on thymidine or thymine. Harrison (7) suggested that thymidylate is dephosphorylated at the cell surface with the simultaneous incorporation of the resulting thymidine, whereas exogenous thymidine is rapidly converted to thymine. Thus, separate routes probably exist for the utilization of dephosphorylated nucleotide and exogenous nucleoside (10).

The results described in this paper demonstrate that cells of *B. cereus* T undergoing outgrowth or vegetative growth contain enzymes capable of converting thymidine to other nucleotides which are subsequently incorporated into RNA. At least a few of these enzymes must be absent in dormant spores and are synthesized de novo. This is illustrated by the fact that no radioactivity from ^{3}H-thymidine is incorporated into cold acid-insoluble material when chloramphenicol is added at zero time (Fig. 1) even though RNA-synthesizing machinery is known to be intact in dormant spores (14). Recently Tanooka et al. (16) presented evidence that several enzymes involved in DNA metabolism are limiting or almost absent in dormant spores of *B. subtilis;* these include thymidine-, deoxycytidine-, and thymidylate kinases, which are synthesized de novo during germination and outgrowth, reaching a maximum at the onset of DNA replication. Our findings indicate that some of the enzymes involved in conversion of thymidine to RNA precursors are also synthesized during outgrowth.

2-Deoxyadenosine inhibits the conversion of thymidine to precursors of RNA synthesis. Thus, the first step involved in this process may be the conversion of thymidine to thymine since 2-deoxyadenosine is an inhibitor of thymidine phosphorylase (4).

The ^{3}H-thymidine used in these experiments was labeled in the methyl group. No radioactivity was found associated with Ap, Up, Gp, or Cp in RNA obtained from cells labeled with thymidine-*methyl*-^{3}*H*. However, the conversion of thymidine-*methyl*-^{3}*H* to

these precursors cannot be ruled out. It could have occurred yet not been detected, because the methyl group would have been lost in this process. The component of RNA which receives the radioactivity from ^{3}H-thymidine appears to be a minor base component. Since the ^{3}H label in thymidine was in the methyl group, it is reasonable to assume that the unidentified labeled minor base component derived from labeled thymidine retains the radioactivity in a methyl or hydroxymethyl group. This also suggests that metabolism of ^{3}H-thymidine in this organism does not involve a demethylation reaction.

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