Regulation of Deoxyribonucleotide Biosynthesis during in Vivo Bacteriophage T4 DNA Replication

INTRINSIC CONTROL OF SYNTHESIS OF THYMINE AND 5-HYDROXYMETHYLICYTOSINE DEOXYRIBONUCLEOTIDES AT PRECISE RATIO FOUND IN DNA

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The kinetics of the de novo formation of pyrimidine deoxyribonucleotides is the same after infection by wild type bacteriophage T4, which generate very low steady state levels of deoxyribonucleotides, and by T4 DNA synthesis-negative mutants (Dna-), which accumulate high levels, suggesting that the control is not by a feedback mechanism. In this study, the ratio of the de novo synthesis of dTMP to HmdCMP derivatives was measured by determining the total thymine and 5-hydroxymethylcytosine (Hmc5Cyt) deoxyribonucleotides synthesized by the reductive pathway from [6-3H]uracil including those in DNA and any degradation products excreted into the medium.

The ratio of the de novo synthesis of Thy/Hmc5Cyt derivatives remained constant at 2.1 ± 0.1 for at least 45 min after infection by wild type phage, i.e. precisely at the Thy/Hmc5Cyt ratio in T4 DNA. On infection by phage mutated in the Dna- genes 32, 41, 44, or 45, the ratio still remained close to 2 to 1 for at least 25 min. Only after the pyrimidine deoxyribonucleotide concentrations reached levels about 100-fold greater than the initial values did the ratio begin to increase. However, a mutant of the structural gene for T4 DNA polymerase showed some increase in ratio by 15 min. Mutants of gene 1 (HmdCMP kinase) were distinct in that the Thy/Hmc5Cyt ratio dropped to about 1.0 by 25 min, and then remained quite constant. Uniquely, in these mutants a significant quantity of 5-hydroxymethyluracil or a derivative was found, about 10% being in the medium. The product was shown to be derived by deamination of a 5-Hmc5Cyt derivative. All Dna- mutants tested excreted 35 to 50% of their thymine derivatives, mostly as thymine, into the medium. Neither thymine nor 5-hydroxymethyluracil derivatives remained constant at 2.1 + 0.1 for at least 45 min after infection by wild type phage, i.e. precisely at the Thy/Hmc5Cyt ratio in T4 DNA. On infection by phage mutated in the structural genes for T4 DNA polymerase, i.e. precisely at the Thy/Hmc5Cyt ratio of 2:1 as an intrinsic property of a complex of enzymes synthesizing and channeling deoxyribonucleotides for T4 DNA replication and not exclusively by effector-sensitive mechanisms.

Previous papers from this laboratory have suggested that enzymes forming the deoxyribonucleotides play a second and direct role in bacteriophage T4 DNA replication by formation of an integrated complex with the polymerizing proteins (2-4). Infected cells rendered permeable to nucleotides by plasmolysis are unable to form DNA if the structural genes for the T4-induced enzymes for dCMP hydroxymethylase and HmdCMP kinase are mutated; i.e. the enzyme blocks cannot be bypassed by their nucleotide products (3). Other laboratories have reported similar findings (5, 6). Recent studies (7-9) have shown that these findings cannot be explained by a failure to form 3'-hydroxyl starting points for DNA replication.

As a part of a program to define the nature of the complexes allowing interaction of the deoxyribonucleotide-synthesizing enzymes and the polymerizing enzymes, this laboratory devised an in vivo assay for the combined activities of dCMP hydroxymethylase and thymidylate synthetase formed after T4 infection (4). It generally has been held that some critical level of polymerizing enzymes and enzymes forming deoxyribonucleotide precursors must be reached before DNA synthesis begins (see Ref. 10). However, studies by the in vivo assay have shown that, in fact, DNA replication and deoxyribonucleotide synthesis are initiated simultaneously at 5 min after infection at 30°C (9). The two activities increase coincidentally and exponentially for about 15 min, then reaching a constant rate (4, 9). As a consequence, the combined rates of synthesis of dTMP and of HmdCMP derivatives in vivo are exactly equivalent to the rates of incorporation of thymine and 5-hydroxymethylcytosine deoxyribonucleotides into the DNA.

