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Replicative Bacteriophage DNA Synthesis in Plasmolyzed T4-Infected Cells: Evidence for Two Independent Pathways to DNA

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Bacteriophage T4-infected *Escherichia coli* rendered permeable to nucleotides by sucrose plasmolysis exhibited two apparently separate pathways or channels to T4 DNA with respect to the utilization of exogenously supplied substrates. By one pathway, individual labeled ribonucleotides, thymidine (TdR), and 5-hydroxy-methyl-dCMP could be incorporated into phage DNA. Incorporation of each of these labeled compounds was not dependent upon the addition of the other deoxyribonucleotide precursors, suggesting that a functioning de novo pathway to deoxyribonucleotides was being monitored. The second pathway or reaction required all four deoxyribonucleoside triphosphates or the deoxyribonucleoside monophosphates together with ATP. However, in this reaction, dTTP was not replaced by TdR. The two pathways were also distinguished on the basis of their apparent Mg^{2+} requirements and responses to *N*-ethylmaleimide, micrococcal nuclease, and to hydroxyurea, which is a specific inhibitor of ribonucleoside diphosphate reductase. Separate products were synthesized by the two channels, as shown by density-gradient experiments and velocity sedimentation analysis. Each of the pathways required the products of the T4 DNA synthesis genes. Furthermore, DNA synthesis by each pathway appeared to be coupled to the functioning of several of the phage-induced enzymes involved in deoxyribonucleotide biosynthesis. Both systems represent replicative phage DNA synthesis as determined by CsCl density-gradient analysis. Autoradiographic and other studies provided evidence that both pathways occur in the same cell. Further studies were carried out on the direct role of dCMP hydroxymethylase in T4 DNA replication. Temperature-shift experiments in plasmolyzed cells using a temperature-sensitive mutant furnished strong evidence that this gene product is necessary in DNA replication and is not functioning by allowing preinitiation of DNA before plasmolysis.

Investigations of DNA synthesis have been facilitated by the recent development of several complex in vitro systems. Unlike the reactions catalyzed by purified polymerases, these systems have been shown to synthesize DNA in a replicative manner and at a rate which more closely approaches that observed in vivo. Moreover, certain of these systems apparently maintain at least some of the controls, as evidenced by the fact that synthesis is blocked by agents that specifically inhibit DNA replication in vivo. In a parallel manner, DNA synthesis in preparations derived from cells carrying dna_{ts} mutations has been shown to be temperature sensitive.

The development of these systems involved either techniques for the isolation of relatively integrated subcellular structures (1, 20, 28, 29, 33) or procedures designed to render intact cells permeable to exogenously supplied substrates and even to macromolecules (15, 27, 41). We find that one of the latter procedures, plasmolysis with high sucrose concentrations (40), is particularly suitable for studying DNA synthesis in cells infected by the lytic bacteriophage T4 (41). The treatment renders these cells permeable to nucleotides and yet stable to subsequent manipulations. In view of the involvement of membrane in T4 DNA replication (31), an obvious and important consideration is that permeabilization appears to be primarily the result of a physical disruption rather than a chemical alteration of the cellular membrane. Several groups have subsequently reported replicative T4 DNA synthesis in cells permeabilized by sucrose plasmolysis (7) or by toluene

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treatment (9, 10, 25). This communication describes two independent pathways for DNA synthesis, both replicative and occurring within the same infected cells. Both reaction systems are dependent on the products of the T4 *dna* (DNA synthesis) genes. The substrates and intermediates for these pathways clearly do not mix, and, in fact, the products of the two pathways can be separated in density experiments. One is a deoxyribonucleoside triphosphate-dependent or substrate-dependent pathway. The second, and by far the predominant route to DNA, incorporates ribonucleotides after conversion to deoxyribonucleotides via the ribonucleoside diphosphate reductase reaction. This sequence of reactions is called the de novo pathway or channel.

(A preliminary account of these experiments was given at the annual meeting of the American Society of Biological Chemists, Minneapolis, 1974 [Fed. Proc. 33:1351].)

MATERIALS AND METHODS

Chemicals. [^{3}H]dATP, [^{3}H]dTTP, [^{3}H]dTMP, and [^{3}H]TdR (thymidine) were obtained from Amersham/Searle and New England Nuclear Corp.; [5-^{3}H]dCMP and [5-^{3}H]dCTP were purchased from Schwarz/Mann Bioresearch; and [6-^{3}H]uridine was from Amersham/Searle. Nonlabeled nucleotides were obtained from the commercial sources described earlier (41), and 5-bromodeoxyuridine 5'-triphosphate (BrdUTP) was from P-L Biochemicals. Micrococcal nuclease was from Worthington Biochemicals Corp. Ethyleneglycol-bis-(2-aminoethyl ether)-*N,N'*-tetraacetic acid (EGTA), *N*-ethylmaleimide (NEM), and rifampin were from Sigma Chemical Co. Isotopically labeled hydroxymethyl-dCMP (HMdCMP) was isolated from radioactive DNA of phage T2 lacking glucose on its hydroxymethylcytidine residues (T2*; reference 18). The latter was produced by the use of *Escherichia coli* K-12 W4597 (uridine diphosphoglucose-pyrophosphorylase-negative). The culture was grown in the medium of Fraser and Jerrel (13) to 5×10^8 cells/ml and infected with a multiplicity of 8 phage. At 10 min after infection, 14 μCi of [6-^{3}H]uridine per ml (5 Ci/mmol) was added. The culture was treated with chloroform at 90 min after infection to complete the lysis, and the phage were purified by differential centrifugation. Phage DNA was extracted by the method of Freifelder (14). The labeled HMdCMP was isolated from the DNA by the procedures of Hurlbert and Furlong (19). Tritium-labeled 5-hydroxymethyl-dCTP (HMdCTP) was prepared by incubating labeled HMdCMP in the presence of ATP in a soluble extract of T4-infected *E. coli* B. The product was isolated by paper chromatography, with isobutyric acid-concentrated ammonium hydroxide-water (66:1:33; vol/vol/vol) as the solvent.

Biological materials. The origins of *E. coli* B (a Su$^-$ strain) and *E. coli* K-12 D110 (*polA$_1$ endoI thy*$^-$) have been described (41). Strain W4597 was from the laboratory of H. Revel via K. Ebisuzaki. All of the mutant phage strains used in this study were derived from phage T4D and are described in Table 2. The origin of most of these mutant strains has been described earlier (41). The mutants *amA453*, *amH2628*, *amN116*, and *amH26* were from R. S. Edgar.

Plasmolysis of phage T4-infected cells. Plasmolyzed phage-infected cells of *E. coli* B were prepared 10 min after infection as previously described (41). To obtain resuspension it is necessary to agitate the mixture vigorously with a Vortex apparatus immediately after adding the plasmolysis buffer to the pellet from the infected culture. Failure to do so often results in severe clumping of the preparation. We prefer to call such preparations, in which the major structural components and presumably the associated regulatory controls remain intact, "in situ systems."

TABLE 1. *Distribution of autoradiographic grains in population of plasmolyzed amH26-infected cells that had incorporated [^{3}H]TdR or [^{3}H]dATP*[a]

Labeled precursor	Distribution	No of cells with:						
		0 Grain	1 Grain	2 Grains	3 Grains	4 Grains	5 Grains	6 Grains
[^{3}H]TdR	Observed	215	146	73	20	7	2	0
	Expected[b]	209	166	71	20	4	1	0
[^{3}H]dATP	Observed	864	238	50	6	0		
	Expected[b]	835	258	40	6	0		

[a] Plasmolyzed preparations of cells infected with T4 phage *amH26* (lysozyme$^-$) were incubated in standard reactions containing ATP and either [^{3}H]dATP (0.5 μCi/nmol), dGMP, dTMP, and HMdCMP or else [^{3}H]TdR (0.5 μCi/nmol) alone. After 30 min at 37°C the cells were treated as described in Materials and Methods. Seventy days were allowed for ^{3}H decay before the slides were developed. An average of 0.85 grain/cell was determined for 461 cells of the [^{3}H]TdR reaction that were counted, and 0.31 grain/cell for 1,138 cells counted from the [^{3}H]dATP reaction. Thus, the ratio of grains associated with cells from the [^{3}H]dATP reaction to grains with cells from the [^{3}H]TdR reaction was 0.40, as compared with a ratio of 0.36 for incorporation into DNA by these same reactions. No tracking was observed in control slides prepared from plasmolyzed *amB22*-infected cells incubated in identical reactions.

