Evidence for a Complex Regulating the in Vivo Activities of Early Enzymes Induced by Bacteriophage T4*

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SUMMARY

An in vivo assay of enzyme activity has been employed to investigate the mechanism of control of enzymes synthesizing deoxyribonucleotides after bacteriophage T4 infection. The assay is based on the release of tritium from 5-labeled pyrimidine nucleotide substrates into water.

At least two phage-induced early enzymes, deoxycytidylate hydroxymethylase and thymidylate synthetase, which are synthesized within a few minutes after infection as measured in extracts, do not function in vivo immediately after their formation. Instead, in vivo these enzyme activities initiate about 5 min after infection at 30°C. The activities initially increase exponentially, and then become linear. This exponential activation process does not require concomitant protein synthesis. A number of experiments rule against substrate limitation, feedback, or isotope dilution as an explanation of these kinetic data. Thus, the same kinetic behavior in vivo is observed on infection by mutants which are unable to synthesize T4 DNA (DO) and accumulate deoxyribonucleotides. However, amber mutants of gene 43, the structural gene for T4-induced DNA polymerase, are exceptions. Infection by these mutants clearly showed a reduced initial rate of 3H release.

Concurrent measurement of DNA synthesis and of in vivo enzyme activity indicates that these two processes follow the same kinetics and coincide in time. These results suggest that the limiting factor in DNA replication is the rate of formation of the deoxyribonucleotide substrates. From these and previous studies we have postulated that dCMP hydroxymethylase and thymidylate synthetase and apparently other enzymes forming deoxyribonucleotides must, to be active in vivo, become part of a complex. We propose that the exponential activation process represents the formation of this complex from its components.

It has been generally accepted that the enzymes responsible for the synthesis of deoxyribonucleotides operate independently of DNA synthesis except insofar as they limit the substrates. Doubt about this independence was suggested from studies with a temperature-sensitive mutant of bacteriophage T4, tsL15, carrying a lesion in the structural gene for the early enzyme, dCMP hydroxymethylase (1). We have proposed that this enzyme, in addition to its catalytic function of forming HMdCMP, has either a second more direct role in DNA synthesis (1, 2), or that it must be activated to function in vivo (1). Thus, cultures infected by tsL15 at 30°C for about 8 min and then shifted to 42°C formed T4 DNA and phage (1). However, if the upward shift was earlier than 5 min after infection, DNA synthesis did not occur even though dCMP hydroxymethylase had already reached about one-third of its ultimate level. We concluded that an active complex stable to 42°C is made between 5 and 8 min at 30°C and that it cannot be formed at 42°C even though the enzyme is present (1). Later, Tomich and Greenberg (3) and Mathews (4) showed that cultures infected by tsL15 at nonpermissive temperatures do not form HMdCMP. As a result of these studies, we reasoned that both in tsL15 and T4H infection, dCMP hydroxymethylase may not function catalytically in vivo until it becomes part of an active complex at least 5 min after infection. To test this hypothesis, we have applied a new simple tool to follow the activity of dCMP hydroxymethylase and of phage-induced thymidylate synthetase. The method is based on the release of 3H from position 5 of the deoxyribonucleotide substrates of these enzymes. This paper demonstrates that while these enzymes are clearly present in extracts within a few minutes after infection, they show no activity in vivo. At 5 min, their activities initiate and then increase exponentially. Moreover, enzyme activation occurs, apparently with the same timing and kinetics, even in the presence of DO mutants which are unable to form T4 DNA. Our studies give insight into the relationship of deoxyribonucleotide synthesis and DNA synthesis.

EXPERIMENTAL PROCEDURE

Materials—Escherichia coli strain GM201 is a low thymine-requiring mutant of strain B described previously (5). The phage mutants, their source, and their phenotypes are given in Table I. The mutant, amN122, which was free of the "m" mutation in DNA polymerase (6), and the mutants, amN66 and amC42, had been selected for a high thymine requirement (3, 4).

1 The abbreviations used are: HMdCMP, 5-hydroxymethyl-dCMP; HMdCTP, 5-hydroxymethyl-dCTP; DO, no DNA synthesis. We have employed the one-letter abbreviations for nucleosides in order to promote continuity and clarity between deoxyribonucleotide metabolism and DNA synthesis.
been purified by John Wiberg, University of Rochester, by three solvent, the compound was eluted using a 1:100 dilution of concentrated NH\textsubscript{4}OH solution, and dried by evaporation. Labeled proportions 66:1:33 (v/v; Solvent I). After evaporating off the 3HOH and other non-charcoal-adsorbable contaminants and is stored at -20\(^\circ\) in 50yo ethanol. Uridine and deoxyuridine were especially important with 5-labeled compounds employed in the 3H release assay in order to minimize the reagent blank values.