More importantly the combined rates of synthesis of thymine and 5-hydroxymethylcytosine deoxyribonucleotides are...
unaltered after infection by Dna- mutants. Since the deoxyribonucleotides reach high levels under these circumstances, it was proposed that feedback effector controls might not play significant roles in their regulation (4, 9). This suggestion was made even though several of the enzymes in the deoxyribonucleotide synthesis pathways are known to respond to positive (11-13) or negative (13) effector action or to product inhibition (14-16). Mutants of the structural gene for T4-induced DNA polymerase show a unique reduction of about 50% in the rate of synthesis of deoxyribonucleotides, and Chiu et al. theorized that this enzyme interacts in some manner with the enzymes forming deoxyribonucleotides (9).

During T4 DNA synthesis deoxyribonucleotides are maintained at low cellular concentrations (4, 17, 18). The apparent lack of feedback control even with the high levels of nucleotides accumulating after infection by DNA- mutants (4, 17, 18), led us to ask whether deoxyribonucleotides are intrinsically synthesized at the ratio of (Adenine + Thymidine)/(Guanine + Hydantoin) found in T4 DNA, i.e. 2:1. The present study gives evidence that, indeed, the ratio of thymine and 5-hydroxymethylcytosine deoxyribonucleotides synthesized de novo is accurately regulated at 2:1, and that this precise regulation is primarily the inherent property of the several constituent enzyme systems.

EXPERIMENTAL PROCEDURES

Biologicals – Escherichia coli strain GM201 (thyA delB) is a low thymine-requiring mutant of strain B from this laboratory (19). Phage T4 lysates were propagated in cultures grown on Fraser-Jerrel medium (20). Amber mutants were grown and tittered on E. coli CR63 (Su+) (4). Either E. coli B (19) or E. coli B' (more repressive to come amber mutants) from J. D. Karam, University of South Carolina, Charleston, was used as the nonpermissive host. The phage were purified by centrifugation and resuspended in Fraser-Jerrel medium. The characteristics and sources of the bacteriophage T4 mutants are listed in Table I.

Materials – The nucleotides, nucleosides, and pyrimidine bases were purchased from Calbiochem, P L Biochemicals, and Nutritional Biochemicals. [5-3H]dUMP and [5-3H]dCMP were obtained from Schwarz/Mann and purified as described previously (4). The [5-3H]uridine, [6-3H]uridine, and [6-3H]uracil were purified by paper chromatography as described except that the solvent was 1-butanol/water in the volume proportions 66:14:4. The purity of the [6-3H]uracil was assayed by two-dimensional thin layer chromatography (Fig. 2). On this basis a correction was made for a contamination of the thymine spot by radioactivity associated with uracil. This correction was about 5% or less of the activity in thymine at 15 min and proportionately much lower at 25 min and later.

Infections with Phase T4 – Cultures of Thy- E. coli were grown at 37° to 2.5 × 109 cells/ml in minimal media (4) supplemented with 5 µg/ml of thymine, 0.1% of vitamin-free enzymatic hydrolysate of casein (Nutritional Biochemicals), and 0.2% glucose. The cells were collected, then washed in minimal media containing casein hydrolysate and glucose, and resuspended at a final concentration of 5 × 109 cells/ml as described (4). The cells were infected at 30°C at a multiplicity of 8. At 45 s the infected cells were diluted 10-fold with minimal media supplemented with glucose and casein, hydrolysate, and containing a labeled base or nucleoside as described in the legends to the figures and tables.

Isolation of Phase T4 DNA – A culture was infected with phage T4D, in the presence of 90 µM [5-3H]uracil (1000 cpm/nmol). At 40 min after infection, a 3-ml sample of the culture was mixed with 0.3 ml of cold 50% trichloroacetic acid and placed on ice. The DNA was purified by the method of Warner and Hobbs (21). After the final wash with cold ethanol, the DNA was dissolved in 0.3 ml of 1 M NH4OH and stored at -20°C.

Preparation of Infection Cultures for Hydrolysis of DNA and Nucleotides to Free Bases – Infected cells were diluted as described with media containing 90 µM [6-3H]uracil at 1.5 × 109 cpm/nmol or as marked in the legends to the figures and tables. At given times the samples were frozen in dry ice/acetone mixture and stored at -20°C.

Formic Acid Hydrolysis – For the hydrolysis of either purified T4 DNA or the infected cell culture, 0.1 ml of the ammonium hydroxide solution prepared as described above was placed in a heavy-walled Pyrex ignition tube (10 x 70 mm). The solution was evaporated to dryness under reduced pressure at about 35°C using a Buchler Evapomix, and the residue was dissolved in 0.5 ml of 89% formic acid. The trichloroacetic acid was removed by evaporation, the residue dissolved in 0.1 ml of NH4OH, and this solution stored at -20°C until hydrolysis. This procedure allows a total recovery of all the thymine and hydroxymethylcytosine derivatives arising by de novo synthesis, both in the cell and the medium including that in DNA.