[b] The distribution of grains expected for a random incorporation by the cell populations was calculated assuming a Poisson type of distribution.

TABLE 2. *Effect of T4 amber mutations on DNA synthesis in plasmolyzed cells*[a]

Gene	Mutant	Phenotype[b]	Defect	Labeled precursor incorporated (pmol)	
				[^{3}H]dATP	[^{3}H]TdR
	T4D	Wild type		153	418
1	*amB24*	DO	Kinase	2	6
32	*amA453*	DO	DNA unwinding protein	15	56
39	*amN116*	DD	Uncharacterized	54	151
41	*amN81*	DS	DNA replication	12	25
42	*amN122*	DO	dCMP hydroxymethylase	13	6
43	*amB22*	DO	DNA polymerase	4	8
44	*amN82*	DO	DNA replication	18	7
45	*amE10*	DO	DNA replication	24	4
46	*amN130*	DA	Host DNA degradation	100	140
59	*amH2628*	DA	Uncharacterized	69	106
e	*amH26*		Lysozyme	160	400

[a] Cultures of *E. coli* were grown at 37°C to 5 × 10^8 cells/ml and infected with a multiplicity of 8. At 10 min after infection, the cells were plasmolyzed, and the preparation was incubated for 30 min in the standard reaction described in Materials and Methods.

[b] DO, No DNA synthesis; DS, some DNA synthesis; DA, arrested DNA synthesis; DD, delayed DNA synthesis (11, 38).

Assay of DNA synthesis. DNA synthesis in plasmolyzed phage-infected cells was measured as incorporation of label from radioactive substrates into acid-precipitable, alkali-stable material as described previously (41). The standard reaction mixture to measure the deoxyribonucleoside triphosphate-dependent pathway contained in a final volume of 0.1 ml: the equivalent of 5 × 10^8 plasmolyzed cells, 3.2 μmol of Tris-hydrochloride (pH 8.4), 0.16 μmol of Mg-acetate; 0.16 μmol of EGTA; 40 μmol of sucrose; 6.4 μmol of KCl; 100 nmol of ATP, and 10 nmol each of dATP, dTTP, dGTP, and HMdCTP, one of the four being labeled at a specific activity of 10 to 30 cpm/pmol. The temperature was 37°C. As indicated in Results and in the figure legends, in certain experiments deoxyribonucleoside monophosphates at the same concentrations were used in place of the triphosphates. To measure the de novo pathway to DNA, the incorporation of labeled TdR was usually followed, using essentially the same reaction system. In some cases ribonucleotides were used in place of TdR. (The rationale for the use of TdR to follow the de novo pathway is developed in the Results section.)

The rate of synthesis by each of the two pathways was proportional to the number of plasmolyzed cells in the range used.

Autoradiographic analysis. Plasmolyzed phage-infected cells were incubated in the standard reaction mixture for 30 min at 37°C and then diluted 10-fold with cold water and collected on nitrocellulose filters. The filters were washed four times with 5 ml of cold water, and the cells were suspended in a cold solution of bovine serum albumin (1 mg/ml). Aliquots of the cell suspensions were spread on slides and fixed by drying under a heat lamp. The fixed cells were further washed by placing the slides first in cold 10% trichloroacetic acid for 10 min and then in cold water. The slides were coated with Kodak NTB-2 liquid emulsion, dried overnight at room temperature under a stream of air, and then stored at 4°C in a dessicated light-proof box during the period of ^{3}H decay. After development, the grains were counted by phase-contrast microscopy.

Preparation of bacteriophage labeled with ^{32}P and 5-BrUdR. As described by Miller et al. (25), a culture of *E. coli* B was grown to 5 × 10^8 cells/ml in TCG medium (20), supplemented with 5-bromodeoxyuridine (BrUdR; 250 μg/ml), 5-fluorodeoxyuridine (5 μg/ml), uracil (25 μg/ml), and carrier-free [^{32}P]orthophosphate (100 μCi/ml), and then infected with T4 bacteriophage. After 120 min, the cells were lysed by adding chloroform, and the phage were purified by differential centrifugation.

Extraction of DNA from plasmolyzed phage-infected cells. Native DNA was extracted by incubating the cells for 30 min at 37°C in a solution containing 0.15 M NaCl, 10 mM EDTA, 40 mM Tris-hydrochloride (pH 7.8), and 0.5% sodium dodecyl sulfate. Denatured DNA for sucrose gradients was prepared by incubating the cells for 30 min at 37°C in a solution containing 0.3 M NaOH, 30 mM EDTA, and 0.5% Sarkosyl.

Centrifugation techniques. Equilibrium CsCl sedimentation was conducted with neutral gradients containing 0.16 M NaCl, 0.04 M Tris-hydrochloride (pH 7.8), and 0.01 M EDTA and in alkaline gradients containing 0.2 M NaOH and 0.01 M EDTA. The samples were centrifuged in a SW50.1 rotor at 33,000 rpm for 66 h at 20°C. Velocity sedimentation was carried out in linear 5 to 20% sucrose gradients that contained 0.3 M NaOH and 30 mM EDTA. These separations were carried out in a SW50.1 rotor at 40,000 rpm for 2 h at 4°C. Fractions were collected by piercing the bottom of the tubes and dripping the solution directly onto Whatman 3MM filter disks. The disks were washed twice with cold 5% trichloroacetic acid, twice with cold ethanol, once with ether, and then dried and counted in a liquid scintillation spectrometer.

RESULTS

Utilization of exogenously supplied deoxyribonucleotides by plasmolyzed phage-infected cells. Plasmolyzed preparations of bacteriophage T4-infected *E. coli* B will utilize exogenously supplied deoxyribonucleoside 5'-triphosphates to synthesize DNA. Figure 1 shows the kinetics of incorporation of radioactivity from [^{3}H]dATP into hot alkali-stable, acid-precipitable material by these cells, by plasmolyzed uninfected *E. coli* B, and also by preparations of cells infected with *amB22*, a mutant defective in the phage-induced DNA polymerase (gene 43). The rate of incorporation by T4-infected cells was twice as great as that observed with uninfected cells. In contrast, no significant incorporation occurred in cells infected by phage carrying an *amber* mutation in the structural gene for DNA polymerase; i.e., all of the synthesis observed with plasmolyzed phage-infected PolA$^+$ cells is catalyzed by the phage-induced DNA polymerase.

As shown in Fig. 2, incorporation of radioactive label from [^{3}H]dATP by plasmolyzed T4-infected cells was severely depressed in the absence of any one of the remaining deoxyribonucleoside triphosphates. This substrate requirement could be met by substituting deoxyribonucleoside 5'-monophosphates in place of triphosphates, provided that ATP was also supplied (Fig. 3). Clearly, the phage and bacterial kinase activities responsible for converting monophosphates to triphosphates are maintained in plasmolyzed cells, and the requirement for added ATP must be as a phosphate

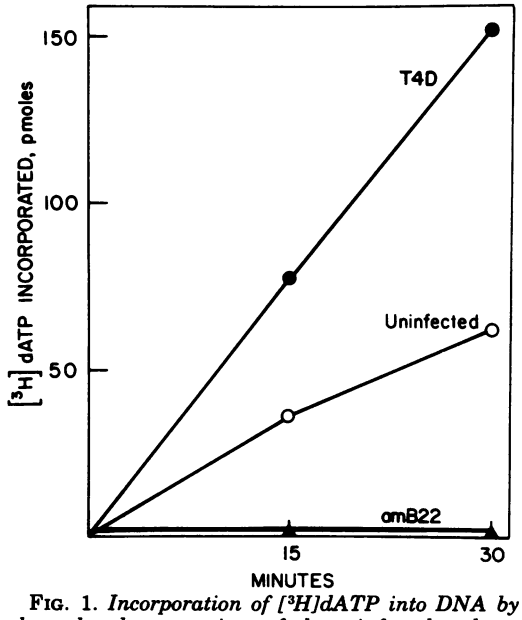

FIG. 1. *Incorporation of [^{3}H]dATP into DNA by plasmolyzed preparations of phage-infected and uninfected E. coli B. The cells were grown, infected, plasmolyzed, and assayed for DNA synthesis as described in Materials and Methods. HMdCTP was the deoxycytidine derivative in the reaction mixture with the phage-infected cells and dCTP was used in the experiments with uninfected plasmolyzed preparations.*

