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Production and Expression of dTMP-Enriched DNA of Bacteriophage SP15

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Normal DNA of *Bacillus subtilis* phage SP15 contains approximately equimolar quantities of dTMP and a hypermodified nucleotide, 5-dihydroxypentyl-dUMP (DHPdUMP). Deoxythymidine (dThd) rescue of phage DNA synthesis in 5-fluorodeoxyuridine (FUdR)-inhibited cultures resulted in the synthesis of SP15 DNA containing enhanced levels of dTMP and correspondingly reduced levels of DHPdUMP. This rescued system was used to probe possible roles of DHPdUMP in phage development. The results suggested that normal levels of DHPdUMP were not required for proper transcription of phage DNA, but normal amounts of DHPdUMP were indispensable for phage assembly and/or DNA maturation. The amount of exogenous dThd required to rescue phage DNA synthesis in FUdR-inhibited cultures was 20-fold higher than the concentration required to rescue cellular replication, whereas the same low concentrations of dThd sufficed to rescue viral and bacterial DNA syntheses in aminopterin-inhibited cultures. Normal SP15 DNA was made in rescued, aminopterin-inhibited cultures. We suggest that FUdR (but not aminopterin) partially suppresses biosynthesis of the hypermodified nucleotide and that there is a barrier to replacement of DHPdUMP by dTMP; therefore, exceptionally large amounts of dThd must be salvaged in FUdR-inhibited cultures to force replacement of the unusual nucleotide by dTMP.

SP15 is the largest known virus that infects *Bacillus subtilis* (27, 30-34). Its DNA weighs at least 250 megadaltons (24; M. Stodolsky, personal communication). Dihydroxypentyl-dUMP (DHPdUMP) replaces half the dTMP in SP15 DNA (4, 13, 22). The presence of the hypermodified nucleotide destabilizes viral DNA, as evidenced by the fact that SP15 nucleic acid has a melting temperature of only 61.5°C in 150 mM NaCl-15 mM sodium citrate (22, 30), compared with 69.3°C for poly(dAMP-dTMP) (23). Three molecules of glucose, as maltose and glucose-1-phosphate, are attached to each DHPdUMP residue (36; C. R. Brandon, Ph.D. thesis, Yeshiva University, New York, N. Y., 1974; M. S. Walker, Ph.D. thesis, University of Texas at Houston, Houston, Tex., 1977). Glucose-1-phosphate confers an unusually high buoyant density on SP15 DNA (22). In CsCl, SP15 DNA has a buoyant density of 1.762 g/ml (22, 24, 36), whereas poly(dGMP-dCMP) has a density of 1.760 g/ml (18). When SP15 DNA is incubated in 0.3 N KOH, glucose-1-phosphate is removed and the buoyant density declines to 1.703 g/ml (22). Release of glucose-1-phosphate is accompanied by fragmentation of the phage DNA (24).

Originally, it was thought that DPHdUMP arose by post-replicational modification of dUMP in nascent DNA (24). Recently, however, DHPdUTP has been identified in formic acid extracts of SP15 phage-infected cells, implying that the hypermodified nucleotide is, in fact, synthesized de novo (36).

Little is known about the biological relevance of DHPdUMP. Walker and Mandel (36) showed that SP15 DNA with a reduced DHPdUMP content could be made during deoxythymidine (dThd) rescue of 5-fluorodeoxyuridine (FUdR)-inhibited cultures. In lieu of mutants defective in the biosynthesis of DHPdUTP, the system devised by Walker and Mandel (36) could supply some preliminary ideas about the role of DHPdUMP in phage development. Accordingly, we studied phage development in dThd-rescued, FUdR-inhibited cultures. The results of these investigations suggest that DHPdUMP may not be required for proper transcription of the SP15 template; however, the hypermodified nucleotide is required for proper replication and DNA maturation. Moreover, the results suggest that, under normal conditions, dTMP and DHPdUMP may not be randomly inserted into the phage genome.

MATERIALS AND METHODS

Phage and bacteria. *B. subtilis* W23 is ATCC 23059. SP15 was obtained from M. Mandel in November 1975.

Medium. Except where noted, the medium used was NBS (21) supplemented with 10 mM glucose. Additional supplements are indicated below.

Conditions of infection. Bacteria were grown at 37°C. When the cells reached 2×10^8/ml, they were harvested by centrifugation at 9,000 × *g* for 1 min and suspended in 0.9 volume of fresh, warm medium that contained 200 μM 6-(*p*-hydroxyphenylazo)-uracil, a selective inhibitor of bacterial DNA synthesis (5, 9, 25). In experiments involving FUdR and dThd, the new medium also contained 1 mg of uridine per ml to prevent any incorporation of 5-fluorouracil into RNA (10) and to suppress dThd phosphorylase activity (3, 6, 34), thereby augmenting salvage of both deoxynucleosides. Five minutes after the cells were suspended, 0.1 volume of medium containing the phage was added. The final multiplicity of infection was 10 PFU per cell.

Preparation of crude extracts. For enzymological studies, 100-ml samples were removed from infected cultures, mixed with an equal volume of chilled (4°C) medium containing 50 μg of chloramphenicol per ml, and centrifuged at 9,000 × *g* for 1 min at 4°C. The pellets were suspended in 2 ml of chilled (4°C) 50 mM Tris-hydrochloride (pH 7.9)–50 mM KCl. The cells were disrupted by sonic treatment with a Branson instrument. Debris was removed by centrifugation at 12,000 × *g* for 30 min at 4°C. The clarified supernatants were dialyzed against three 1,000-volume changes of the buffer mentioned above and stored frozen at −40°C.

Purification of phage. Conditions of infection were basically the same as those described above, except cells were not harvested and suspended in new medium before infection. After lysis was complete, phage were initially concentrated by the salt-polyethylene glycol procedure (35). Precipitated phage were collected by low-speed centrifugation and suspended in 0.01 volume of 50 mM Tris-hydrochloride (pH 7.9)–300 mM KCl–1 mM $MgCl_2$–5 μM $ZnSO_4$ (phage buffer). Phage were banded in CsCl (2, 36), dialyzed into phage buffer, and stored over $CHCl_3$ at 4°C.

Isolation of DNA from mature phage. Phage were first dialyzed into 50 mM Tris-hydrochloride (pH 7.9)–10 mM sodium-EDTA (pH 7.9). The suspension was warmed to 37°C, and sodium dodecyl sulfate was added to 1%. The suspension clarified and became viscous immediately. Five minutes later, Pronase was added to 50 μg/ml, and the mixtures were incubated at 37°C for 2 h. After one extraction with cold phenol–isoamyl alcohol–$CHCl_3$ (25:24:1), the DNA was ethanol precipitated (36), dissolved into 300 mM NaCl–30 mM sodium citrate (pH 7.5), and dialyzed against three 1,000-volume changes of the same buffer. DNA solutions were stored over $CHCl_3$ at 4°C.

Isolation of DNA from infected cells. Infected cells were harvested by centrifugation and suspended in 0.2 volume of 50 mM Tris-hydrochloride (pH 7.9)–10 mM sodium-EDTA (pH 7.9)–1 mg of egg white lysozyme per ml–1% sodium dodecyl sulfate. The mixtures were incubated at 37°C for 1 h and extracted three times with cold phenol–isoamyl alcohol–$CHCl_3$ (25:24:1). The rest of the procedure is described above.