Formic Acid Hydrolysis – For the hydrolysis of either purified T4 DNA or the infected cell culture, 0.1 ml of the ammonium hydroxide solution prepared as described above was placed in a heavy-walled Pyrex ignition tube (10 x 70 mm). The solution was evaporated to dryness under reduced pressure at about 35°C using a Buchler Evapomix, and the residue was dissolved in 0.5 ml of 89% formic acid. Distilled from Baker reagent grade formic acid (90.4%). The ignition tube was sealed approximately 1.5 cm from the top and placed in a screw-capped pipe bomb which had been preheated to 175°C. The bomb was placed in a 172-175°C oven for 30 min (27, 28), then removed and rapidly cooled on ice. The sealed end of the tube was submerged in a dry ice/acetone mixture to freeze the formic acid. The opposite (rounded) end of the tube was then heated in a hot pin point flame until the pressure inside the tube was released through a small hole in the glass wall. A larger opening was made by the flame, the hydrolysate washed into a 12-ml conical centrifuge tube, and the solution evaporated to dryness. The residues from the hydrolyzed DNA were dissolved in 1 ml of 1 N HCl while the cell culture was dissolved in 0.05 ml of 1 N HCl. This hydrolysis completely converts pyrimidine deoxyribonucleotides (but not pyrimidine ribonucleotides) to their free bases (see Fig. 1A).

To measure the absolute quantities of thymine and hydroxymethylcytosine after hydrolysis rather than the ratio of these two compounds, it is necessary to account for volume changes which result from the presence of salts from the media. After the formic acid hydrolysis steps, the resulting residue of salts from the media comprises a relatively large percentage of the final volume. To estimate this contribution to the volume, 89% [6-H]uracil was carried through the described hydrolysis and chromatography procedures. No loss of label from [6-H]uracil occurred during hydrolysis. From the recovery of radioactivity per ml, a factor of 9.44 was obtained for volume correction (but see text to Figs. 5 and 6).

Two-dimensional Thin Layer Chromatography – Plastic sheets precoated with cellulose (20 × 20 cm, Eastman Kodak) were used for...
thin layer chromatography. Materials were applied at the lower left hand corner about 3.8 cm from each edge using 1 to 10 µl of the solution containing the hydrolysis product and 20 nmol of each chromatographic standard. About 0.5 cm of the cellulose layer was scraped from the right and left hand edges, and the sheets were developed with 1-butanol:methanol:water:concentrated HCl in the volume proportions of 70:10:15:5 (Solvent I) until the solvent reached the top of the chromatogram (about 5 h). After development, the chromatograms were allowed to dry overnight or dried first with cool and then with warm air. For development in the second dimension, the right and left hand edges were again scraped, and a 4.5-cm paper wick of Whatman No. 1 was machine sewn to the top of the plastic sheet using white polyester thread. The paper wick was folded twice such that it would project beyond the front of the cellulose layer and was separated from the next chromatogram by the glass plate used for its support. The chromatograms were developed in 1-butanol:methanol:water:saturated NH₄OH:saturated formic acid solution in the volume proportions of 60:20:1:20 (Solvent II) until the solvent reached the end of the paper wick (about 6 h). For good separation with this solvent, it is necessary to allow the tank to equilibrate with the solvent for several hours before use. After development the positions of standards were marked under the shortwave ultraviolet lamp. Each fraction was cut out 3 mm beyond its edges and placed directly into a scintillation vial. The radioactive material was eluted with 0.2 M HCl solution containing 0.2 M HCl, with vigorous blending on a Vortex mixer for several minutes. Absolute ethanol (4.5 ml) and 10.5 ml of scintillation fluid consisting of 0.4 g of 2,5-diphenyloxazole and 0.05 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene per 100 ml of toluene were added, and the mixture was counted in a Beckman LS-200 scintillation spectrometer.

As shown in Fig. 2, this procedure gives complete separation of thymine and hydroxymethylcytosine from the other labeled compounds and also allows hydroxymethyluracil to be quantitated. In addition, little radioactive material is found in the areas surrounding the thymine and hydroxymethylcytosine spots; i.e. the latter are not contaminated by trails of other radioactive compounds.