The solvent was isobutyric acid-concentrated NH\textsubscript{4}OH-H\textsubscript{2}O in the 3H release assay using Whatman No. 3MM filter paper. This purification removes from General Biochemicals. The solid was dissolved under nitro-burgh ALtigated Carbon Co., Pittsburgh, Pennsylvania, &as used nH 7.8 (25\(^\circ\)) and stored at -20\(^\circ\). Charcoal, type “CAL.” Pitts-gen in 1.3

Nutritional Biochemical Co. Tetrahvdrofolate was purchased in the

in the

viva 3H release assay. The collected material was washed with three 5-&l aliquits of 2-mercaptoethanol, 1.0 mg of dZ,L-tetrahydrofolic acid, 1 pmole of trichloroacetic acid containing 0.1 m sodium pyrophosphate. The collected material was washed with three 5-mi aliquots of NaOH, and then filtered through the same steps as described by Ou et al. (1) and labeled dUMP on Dowex-1-formate columns (7). These compounds were stored at -20\(^\circ\) in 50% ethanol. Uridine and deoxyuridine were purchased from Schwarz-Mann and P. L. Biochemicals, Inc., respectively. Vitamin-free enzymic digest of casein was from Nutritional Biochemical Co. Tetrahydrofolate was purchased from General Biochemicals. The solid was dissolved under nitro-burgh ALtigated Carbon Co., Pittsburgh, Pennsylvania, was used in the tritium release assay. The coarse charcoal was ground in a ball-mill for about 2 days and sieved (80 mesh). The fine charcoal was then treated successively with 4 x HCl, 4 x NaOH, and again with 4 x HCl, washing with water between each treatment. After a final thorough washing with water, the charcoal was dried at 80\(^\circ\). This charcoal shows high capacity for adsorption of nucleotides, and dCMP can be desorbed only in very low yield. Norite A charcoal-ashed by the same procedure was used in the in vivo 3H release assay.

Monitoring Enzyme in Vivo by 3H Release—Thy\textsuperscript{r} E. coli was grown at 37\(^\circ\) to 2.5 x 10\(^8\) cells per ml in Vogel-Bonner minimal media (8), supplemented with 5 \mu g per ml of thymine, 0.1% casein hydrolysate, and with 0.2% glucose as the carbon source. Cells were collected at 5000 x g at 4\(^\circ\), washed with 0.5 volume of minimal media, reentrifuged, and suspended in 0.05 volume of minimal media, and then equilibrated at 30\(^\circ\). One minute prior to infection, L-tryptophan was added to a concentration of 50 \mu g per ml. T4 phage was added at a multiplicity of infection of 8:1. At 45 s after infection, the culture was diluted to 1 x 10\(^8\) cells per ml with minimal medium containing 0.1% casein hydrolysate, 0.2% glucose, and [5-\textsuperscript{3}H]U, or [6-\textsuperscript{3}H]U, or [5-\textsuperscript{3}H]dU, with carrier U or dU at 0.091 m. The results are linear over the range 10\(^4\) to 10\(^6\) cells per ml. When 5-\textsuperscript{3}H-labeled deoxyuridine was used, unlabeled uridine was added at a 4.0 mm final concentration to prevent breakdown of dU via thymidine phosphohydrolase (9, 10) and to dilute uracil which might leak through. Aliquots (0.5 mI) of culture taken at various times after infection were added to 0.6 ml of a 4% perchloric acid-charcoal suspension prepared by adding 20 g of charcoal to 100 ml. The mixture was allowed to stand 30 min or longer with frequent mixing. The samples were filtered through Schleicher and Schuell nitrocellulose filters (0.45 pm, 24 cm), and 0.5 ml of the filtrate was counted in the ethanol-toluene scintillation fluid employed earlier (7) using a Beckman LS-230 scintillation spectrometer.

This procedure was based on that developed by Ou et al. (4) for the measurement in vivo of thymidylate synthetase activity in uninfected E. coli cultures. Since equivalent infection does not always occur, the process was monitored by measuring the activity of dCMP hydroxymethylase, dTMP synthetase, or deoxy- cystidine triphosphatase 10 min after infection. The results obtained in the in vivo tritium release assays with DO mutants were corrected to the wild type (T4D) enzyme values as described by Shapiro et al. (11). While these enzyme activities may be altered in certain mutants, the usefulness of the corrections is manifested by the finding that in most instances, the corrected 3H release values with DO infections are quite close to that shown by the wild type T4D infection. This correction, of course, does not affect the form of the kinetics of 3H release. The corrections were usually less than 25\%, the greatest being 2-fold. The enzymes used as standards are referred to in the legends to figures.

RNA and DNA Synthesis—When [5-\textsuperscript{3}H]uridine is employed in T4-infected cultures, all acid-precipitable material will be RNA, since deoxyribonucleic acid is displaced in the synthesis of thymidine hydroxymethyl dCMP. In measuring RNA synthesis, aliquots of a culture were treated by treatment with cold 2% perchloric acid and kept on ice. The samples were filtered through Schleicher and Schuell filters previously washed with 5 ml of cold 5% trichloroacetic acid, and the filtrates were collected in a 1.5 ml vial containing 200 mg of specially prepared high capacity charcoal. The filtrates were allowed to proceed at room temperature for at least 15 min with frequent mixing. The aqueous filtrate was collected by filtering through a paper filter, and the filters were dried under an infrared lamp and counted in toluene-based scintillator (12). To measure incorporation into DNA using [6-\textsuperscript{3}H]uridine, 1 ml of infected culture was mixed with 1 ml of 2% sodium dodecyl sulf-sate-1.0 n NaOH. After heating for 20 min at 80\(^\circ\), the mixture was cooled in an ice bath, and 1 ml of cold 50% trichloroacetic acid was added. The precipitated material was processed and counted as in the case of the RNA samples.

Enzyme Assays in Extracts—Thymidylic synthetase was measured at 25\(^\circ\) in extracts by the tritium release assay (7) as modified earlier (13), except that the ethanol-toluene scintillation mixture was used. It is important that [5-\textsuperscript{3}H]dUMP be purified to reduce the blank (see “Materials”).