Other procedures. Protein concentration was determined by the method of Lowry et al. (17). Serum blocking power of extracts was measured by the method of DeMars (8) and is reported in units of phage equivalents per infected cell. Antiserum specific for SP15 (serum value, 12,000) was prepared by J. Konvicka and supplied by M. Mandel. The lytic enzyme assay is detailed elsewhere (9). Incorporation of labeled precursors into DNA was determined by the method of Lembach and Buchanan (16).

Materials. All unlabeled chemicals were obtained from Sigma. All labeled compounds were obtained from Schwarz/Mann, except $^{32}P_i$ which was purchased from New England Nuclear.

RESULTS

Inhibition of DNA synthesis by FUdR and rescue of DNA synthesis by dThd. FUdR is taken up by cells and phosphorylated to fluoro-dUMP by dThd kinase (6, 14, 34). Flouro-dUMP binds covalently to dTMP synthetase, thereby inhibiting the de novo synthesis of dThd nucleotides and, in turn, DNA replication (14). DNA synthesis can be restored by supplying an exogenous source of dThd (6, 14, 34). Such dThd-rescued conditions do not result from reactivation of dTMP synthetase; rather, the cell can now generate dTMP (and, hence, dTTP) via the salvage pathway, a pathway unaffected by FUdR (6, 14, 34).

Bacterial and viral DNA syntheses were equally sensitive to inhibition by FUdR (Fig. 1 and 2), and the sensitivity of viral replication to FUdR remained unchanged throughout the program (Fig. 2). Replication of the phage genome stopped or declined manyfold within 2 min of FUdR addition (data not presented).

Concentrations of exogenous dThd as low as 1 or 2 μg/ml provided measurable rescue of bacterial DNA synthesis, although 50 to 100 μg of dThd per ml was required for complete restoration of cellular replication (Fig. 3 and 4). Somewhat surprisingly, SP15 DNA synthesis proved harder to rescue than did bacterial replication. Concentrations of exogenous dThd as high as 50 μg/ml rescued SP15 DNA synthesis to only barely detectable levels, and even 5 mg of dThd per ml failed to completely restore viral replication (Fig. 3 and 4). For the experiments shown in Fig. 3, FUdR and dThd were added at 5 min preinfection. In many other experiments, the time of addition of deoxynucleosides relative to the time of infection was varied; in all cases, the degree to which a given concentration of dThd rescued viral DNA synthesis was the same (Fig. 4).

Buoyant density of replicating SP15 DNA in dThd-rescued, FUdR-inhibited cultures. Walker and Mandel (36) reported that SP15 DNA made under rescued conditions contained enhanced levels of dTMP and correspondingly

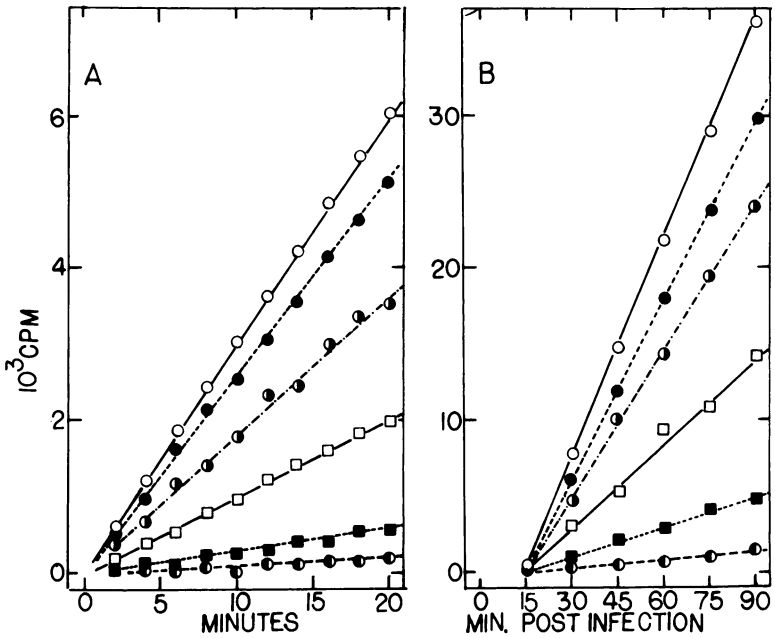

FIG. 1. *Effect of FUdR on bacterial and SP15 DNA syntheses. (A) Bacterial DNA synthesis. Ten-milliliter portions of a bacterial suspension were harvested by centrifugation and suspended in 9 ml of warm (37°C) medium containing FUdR. Five minutes later, 1 ml of medium containing [G-^{3}H]adenine (50 μCi/ml) was added. Samples (1 ml) were removed periodically over a 20-min interval, and the amount of label in DNA was estimated (16); controls showed that incorporation of adenine deviated from linearity after 20 min. (B) SP15 DNA synthesis. The cells were harvested and suspended in fresh medium as described above except that 200 μM 6-(p-hydroxyphenylazo)-uracil was also present. Five minutes later, 1 ml of medium containing SP15 and labeled precursor was added. Symbols: no FUdR (○); FUdR at 5 (●), 10 (◑), 20 (□), 50 (■), and 100 (◐) μg/ml.*

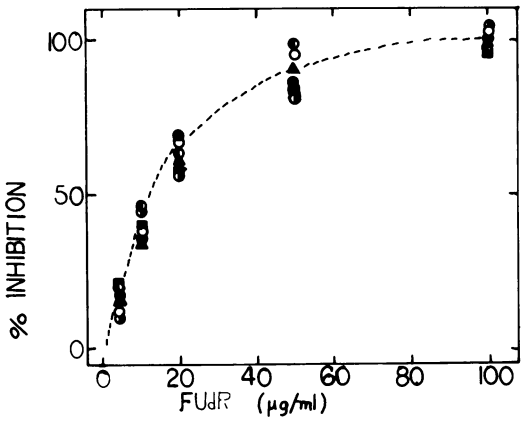

FIG. 2. *Effect of time of addition of FUdR on SP15 DNA synthesis. Ten-milliliter cultures were infected in the absence of FUdR as described in the text. At various times postinfection, 0.2 ml of medium containing FUdR was added, followed 5 min later by 0.1 ml of medium containing [G-^{3}H]adenine (500 μCi/ml). At intervals, samples were removed and the amount of label in DNA was determined. It should be pointed out that the interval over which incorporation of label into DNA was measured changed, depending upon the time of addition of FUdR. Thus, when FUdR was added at, say, 15 min postinfection, label was added at 20 min and samples were removed at 21, 31, 41, 51, 61, 71, 81, and 90 min postinfection; when FUdR was added at 75 min, label was added at 80 min and samples were removed at 81, 82, 83, 84, 85, 86, 87, 88, and 90 min postinfection. Incorporation of label was linear over any interval tested between 20 and 90 min postinfection (Fig. 1B). Relative rates of DNA synthesis are based upon data obtained with parallel cultures that received a sham addition of FUdR at each of the times in question. Symbols: bacterial DNA synthesis (data taken from Fig. 1A) (▲); SP15 DNA synthesis when FUdR was added either 5 min before infection (data from Fig. 1B) (○) or 15 (●), 35 (◑), 55 (◐), and 75 (■) min postinfection.*

reduced amounts of DHPdUMP. This substitution was reflected by the production of DNA with a reduced buoyant density (36). These earlier experiments were limited to conditions that yielded fairly high levels of rescue. Therefore, we decided to see if the base composition and buoyant density of viral DNA made under rescued conditions were functions of exogenous dThd concentration.