In Vivo Tritium Release Assay—The cells were grown, infected, and diluted into medium containing [6-3H]uridine (4 x 10⁶ cpm/ml), into water was measured by methods described earlier (4).

DNA Synthesis—The conditions of infection and dilution were as described above, but using [6-3H]uridine (4 x 10⁶ cpm/ml), 90 µM). At given times after infection, 0.5-ml aliquots of the culture were mixed with 0.5 ml of 1% sodium dodecyl sulfate and 0.5 n NaOH and treated as described (3, 4).

RESULTS

Thy/HmCyt Ratio in Phage T4 DNA—In these and all subsequent experiments, the host was Thy⁻. Phage T4 DNA labeled by administering [6-3H]uracil during infection was purified and hydrolyzed as described under "Experimental Procedures." The Thy/HmCyt ratios found in three chromatograms of the hydrolyzed T4 DNA are shown in Table II. The average value of 2.02 is in good agreement with previously published base ratios for T4 DNA which range from 1.88 to 2.16 (27-29). Table II also shows that the administered [6-3H]uracil specifically labeled the thymine and hydroxymethylcytosine residues and that no significant labeled cytosine or hydroxymethyluracil was found in the phage DNA.

Thy/HmCyt Ratio in Infected Cell Culture—When [6-3H]uracil is administered to cultures infected by phage T4D, TMP, HmdCMP, and all subsequent compounds in the pathway will be labeled (see Fig. 3) (4). A variable dilution of the labeled compounds by the de novo synthesis of pyrimidine ribonucleotides is prevented by adding sufficient amounts of carrier uracil to ensure feedback inhibition of that pathway (30). The ratio of the enzymatic activities leading to the synthesis of dTMP and HmdCMP from administered [6-3H]uracil is then determined by measuring the labeled thymine and hydroxymethylcytosine after hydrolysis of the whole cell culture. The method measures all compounds derived from labeled dTMP and HmdCMP including their degradation products (see below). Deoxyribonucleotides arising by host DNA degradation do not mix with this de novo pathway synthesizing T4 DNA (8).

The Thy/HmCyt ratios found for T4D-infected cell cultures in two separate infections are shown in Table III. The average value of 2.02 is very close to that for purified T4 DNA in Table II. Thus, the synthesis of dTMP and HmdCMP is precisely regulated in vivo to maintain the same ratio to each other as labeled uracil and uridine give equivalent results in the 'H release assay and in the measurement of DNA synthesis. [6-3H]Uracil is employed in the determination of the Thy/HmCyt ratio to reduce interference in the thin layer chromatography (see Fig. 2).
the Thy/HmCyt ratio in Table IV, the substantial agreement between these two methods provides further evidence for the validity of the tritium release assay as a means of monitoring in vivo enzyme activity.

**dTMP and HmdCMP Synthesis after Infection with Dna- Mutants**—The results in Fig. 4 show that the Dna- mutants used in this study exhibit the expected phenotype with respect to DNA synthesis. Cells infected with these mutants were compared with cells infected by the wild type phage to determine the effect of inhibition of DNA synthesis and the resultant accumulation of deoxyribonucleotides on the regulation of dTMP and HmdCMP biosynthesis. Amber mutations in genes 1, 41, 44, and 45, responsible for the Dna- phenotype, have little or no effect on the combined rates of synthesis of dTMP and HmdCMP as measured by the rate of tritium release from labeled precursors (4). The results in Fig. 5 confirm that the kinetics of tritium release is very similar for cells infected with different Dna- amber mutants of phage T4. The incorporation of [6-3H]uridine into alkali-stable, acid-insoluble material was measured as described under "Experimental Procedures."
The results with several other DNA mutants (amNS2, amNS1, and amA453) were very similar to those for amE10 (see Table V). In the absence of DNA synthesis the Thy/HmCyt ratios remained very close to the wild type values during the first 25 min of infection and then increased to average values of 3.0 at 35 min and 4.2 at 45 min. Thus, the accumulation of deoxyribonucleotides appears to have little effect on the regulation of dTMP and HmdCMP synthesis at least until very high levels are reached, i.e. about 8 to 10 nmol of Thy + HmCyt/10^6 cells. With gene 43 amber mutants the ratios increased somewhat earlier after infection. This observation is particularly interesting. Our previous studies had shown that in gene 43 amber mutants, in contrast to other DNA mutants, the total tritium release activity is decreased, and we suggested that DNA polymerase may interact with the deoxyribonucleotide-synthesizing enzyme complex (4, 9).

