*ENZYMATIC SYNTHESIS OF DEOXYRIBONUCLEIC ACID. VI. INFLUENCE OF BACTERIOPHAGE T2 ON THE SYNTHETIC PATHWAY IN HOST CELLS**

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Information now available about DNA[1] synthesis by *Escherichia coli* enzymes[2–4] has encouraged an inquiry into the biochemical basis for the observation that a phage-infected *E. coli* cell ceases to produce its own DNA and makes instead the DNA characteristic of the infecting phage.[5] This general problem poses several rather specific questions which may be summarized as follows:

1. T2, T4, and T6 DNA differ from the DNA of *E. coli*, as well as from that of other sources which have been examined, in containing hydroxymethylcytosine (HMC) but no cytosine.[6] Flaks and Cohen[7] have already shown that within several minutes after infection by phage T2, T4, or T6, a new enzyme which hydroxymethylates deoxycytidine 5′-phosphate is produced. Is there an enzyme for converting the resulting dHMC-5-P to the triphosphate level in order to provide a functional substrate for DNA synthesis?

2. With respect to the exclusion of cytosine from the DNA of phage T2, T4, and T6, is there a mechanism in the infected cell for removal of deoxycytidine triphosphate from the site of polymerase action?

3. The DNA's of T2, T4, and T6 contain glucose linked to the hydroxymethyl groups of the HMC in characteristic ratios,[8, 9, 10] although it is clear that in T2 and T6 some of the HMC groups contain no glucose.[9] According to our present understanding of DNA synthesis,[4] it is difficult to conceive how these constant ratios are achieved if the incorporation were to occur via glucosylated and non-glucosylated HMC nucleotides. Is there an alternative mechanism involving direct glucosylation of the DNA even though direct substitutions on intact DNA have been hitherto unknown?

4. Following phage T2 infection there is a temporary halt followed by a resumption of DNA synthesis at about 5 times the rate shown by the uninfected cell.[11] However, measurements with extracts of infected cells, using standard substrates, revealed much diminished rather than the anticipated augmented levels of DNA-synthesizing activity.[12] What are the altered conditions for assay of DNA synthesis in infected cell extracts which would elicit the high levels of activity expected from the physiologic studies?

We have explored these questions and have found that following infection with phage T2 several new enzymes appear.[13] These are (1) an enzyme which phosphorylates dHMC-5-P, leading to the synthesis of hydroxymethyldeoxycytidine triphosphate (dHMC-TP), (2) an enzyme which removes the terminal pyrophosphate group from dCTP, and (3) an enzyme which transfers glucose from UDPG directly to the HMC in DNA. Measurements of DNA synthesis, using dHMC-TP in place of dCTP, revealed about a 12-fold increase in activity in extracts of infected cells over the levels observed in uninfected cell extracts.

METHODS AND MATERIALS

Preparation of Cell Extracts.—E. coli B was grown at 37° with vigorous aeration in M-9 medium[14] modified to contain per liter: KH_2PO_4, 3 gm, Na_2HPO_4, 6 gm, NH_4Cl, 1 gm, $MgSO_4 \cdot 7H_2O$, 0.49 gm, glucose, 5.0 gm, $FeSO_4 \cdot 7H_2O$, 0.5 mg, $CaCl_2$, 55 mg. Growth at a logarithmic rate continued to 4–5 $\times$ 10^9 cells per ml with a generation time of about 50 min.

Cell extracts were prepared in two ways:

Method I: Cultures grown to 2 $\times$ 10^8 cells per ml were chilled, centrifuged, and the cells resuspended at 4 $\times$ 10^9 cells per ml in cold growth medium. Five $T2r^{+15}$ or T5 per cell were added and after a 4-min adsorption period at 0°, the culture was diluted twenty-fold into fresh growth medium at 37° and aeration continued. The time of dilution was taken as "zero minutes." 50-ml aliquots were pipetted rapidly onto crushed ice at intervals. The cells were sedimented by centrifuging for 5 min at 10,000 $\times$ g, were resuspended in 1 ml. of 0.5 *M* glycylglycine buffer, pH 7.0, containing 0.001 *M* glutathione, and were stored for 1 to 3 days at −15°. The cells were disrupted in a 10 kc Raytheon sonic oscillator. After removal of a small amount of debris by centrifugation, the extracts contained about 2 mg of protein per ml.

Method II: Cultures were grown to 2 $\times$ 10^9 cells per ml, in the modified medium without $CaCl_2$ and four $T2r^+$ per cell added ("zero minutes"). 50-ml aliquots were pipetted rapidly onto crushed ice at intervals. The cells were sedimented and resuspended in 4 ml of 0.05 *M* glycylglycine buffer, pH 7.0, containing 0.001 *M* glutathione and disrupted as above. Extracts containing about 6 mg of protein per ml were obtained after centrifugation.