For these experiments, parallel cultures of infected cells received FUdR and dThd at 20

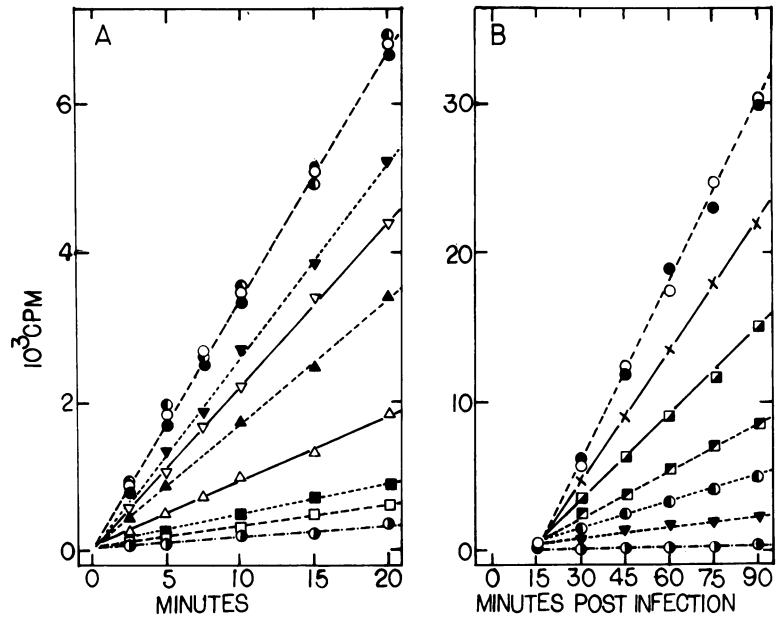

FIG. 3. *dThd rescue of DNA synthesis in FUdR-inhibited cultures. Cultures were either sham infected (A) or infected with SP15 (B) as described under Fig. 1 except that dThd was added with the FUdR (100 μg/ml). Symbols: no FUdR or dThd (○); dThd (5 mg/ml) alone (●); FUdR alone (◑); FUdR plus 1 μg (□), 2 μg (■), 5 μg (△), 10 μg (▲), 20 μg (▽), 50 μg (▼), 100 μg (◐), 500 μg (◩), 1 mg (◪), and 5 mg (×) of dThd per ml.*

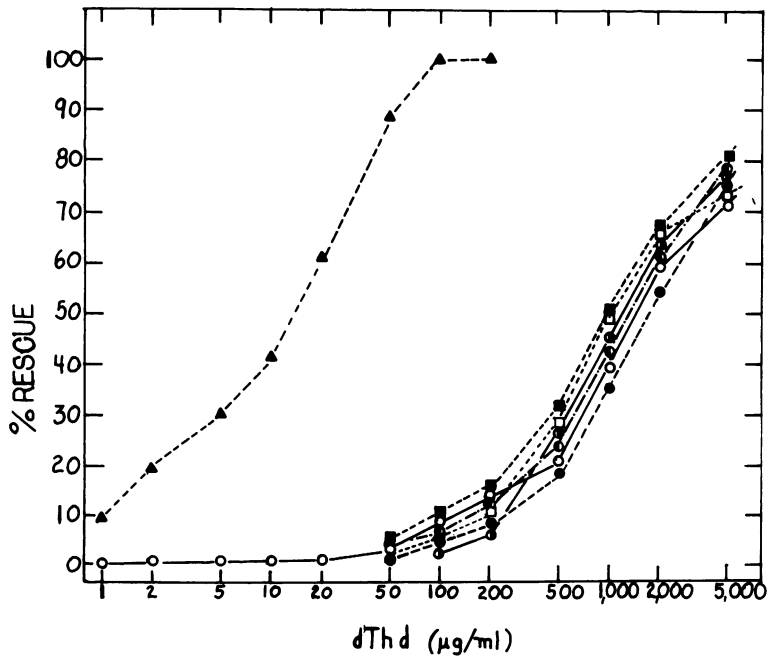

FIG. 4. *Effect of dThd concentration on DNA synthesis in sham- and SP15-infected cultures. Protocols are given in the legends to Fig. 1 through 3. Percent rescue of DNA synthesis = $[(R_T - R_F)/(R_N - R_F)] \times 100$, where R_N, R_F, and R_T are the respective rates of DNA synthesis in cultures that received only dThd (5 mg/ml), only FUdR (100 μg/ml), or FUdR plus the indicated amount of dThd. Symbols: bacterial DNA synthesis (▲); SP15 DNA synthesis when FUdR and dThd were added either 5 min preinfection (○) or 10 (●), 20 (◑), 40 (◐), 60 (□), or 75 (■) min postinfection.*

min postinfection. The cells were labeled with $^{32}P_i$ from 25 until 55 min. Cellular DNA was extracted. A small portion of the labeled DNA was banded in CsCl; the remainder was hydrolyzed to mononucleotides, and the distribution of label among the nucleotides was determined.

The small amount of DNA synthesized at 50 μg of dThd per ml had a buoyant density comparable to normal, mature SP15 DNA (Fig. 5A and B). DNAs made in the presence of higher concentrations of dThd showed a polydisperse distribution in CsCl gradients and steadily decreasing average buoyant densities (Fig. 5C through F). SP15 DNA containing only dTMP should have a buoyant density of 1.698 g/ml (18), but the least dense DNA made under rescued conditions had a density of 1.729 g/ml (Fig. 5F), indicating that viral DNA containing only dTMP could not be made, at least, not in measurable quantities.

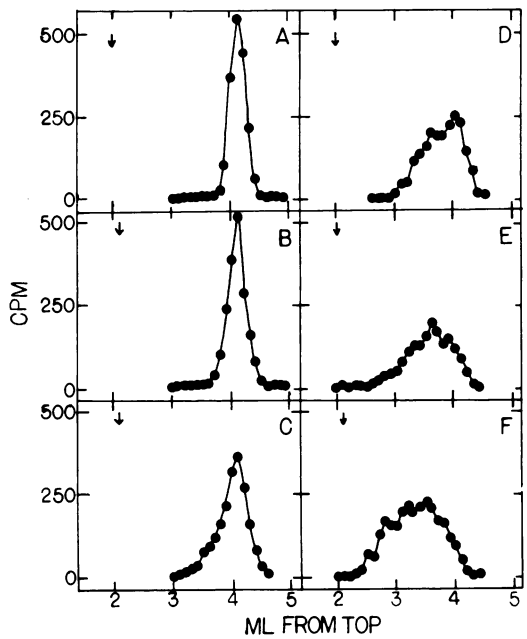

FIG. 5. *Buoyant density of replicating SP15 DNA made in dThd-rescued cultures. Parallel 100-ml cultures of B. subtilis W23 were infected with SP15 as described in the text. At 20 min postinfection, the cultures were harvested by centrifugation and suspended in warm medium containing FUdR (100 μg/ml) and dThd (50 μg/ml to 5 mg/ml). Five minutes later, 1 ml of medium containing 1 mCi of $^{32}P_i$ was added. Thirty minutes later, the cells were harvested and DNA was isolated. [^{32}P]DNA (2,000 cpm in 0.1 ml) was layered over 5.9 ml of a CsCl solution (density = 1.74 g/ml) in a 0.5- by 2-inch (ca. 1.3- by 5.1-cm) cellulose nitrate tube. The tubes were centrifuged at 39,000 rpm in an SW50.1 rotor at 10°C for 36 h. Fractions (0.1 ml) were collected from the top with an ISCO density gradient fractionator. Arrows mark the position of 0.5 μg (1,000 cpm) of bacterial [^{14}C]DNA run in the same tubes. (A) DNA isolated from normal virions. (B through F) DNAs isolated from FUdR-inhibited cultures rescued with 50, 100, 500, 1,000, and 5,000 μg of dThd per ml, respectively.*

As expected (36), decreasing buoyant densities were accompanied by an increase in the amount of label recovered from DNA as dTMP and a commensurate drop in the amount of label in DHPdUMP (Table 1). The relative abundances of dAMP, dGMP, and dCMP in SP15 DNA were unaffected by rescue conditions (data not shown).