In these analyses the values represent the total dTMP and HmdCMP derivatives synthesized de novo. Based on the work of Mathews (17, 31) and Warner and Hobbs (18), after infection by DNA mutants deoxyribonucleotides appear to be present in the cell primarily as the triphosphates, generally with less of the diphosphates (31) and much less of the monophosphates. In this paper (see "Discussion") the intracellular concentrations of dTTP and HmdCTP are estimated from the 3H release and ratio values, accounting for the excretion of thymine and HmCyt derivatives into the medium (see below) and the reported distribution of the deoxyribonucleotide species in the cell.

dTMP and HmdCMP Synthesis after Infection by Gene 1 Mutant (HmdCMP kinase) - The Thy/HmCyt ratios with the gene 1 mutant, amB24, were distinctly different from the results with other DNA mutants (Table V). At 15 min the ratio was about the same as the values found with the other DNA mutants, i.e. about 2, but by 25 min the ratio had dropped to a value of 1.3, remaining constant to at least 45 min. The amounts of dTMP and HmdCMP synthesized were again calculated by combining tritium release and ratio data, and the results of this calculation are given in Fig. 7. In addition to the change, the kinetics is quite different from that for amE10 in Fig. 6. For amB24 the amounts of dTMP and HmdCMP synthesized continued to increase linearly after the initial exponential period. With amE10 and the other DNA mutants, dTMP synthesis was stimulated, and HmdCMP synthesis was inhibited during later infection times. The results in Fig. 7 also show that a significant quantity of 5-hydroxymethyluracil is formed after amB24 infections (see below). When hydroxymethyluracil is taken into account, the ratio of dTMP to HmdCMP synthesis becomes about 1.8 at 15 min, and 1.0 at 25 min, remaining quite constant to 45 min (see Table V). Since the tritium release

**Table V**

<table>
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<tr>
<th>Phage mutant</th>
<th>Gene</th>
<th>Thy/HmCyt ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Minutes after infection</td>
</tr>
<tr>
<td></td>
<td></td>
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<tr>
<td>T4D</td>
<td>W.T.</td>
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<tr>
<td>amNS2</td>
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<td>2.4</td>
</tr>
<tr>
<td>amNS1</td>
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<td>2.1</td>
</tr>
<tr>
<td>amA453</td>
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</tr>
<tr>
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<tr>
<td>amB24*</td>
<td>1</td>
<td>0.8*</td>
</tr>
</tbody>
</table>

*The values given here represent the amB24 data corrected for 5-HmUra formation i.e. they are Thy/(HmCyt + HmUra) ratios (see Fig. 7).
activity with amB24 infections is essentially the same as with the wild type control (4), the decrease in the ratio to 1.0 indicates that dTMP synthesis has decreased by about 25% and HmdCMP synthesis has increased by 50%. Thus, a kinase mutation, in contrast to all other Dna- mutants, however unlikely (32), or by oxidation of the methyl group of thymine, [6-3H]deoxyuridine was administered to an amB24-infected culture. Labeled deoxyuridine is converted directly to labeled dUMP and dTMP (4) without forming labeled dCMP and HmdCMP (see Fig. 3). Unlabeled uridine was added to a concentration of 4 mM to prevent the breakdown of deoxyuridine to uracil by thymidine phosphorylase and to dilute any [3H]uracil entering the de novo pathway (33). At 45 min after infection, a sample of the amB24 culture was hydrolyzed and chromatographed. An aliquot of the resulting hydrolysate contained 821 cpm in thymine, 59 cpm in hydroxymethylcytosine, and 0 cpm in hydroxymethyluracil. The results in Fig. 7 indicate that about 30% of the counts occurring as thymine should be found in labeled hydroxymethyluracil if the latter were derived from labeled dUMP or dTMP. Since none was found, the hydroxymethyluracil formed in amB24 infections must be derived from the deamination of a HmCyt derivative. Movement of Thymine and Hydroxymethylcytosine into Media after Infection by Dna- Mutants—Table VI shows an experiment in which the growth media were examined for free thymine and hydroxymethylcytosine after infection by T4D and Dna- mutants. After 48 min, samples of the media, freed of cells by centrifugation, were chromatographed directly without hydrolysis. No labeled thymine or hydroxymethylcytosine was found in the media after infection with the wild type phage. With all of the Dna- mutants, however, an average of about 40% of the dTMP synthesized by 48 min was found in the media as labeled thymine. While the described procedure does not give complete separation of thymine and thymidine, using internal standards two-dimensional thin layer chromatography indicates that no more than 15% of the labeled material in the media is actually thymidine.