3H hydroxymethyl dCMP hydroxymethylase was measured by a modification of the method of Yeh and Greenberg (14) following the conversion of the 3H atom of [5-\textsuperscript{3}H]dUMP to tritiated water in the absence of formaldehyde. Again, it is necessary that [5-\textsuperscript{3}H]dCMP be purified (see “Materials”). The details of the modified 3H release assay are as follows. The reaction mixture contained 20 amoles of potassium phosphate buffer, pH 7.6, 10 amoles of mercaptoethanol, 1.0 mg of dl-L-tetrahydrofolic acid, 1 amole of [5-\textsuperscript{3}H]dCMP, and extract in a total volume of 0.5 ml. The reaction temperature was 30\(^\circ\), the time, 20 min. The reaction was terminated by the addition of 0.7 ml of a stirred suspension containing 200 mg of specially prepared high capacity charcoal per ml (w/w) in 10\(^{-3}\) m phosphate (see “Materials”) and 10\(^{-9}\) m pyrophosphate buffer, pH 7.6. The adsorption to charcoal was allowed to proceed at room temperature for at least 15 min with frequent shaking. The aqueous filtrate was collected by passing the charcoal-treated solution through a membrane filter (0.45 \mu m, Schleicher and Schuell, Inc.) under vacuum. The 3H activity was measured on 0.3 ml of the filtrate in a liquid scintillation counter using the ethanol-toluene scintillation fluid. Deoxyxystidine triphosphate hydrolyase (dCTPase) was measured at 30\(^\circ\) as described by Price and Warner (15).

Isolation of dUMP and dCMP—Labeled dUMP formed from [5-\textsuperscript{3}H]dU (Fig. 7) was isolated by charcoal treatment and elution as described previously (8), and characterized by chromatography with Solvents I (see “Materials”) and II, isopropyl alcohol-concentrated NI\textsubscript{4}OH-0.1 m H\textsubscript{3}PO\textsubscript{4} at 70:10:90 (v/v). Labeled

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### Table I

**Table I**  
**Daudi phage T4 mutants**

<table>
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<tr>
<th>Gene</th>
<th>Mutant</th>
<th>Defect</th>
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<td>a</td>
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<td>amB65 frd1</td>
<td>Ribonucleoside diphosphate reductase</td>
<td>c</td>
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</tbody>
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* a, R. S. Edgar; b, J. S. Wiberg; c, I. Tesman; d, Carol Bern-

stein.

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Formation of labeled dCMP and HMdCMP after infection by T4 mutant, amB24

The isolation and characterization of the nucleotides are described under "Results." The specific activity of [5-3H]uridine was 3.9 x 10^6 cpm per nmole. The intracellular concentration of radioactive nucleotides was calculated on the assumption that the cell volume is 0.9 x 10^-12 ml (4). To obtain nanomoles of labeled nucleotide per ml of culture, multiply the intracellular concentration in molarity by 900.

<table>
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<tr>
<th>Minutes after Infection</th>
<th>Intracellular concentration</th>
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<td>dCMP</td>
</tr>
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<td></td>
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<tr>
<td>10</td>
<td>1.06</td>
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<tr>
<td>15</td>
<td>2.36</td>
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dCMP formed from [5-3H]U (Table II) was characterized according to our earlier procedure (9).

RESULTS

Activation of dCMP Hydroxymethylase and dTMP Synthetase Measured in Vivo by Tritium Release—If [5-3H]uridine is administered to Escherichia coli infected with bacteriophage T4D, label will appear in position 5 of dUMP and dCMP by the pathways shown in Fig. 1. The two labeled compounds, [5-3H]dCMP and [5-3H]dUMP, will release 3H in the dCMP hydroxymethylase and thymidylate synthetase reactions, respectively. By employing a Thy- host, the 3H release is almost exclusively due to enzymes induced by the phage infection. This procedure measures the activities of these enzymes in vivo at the prevailing concentrations of the substrates. A limitation of the method is that a finite time is required to saturate the nucleotide pools, and in addition, de novo synthesis and host DNA breakdown can cause dilution. However, de novo synthesis of pyrimidine nucleotides is limited by feedback (16). The technique is not suggested as a quantitative method akin to an in vitro assay of an enzyme saturated by its substrates. Instead, it is considered as a relative tracing of a pathway to show qualitative changes. Under appropriate conditions, the comparative analysis can be quantitative. Substrate level is considered under "Results" and "Discussion."

The rate of 3H release when [5-3H]uridine was administered to a Thy- host on infection at 30° by phage T4D is shown in Fig. 2. The values for dCMP hydroxymethylase and dTMP synthetase as measured in extracts prepared by sonication up to 10 min after infection by T4D are also presented. At this time, these enzymes have reached 60 to 80% of their maximum activities (1, 11). A striking difference is found between the in vivo and the in vitro assays. dCMP hydroxymethylase activity is observed in extracts almost immediately after infection, and dTMP synthetase activity begins between 3 and 4 min. However, the release of 3H in vivo, which results from the activities of both dCMP hydroxymethylase and dTMP synthetase, becomes clearly apparent only about 7 to 8 min after infection. In Fig. 2, the dCMP hydroxymethylase and dTMP synthetase values in vivo are expressed as rates, i.e. nanomoles of 3H released per min per 10^9 cells, whereas the in vitro data represent the total 3H released per 10^8 cells with increasing time. Accordingly, in the inset to Fig. 2, the first derivatives of the in vivo release values are expressed, i.e. the rates, are presented for a direct comparison with the activity of dCMP hydroxymethylase and dTMP synthetase in extracts. The rate of 3H release in vivo increases until about 20 min after infection and then remains constant. These data show first, that the combined rates of dCMP hydroxymethylase and of dTMP synthetase measured between 6 and 10 min after infection and under conditions of saturation of the enzymes by their substrates are 1 to 2 orders of magnitude greater than the rate of 3H release in vivo. Second, in vivo the rate of 3H release increases exponentially, whereas in extracts, dCMP hydroxymethylase and dTMP synthetase activities increase approximately linearly. The significance of the finding that in T4D infection the rate of tritium