Phage production in rescued, FUdR-inhibited cultures. Normal infections yielded a burst of 50 to 80 PFU per cell, but few, if any, viable progeny were made in FUdR-inhibited cells (Table 2). In rescue experiments, the concentration of dThd used was sufficient to restore DNA synthesis to 70% of its normal value (Fig. 3 and 4) and to give normal cell lysis (data not shown); under the same conditions, however, the viable progeny yields were subnormal (Table 2). The exact extent to which viable phage production was reduced under dThd-rescued conditions is difficult to gauge from the data at hand, inasmuch as estimates of burst sizes varied considerably between experiments; notwithstanding, it does appear that under dThd-rescued conditions, viable phage yields did not exceed

TABLE 1. *Relative abundance of DHPdUMP and dTMP in SP15 DNA isolated from rescued cultures*[a]

dThd concn (μg/ml)	% of normal abundance[b]	
	dTMP	DHPdUMP[c]
50	100	100
100	100	100
200	118	84
500	133	63
1,000	150	48
2,000	165	35
5,000	160	36

[a] The same [^{32}P]DNAs described in the legend to Fig. 5 were used, as well as DNAs obtained in separate trials. The DNAs were incubated in alkali to remove glucose-1-phosphate and were then enzymatically degraded to mononucleotides (36; Walker, Ph.D. thesis, University of Texas at Houston, 1977). The nucleotides were fractionated by two-dimensional chromatography on cellulose thin layers (36). Positions of the spots were determined by autoradiography. Nucleotide material was eluted, and the amount of label in each spot was determined.

[b] Relative values are based upon the mole fraction of a particular nucleotide in normal DNA.

[c] It should be pointed out that the procedure used actually yields diglucosylated DHPdUMP (36; Walker, Ph.D. thesis, University of Texas at Houston, 1977).

TABLE 2. *Viable phage yields in SP15-infected cultures*[a]

Condition	PFU/cell
Normal	50, 84, 63, 52, 44
FUdR (100 μg/ml)	<0.01 in all trials
FUdR (100 μg/ml) and dThd (5 mg/ml)	8, 29, 21, 11, 16

[a] The experimental protocol was identical to the one described by Walker (Ph.D. thesis, University of Texas at Houston, 1977). The results of five independent trials are shown. FUdR and dThd were added at 5 min preinfection.

30% of the normal values. The eclipse period (9) was normal in rescued cultures (data not given).

Reduced viable progeny yields could reflect either the production of fewer phage with normal infectivity or the production of normal numbers of phage with reduced infectivity. To distinguish between these two alternatives, the following experiment was done. Parallel cultures were infected under normal and rescued conditions. The cells were labeled with [1-^{14}C]leucine throughout the last 30 min of the latent period. After lysis, aliquots were centrifuged through CsCl gradients, and the distributions of viable phage and radioactivity were determined.

Both cultures incorporated comparable amounts of label into protein (data not presented). In the case of normal lysates, all infectious phage and 30% of the label was recovered in a narrow band at 1.556 g/ml (Fig. 6A). In the case of rescued lysates, however, two discrete bands, containing nearly equal amounts of infectious phage and label, were observed (Fig. 6B). Only 11% of the label in rescued lysates cobanded with infectious phage, implying that fewer progeny were indeed made under the conditions in question. The ratio of PFU to radioactivity should reflect the infectivity of SP15 populations. If this criterion is valid, then the low-density phage made under rescued conditions had normal infectivity (Fig. 6B). Collectively, these data indicate that the reduced burst sizes obtained under rescued conditions (Table 2) resulted principally from the production of fewer progeny viruses.

Buoyant density of DNA isolated from mature phage. The low-density phage made under rescued conditions presumably contain dTMP-enriched DNA. To confirm this, [^{32}P]-DNA was isolated from phage and banded in CsCl. Normal-density phage made under rescued conditions contained normal-density DNA (Fig. 7A), whereas the DNA isolated from low-density phage had the reduced density characteristic of dTMP-enriched DNA (Fig. 7B). It is interesting to note that half the DNA isolated

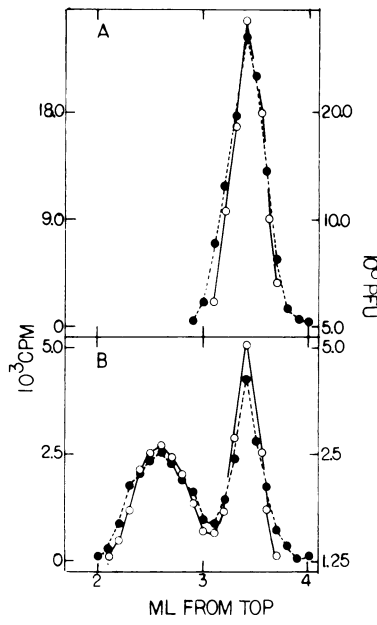

FIG. 6. *Total and viable phage productions in SP15 phage-infected cells. One-milliliter cultures of B. subtilis W23 were infected as described in the text and the legend to Fig. 3. [1-^{14}C]-leucine (5 μCi/ml) was added at 60 min postinfection. After lysis, large debris was removed by centrifugation, and 0.2-ml portions of the lysates were layered onto 5.9-ml CsCl solutions (density = 1.500 g/ml). The tubes were centrifuged as described in the text. A 0.01-ml portion of each fraction was diluted and plated; the remainder was assayed for hot-acid-precipitable radioactivity. (A) Cells infected in the presence of dThd (5 mg/ml). (B) Cells infected in the presence of FUdR (100 μg/ml) and dThd (5 mg/ml). Symbols: ○, PFU; ●, counts per minute.*

from phage made in rescued cultures had a normal density (Fig. 7C), but, within the intracellular replicating pool, only a small amount of the DNA had a normal density (Fig. 5F). This must mean that there is a preferential packaging of normal-density DNA into phage.

Synthesis of lytic enzymes and structural proteins under dThd-rescued, FUdR-inhibited conditions. Inhibiting viral DNA synthesis with FUdR delayed the onset of lysis by 30 to 40 min (data not presented). This indicated that synthesis of the lytic enzymes (and probably other proteins) was affected by the absence of viral replication. Accordingly, we studied the syntheses of lytic proteins and structural proteins under FUdR-inhibited and rescued conditions. These are the only known SP15 proteins whose syntheses occur after the onset of viral replication (9).