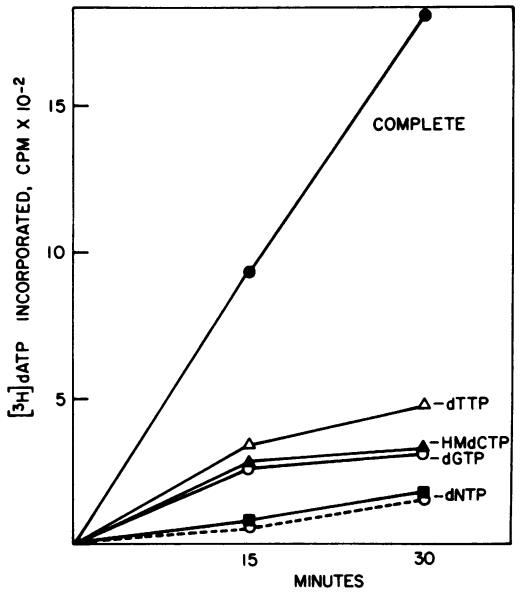

FIG. 2. *Requirement of the entire complement of deoxyribonucleoside triphosphates for incorporation of [^{3}H]dATP in plasmolyzed T4-infected cells. The assay conditions were as described in Materials and Methods but with the substrates present as shown.*

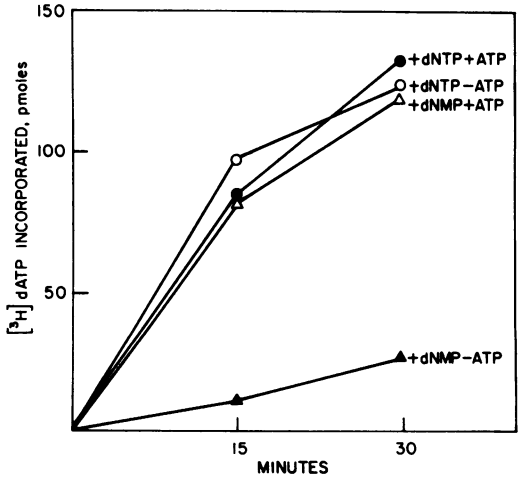

FIG. 3. *Deoxyribonucleoside monophosphates plus ATP support incorporation of [^{3}H]dATP. The assay conditions were as in the legend to Fig. 2 but with the substrates as shown.*

donor in these kinase reactions. On the other hand, unlike several other permeabilized bacterial systems that show a strict requirement for ATP in the synthesis of DNA (15, 27, 37, 40), the addition of ATP to reactions containing all of the deoxyribonucleoside triphosphates showed little or no effect.

In this reaction, dCTP could substitute for hydroxymethylcytosine deoxyribonucleotide (not shown). Although dCTPase activity (gene 56 product) is present, the product, dCMP, did not replace the requirement for HMdCMP. The explanation for this finding is unclear. [5-H^3]dCTP does not enter DNA as [5-^{3}H]dCMP (41). However, after administering [5-^{3}H]dCTP, tritium is released at a rate that could account for the formation of HMdCMP (not shown). It is also possible that external dCMP, per se, cannot enter the pathway to form HMdCMP. Neither dUMP nor dUTP replaced thymine-containing deoxyribonucleotides. Ribonucleotides also failed to replace the corresponding deoxyribonucleotides as substrates for this reaction.

Essentially the same reaction characteristics as described above for the incorporation of label from [^{3}H]dATP were also observed with [^{3}H]HMdCTP and [^{3}H]dTTP (results not presented).

Labeled TdR monitors a second pathway to DNA in plasmolyzed phage-infected cells—the de novo pathway. Figure 4A presents data demonstrating that TdR substituted only poorly for thymine-containing deoxyribonucleotides in support of the incorporation of labeled dATP in plasmolyzed infected cell preparations. Nevertheless, radioactive TdR was readily incorporated into DNA by the same preparation of plasmolyzed cells (Fig. 4B). The apparent rate of this reaction was two to three times greater than that observed with [^{3}H]dATP, and the high rate of incorporation was only slightly affected by the absence of an exogenous supply of the other deoxyribonucleotide precursors of DNA. Furthermore, addition of dTTP had no diluting effect on the incorporation of labeled TdR.

The behavior of labeled TdR suggested that it was monitoring the de novo pathway to DNA in these cells. To test this possibility, assays were run in the presence of hydroxyurea (HU), an agent that inhibits ribonucleoside diphosphate reductase (32, 39). Figure 5 shows that the addition of 0.01 M HU to the standard assay mixture greatly depressed the incorporation of [^{3}H]TdR even in the presence of the other deoxyribonucleotide substrates and ATP. By contrast, HU had little effect on the incorporation of label from [^{3}H]dATP or other ^{3}H-labeled

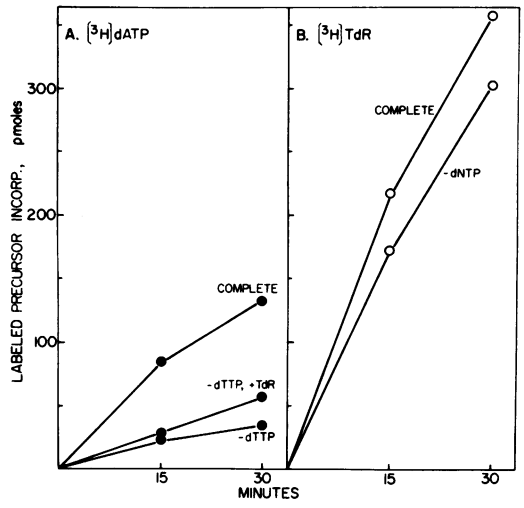

FIG. 4. *Comparison of the incorporation of [^{3}H]dATP and [^{3}H]TdR. The conditions were as in the legend to Fig. 2. Aliquots of the same plasmolyzed preparation of infected cells were assayed in the standard reaction mixture containing either (A) [^{3}H]dATP or (B) [^{3}H]TdR plus the other substrates as shown.*

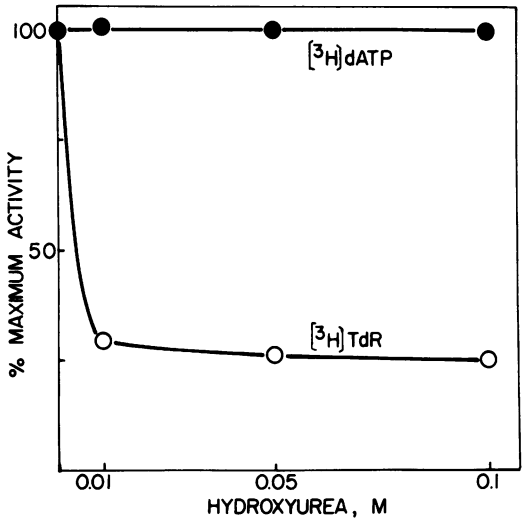

FIG. 5. *Differential effect of HU on the incorporation of labeled dATP and TdR into DNA. Aliquots of the same preparation of plasmolyzed, infected cells were assayed for DNA synthesis using either [^{3}H]dATP or [^{3}H]TdR as the tracing compounds as in Fig. 4. HU was added at the concentrations shown, and the radioactivity incorporated into the DNA fraction was measured after 15 min at 37°C.*

deoxyribonucleoside triphosphates (not shown) under the same assay conditions.

Labeled ribonucleotides were also readily incorporated into DNA by the same cell prepara-

tions (not shown). As with labeled TdR, this incorporation was also unaffected by the presence or absence of the other deoxyribonucleotides but was completely inhibited by the addition of hydroxyurea. These experiments, taken together with those in the previous section, show that ribonucleotides do not support the deoxyribonucleoside triphosphate-dependent pathway to DNA. Instead, they enter DNA via the reductive de novo pathway, and TdR enters and monitors this pathway at another level.