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**Table II**

| TABLE II |
| Formation of labeled dCMP and HMdCMP after infection by T4 mutant, amB24 |

The isolation and characterization of the nucleotides are described under "Results." The specific activity of [5-3H]uridine was 3.9 x 10^6 cpm per nmole. The intracellular concentration of radioactive nucleotides was calculated on the assumption that the cell volume is 0.9 x 10^-12 ml (4). To obtain nanomoles of labeled nucleotide per ml of culture, multiply the intracellular concentration in molarity by 900.

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**Fig. 1.** Pathways converting labeled uridine and deoxyuridine to dUMP, dCMP, and DNA after T4 phage infection. Enzymes which are induced by phage infection are indicated as ; in some cases, a comparable host enzyme exists (29).

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**Fig. 2.** Comparison of tritium released in vivo using [5-3H]uridine with the specific activities of dCMP hydroxymethylase and dTMP synthetase in extracts. A Thy- Escherichia coli culture was grown and infected as described under "Experimental Procedure." The specific activity of [5-3H]uridine was about 10^4 cpm per nmole in the different infections, and the concentration was 0.001 mm. The levels of infection by T4D and by the double mutant id8 amN122 were normalized by reference to the levels of dCTPase activities (see "Experimental Procedure"). The scale for thymidylate synthetase activity has been expanded. The two enzyme measurements were carried out on separate infected cultures. At 10 min, the specific activity of dTMP synthetase normally is 1/4 to 1/2 that of dCMP hydroxymethylase (11). In the in vivo assay, the rate of 3H release in vivo after T4D infection presented as natoms per 10^8 cells per min is plotted versus time of infection.

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**RESULTS**

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FIG. 3 (left). Rates of release of $^3$H from [5-$^3$H]uridine in vivo after infection by various DO mutants compared with T4D. The conditions were as in Fig. 2. The dotted line (T4D) was taken from Fig. 2. The different infections were normalized to T4D as follows. $amEl0$, $amN82$, $amN81$, and $amC42$ were corrected by references to the thymidylate synthetase activities in a simultaneous infection by T4D; $amB24$, $amB24$, was normalized by use of thymidylate synthetase, and $amB24$, $amB24$, was normalized by dCTPase measurements. Normalization of $amB22$ by both dCTPase and thymidylate synthetase levels gave identical results.

FIG. 4 (center). $^3$H release from [5-$^3$H]uridine after infection by phage mutants with lesions in dCMP hydroxymethylase or thymidylate synthetase. The conditions were as in Fig. 2. The release increases exponentially in the initial phase of the reaction which is considered in subsequent sections of this paper.

Infection by the double mutant, $amN182$ $td8$, which induces neither dCMP hydroxymethylase nor dTMP synthetase, shows no $^3$H release after about 3 min. The slight activity seen in the first few minutes with this double mutant and with other phage infections perhaps represents $^3$H release associated with the methylation of UMP residues and with the pseudo-uridylic acid rearrangement during the synthesis of phage-induced (17, 18) and residual host tRNA (19).

Release of $^3$H after Infection by DO Phage Mutants—One possible interpretation of the delay in $^3$H release and the exponential kinetics observed in vivo is that the enzyme activities leading to release of $^3$H from position 5 of the pyrimidine nucleotide precursors are regulated through allosteric control by deoxyribonucleotide products, perhaps at the level of ribonucleoside diphosphate reductase (20, 21), or that the activities of dCMP hydroxymethylase and thymidylate synthetase are inhibited by the accumulation of their respective nucleotide products (22, 23). By this idea, these enzymes would not be able to function until T4 DNA synthesis initiates and removes the accumulated nucleotides.

Accordingly, the kinetic behavior of $^3$H release was explored after infection by several DO mutants in which deoxyribonucleotides accumulate as mono-, di-, and triphosphates with characteristic profiles rather than entering into DNA synthesis (4, 24). After infection by amber mutants of genes 1, 41, 44, and 45, the kinetic process of release of $^3$H in vivo was very similar to that with the wild type control (Fig. 3). Both mutants of gene 1 expected values were calculated as in the text. Infection by $amN55$ was equivalent to infection by T4D based on both dCTPase (○) and thymidylate synthetase (●) activities, and infection by $td8$ and T4D was equivalent based on dCMP hydroxymethylase activities.

FIG. 5 (right). Addition of labeled precursor at different times after infection. T4D-infected E. coli cells were divided into three equal aliquots. As shown by the arrows, [5-$^3$H]U was added at 0.75 (A), 7.8 (B), and 15.8 (C) min after infection. The conditions were as in Fig. 2. The data are plotted on a semilog scale, exponential parts of the reactions being represented by the solid segments and the linear part of the reaction and the transient region between these rates by the dotted lines. (amB24 and $amC42$) fell in this range. On the other hand, a gene 43 mutant, $amB22$, exhibited a lesser rate. The release of $^3$H on infection by this mutant and other amber mutants of gene 43 has been found to be consistently lower than in T4D infection.

The conclusions from these findings may be summarized as follows.