Absence of SP15 DNA synthesis delayed the

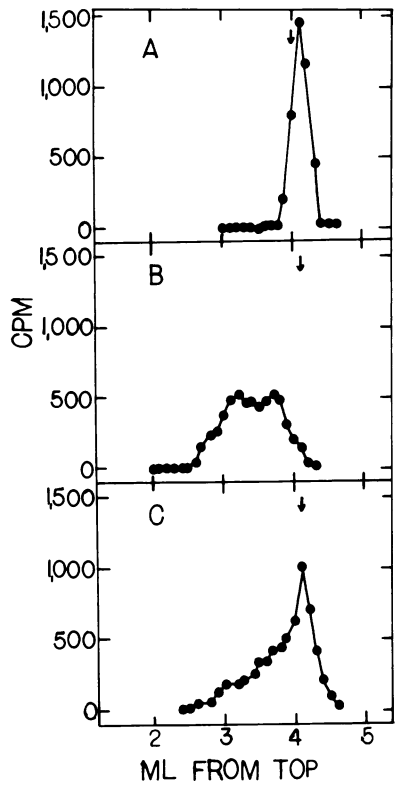

FIG. 7. *Buoyant density of DNA isolated from phage made under dThd-rescued conditions. A 600-ml culture of cells was infected in the presence of FUdR (100 μg/ml) and dThd (5 mg/ml). At 20 min postinfection, 6 ml of medium containing 1.5 mCi of $^{32}P_i$ was added, and the culture was incubated until lysis. The radioactively labeled lysate was mixed with an unlabeled 3-liter lysate. Phage were concentrated and banded in CsCl as described in the text and the legend to Fig. 6. Low- and normal-density phage (Fig. 6) were pooled separately, dialyzed into phage buffer, rebanded in CsCl, and dialyzed into phage buffer. DNA was isolated and subjected to isopycnic centrifugation as described in the legend to Fig. 5. Each gradient received (in 0.1 ml) 0.5 μg each of [^{32}P]DNA (ca. 5,000 cpm) isolated from phage made in rescued cultures and [^{3}H]DNA (ca. 1,200 cpm) isolated from normal phage. The position of the latter DNA at equilibrium is marked by an arrow. (A) DNA from purified normal-density phage in rescued lysates. (B) DNA from purified low-density phage in rescued lysates. (C) DNA from phage in rescued lysates where CsCl gradient fractions containing low- and normal-density phage were pooled together.*

appearance of lytic enzymes and structural proteins (i.e., those proteins in extracts responsible for serum blocking power) by 20 to 30 min (Fig. 8A and B). Once synthesis of these proteins began, however, they accumulated at normal rates to essentially normal levels. Under rescued conditions, synthesis of the two classes of protein appeared normal in all respects (Fig. 8A and C).

Delayed appearance of certain proteins under FUdR-inhibited conditions could reflect delayed transcription or translation or both. To obtain additional information on this point, experiments were done that measured the dependence of protein synthesis on the time of addition of antibiotic inhibitors of transcription (9, 21). The antibiotics used were rifampin and streptolydigin, selective inhibitors of RNA chain initiation and elongation, respectively (7, 29, 37).

Basically, aliquots of cultures are removed various times postinfection and transferred to flasks that contain one of the drugs. These subcultures are subsequently incubated for a time sufficient to yield the highest possible levels of a given protein. Synthesis of a protein remains inhibitable by rifampin or streptolydigin provided the drug is added before initiation or completion, respectively, of the corresponding mRNA. The time at which synthesis of a protein becomes rifampin refractile relative to the time at which synthesis becomes streptolydigin refractile is probably dependent upon a variety of factors, including rate of transcription, size of the gene, and position of the gene within the transcriptional unit. Comparing the time at which synthesis becomes streptolydigin refractile with the time of appearance of the protein within the cell reflects the interval required to translate the mRNA. This latter parameter probably includes the time needed for the lead ribosomes to transit the entire mRNA as well as the interval between completion of the mRNA and binding of the lead ribosomes. In some procaryotic systems, translation begins before transcription is completed, so synthesis of a protein becomes streptolydigin refractile only several minutes before active protein makes its appearance in the cell (see, e.g., reference 21). However, certain SP15-coded mRNAs are evidently completed many minutes before their translation begins (see below and reference 9).

In a normal program, mRNA's for lytic enzymes were not present until 25 min, as evidenced by the fact that enzyme synthesis was antibiotic inhibitable at earlier times (Fig. 9A). Enzyme synthesis became refractile to both drugs simultaneously, implying a comparatively short interval between initiation and completion of these mRNA's. Active enzymes themselves were not present until 50 min (Fig. 9A). Therefore, potentially translatable messengers for lytic enzymes were present well in advance of their protein products (9). In FUdR-inhibited cultures, mRNA's appeared at 45 min, followed by the proteins at about 70 min (Fig. 9B). Hence,

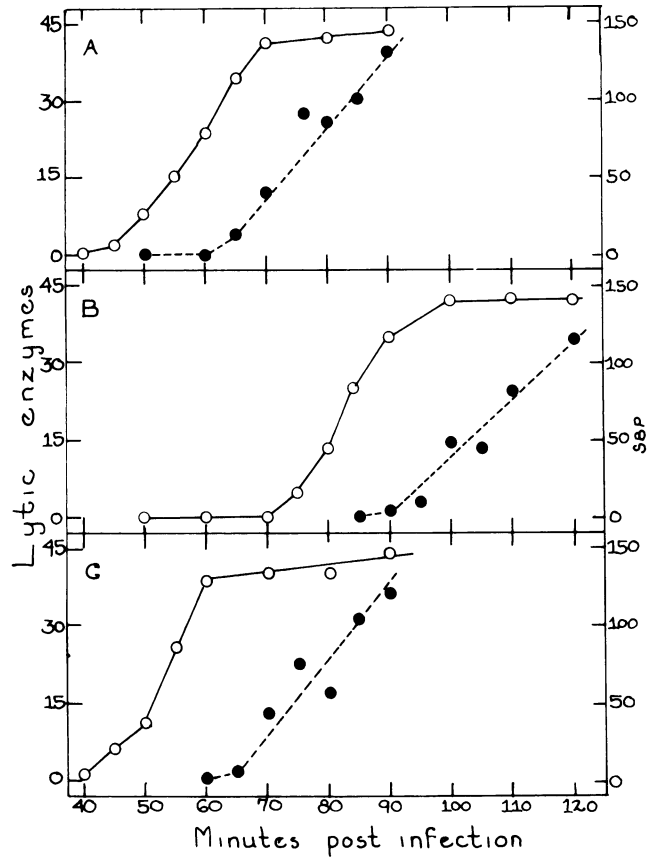

FIG. 8. *Effect of FUdR on syntheses of lytic enzymes and structural proteins. A 2-liter culture of B. subtilis W23 was infected as described in the text and the legend to Fig. 1. At the times specified in the graph, 100-ml samples were removed and extracts were prepared. Extracts were assayed for lytic enzymes and structural proteins. (A) dThd (5 mg/ml) added at 5 min preinfection. (B) FUdR (100 μg/ml) added at 5 min preinfection. (C) FUdR and dThd added at 5 min preinfection. Symbols: ○, lytic enzymes; ●, serum blocking power (SBP) (structural proteins).*

absence of SP15 DNA replication delayed the initiation of mRNA by 20 min, but there was no remarkable effect on the interval required for either mRNA completion or enzyme appearance (Fig. 9A and B). Under rescued conditions, mRNA's and proteins appeared at their normal times (Fig. 9A and B). Similarly, the delayed appearance of structural proteins (serum blocking power) in the absence of DNA synthesis was attributable to delayed initiation of mRNA transcription (Fig. 10). Unlike the situation with lytic enzymes, however, a nearly 10-min interval separated the times at which synthesis of structural proteins became rifampin and streptolydigin refractile (Fig. 10); this seems to be a common characteristic of SP15 mRNA's that make their initial appearance at times later than 40 min (9).