Only a trace of labeled free 5-hydroxymethylcytosine or none was found in the media after infection with the Dna- mutants. As an indication of the total hydroxymethyldeoxycytidine derivatives excreted, a sample of medium taken at 45 min after infection with amR10 was subjected to hydrolysis before chromatography. Hydroxymethylcytosine equal to about 4% of the total HmdCMP synthesized was found (as compared to 0.1% before hydrolysis). Somewhat more free HmCyt was found after infection by amB24. In addition about 40% of the 5-hydroxymethyluracil derivatives formed in accumulation. The Dna-arrest mutant differed from Dna- mutants by about a 10-min delay in the increase in ratio, possibly corresponding to the initial 10 min of DNA synthesis.

Synthesis of 5-Hydroxymethyluracil—To obtain accurate ratios, it is necessary to account for all of the dTMP and HmdCMP synthesized. The loss of 5-hydroxymethylcytosine by deamination to 5-hydroxymethyluracil would result in high ratios. The results in Table II indicate that 1% or less of the hydroxymethylcytosine in T4 DNA is converted to hydroxymethyluracil during formic acid hydrolysis. The formation of hydroxymethyluracil is shown in Fig. 9. With the wild type phage, no detectable formation of hydroxymethyluracil occurs during the entire infection. After infection with the Dna- mutants, only a small amount of hydroxymethyluracil is formed, primarily at late times in the infection. With amB24, however, a large quantity of hydroxymethyluracil is formed over the entire infection period. From the data in Fig. 7 the rate of hydroxymethyluracil formation is about 30% of the total rate (hydroxymethylcytosine + hydroxymethyluracil) during the linear period from 25 to 45 min.

To establish that hydroxymethyluracil was not formed in some unknown way by the hydroxymethylation of dUMP, however unlikely (32), or by oxidation of the methyl group of thymine, [6-3H]deoxyuridine was administered to an amB24-infected culture. Labeled deoxyuridine is converted directly to labeled dUMP and dTMP (4) without forming labeled dCMP and HmdCMP (see Fig. 3). Unlabeled uridine was added to a concentration of 4 mM to prevent the breakdown of deoxyuridine to uracil by thymidine phosphorylase and to dilute any [3H]uracil entering the de novo pathway (33). At 45 min after infection, a sample of the amB24 culture was hydrolyzed and chromatographed. An aliquot of the resulting hydrolysate contained 821 cpm in thymine, 59 cpm in hydroxymethylcytosine, and 0 cpm in hydroxymethyluracil. The results in Fig. 7 indicate that about 30% of the counts occurring as thymine should be found in labeled hydroxymethyluracil if the latter were derived from labeled dUMP or dTMP. Since none was found, the hydroxymethyluracil formed in amB24 infections must be derived from the deamination of a HmCyt derivative.
Excretion of Thy and HmCyt derivatives from infected cells into media

At 48 min after infection, aliquots of the cultures from the experiments described in Table V were analyzed. The culture was placed in ice, the cells removed by centrifugation, and the resulting media chromatographed directly without hydrolysis.

Table VI
Excretion of Thy and HmCyt derivatives from infected cells into media

<table>
<thead>
<tr>
<th>Phage mutants</th>
<th>Per cent of each deoxyribonucleotide synthesized de novo occurring in medium as free base*</th>
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<tbody>
<tr>
<td>T4D</td>
<td>0.0</td>
</tr>
<tr>
<td>amB24</td>
<td>0.0</td>
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<td>amN82</td>
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<tr>
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<td>50</td>
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* See text for description.

amB24 infection was excreted into the medium.

Examining the appearance of labeled thymine and thymidine in the medium after infection with amE10 (not presented) showed that between 15 and 45 min consistently about half of the dTMP derivatives synthesized was converted to the nucleoside (34) and the free base and removed from the cell.

Duration of Tritium Release Activity—The experiments in Fig. 10 show that the release of tritium from 5-labeled pyrimidine in the medium after infection with amB24 (not presented) was excreted into the medium.