1. In the absence of the products of the DO genes 1, 41, 44, and 45, the activation of the two enzymes still follows the exponential kinetics displayed by wild type phage. The levels of the deoxyribonucleotides appear to have no effect on the kinetics of $^3$H release. Therefore, allosteric or feedback controls may not play an important function in the regulation of these enzymes in vivo.

2. It follows that the products of these DO genes apparently are not necessary for the release of $^3$H by the dCMP hydroxymethylase and thymidylate synthetase reactions in vivo.

As will be shown in an ensuing section, the rates of $^3$H release and of DNA synthesis are nearly equivalent in T4D infection. The level of nucleotide accumulation in wild type infection is very low (4, 24). Therefore, the $^3$H released in the in vivo synthesis of dTMP and of 5-hydroxymethyl-dCMP must closely reflect the thymine to 5-hydroxymethylcytosine ratio in T4 DNA, which is 1.89:1 (25). Inasmuch as most of the DO mutants show $^3$H release rates which appear to be unchanged from that after T4D infection, we conclude that the ratio of the rates of dTMP to 5-hydroxymethyl-dCMP synthesis still approaches that in wild-type infection.

P. K. Tomich, unpublished experiments.
Tritium Release after Infection by Phage Mutants Unable to Induce Either Thymidylate Synthetase or dCMP Hydroxymethylase Activities—In an effort to quantitate the contributions of the activities of thymidylate synthetase and dCMP hydroxymethylase to the $^3$H release values in vivo (see previous section), we employed phage mutated in these functions. Fig. 4 shows a set of experiments in which the $^3$H release after infection by amN55, a mutant lacking dCMP hydroxymethylase activity, is compared with the $^3$H release after T4D infection. Such a mutant would show only thymidylate synthetase activity. In the same figure are shown the results of infection by tdS, a mutant lacking thymidylate synthetase activity and thereby exhibiting only dCMP hydroxymethylase activity. The expected values shown in the figure are calculated on the assumption discussed in the previous section, i.e. that thymidylate synthetase and dCMP hydroxymethylase activities in vivo after wild type infection reflect the thymine to 5-hydroxymethylcytosine ratio in DNA. The values found for tdS infection do not appear to be significantly different from the calculated values. However, the observed values for the amN55 infection were much lower than the expected activity, being about $1/4$ the value at 20 min. This loss of activity appears to point to a direct effect of dCMP hydroxymethylase on dTMP synthesis. Furthermore, this conclusion was corroborated by a direct assay of dTMP synthetase activity by measurement of the $^3$H released from $[5-^3$H]dUMP; see below). In a culture infected by a temperature-sensitive mutant of gene 49, $^3$H release was decreased in nearly direct proportion to the loss of activity of dCMP hydroxymethylase as the culture was raised stepwise toward permissive temperatures.

Thus, it appears that a lesion in the dCMP hydroxymethylase gene had a profound effect on the thymidylate synthetase activity observed in vivo. By contrast, a mutant in the structural gene for the phage-induced thymidylate synthetase had no apparent effect on dCMP hydroxymethylase activity.

Labeled Substrates for Enzymes Are Not Limiting—Berglund et al. (20) have reported that phage-induced ribonucleoside diphosphate reductase is not detectable until 5 min after infection. It could be argued that the lack of early tritium release results from a limitation of substrate; that is, until about 5 min after infection, the levels of $[5-^3$H]dUMP and $[5-^3$H]dCMP might be too low for enzymatic activity. This argument would have to assume that the host reductase would not be functioning immediately after infection. It is known that T4 reductase mutants are not lethal, and therefore, the host enzyme must be capable of functioning.

Nevertheless, the possibility that reductase activity was the limiting factor was tested by the following experiment. A 100-ml culture of E. coli Thy$^{-}$ ooc was infected by amB24, and $[6-^3$H]uridine was added as described under "Materials." At various times after infection, 20-ml aliquots were taken and dCMP and HMDCMP were isolated and characterized as described in a previous paper (3). In this experiment, amB24 was chosen since Warner and Hobbs (24) have shown that high levels of both dCMP and HMDCMP accumulate after infection by this mutant. The intracellular concentrations of labeled dCMP and HMDCMP are shown in Table II. Radioactive dCMP was already present essentially at its equilibrium concentration at 1 min after infection. However, no HMdCMP was detected even at 5 min, the earliest point measured at which labeled HMdCMP was detected being 10 min. In these studies, less than $0.02 \times 10^{-4}$ m labeled HMdCMP could have been detected.

We conclude from this experiment that in the first 5 to 7 min after infection, the concentration of labeled dCMP is not a limiting factor in the detection of $^3$H release resulting from dCMP hydroxymethylase activity. While the levels of dCMP reached were less than the concentration giving maximum velocity with purified enzyme (14, 22), i.e. about $1/4$ to $1/2$ the $K_m$, at this concentration, the enzyme activity would be expected to be easily measured by the sensitive $^3$H release technique. More importantly, in wild type infection, the level of dCMP formed by de novo synthesis was found by Warner and Hobbs to be less than 0.01 nmole per 5 x $10^6$ infected cells or less than $1 \times 10^{-3}$ m intracellularly. Yet, this steady state level of dCMP is capable of maintaining sufficient dCMP hydroxymethylase activity to permit a normal rate of T4 DNA synthesis. Warner and Hobbs showed this rate of DNA synthesis to be of the order of 0.01 nmole of hydroxymethylcytosine nucleotide incorporated per min per $5 \times 10^6$ cells (24).