In passing, we note that production of SP15 DNA-dependent DNA polymerase and DNA-glucosylating enzyme was unaffected by the absence of phage DNA synthesis (data not presented). These latter two enzymes make their initial appearance before the onset of replication, so the effects of FUdR on protein synthesis are seemingly limited to a particular subclass of SP15 phage-coded functions.

Dependence of lytic enzyme and structural protein synthesis upon the time of addition of FUdR. The foregoing results showed that synthesis of lytic enzymes and structural proteins was, to some extent, dependent upon DNA synthesis. To determine the duration of DNA synthesis required for proper expression of these functions, the dependence of protein synthesis on time of FUdR addition was monitored.

Up to 10 min postinfection, addition of FUdR delayed the appearance of lytic enzymes and

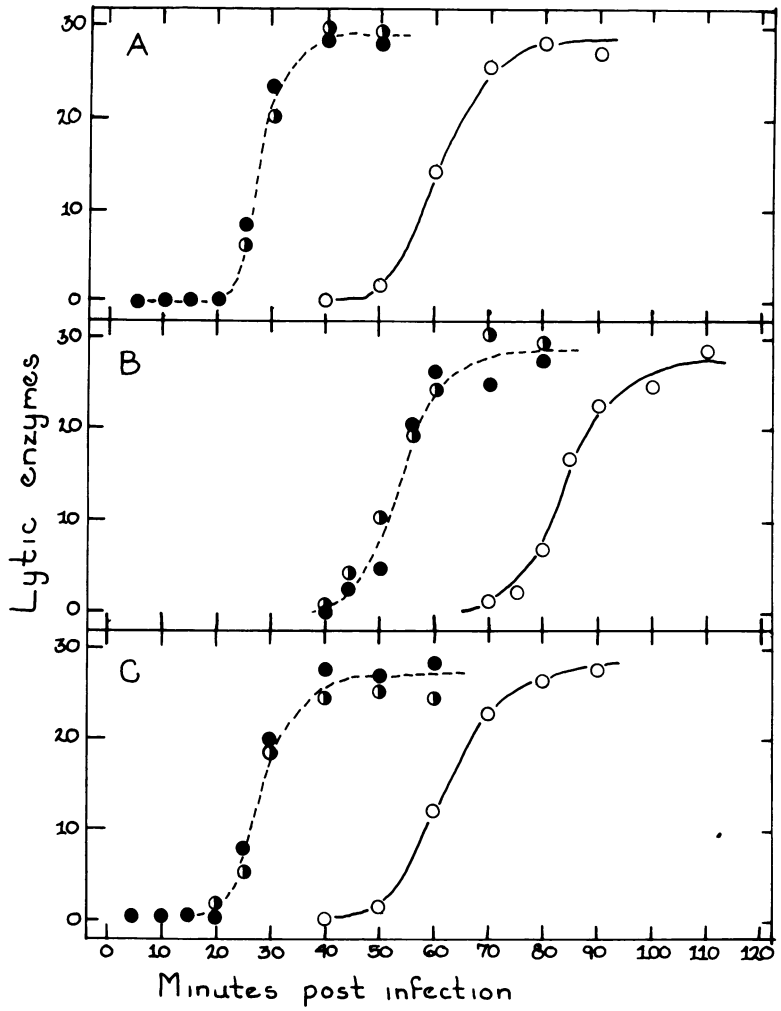

FIG. 9. *Effect of transcriptional inhibitors on appearance of lytic enzymes. Two-liter cultures were infected. At the times indicated, three 100-ml portions were removed. One portion was harvested immediately. The other two portions were transferred to fresh vessels containing sufficient rifampin or streptolydigin to give a final antibiotic concentration of 10 μg/ml. Antibiotic-treated portions were incubated until either 89 min postinfection (normal or dThd-rescued infections) or 119 min postinfection (FUdR-inhibited cultures). Extracts were prepared and assayed for lytic enzymes. (A) Normal infection. (B) FUdR added at 5 min preinfection. (C) FUdR and dThd added at 5 min preinfection. Symbols: ○, samples harvested immediately; ●, samples incubated with rifampin; ◑, samples incubated with streptolydigin.*

structural proteins by about 30 min (Fig. 11). Adding the analog at later times generated progressively shorter delays until, by 20 min, protein synthesis was unaffected by FUdR. Between 10 and 20 min postinfection, SP15 DNA accumulated to only 5% of its maximum level (9; Fig. 1 and 3), suggesting that only small amounts of replication suffice for proper expression of the functions under consideration.

Effect of aminopterin on viral DNA synthesis and rescue of DNA synthesis with dThd. There is reason to believe that FUdR partially inhibits synthesis of DHPdUTP (36). Consequently, the large amount of dThd required to restore SP15 replication in FUdR-inhibited cultures (and its attendant production of dTMP-enriched DNA) may be engendered by the limited availability of DHPdUTP under such conditions. From this, it follows that lower concentrations of dThd should rescue phage replication in systems where de novo synthesis of dTMP is inhibited without affecting synthesis of the hypermodified nucleotide; for reasons discussed below, it does not necessarily follow that

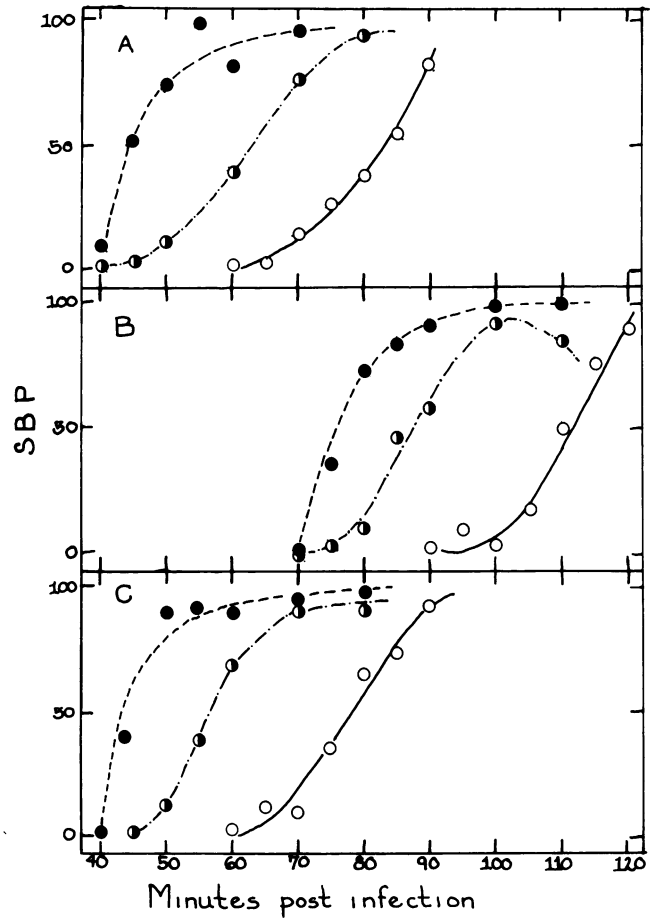

FIG. 10. *Effect of transcriptional inhibitors on synthesis of structural proteins (serum blocking power [SBP]). The same extracts described in the legend to Fig. 9 were used. (A) Normal infection. (B) FUdR present from 5 min preinfection. (C) FUdR and dThd present from 5 min preinfection. Symbols: ○, samples harvested immediately; ●, samples incubated with rifampin; and ◑, samples incubated with streptolydigin.*

the conditions in question would generate DHPdUMP-enriched DNA. Aminopterin inhibits de novo synthesis of dTMP (see below), but, evidently, it has little or no effect on DHPdUTP biosynthesis (Walker, Ph.D. thesis, University of Texas at Houston, 1977). Therefore, dThd rescue of phage replication in aminopterin-inhibited cultures was studied.