Exogenously supplied HMdCMP incorporated by both pathways to DNA. Although exogenously supplied deoxyribonucleoside triphosphates are not incorporated into DNA in plasmolyzed phage-infected cells by the [^{3}H]TdR-monitored pathway described above, it is clear that HMdCMP can be utilized by this pathway. Although the incorporation of label from [^{3}H]HMdCTP, like [^{3}H]dATP, was almost completely dependent upon an added supply of the other deoxyribonucleoside triphosphates (previous section), HMdCMP incorporation showed a different pattern. Figure 6 shows the incorporation of HMdCMP into DNA in the presence (A) and in the absence (B) of ATP. With both ATP and deoxyribonucleoside 5'-monophosphates present, HMdCMP entered the triphosphate-dependent reaction, since HU had little effect (Fig. 6A). In the presence of ATP but in the absence of the deoxyribonucleoside 5'-monophosphates, a somewhat reduced incorporation of labeled HMdCMP occurred. Under these conditions, HU markedly inhibited the incorporation. The HU-sensitive fraction is considered to be de novo synthesis.

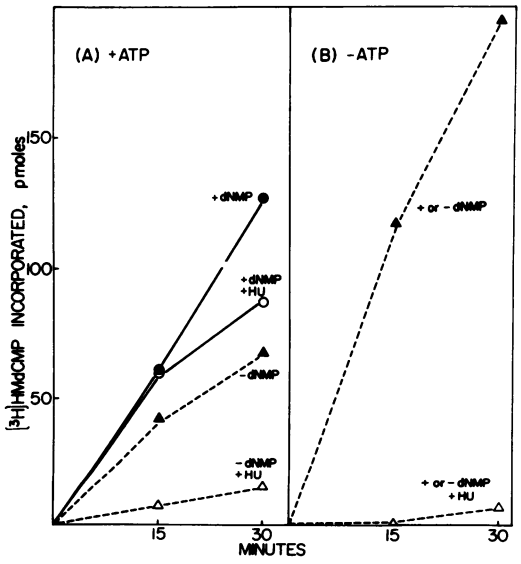

FIG. 6. *[^{3}H]HMdCMP is incorporated into DNA by both pathways; effect of ATP. The reaction mixtures were as described in Materials and Methods but with dAMP, dGMP, dTMP, and ATP present as shown. HU was added as indicated to a concentration of 0.05 M.*

In the absence of ATP and independent of the other deoxyribonucleotides (Fig. 6B), labeled HMdCMP was funneled almost quantitatively into the HU-sensitive de novo pathway to DNA and, in fact, this incorporation occurred at an even greater rate than was observed in the triphosphate-dependent reaction. A reasonable interpretation of these findings is that HMdCMP can be incorporated into DNA by either pathway. Previous studies have shown that HMdCM^{32}P is incorporated intact into DNA via the dNTP-dependent pathway (41).

Since labeled dAMP and dGMP were not tested directly, it is not known whether they are incorporated into the de novo pathway. Labeled dTMP could not be tested easily because it can be converted to TdR which, of course, enters the de novo pathway.

Substrates utilized by the two pathways in plasmolyzed phage-infected cells are incorporated into separate DNA products. The characteristics of incorporation of various added substrates clearly indicate two distinct pathways to DNA in plasmolyzed preparations of phage-infected cells. The following experiment was conducted to determine whether these pathways represented separate channeling of different substrates into a common synthetic reaction or into mechanisms synthesizing separate products. Plasmolyzed T4-infected cells were incubated in the standard reaction mixture containing [^{14}C]TdR plus [^{3}H]dATP, BrdUTP, dGTP, and HMdCTP. After 30 min at 37°C, the cells were lysed as described under Materials and Methods, and the reaction products were centrifuged to equilibrium in alkaline CsCl density gradients. All of the ^{14}C-labeled DNA synthesized in this reaction was found as a very sharp peak at the fully light position in the gradient (Fig. 7). In contrast, the [^{3}H]DNA banded as a significantly heavier and broader peak, with some of the material extending even to the bottom of the gradient.

These results clearly demonstrate that the substrates utilized by these two pathways are incorporated into separate products. The sharpness of the peak containing the ^{14}C-labeled material also indicates that little or no genetic recombination occurs between the products of the two reactions. The broad banding pattern of the [^{3}H]DNA, with some also found at the light position in the gradient, suggests that the BrdUTP utilized in this reaction may have mixed with an endogenous source of thymine-

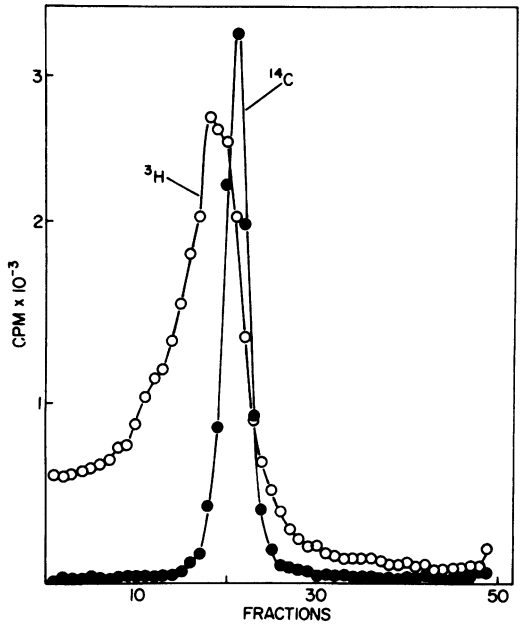

FIG. 7. *Incorporation of [³H]dATP and [¹⁴C]TdR into separate DNA products by plasmolyzed T4-infected cells. The reaction mixture contained the standard additions including 100 nmol of ATP and 10 nmol each of [³H]dATP (0.5 μCi/nmol), [¹⁴C]TdR (0.05 μCi/nmol), BrdUTP, dGTP, HMdCMP, and 5 × 10⁸ plasmolyzed cells. After 30 min at 37°C, the cells were lysed and the DNA banded in an alkaline CsCl density gradient (initial density = 1.74 g/cm³) as described in Materials and Methods.*

containing substrates, either from host DNA breakdown or by leakage from the de novo pathway (Fig. 2).

Two pathways occur within the same cells, not separate populations. A possible explanation for the two apparent pathways to DNA in plasmolyzed cells is that they represent separate reactions by two different subpopulations in the total cell preparation. For example, the de novo pathway described above might represent synthesis by relatively intact cells, whereas the utilization of exogenous triphosphates might be restricted to those cells that were rendered permeable and perhaps more severely disrupted by the plasmolysis procedure. However, several lines of evidence argue against this possibility.

First, both modes of synthesis were similarly depressed in plasmolyzed preparations of Su⁻ cells infected with phage bearing *amber* mutations in various DNA synthesis genes (see Table 2 in the next section). This indicates that both pathways represent undisturbed systems requiring the gene products necessary in vivo. The next section elaborates on this idea. It may be noted that both pathways were observed in cells infected with the *amber* mutant H26 (gene e); these plasmolyzed infected cells were extremely stable to manipulation after plasmolysis, suggesting that the two types of synthesis did not occur as a consequence of some inherent instability of a major portion of the infected-cell population.

Direct evidence in support of this conclusion was obtained by autoradiographic analysis of plasmolyzed *amH26*-infected cells. Portions of the same preparation were incubated in the standard incorporation mixture containing either [³H]dATP plus the other three nucleotide substrates or else [³H]TdR as the sole exogenously supplied DNA precursor and then treated as described in Materials and Methods. The observed distribution of grains among cells from each incorporation reaction was very close to that expected for a random distribution (Table 1). We conclude that the fraction of plasmolyzed cells synthesizing DNA by each pathway is close to one. Not more than a few percent of a second species could be present (23). The results are consistent with the concept that the two pathways occur in the same cell.