The degradation of host DNA to dCMP and, via dCMP deamination, to dUMP, might reasonably be expected to dilute the 5-$^3$H precursors in the $^3$H release process (26). On infection by T4 phage at 37°, host DNA breaks down to acid-soluble derivatives after 5 min, most rapidly in the period between about 10 and 20 min (27). Nevertheless, the exponential increase in the rate of $^3$H release from administered $[5-^3$H]uridine, beginning at about 5 min at 30°, occurs at a time when the dCMP from host DNA could be expected to dilute the labeled pool of nucleotides. That is, while the specific activity of the pool should be decreasing, the rate of formation of 5-hydroxymethyl-dCMP (and of dCMP) increases to be increasing (Fig. 2). T4 mutants with lesions in gene 46 are unable to degrade the host DNA beyond fragments with molecular weights of about $10^4$ to $10^5$ (28, 29), and therefore do not give rise to deoxyribonucleotides. Infection by amN130 (a gene 46 mutant) showed kinetics of $^3$H release from administered $[5-^3$H]uridine which were superimposable on the values after wild type infection. Such experiments and studies with a phage-infected system rendered permeable to nucleotides by sucrose treatment (2) suggest that the deoxyribonucleotides formed de novo and those derived from host degradation do not mix (30). Accordingly, $^3$H release appears to be unaffected by the host DNA degradation. It may be added that the experiments shown in Figs. 5 and 6 are not compatible with a varying dilution from host DNA degradation.

Addition of Tracer Compound at Different Times after Infection.—The following experiment was designed as an additional test to determine whether the delay of $^3$H release and the exponential kinetics are explained by dilutions of labeled precursors. In the experiment shown in Fig. 5, $[5-^3$H]uridine was added at the indicated times after infection. The $^3$H release was measured and the results plotted on semilogarithmic paper.

Tracer compound added at 7.8 or at 15.8 min after infection shows rates of $^3$H release which almost immediately correspond to the kinetics shown at each of these times when the labeled compound was added at the time of infection. If dilution of the isotopic compound accounted for the delay and the exponential kinetics of $^3$H release (Fig. 2), addition of labeled compound at different times should always show such kinetics. Therefore, we

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4 A. Eckhardt, unpublished results.

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1 Infection of a host with DNA prelabeled with $^3$H at position 5 of its dCMP residues leads to liberation of $^3$H beginning about 5 min after infection (C.-S. Chiu, unpublished results).

4 P. K. Tomich and C.-S. Chiu, unpublished results.
Infection by tsL13 at 30° but not at 42°, the dCMP hydroxymethylase formed at 30°, is capable of forming product and DNA at 42°. Since more active complex cannot be formed at 42°, the rate is not exponential. When shift-down occurs, new enzyme is formed, and the complex is synthesized at an exponential rate unless chloramphenicol is added to prevent the synthesis of active dCMP hydroxymethylase.

FIG. 6 (left). Inability of tsL13 to form an active complex at nonpermissive temperatures. The infection procedure was as described under "Experimental Procedure" except that the final cell concentration was 6 x 10⁶ per ml. [5-3H]Juridine was employed as in Fig. 2A, at 10 min after infection, half of the culture was shifted to 42° (□) as described previously (1). The time to reach 42° was about 20 s, and this point is indicated by the arrow. B, in a second experiment, infection was again carried out at 30°. At 9 min after infection, part of the culture was shifted to 42° (dotted line) and the remainder maintained at 30° (●). At 14 min, a portion of the 42° culture was divided into two parts. It was shifted back to 30° (○); the other half was shifted to 30°, and chloramphenicol was added to a final concentration of 120 μg per ml (▲). 

FIG. 7 (right). Tritium release and dUMP accumulation in cultures, labeling by [5-3H]deoxyuridine. In these experiments, [5-3H]dU was used at 0.091 mM and at a specific activity of 3.3 x 10⁶ cpm per nmole with the T4D infection and 5.4 x 10⁶ cpm per nmole with amB24. The intracellular concentration of dUMP in the amB24 infection was calculated as described in the legend to Table II.

Measurement of Thymidylate Synthetase Activity in Vivo through Bypass of Ribonucleoside Diphosphate Reductase; dUMP Is Not Limiting Factor—A direct way to bypass the phage-induced ribonucleoside reductase takes advantage of [5-3H]deoxyuridine which is converted to [5-3H]dUMP via thymidine kinase (31), as shown in Fig. 1. Phage infection also induces a second thymidine kinase (32). In the following experiment (Fig. 7), deoxyuridine was protected against breakdown due to thymidine phosphorylase (9) by inhibiting this enzyme with uridine at a 4.0 mM final concentration (10). As an indication of the quantity of [5-3H]dU converted to uracil and then passing through the large uridine pool, RNA synthesis was followed. Based on the negligible incorporation into RNA, label entering the uridylic acid pool by such a pathway was insignificant.