The major finding of this in vivo study is that in phage T4 infection, dTMP and HmdCMP are synthesized via the de novo pathway in the exact proportion in which they occur in T4 DNA, i.e. 2:1, respectively. An earlier study had shown that the combined rates of the de novo syntheses of HmdCMP and dTMP were precisely equal to the rates of utilization of these nucleotides for DNA synthesis (4). The ratio of Thy/HmCyt deoxyribonucleotides synthesis and the rates of synthesis (4) are not dependent on concomitant DNA synthesis. Thus, after infection by Dna- mutants the ratio is maintained at 2:1 for periods of about 25 min. This resistance to variation is all the more remarkable since the ratio does not begin to change until the accumulating nucleotides reach levels many times greater than either the concentration occurring at the initiation of deoxyribonucleotide synthesis (4) or in the steady state conditions prevailing in wild type infection (17, 18). The ultimate rise in the ratio appears to be by product inhibition of dCMP hydroxymethylase, a process which is unlikely to be part of a normal control mechanism (see below).

We suggest that the regulation of the rates of synthesis of dTMP and HmdCMP is an inherent property of a complex of enzymes, proposed earlier (2-4, 9), which synthesizes deoxyribonucleotides and channels (8) the products directly into the complex bringing about polymerization to DNA (22). Final proof for this suggestion must await isolation of the proposed complex. However, in another study we have shown that the input phage T4 DNA has a direct role in controlling the synthesis of the pyrimidine deoxyribonucleotides (1), and this factor may represent the intrinsic regulatory characteristic of the complex. The present studies appear to rule against regulation by feedback-sensitive enzymes. The arguments for this position are presented below.

A proposal for a new regulatory system should consider the known control mechanisms. Possible points of control in the synthesis of deoxyribonucleotides include ribonucleoside diphosphate reductase (11, 12) and dCMP deaminase (13), which are effector-sensitive enzymes and dCMP hydroxymethylase and dTMP synthetase, which are product-inhibited (14-16). However, we propose that deoxyribonucleotide synthesis in vivo is not regulated exclusively by effector control of ribonucleoside diphosphate reductase and dCMP deaminase and that dTMP synthetase is not subject to product-inhibition in vivo.

These controls are discussed in the following paragraphs.

Scocca et al. carried out careful studies of highly purified T4-induced dCMP deaminase (13). HmdCTP (or dCTP) is a positive effector and dTTP a competitive inhibitor of the enzyme. At a constant ratio of dTTP to dCTP, the enzyme activity increases as the concentrations of the two effector compounds increase. Therefore, on infection by a Dna- mutant the increase in dTTP and HmdCTP at a constant ratio would ultimately form more dTTP. An accumulation of dTTP would be expected to inhibit dCMP deaminase, thereby reducing the synthesis of dUMP and hence of dTMP and of dTTP, while simultaneously increasing the level of dCMP and hence of HmdCMP and finally of HmdCTP (Fig. 3). In the reverse manner an excess of HmdCTP should lead to a stimulation of dCMP deaminase forming additional dTTP and reducing the available dCMP for formation of HmdCTP.

However, after infection by Dna- mutants, HmdCTP and dTTP reach very high levels before the ratio begins to change. By combining the present studies with the results of Mathews (17, 31) and Warner and Hobbs (18), we estimate that dTTP reaches an intracellular concentration of 10^-3 M at 25 min after infection by a Dna- mutant (see comment on dTTP and HmdCTP concentrations under "Results.") After about 35 min the synthesis of HmCyt derivatives ceases. Nevertheless, the synthesis of dTMP derivatives continues for 6 h (Fig. 10). Since in the absence of dCMP deaminase (cd mutants) the Thy/HmCyt ratio decreases greatly, the activity of this enzyme (and ribonucleoside diphosphate reductase) therefore must continue unabated after infection by Dna- mutants. Thus, while dCMP deaminase basically has the ability to regulate the Thy/HmCyt ratio up or down, the system does not respond in situ. Finally, this control, per se, does not appear to embrace an integrated mechanism for sensing a variation and accurately correcting to a 2:1 ratio.