Thus far it could be argued that the de novo pathway to DNA occurs in only a few percent of the cells that survive plasmolysis and that the dNTP-dependent pathway occurs in broken cells. Figure 8 presents an experiment which shows that in the plasmolyzed preparation the incorporation of labeled TdR or uridine (after

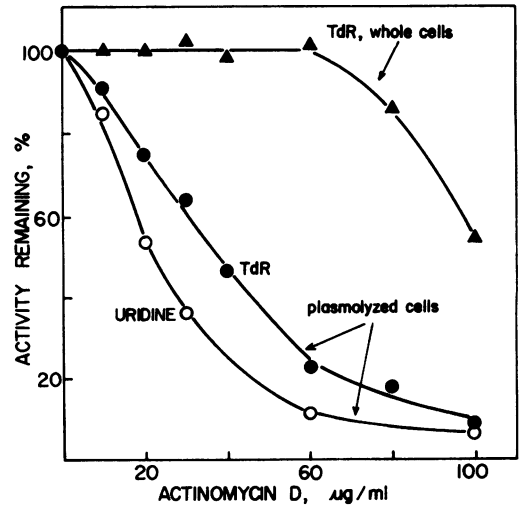

FIG. 8. *Differential effects of actinomycin D on the de novo pathway to DNA in plasmolyzed and whole infected cells. The conditions were as described in Materials and Methods using either labeled TdR or uridine with ATP but without deoxyribonucleoside triphosphates.*

conversion through the reductive pathway) into DNA is susceptible to low levels of actinomycin D, whereas in whole cells these levels are without effect. This experiment is a strong argument that the TdR and uridine labels are not incorporated only by whole cells.

Control of DNA synthesis in plasmolyzed phage-infected cells; effect of *amber* and temperature-sensitive mutations on each reaction. Table 2 shows the level of incorporation by each reaction in plasmolyzed preparations of cells infected with various *amber* mutants of T4. For the most part, DNA synthesis by each reaction in this system resembled that observed in vivo in cells infected by these mutants. Thus, synthesis was drastically reduced in the absence of a functional phage-induced polymerase (gene 43) or the products of the *dna* genes 41, 44, and 45 (1, 26). It is especially significant that in both pathways mutants in genes 39 and 59 showed only partially reduced levels of DNA synthesis in the in situ system, in agreement with their in vivo characteristics. This clearly distinguishes the dNTP-dependent pathway from the in vitro system of Alberts and coworkers, which is dependent only on the products of genes 32, 41, 43, 44, 45, and 62. Table 2 also shows that a mutant in gene 46 caused a greater effect on the de novo pathway than on the substrate-dependent pathway.

As we have described (41), dNTP-dependent DNA synthesis was negligible in plasmolyzed cells infected with phage bearing *amber* mutations in the structural genes for dCMP hydroxymethylase or the phage-induced deoxyribonucleoside monophosphate kinase. Similar findings have also been observed by other workers in both plasmolyzed (7) and toluenized (9) T4-infected *E. coli*. Clearly, both plasmolyzed and toluenized phage-infected cells incorporate exogenous deoxyribonucleotides into DNA, and we have interpreted the inability to bypass these two enzymes with their products as evidence that they play more direct roles in DNA replication. A simpler explanation which has been suggested (9) is that DNA synthesis in these cells is dependent upon the establishment of growing points before treatment; since initiation had never occurred in cells infected with these mutants, it follows that DNA synthesis in situ would be low. To test this possibility, cells were infected under permissive conditions with the temperature-sensitive gene 42 mutant, *LB1*. After DNA synthesis had initiated in vivo, the infected cells were plasmolyzed and assayed for incorporation by both the de novo pathway (Fig. 9) and the triphosphate-dependent reaction (Fig. 10). Incorporation of [^{3}H]HMdCMP by the de novo pathway was

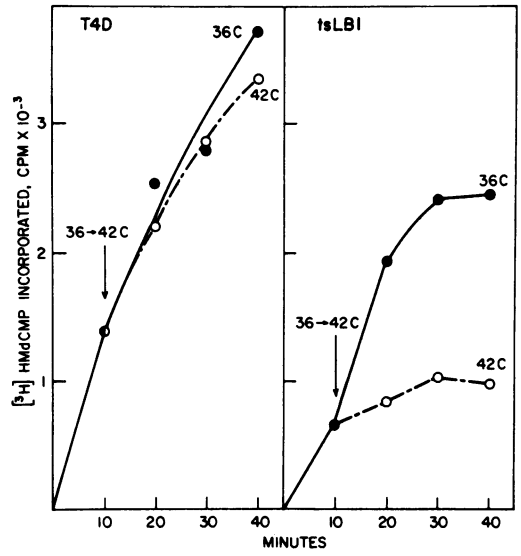

FIG. 9. *Incorporation of [^{3}H]HMdCMP into DNA via the de novo pathway in plasmolyzed cells infected with a temperature-sensitive mutant of gene 42. A culture of E. coli B was grown at 36°C to a density of 5 × 10^8 cells/ml and then divided into two portions. One portion was infected with T4, and the other was infected with tsLB1. At 10 min after infection, the cells were plasmolyzed and incubated at 36°C in a standard reaction mixture containing [^{3}H]HMdCMP (8 cpm/pmol) as a sole supplied substrate and without ATP. After 10 min, a portion of each reaction mixture was shifted to 42°C, a nonpermissive temperature for the tsLB1-infected cells. Samples of 0.1 ml (5 × 10^8 cells) were taken at the times shown.*

very low at the nonpermissive temperature of 42°C (not shown). Figure 9 shows that when the temperature was shifted from the permissive temperature of 36 to 42°C, the incorporation was rapidly reduced. Synthesis by plasmolyzed cells infected with wild-type phage was unaffected by the temperature shift. This result is not explained by the shutoff of dTMP synthesis reported by Tomich et al. (36). In our previous studies, addition of thymine derivatives did not allow incorporation of HMdCMP or HMdCTP in *amber* mutants of gene 42 (41). Clearly, in the de novo pathway, a functioning dCMP hydroxymethylase is required to incorporate HMdCMP into DNA even after DNA synthesis has commenced.

However, incorporation of labeled HMdCMP or HMdCTP by the deoxyribonucleoside triphosphate-dependent reaction in plasmolyzed *tsLB1*-infected cells was not temperature sensitive (Fig. 10). This reaction appears also not to be dependent upon growing points established before permeabilization, since plasmolyzed preparations of *tsLB1*-infected cells carry out

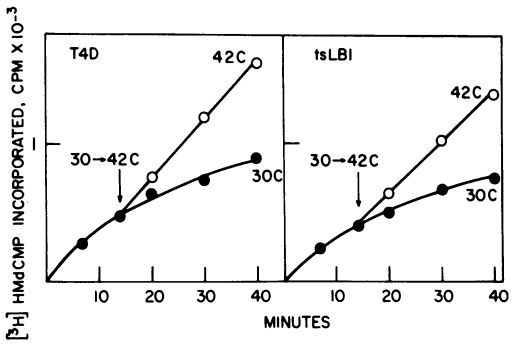

FIG. 10. *Incorporation of [³H]HMdCMP into DNA via the deoxyribonucleoside triphosphate-dependent reaction in plasmolyzed cells infected with a temperature-sensitive mutant of gene 42. Infected cells were prepared and assayed as described in Fig. 9 except that the infections and initial incubation were at 30°C, and the reactions contained ATP and the other deoxyribonucleoside monophosphates in addition to [³H]HMdCMP.*

immediate and normal levels of synthesis even when prepared under strictly nonpermissive conditions, i.e., infecting at 42°C and then adding chloramphenicol before harvesting and plasmolysis (data not shown). It is not clear why a *ts* mutant of gene 42 (LB1) had no effect on the substrate-dependent pathway, but blocked the de novo pathway. Wovcha et al. had shown previously that an *amber* mutant of gene 42 could not be bypassed by HMdCMP or HMdCTP under conditions in which the dNTP-dependent pathway was being assayed (41). Conceivably the structure of the de novo complex has a more rigorous requirement for the correct configuration of dCMP hydroxymethylase.

In contrast to our results with the gene 41 mutant *amN81*, Collinsworth and Mathews found that cells infected with this same phage gave nearly normal synthesis in the plasmolyzed system as compared to wild-type infections (7). They also reported that a mutant in gene 1, *amE957*, gave normal synthesis in situ when triphosphates were supplied, whereas another gene 1 mutant, *amB24*, did not synthesize DNA in this system. In our hands, cells infected with either *amB24* or *amE957* were blocked in DNA synthesis in situ. These workers used *E. coli* D110 as the host bacterium, whereas our infections were done with an *E. coli* B strain, and the discrepancy between our findings and theirs is apparently related to differences between these two strains. Thus, we also found that incorporation by *amN81*-infected D110 in this system was only slightly less than that observed with wild type-infected D110 but much greater than that observed with *amN81*-infected *E. coli* B (data not shown).