Aminopterin is an analog of dihydrofolic acid, the immediate precursor to tetrahydrofolate (1). Aminopterin competitively inhibits dihydrofolate reductase, the enzyme that reduces dihydrofolate to tetrahydrofolate (1). Tetrahydrofolate is involved in the biosynthesis of *N*-formylmethionine and other amino acids as well as the de novo synthesis of purine nucleotides and dTMP (1, 19). Consequently, aminopterin inhibits protein synthesis and nucleic acid synthesis. This posed a problem in that we wished only to consider the effects of aminopterin on SP15 DNA synthesis that were attributable to inhibiting de novo synthesis of dTMP. Inhibition of purine biosynthesis can be compensated for by adding guanine and adenine to the medium (1, 12). The effects of aminopterin on protein synthesis are more difficult to reverse (12). In many phage systems, replication of the viral genome becomes independent of new protein synthesis soon after infection, but, so far as SP15 is concerned, replication of the viral genome remains dependent upon new protein synthesis throughout most of the latent period (9). This problem is especially acute at times later than 60 min, when inhibition of protein synthesis arrests replication almost immediately (9). Therefore, our experiments had to be limited to times when cessation of protein synthesis would not immediately upset phage DNA synthesis. For the experiments reported here, aminopterin was added at 40 min postinfection; terminating pro-

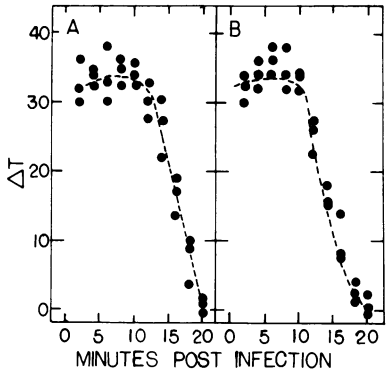

FIG. 11. *Effect of time of FUdR addition on delayed appearance of lytic enzymes and structural proteins. Parallel 2-liter cultures were infected as described in the text. At the times specified in the figure, 40 ml of medium containing 5 mg of FUdR per ml was added; a control culture received a sham addition of FUdR at 1 min postinfection. Between 40 and 120 min postinfection, 50-ml samples were removed at 2.5-min intervals. Extracts were prepared and assayed for lytic enzymes and serum blocking power (structural proteins). The parameter ΔT is the difference between the time of appearance of lytic enzymes or structural proteins in cultures that received FUdR and the time of their appearance in the control culture. The results of three independent experiments are shown. (A) Lytic enzymes; (B) structural proteins.*

tein synthesis at that time permits DNA synthesis to proceed for about 20 min (9). Provided that the absence of protein synthesis does not affect the requirement of replication for dThd nucleotides and that an exogenous source of purines is supplied, then any effect of aminopterin on DNA synthesis should, in principle, be attributable to inhibition of the de novo synthesis of dTMP.

Under the conditions employed, aminopterin was a potent inhibitor of SP15 DNA synthesis (Fig. 12), and viral replication was rescuable by supplying an exogenous source of dThd (Fig. 12). More importantly, the amount of dThd required to restore phage DNA synthesis in aminopterin-treated cultures was 20-fold lower than the amount of dThd required to rescue FUdR-treated cells (Fig. 3, 4, and 12). Indeed, the amount of exogenous dThd required to rescue phage DNA synthesis in aminopterin-inhibited cultures was comparable to the low levels of dThd required to rescue replication in FUdR- and aminopterin-inhibited uninfected cells (Fig. 3; our unpublished data).

DISCUSSION

Inhibiting de novo synthesis of dThd nucleotides with either FUdR or aminopterin reduced SP15 DNA synthesis by at least 95% (Fig. 1 through 4 and 12). Manyfold higher concentrations of exogenous dThd were required to rescue DNA synthesis in FUdR-inhibited cultures, compared with aminopterin-inhibited cultures (Fig. 4 and 12). Furthermore, rescue of aminopterin-treated cultures yielded normal phage DNA (Walker, Ph.D. thesis, University of Texas at Houston, 1977; H. Witmer, unpublished data), whereas phage DNA made in rescued FUdR-inhibited cultures was somewhat enriched for dTMP (36; Fig. 5; Table 1). Transposed into other terms, dThd rescue of FUdR-inhibited cultures resulted in the synthesis of phage DNA containing dTMP at sites normally occupied by DHPdUMP. FUdR, but not aminopterin, seems to partially suppress synthesis of DHPdUTP (36). Consequently, replication in dThd-rescued, FUdR-inhibited cultures probably occurred against a limiting pool of DHPdUTP; as a result, abundant DNA synthesis, in this case, apparently required some replacement of DHPdUMP by dTMP. Perhaps dTMP cannot be inserted into sites normally occupied by the hypermodified nucleotide unless dTTP is present at a certain molar excess relative to DHPdUTP. This hypothesis explains why more dThd is required to restore replication in FUdR-inhibited cultures; i.e., sufficient dTMP (and, hence, dTTP) must be generated to overcome the postulated barrier to replacement of the hypermodified nucleotide by dTMP.

The above hypothesis would also explain why the degree of replacement of DHPdUMP by dTMP is dependent upon the concentration of dThd used to rescue FUdR-inhibited cultures (Fig. 5; Table 1). Thus, as dThd concentration rises, the intracellular ratio of dTTP to DHPdUTP increases. At low levels of dThd (e.g., 50 μg/ml), the ratio is comparable to that maintained in normally infected cells, so DNA with an essentially normal base composition is generated; the low levels of DNA synthesis observed under these conditions presumably stem from a limiting pool of DHPdUTP coupled with dTTP concentrations too low to "force" insertion of the conventional nucleotide at sites normally occupied by DHPdUMP. With higher concentrations of dThd, DHPdUTP remains at the same subnormal level because dThd cannot rescue synthesis of the hypermodified nucleotide (36). Under these conditions, however, dTTP levels are sufficient to enable some replacement of DHPdUMP by dTMP. A systematic study of dTTP and DHPdUTP pools under normal and rescued conditions is obviously required, as suggested by Walker and Mandel (36).