The results in Fig. 7 show tritium released from [5-3H]dU in both wild type and amB24-infected Thy- cells. This assay measures only the phage-induced thymidylate synthetase, and would show no blank due to tRNA synthesis. The 4H values were not significantly different from the zero time blank in the first 7 or 8 min. On infection by wild type phage, kinetic behavior similar to that obtained with [5-3H]U is observed. The rate of release of [5-3H]U was much lower after infection by amB24 than by T4D. In such experiments, T4D and amB24 gave similar infection as measured by early enzyme levels. In Fig. 3, it was demonstrated that cultures infected by T4D and amB24 showed very similar kinetics of [5-3H]U release when [5-3H]U was the labeled compound. Comparison of the results shown in Fig. 7 with those in Figs. 2 to 5 shows that after T4D infection, [5-3H]U is considerably slower than from [5-3H]U. The actual explanation for the difference between the results found with [5-3H]deoxyuridine and [5-3H]Juridine has not been resolved. One possibility is an effect on thymidine kinase activity, and another is a differential isotopic dilution of [5-3H]-dUMP because of an apparent enhancement of ribonucleoside diphosphate reductase by the amB24 mutation (24). The concentration of [5-3H]dUMP which accumulated after the amB24 infection was measured and is also shown in Fig. 7.
FIG. 8. Determination of time of initiation of $^3$H release from 5-3H precursors in vivo by use of chloramphenicol. The graph is the semilog plot of the data. The lines, representing the addition of chloramphenicol at various times after infection, cross at 4.8 min. A 90-ml culture of Ty$^-\,$E. coli was infected by T4D at $30^\circ$ using 0.091 mM [5-3H]uridine at a specific activity of $2.5 \times 10^4$ cpm per nmole. At the indicated times after infection, 15-ml aliquots were pipetted into 50-ml flasks, in a rotary shaker bath at $30^\circ$, containing 0.5 ml of 3.0-mg chloramphenicol per ml. Tritium release was measured as before.

This labeled compound rapidly reached a fairly constant intracellular concentration which was 10 to 20 times the K$_m$ of T4D-induced thymidylate synthetase for dUMP based on studies with the closely related T2 and T6 enzymes (22). Thus, even though both the enzyme and its substrate are present early in the infective process, enzyme activity does not appear to start until later. It is most unlikely that initially the pool of labeled dUMP was so highly diluted by unlabeled dUMP that thymidylate synthetase was not detectable. At 10 min after infection, $^3$H release from dUMP is readily measurable, and this reaction must call on the pool of labeled dUMP present before 10 min.

On infection of a Thy$^+$ host by a Thy$^+$ phage, $^3$H release could be detected in vivo from [5-3H]deoxyuridine within less than 1 min after infection (not shown). Therefore, [5-3H]dUMP and 5,10-methylenetetrahydrofolate are available for the host thymidylate synthetase activity long before phage-induced thymidylate synthetase activity can be detected in vivo (Fig. 2).

Effect of Chloramphenicol on $^3$H Release: Measurement of Time of Initiation of Enzyme Activation—A series of experiments was carried out by adding chloramphenicol at various times after infection and measuring $^3$H release from [5-3H]uridine added to the culture. The results are plotted on a semilogarithmic graph (Fig. 8). Slight enzyme activation occurred even when chloramphenicol was added at 3 min. It is significant that the enzyme activity after chloramphenicol treatment still followed exponential kinetics initially. The lines extrapolate to approximately the same point (4.8 min). This value is estimated to be accurate to within $\pm 0.5$ min. The scattering of points at early times is attributed to the inaccuracies at the low activities.

Since the conditions at each chloramphenicol concentration were identical except for the amount of protein synthesis, the results in Fig. 8 are interpreted as evidence that an active enzyme complex begins to form at about 5 min after infection. The different apparent first order rates may represent a dependence on the concentrations of the subunits of the complex. It does not appear that activation of these enzymes depends on protein synthesis, other than the obvious requirement for the synthesis of the enzymes themselves and of the enzymes converting radio-active precursors to the appropriate substrates.

Tritium Release Kinetics and DNA Synthesis Kinetics Coincide—Since tritium release occurred at the time DNA synthesis normally initiates even in the absence of DNA synthesis (Fig. 3), we carried out the following experiment to determine whether the two events were correlated. A mixture of [5-3H]uridine and [6-3H]thymidine was used. In cultures infected by T4 wild type-phage, both of these precursors are converted to a [5,6-3H]dTMP mixture and to a [5,6-3H]dCMP mixture as described previously. Those dUMP molecules passing through dTMP synthetase or dCMP molecules forming HMdCMP release $^3$H from position 5. The remaining [6-3H]dTMP and [6-3H]HMdCMP simultaneously find their way into DNA. Thus, the enzyme activities and DNA synthesis can be followed concurrently and with identical isotope dilutions. The results of such an experiment are shown in Fig. 9.

Both $^3$H release (from dTMP synthetase and dCMP hydroxymethylase) and DNA synthesis appear to initiate simultaneously in this experiment. In the inset to this figure, both DNA synthesis and $^3$H release are plotted on a semilogarithmic scale during the period between 5 and 15 min. From this in vivo experiment, DNA synthesis and the activation of dCMP hydroxymethylase and dTMP synthetase appear to show identical exponential kinetics. The results suggest but do not prove that the activation of these enzymes and of DNA synthesis represents the same process.

DISCUSSION

The most obvious results presented in this paper are: (a) thymidylate synthetase and dCMP hydroxymethylase are not active
in *vivo* immediately after their formation, but at 30° are activated at about 5 min after infection, (b) after activation occurs, the *\textit{in vivo}* activities of these enzymes increase exponentially for an additional 10 to 15 min and then become linear; (c) the activities of the two enzymes are the same whether the infection is by wild type phage or DO mutants. Amber mutants of gene 43 (T4 DNA polymerase) are exceptions, since on infection by such mutants, activation of the enzymes is slower; (d) gene 42 mutants greatly decrease the activity of thymidylate synthetase as measured *\textit{in vivo};* however, a td mutant had no apparent effect on the release of \(^{14} \text{H}\) by the dCMP hydroxymethylase reaction; (e) activation of dCMP hydroxymethylase, of dTMP synthetase, and of T4 DNA synthesis appears to show identical and coincidental kinetics.

dCMP hydroxymethylase belongs to the class of immediate early enzymes (34) and has been detected as early as 1 min after infection, and thymidylate synthetase can be detected before 4 min (Fig. 2). Nevertheless, *\textit{in vivo* the two enzymes do not function until after about 5 min, and then their activities increase exponentially. The rate of \(^{14} \text{H}\) release in *\textit{vivo*}, which is a measure of both enzymes, is far lower between 6 and 10 min than the combined activities of the two enzymes in *\textit{vivo*} (Fig. 2).