Other characteristics of the reactions. Besides the observed differences in exogenous substrate utilization, the two modes of synthesis were found to differ with respect to certain other reaction characteristics (Table 3). Thus, deoxyribonucleoside triphosphate incorporation was severely depressed in the absence of added Mg^{2+} whereas the de novo pathway was unaffected. Even the addition of EDTA failed to block this latter reaction, suggesting that any divalent cations required for this incorporation must be endogenously supplied and either inaccessible to this chelating agent or else tightly bound to the proteins involved. Neither reaction was affected by the addition of pancreatic DNase, indicating that this system is not readily permeable to macromolecules of this size (molecular weight, 31,000 [24]) at least. This is in contrast to the observed inhibitory effect of this enzyme on DNA synthesis in plasmolyzed uninfected cells (40). Micrococcal nuclease, which has a molecular weight of 16,800 (35), inhibited the substrate-dependent reaction but not the de novo pathway, suggesting that these reactions are occurring at different sites within the cell. Rifampin and nalidixic acid had no demonstrable effect on synthesis by either reaction; the latter does not, however, inhibit T4 DNA synthesis in vivo. NEM is a potent inhibitor of T4 DNA polymerase (17) and of DNA replication in other permeabilized T4-infected (7, 9) and uninfected (15, 27, 37, 40) systems. In

TABLE 3. *Characteristics of incorporation by each reaction in plasmolyzed phage-infected cells*[a]

Condition	Relative incorporation of:	
	[³H]dATP	[³H]TdR
Complete	100	100
− Mg^{2+}	24	100
− Mg^{2+} + EDTA (20 mM)	0	118
+ Micrococcal nuclease (100 μg/ml)	45	100
+ Pancreatic DNase (100 μg/ml)	107	100
+ Rifampin (100 μg/ml)	100	100
+ Nalidixic acid (100 μg/ml)	84	85
+ NEM (5 mM)	142	6

[a] Reactions were as described in Materials and Methods. Incubation was at 37°C for 30 min. In the experiments with micrococcal nuclease and pancreatic DNase, the cells were preincubated with the enzyme (or buffer as a control) for 5 min at 37°C, and the remaining reaction components were then added.

plasmolyzed T4-infected *E. coli* B cultures this agent effectively blocked the de novo pathway but actually stimulated synthesis by the substrate-dependent reaction. However, we found that both reactions were blocked by this compound in T4-infected *E. coli* D110 (*polA*$^-$; data not shown), suggesting that the incorporation noted in T4-infected *E. coli* B in the presence of NEM was catalyzed by DNA polymerase I. Furthermore, any sulfhydryl enzyme in the de novo pathway might be blocked by NEM.

The possible role of host DNA polymerase I raised the question of the specificity of the DNA synthesized by these reactions in T4-infected PolA$^+$ cells. To determine the degree of specificity, the DNA product formed in each reaction was isolated and hybridized against T4 and *E. coli* DNA. As seen in Table 4, the DNA formed by the de novo reaction, and by the substrate-dependent reaction in the absence of NEM, was largely phage DNA. In the presence of NEM, an equivalent amount of phage DNA was synthesized in the substrate-dependent reaction, but now a sizeable amount of *E. coli* DNA was also formed. It is the synthesis of this *E. coli* DNA that presumably accounts for the stimulation of incorporation observed in the presence of NEM.

TABLE 4. *Specificity of the DNA synthesized by each reaction in plasmolyzed cells*[a]

Source of product DNA	Label incorporated	Radioactivity hybridizable with:	
		T4 DNA	*E. coli* DNA
T4-infected *E. coli*	[^{3}H]dATP	4,200	764
T4-infected *E. coli* (+5 mM NEM)	[^{3}H]dATP	5,840	3,120
T4-infected *E. coli*	[^{3}H]TdR	13,800	2,950
Uninfected *E. coli*	[^{3}H]dATP	242	1,050
Uninfected *E. coli*	[^{3}H]TdR	0	1,540

[a] Plasmolyzed preparations of T4-infected and uninfected *E. coli* B were incubated in reactions as described in Fig. 10. After 30 min at 37°C the reaction mixtures were diluted 10-fold with cold 0.15 M NaCl-0.015 M sodium citrate (pH 7.0) and sonicated, and the DNA was extracted with phenol. The newly synthesized DNA was hybridized against DNA from T4 or *E. coli* as described by Denhardt (8). The radioactivity is expressed as counts per 10 min.

CsCl density gradient analysis of the product DNA. The following experiments demonstrate that both of the pathways described above in plasmolyzed phage-infected cells incorporate exogenously supplied substrates into DNA in a replicative manner. *E. coli* B was infected with T4 phage containing DNA labeled with ^{32}P and rendered more dense by administration of 5-BrUdR. The infected cells were harvested at 10 min, plasmolyzed, and incubated at 37°C in the standard reaction mixture containing either [^{3}H]dATP plus the other substrate nucleotides and ATP, or else [^{3}H]TdR alone. After 30 min, the cells were lysed as described in Materials and Methods, and the reaction products were centrifuged to equilibrium in CsCl density gradients. Most of the newly synthesized ^{3}H-labeled material in each reaction mixture banded in neutral gradients at the position of fully light DNA (Fig. 11). A lesser amount banded at the hybrid position, and very little ^{3}H label was found at the heavy parental location. When centrifuged to equilibrium in alkaline CsCl gradients, all of the ^{3}H-labeled DNA from the reactions described above now banded at the fully light position (Fig. 12).

Clearly, and in contrast to synthesis by a repair type mechanism, the progeny DNA was not covalently attached to the parental DNA in either reaction. In addition to indicating a replicative mode of synthesis, these results, which were obtained without extensive fragmentation of the DNA, again demonstrate that recombination is much lower in the plasmolyzed system than in vivo (compare Fig. 7 and reference 21). The fact that newly synthesized DNA was found at the light position in the neutral gradients as well as at the hybrid location argues that reinitiation must occur in the plasmolyzed T4-infected system, in agreement with the reported characteristics of DNA synthesis in plasmolyzed M13-infected *E. coli* (34).

Since under these conditions a considerable quantity of light-labeled progeny DNA is synthesized in the first 10 min after infection, it could be argued that the banding patterns described above are a result of preferential replication (or repair) of this progeny DNA and thus do not accurately reflect the relationship between DNA synthesized after plasmolysis and its template-primer. To test this possibility cells, both grown for several generations and infected in the presence of BrUdR, were plasmolyzed, assayed, and analyzed as above. Figure 13 shows the resulting profiles in alkaline CsCl gradients of the newly synthesized and parental DNA in each reaction. As in the experiment shown in Fig. 12, a considerable portion of DNA synthesized in situ in each reaction banded at the fully light position of the gradient. The occurrence of some ^{3}H-labeled material at hybrid locations is expected, since part of the DNA synthesized in situ would represent elongation of strands already initiated at the time of plasmolysis. These results clearly support the conclusion that both of the pathways to