Aminopterin inhibits de novo synthesis of dTMP (1, 14), but, as noted above, it apparently

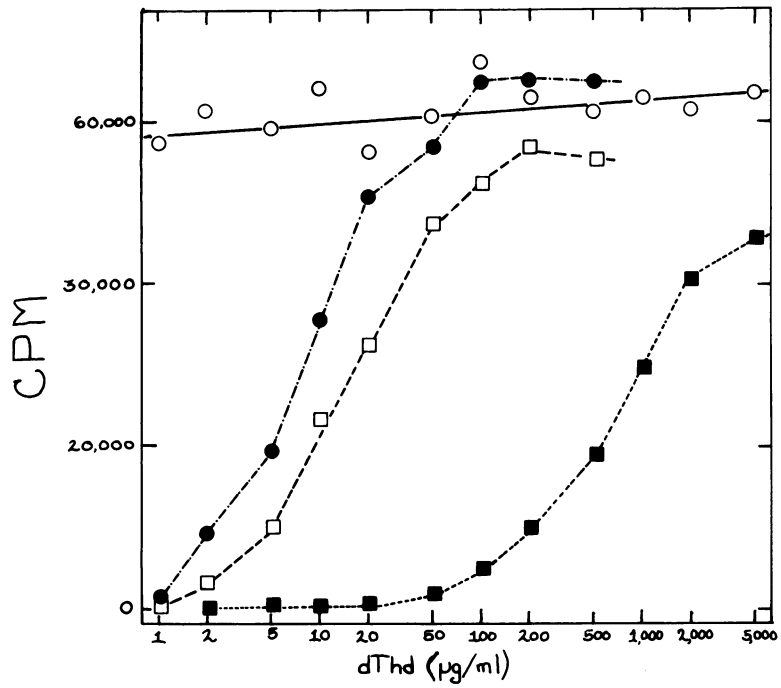

FIG. 12. *dThd rescue of aminopterin-inhibited cultures. Four parallel cultures were infected as described in the text, except that NLM medium (24) supplemented with guanine and adenine (20 μg of each per ml) was used. At 40 min, the cells were harvested by centrifugation and suspended in fresh, warm medium that contained either chloramphenicol (50 μg/ml) alone* (○), *aminopterin (5 μg/ml) alone* (●), *chloramphenicol and aminopterin* (□), *or chloramphenicol plus FUdR (100 μg/ml)* (■). *Two minutes later, 1-ml portions of the four cultures were transferred to tubes that contained fresh medium additionally supplemented with 100 μCi of $^{32}P_i$ and various concentrations of dThd. The subcultures were labeled for 20 min, and the amount of radioactivity in DNA was determined.*

has no major effect on DHPdUTP biosynthesis. Nevertheless, SP15 DNA synthesis is inhibitable by aminopterin (Fig. 12; Walker, Ph.D. thesis, University of Texas at Houston, 1977), implying that DNA containing only the hypermodified nucleotide cannot be made. Although a variety of explanations would account for this, there are good stereochemical reasons for believing that hypermodified nucleotides cannot be nearest neighbors to each other (M. Mandel, personal communication). Indeed, when aminopterin-treated cultures are rescued with subsaturating concentrations of dThd, phage SP15 DNA with a normal density is made (Witmer, unpublished data). Therefore, at present, it seems that SP15 DNA with an augmented DHPdUMP content cannot be generated.

The mechanism that selects which pyrimidine nucleotide, dTMP or DHPdUMP, to pair with dAMP remains unclear. If dTMP cannot, in fact, be readily inserted into sites normally occupied by the hypermodified nucleotide (Fig. 3 and 4), it could well be that, to a large extent, the selection is nonrandom. This line of reasoning could help explain the hypermodified character of the base that partially replaces thymine in the DNAs of SP15 and φW-14 (4, 13, 15). Basically, these viruses must incorporate two bases with identical H-bonding properties into DNA in a precise pattern. A complex substituent at the 5 position may provide sufficient distinction to insure errorless discrimination between the two bases.

The role of DHPdUMP in phage development is conjectural, and definite answers on this subject cannot be forthcoming without mutants defective in the biosynthesis of the hypermodified nucleotide. Notwithstanding, the results presented here provide some insights into this important problem. DNA containing normal amounts of DHPdUMP is apparently not required for proper expression of viral genes. Evidence for this conclusion comes from studies with dThd-rescued, FUdR-inhibited cultures, which showed that the appearance of certain virus-coded proteins was normal under the conditions stated (Fig. 8 through 10), even though DNA with a subnormal content of DHPdUMP

was made (Table 1). Parenthetically, we note that recent experiments have shown that synthesis of SP15 DNA-dependent DNA polymerase and DNA-glucosylating enzyme is normal under rescued conditions (M. Dosmar and H. Witmer, unpublished data). Proper maturation apparently requires DNA with a normal concentration of DHPdUMP. This is evidenced by the fact that progeny yields were considerably reduced in rescued FUdR-inhibited cultures (5; Fig. 6; Table 2). Moreover, half the progeny viruses assembled under rescued conditions contained DNA with a normal buoyant density (Fig. 7C), despite the fact that little replicating DNA had a normal density (Fig. 5F). Such data imply that, despite its normal infectivity (Fig. 6B), dTMP-enriched DNA either matures inefficiently or is packaged inefficiently. Walker and Mandel (36) recently proposed that DHPdUMP is required for proper binding to DNA of certain proteins concerned with phage assembly. SP15 codes for 13 to 16 DNA-binding proteins (Witmer, unpublished data), and it will be informative to compare the bindings of these proteins to normal and dTMP-enriched DNA.

It is interesting to note that the impact of replacing an unusual base with its normal analog differs widely among *B. subtilis* phage. For example, replacing uracil with thymine in PBS2 DNA considerably reduced phage DNA synthesis and progeny production (26). On the other hand, as much as 20% of the 5-hydroxymethyluracil in ϕe DNA can be replaced by thymine without affecting expression of the phage genome or progeny production (20, 28). SP15 seems to sit somewhere between these two extremes.

Absence of phage DNA synthesis delayed the appearances of certain SP15 proteins (Fig. 8). The available evidence suggested that the absence of replication delayed initiation of the relevant messengers but that, once initiated, transcription of these mRNA's proceeded to normal levels (Fig. 9 and 10).

DNA synthesis between 10 and 20 min postinfection is critical for proper synthesis of lytic enzymes and structural proteins (Fig. 11). It is easy to see, at least qualitatively, how DNA synthesis during this interval could be involved with production of lytic enzymes, since transcription of the pertinent messengers starts at 20 min postinfection (9; Fig. 9). It is harder to see how DNA synthesis between 10 and 20 min postinfection could directly affect elaboration of structural protein messengers, since transcription of these species does not begin until 20 to 30 min later (Fig. 10). On the one hand, it is possible that initiation of structural protein messengers is dependent upon prior synthesis of a protein whose messenger, in turn, is not initiated at its proper time without DNA synthesis between 10 and 20 min postinfection. On the other hand, it may be that certain messengers are properly transcribed only after SP15 DNA has assumed some special conformation and/or has become associated with some cellular structure, such as the membrane; according to such a model, conformational changes and/or association with cellular structures requires a brief interval of DNA synthesis, but, once effected, these transformations are irreversible, even by inhibiting further DNA synthesis. Although DNA synthesis between 10 and 20 min postinfection is essential for proper synthesis of lytic enzymes and structural proteins, it seems that normal rates of DNA synthesis during the interval in question are not required to insure correct timing of late events in the program. This latter conclusion is evidenced by the fact that lytic enzymes and structural proteins are made normally in FUdR-inhibited cultures rescued with 100 μg of dThd per ml (our unpublished data), i.e., a concentration of dThd that yielded only 15 to 20% rescue of DNA synthesis (Fig. 3 and 4).

Under nonpermissive conditions, conditionally lethal, DNA-negative mutants of SPO1 show delayed lysis, and this characteristic has been exploited to enrich for such mutants (11). DNA-negative mutants of SP15 should also exhibit delayed lysis (Fig. 8). Attempts to isolate DNA-negative mutants of SP15 are currently in progress; a subclass of such mutants should be those defective in the biosynthesis of DHPdUTP.

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