We have been particularly sensitive to the possibility that the delay in the *\textit{in vivo*} activation of dCMP hydroxymethylase and of dTMP synthetase and the exponential character of the \(^{14} \text{H}\) release reflecting this activation could be explained by factors other than the formation of a complex. Our experiments rule against nonavailability of substrates for these enzymes (Table II and Fig. 7), isotope dilution by de novo synthesis (Fig. 5), or by host DNA breakdown (see Footnote 6), or allosteric feedback against nonavailability of substrates for these enzymes (Table II). Experiments using T4 phage mutated in a gene for ribonucleoside diphosphate reductase as well as studies with hydroxyurea, an inhibitor of this enzyme (27), have shown qualitative changes in the incorporation of deoxyribonucleotides into DNA in plasmolyzed preparations. Thymidylate synthetase can be detected before 4 min after infection,3 and thymidylate synthetase can be detected before 4 min (Fig. 2). Nevertheless, *\textit{in vivo* the two enzymes do not function until after about 5 min, and then their activities increase exponentially. The rate of \(^{14} \text{H}\) release in *\textit{vivo*}, which is a measure of both enzymes, is far lower between 6 and 10 min than the combined activities of the two enzymes in *\textit{vivo*} (Fig. 2).

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does not reach its maximum inhibitory effect for at least 1 min after addition; these results do not rule against involvement of reductase and T4 DNA polymerase in the complex. It is also not known whether discrepancies in the actual initiation times of these enzymes exist or whether a host function could substitute in the complex.

By our experiments, DNA synthesis appears to coincide with the in vivo ³H-releasing activity (Fig. 9). This finding in itself may arise only because DNA synthesis is limited by the rate of synthesis of deoxyribonucleotides. Since the rate of ³H release to 30 min after infection is identical whether DNA synthesis occurs or not (i.e. with DO mutants) and since we have demonstrated an intimate role of the dCMP hydroxymethylase enzyme in DNA synthesis (2), it is reasonable to suggest that the complex of enzymes formed at about 5 min after infection also contains the components to initiate DNA synthesis. However, although our data show coincidence of DNA synthesis and ³H release after 5 min, further effort is necessary to establish that these two processes actually begin at the same time. Perhaps it is relevant that sucore-plasmolyzed T4-infected cells (2) begin to exhibit the ability to synthesize DNA at about 5 min after infection and that Miller (42) has recently suggested that T4 DNA attaches to the membrane at about 5 min after infection.

The precise time at which DNA synthesis initiates has remained unclear, although time course curves have been reported over many years by many investigators (43-46). Aside from obvious differences because of temperature, the exponential character of the rise in activity may be responsible for the difficulty in measuring the synthesis in the first few minutes.

The cell membrane may be an integral part of this deoxyribonucleotide-synthesizing complex. Siegel and Schaechter (47) recently have summarized the growing literature supporting the concept of a DNA-membrane complex in T4 DNA replication. Several observations implicate the cell membrane in the complex described in the present work. For example, treatment of plasmolyzed T4-infected preparations (2) with low levels of Triton X-100 (0.01 to 0.02%) causes a loss of the requirement for dCMP hydroxymethylase in DNA synthesis.7 Consistent with the idea of a dCMP hydroxymethylase complex is the observation7 that more than 10% of the total activity of this enzyme is found in the particulate fraction obtained by BRIJ-lysozyme treatment (48). This binding to the membrane fraction does not occur after tsL18 infection at nonpermissive temperature even though the enzyme is still active. Binding occurs at permissive temperature, and wild type enzyme added to uninfected particulate fraction does not bind.

In contrast to the types of results outlined above, in concentrated preparations from T4-infected cells such as those described by Barry and Alberts (49), which are presumably free of membrane and which are dependent on various DO gene products for DNA synthesis, no requirement for dCMP hydroxymethylase has been reported.

Recently Chiu and Greenberg (50) have demonstrated that certain temperature-sensitive mutants of gene 42 increase the reversion rate of some rII mutants in the same genome by about 4 to 10 times. Whether or not this mutagenic effect derives from the role of dCMP hydroxymethylase in the complex and in DNA synthesis remains to be determined.

In summary, dCMP hydroxymethylase may be considered to have two intimately related functions. It acts as part of the DNA-synthesizing apparatus (2), and, as shown in this paper, it is one of at least two enzymes which is activated with kinetics appearing to be identical with the kinetics describing the initial stages of DNA synthesis. By the process described here, a tight control can exist between the enzymes synthesizing the precursors and the DNA polymerization system.

Note Added in Proof—We find that several DO mutants show a slightly increased T/HMC ratio even though the total ³H release remains the same. Although this finding may suggest a control at ribonucleoside diphosphate reductase, it does not account for the exponential character of the release of ³H.

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Evidence for a Complex Regulating the \textit{in Vivo} Activities of Early Enzymes Induced by Bacteriophage T4

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