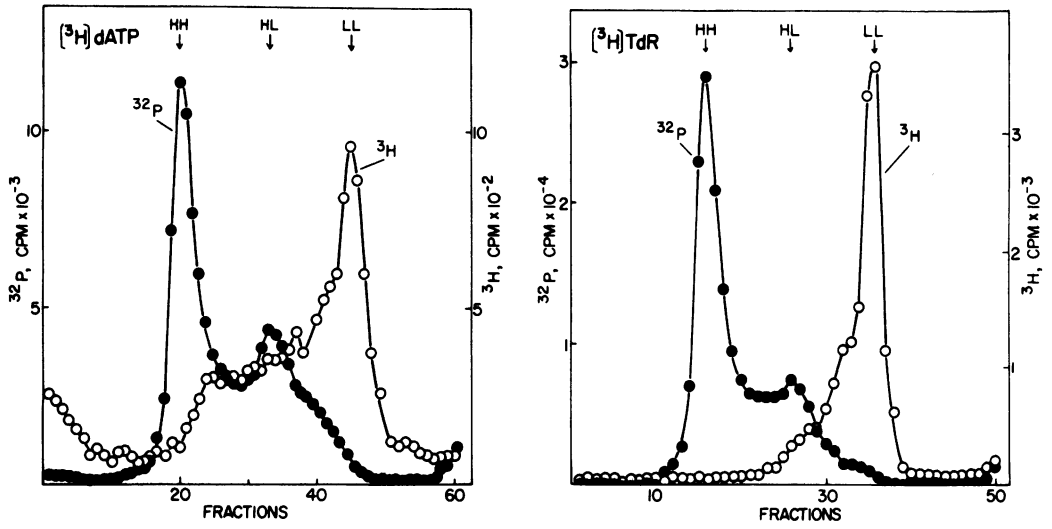

FIG. 11. *Neutral CsCl density gradient analysis of the DNA synthesized in the de novo and in the deoxyribonucleoside triphosphate-dependent reactions in plasmolyzed T4-infected E. coli B. The culture was grown at 37°C to a cell concentration of 5 × 10⁸/ml and then infected at a multiplicity of 5 with T4 that had been labeled with ³²P and 5-BrUdR. After 10 min of infection, the cells were harvested, plasmolyzed, and then incubated in standard reactions containing ATP and either the combined four nucleotides, [³H]dATP (0.5 μCi/nmol), dGMP, dTMP, and HMdCMP or [³H]TdR (0.33 μCi/nmol) alone. After incubation for 30 min at 37°C, the plasmolyzed cells were lysed, and the DNA banded in neutral CsCl gradients (initial density = 1.74 g/cm³) as described in Materials and Methods. The indicated reference positions were determined by centrifugation of light (LL) and fully-heavy (HH) T4 DNA under identical conditions.*

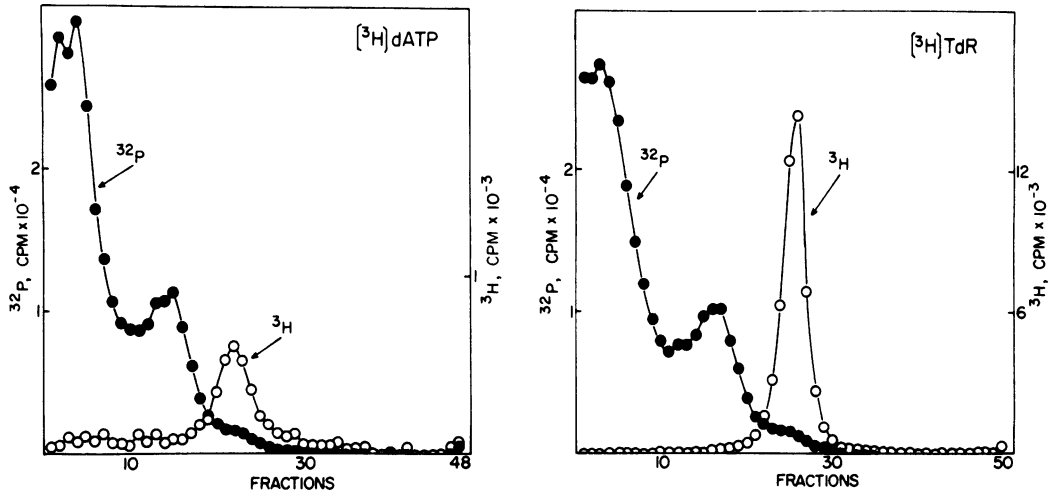

FIG. 12. *Alkaline CsCl density gradient analysis of the DNA synthesized by the two pathways. DNA was prepared and banded in alkaline CsCl gradients as described in Fig. 11.*

DNA observed in plasmolyzed T4-infected cells primarily carry out replicative DNA synthesis.

The sharp, rather evenly spaced peaks observed in Fig. 13 ([³H]ATP) are unexplained. They were observed in other analyses by neutral and alkaline CsCl gradients of the deoxyribonucleoside triphosphate reaction products. No such pattern was found with material synthesized in the de novo reaction.

Size of the product DNA. Plasmolyzed preparations of cells infected with ³²P-labeled phage were incubated in the standard reaction mixture containing either [³H]dATP plus the other substrate deoxyribonucleotides and ATP or

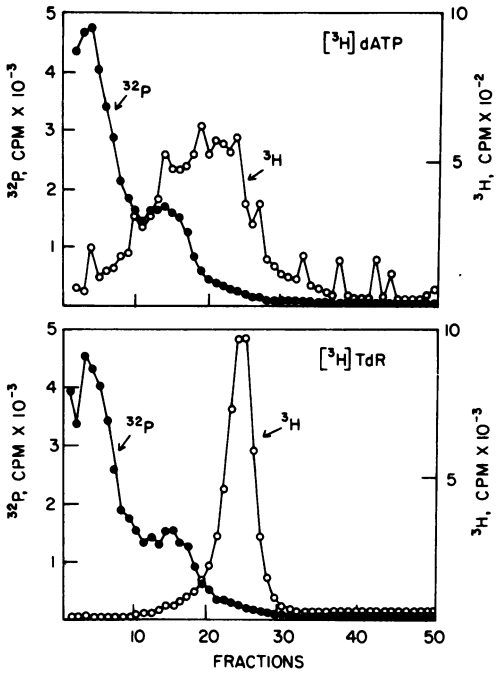

FIG. 13. *Alkaline CsCl density gradient analysis of the DNA synthesized by the two pathways. The experimental conditions were as described in Fig. 11 except that the cells were grown for several generations, and infected, in medium containing 5-BrUdR (250 μg/ml), 5-fluorodeoxyuridine (5 μg/ml), and uracil (25 μg/ml).*

[3H]TdR alone. After 30 min at 37°C, the cells were lysed as described in Materials and Methods, and the reaction products were analyzed by sedimentation through alkaline sucrose gradients (Fig. 14). Part of the [3H]TdR incorporated under these conditions corresponded in size to mature T4 DNA and also occurred as a rather broad peak of somewhat smaller pieces. By contrast, all of the label incorporated into DNA in the [3H]dATP-containing reaction was found in a uniform peak of slowly sedimenting fragments. Unlike the results reported by Miller et al. for deoxyribonucleoside triphosphate-dependent DNA synthesis in toluene-treated phage-infected cells (25), the size of the product of this reaction was not affected by the concentration of dNTP in the reaction mixture. The possibility that the lower-molecular-weight material is converted to mature phage DNA needs to be explored. It is also evident from Fig. 14 that some of the parental DNA was extensively fragmented in these experiments.

DISCUSSION

This paper has presented evidence that two independent, nonmixing deoxyribonucleotide pathways or channels within the same infected cell lead to bacteriophage T4 DNA replication and that the DNA products of the two modes of synthesis are separable. One of these channels, carrying the bulk of the synthesis, is the de novo pathway to deoxyribonucleotides. The other is the deoxyribonucleoside triphosphate- or substrate-dependent pathway.

These conclusions are based on the following findings. (i) Deoxyribonucleoside triphosphate incorporation into DNA in plasmolyzed, infected cells is dependent on the presence of all four deoxyribonucleotides. TdR (which monitors the de novo pathway) and ribonucleotides are incorporated even more rapidly into DNA

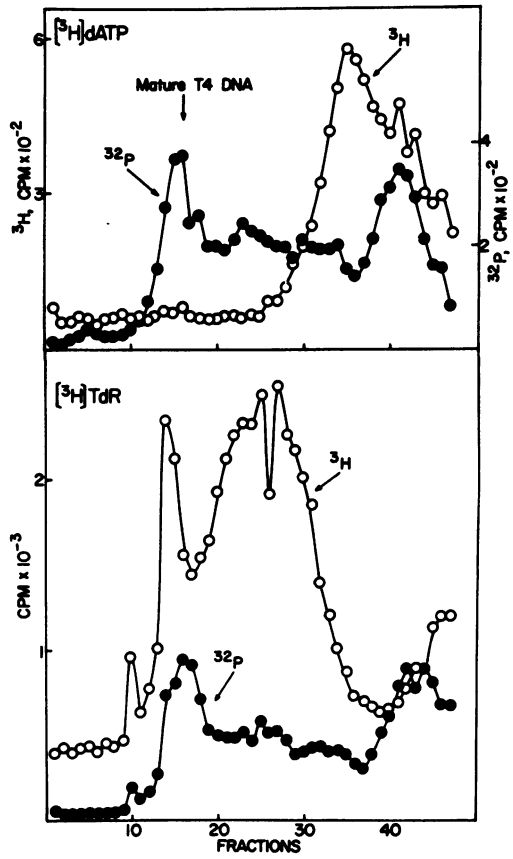

FIG. 14. *Sedimentation analysis of the product and parental DNA in plasmolyzed T4-infected cells. A culture of E. coli B was infected with 32P-labeled T4 and then plasmolyzed and incubated under conditions similar to those described in Fig. 11. The cells were lysed, and the DNA was sedimented in 5 to 20% alkaline sucrose gradients as described in Materials and Methods. The position of the indicated reference marker for mature T4 DNA was determined by sedimentation in a parallel gradient.*

in the same cell. However, the de novo pathway does not provide the deoxyribonucleoside triphosphates required for in the incorporation of labeled dATP. Thus, TdR will not replace dTTP in the deoxyribonucleoside triphosphate-dependent pathway (Fig. 4). Furthermore, the incorporation of labeled TdR into DNA is unaffected by the addition of deoxyribonucleoside triphosphates, including dTTP.