From the known properties of ribonucleoside diphosphate reductase it appears unlikely that this regulation of this enzyme is responsible for the high Thy/HmCyt ratios, such as occur with Dna- mutants, or the low ratios resulting from amB24 infection. Berglund has demonstrated that T4-induced ribonucleoside diphosphate reductase is activated by dATP, HmdCTP, and dTTP. Unlike the host reductase (36), no primary feedback inhibition occurs (11, 12). The K_M values for dATP as a positive effector for CDP and UDP reduction were 2 and 4 x 10^-7 M, respectively. The values for HmdCTP and dTTP as positive effectors for both CDP and UDP were 1 x 10^-3 M and 2 x 10^-6 M, respectively. In the presence of effectors, the K_M value for UDP was about twice that for CDP, but the V_max values with both substrates were essentially equal (11, 12). Accordingly, the levels of the ribonucleoside diphosphates may need to be investigated as factors contributing to the control. Because of the great intracellular concentrations of deoxyribonucleoside triphosphates reached by 25 min after infection with Dna- mutants (17, 18, 31), it is improbable that a further increase in the concentrations of these three deoxyribonucleotides could show any effect. In fact, the low steady state levels which obtain for these nucleotides during DNA synthesis in wild type infection appear to fully activate the reductase.

We interpret the rise in Thy/HmCyt ratio ultimately occurring after infection by Dna- mutants to be the result of product inhibition of dCMP hydroxymethylase by accumulating HmdCMP (15, 16) under conditions which allow dCMP deaminase (Fig. 3) to siphon off the competitive dCMP. As a consequence, all of the dCMP normally converted to HmdCMP derivatives is diverted to dUMP and thence to dTMP derivatives. By this process the resulting dTMP compounds are equal to the sum of HmCyt + Thy derivatives formed in wild type infection or before the ratio change. Product inhibition of dTMP synthetase (14) does not seem to be involved in vivo, possibly because of degradation of dTMP (34).

The low Thy/HmCyt ratio occurring after infection with a mutant defective in the structural gene for HmdCMP kinase, amB24, is unique. This effect probably results from the limitation of HmdCMP for the activation of dCMP deaminase (33).

It is unlikely that the results of the present study can be explained by the leakage of excess deoxyribonucleotides out of the channel normally leading to DNA growing points and that only a constant level of nucleotides remains to control the feedback-sensitive enzymes. An accurate regulation of spillover or the appropriate overflow structure would have to obtain to maintain desired levels of effector nucleotides, and in wild type infection no evidence of overflow is seen (Table VI and Ref. 4). The recent studies of Mathews appear to support the concept that the deoxyribonucleotide pools accumulating after infection by Dna- mutants are available for DNA synthesis on reversal of the DNA block (31).

This study has not addressed itself to the purine deoxyribonucleotides (11). It seems justifiable to assume that dADP and dGDP are formed via ribonucleoside diphosphate reductase in the complex and commensurate with the rates of formation of dTTP and HmdCTP, respectively.

We have suggested that deoxyribonucleotides from host DNA breakdown do not mix with the pool derived by de novo synthesis (4). Thus, the kinetics of H release from administered [5-3H]uridine neither shows perturbation by the transient peak of deoxyribonucleotides derived from host DNA degradation (37) nor is obviously affected by a genetic block in the breakdown pathway (4). Recently, Wovcha et al. using sucrose-plasmolyzed, permeable preparations have demonstrated that two minimizing pathways from deoxyribonucleotides to DNA exist within the same T4-infected cells, one a de novo pathway and the other polymerizing added deoxyribonucleoside triphosphates (8). It was suggested that the latter may be the pathway utilizing deoxyribonucleotides derived from host DNA breakdown. Actually Snustad et al. very recently have suggested from an electron microscopy study that nuclear breakdown may be separated, by compartmentalization, from phage DNA synthesis (38).

Our previous reports have suggested that in T4 infection deoxyribonucleotide synthesis occurs by a complex of enzymes tightly geared to the enzymes catalyzing DNA replication. It now seems clear that the evolution of this complex of enzymes ensures the most rapid and precisely regulated synthesis of deoxyribonucleotides to fulfill the needs of the replicating point.

It is conceivable that altered Thy/HmCyt ratios are related to the mechanism of mutagenesis (39). The idea is supported by evidence that either thymine excess (40) or deprivation (40, 41) is mutagenic. In combination with base-pairing (42) and such repair mechanisms as the editing function of polymerase (43), a primary function of this kind of complex may be to promote the highest fidelity of replication (44, 45).
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Note Added in Proof—Gillin and Nossal (46) have now shown that T4 DNA polymerases from mutator, antimutator, and wild type phage differed greatly in their accuracies of nucleotide selection. It seems reasonable to suggest that an alteration in the ratio of synthesis of the deoxyribonucleotides could either aggravate or improve the error frequency of a mutator or an antimutator polymerase.

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