(ii) The de novo pathway is inhibited by HU, whereas the deoxyribonucleoside triphosphate-dependent system is unaffected (Fig. 5).

(iii) The two pathways differ greatly in their response to various agents and their dependence on added Mg^{2+} (Table 3).

(iv) Both channels are dependent on the same T4-induced *dna* gene products for DNA replication.

(v) DNA is formed in both pathways by replicative synthesis, with little or no repair synthesis, and the bulk of the DNA products of the two pathways hybridize with T4 DNA.

(vi) Autoradiographic analysis gives evidence that the two pathways both occur within the same cell and not two separate cell populations.

(vii) Incorporation of labeled TdR or uridine (via the reductive pathway) into DNA in plasmolyzed preparations is inhibited by low concentrations of actinomycin D, whereas infected whole cells are resistant to this antibiotic over the same concentration range. This is a strong argument that the de novo system occurs in plasmolyzed cells.

(viii) The DNA products formed in the same culture by the two pathways can be separated from one another by use of a density label.

The properties of the two pathways are summarized in Table 5.

The channeling of nucleotides into the replication complex needs to be considered in terms of our previous studies. Chiu and Greenberg (6), using a temperature-sensitive mutant of phage T4 in gene 42, presented evidence that dCMP hydroxymethylase has an intimate role in DNA replication. Wovcha et al. (41) and Collinsworth and Mathews (7) demonstrated that *amber* mutations in the T4 structural gene for dCMP hydroxymethylase or HMdCMP kinase could not be bypassed by their deoxyribonucleotide products in infected cells rendered permeable by plasmolysis with concentrated sucrose. From these studies we concluded that these enzymes were an integral and obligatory part of the DNA replication complex.

More recently, Tomich et al. have described in vivo experiments which show that the synthesis of HMdCMP and dTMP after phage T4 infection at 30°C begins at about 5 min (36). The rate of synthesis of these deoxyribonucleotides increases in an exponential manner until about 20 min and then becomes constant. Concurrently, the kinetics of DNA synthesis coincide exactly with that of the deoxyribonucleotides in exponential character and timing (5, 36). A model was suggested in which the enzymes forming deoxyribonucleotides via reduction of ribonucleotides are complexed with the polymerization enzymes (5, 36, 41). The present studies extend this model by demonstrating a channel from ribonucleotides to deoxyribonucleotides and thence to DNA (Fig. 15). This channel shows little or no mixing with deoxyribonucleoside triphosphates added to the system. Most recently, J. B. Flanegan (Ph.D. thesis, Univ. of Michigan, Ann Arbor, 1975) has shown that the synthesis of the deoxyribonucleotides via the de novo pathway is carefully regulated at a ratio of thymine to hydroxymethylcytidine derivatives synthesized throughout the infection process of close to 2:1, exactly the ratio in T4 DNA. This ratio is maintained even with T4 *dna* mutants for at least 25 min after infection. It was shown earlier that the rate of synthesis of the sum of the dTMP and HMdCMP derivatives is exactly

TABLE 5. *Comparison of the two pathways to T4 DNA*

Characteristic	De novo path	dNTP path
Inhibited by HU	Yes	No
Requires addition of Mg^{2+}	No	Yes
Sensitive to NEM	Yes	No
Affected by mutants of T4 genes 1, 32, 39, 41, 43, 44, 45, 46[a] and 59	Yes	Yes
Requires addition of the 4 deoxyribonucleotides	No	Yes
Represents repair functions	No	No
Replicative synthesis	Yes	Yes

[a] See Results.

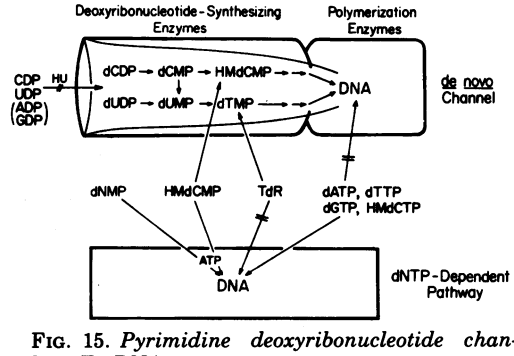

FIG. 15. *Pyrimidine deoxyribonucleotide channels to T4 DNA.*

the same in the presence or absence of DNA synthesis. Thus, the major control of deoxyribonucleotide synthesis does not seem to be dependent on the level of deoxyribonucleotides. Since the levels increase very greatly in DNA mutants, these findings seem to be more in keeping with a regulatory system dependent on the formation of a specific complex of enzymes and less with a feedback or effector mechanism, though the T4-induced ribonucleoside diphosphate reductase has been shown by the studies of Berglund and co-workers to be beautifully controlled by effector deoxyribonucleoside triphosphates (2, 3), and though Bessman and coworkers (12, 30) demonstrated that T4-induced dCMP deaminase is precisely regulated by the relative concentrations of dTTP, which is an inhibitor of the enzyme, and HMdCTP, which is an activator.

In another study Flanegan and Greenberg (manuscript in preparation) have suggested that the relative resistance of the system to the accumulation of deoxyribonucleotides occurs because the enzymes are in a complex and do not readily respond to feedback effector compounds. An alternative explanation that the enzymes are physically separated from the main body of the deoxyribonucleotides is, in fact, another description of a complex.

It is striking that two channels for synthesizing T4 DNA appear to exist within the same cell. It may be that the deoxyribonucleoside triphosphate-dependent reaction represents a separate mechanism for synthesizing phage DNA from salvaged deoxyribonucleotides such as, for example, those derived from the breakdown of the host chromosome. Nucleotides derived from this source do not mix with those synthesized de novo (36). In this regard it is also interesting to note that T4 mutants have recently been described which show normal host DNA degradation and yet are HU sensitive (16). Among these mutants are some that apparently cannot reincorporate the nucleotides derived from the bacterial chromosome into phage DNA. These mutants conceivably could have defects in a component(s) required specifically for this reaction but not for the de novo pathway. If, in fact, the triphosphate-dependent reaction represents the host breakdown pathway, a reasonable prediction is that two species of phage will be formed in a recombination-deficient mutant, one containing DNA primarily from the host DNA breakdown and the other from de novo synthesis.

We note that Billen et al. (4) have observed a differential utilization of TdR versus dTTP in *B. subtilis* rendered permeable by a freeze treatment. Their results also indicated that these substrates were incorporated into DNA by independent reactions occurring at separate sites within the cell. However, a major difference is that the triphosphate reaction which they observed was shown to be repair synthesis, whereas both reactions in plasmolyzed T4-infected cells appear to be replicative.

ACKNOWLEDGMENTS

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We are grateful to Helen Gay of the Department of Zoology for her direction in carrying out the autoradiography studies and to Mary Wovcha for calculating the Poisson distribution curves.

ADDENDUM IN PROOF

Since the submission of this paper, North et al. (T. W. North, M. E. Stafford, and C. K. Mathews, J. Virol. 17:973-982, 1976) have shown that dNTP-dependent DNA synthesis is blocked in temperature-shift experiments with *tsLB1*-infected plasmolyzed cells. However, in our hands the de nova, but not the dNTP-dependent, reaction was blocked by this mutant.

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