All results refer to extracts prepared by Method I unless otherwise stated. While Method II was less effective for phage multiplication (see below), a description of this method is included since it provided an alternative and efficient technique for obtaining concentrated extracts in kinetic studies. The activities per mg protein for the several enzymes studied were found to be at levels similar to those obtained by Method I.

Bacteriophage Determinations.—Bacteriophage was assayed by standard techniques.[16] Intracellular phage was measured after "lysis from without" essentially as described by Doermann.[17] The formation of infectious units in both $T2r^+$ and T5 infected cells (Fig. 1) was found to proceed in normal fashion in cells infected as described in Method I. $T2r^+$-infections produced by Method II yielded only about 2 phage per original cell at 25 min when measured after "lysis from without," although after clearing of the culture a yield of several hundred phage per original cell was obtained.

Enzyme Assays and Preparations.—Phosphorylation of the deoxynucleoside monophosphates (kinase activities) was measured, as described before,[2] by using a 5′-P^{32}-labeled mononucleotide as substrate and assaying the amount of label which becomes resistant to the action of semen phosphatase.[18] Formation of dHMC-5-P from dC-5-P (hydroxymethylase) was assayed according to Flaks and Cohen.[7] The assay of DNA synthesis ("polymerase") was measured by the conversion of a C^{14}-labeled deoxynucleoside triphosphate into an acid-insoluble product.[2] "Polymerase" fraction VII from uninfected *E. coli* was prepared as previously described.[2]

Substrates.—Deoxynucleotides and samples of native and enzymatically synthesized DNA were prepared as in earlier studies.[2, 4] dHMC-5-P was synthesized according to Flaks and Cohen[7] using a 230-fold purified hydroxymethylase[19]; C^{14}-dHMC-5-P was obtained by using C^{14}-formaldehyde (Volk Radiochemical Co.) in the hydroxymethylation reaction, and P^{32}-dHMC-5-P was prepared by using P^{32}-dC-5-P. The E_m value determined for the nucleotide in this preparation was 13.5×10^3 at 284 mμ at pH 1. Since this value conflicts with that of 11.7×10^3 given by Flaks and Cohen,[7] it is regarded as provisional and requires further

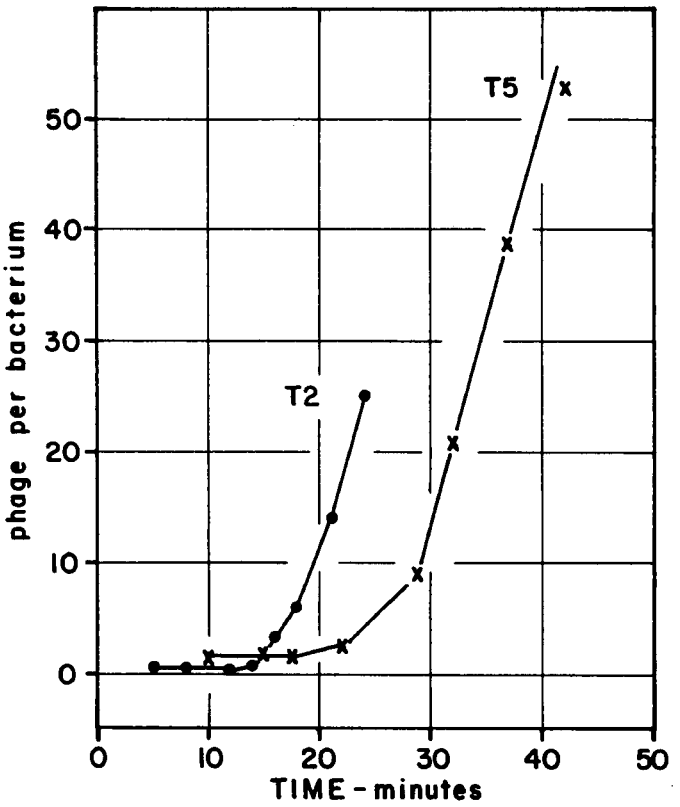

FIG. 1.—Appearance of phage in T2- or T5-infected cells. Infected cultures were prepared by Method I. Infectious units were measured after "lysis from without."

checking. C^{14}-glucose 6-phosphate was prepared by hexokinase action on uniformly labeled C^{14}-glucose (Isotope Specialties Co.). C^{14}-UDPG was prepared from C^{14}-glucose 6-phosphate and uridine triphosphate by the action of phosphoglucomutase and UDPG pyrophosphorylase as outlined by Glaser and Brown.[20] Unlabeled UDPG was a product of the Sigma Chemical Company.

RESULTS

An Enzyme which Phosphorylates dHMC-5-P.—At about 4 min after infection of *E. coli* with phage T2, it was possible to detect in the extracts an enzyme which catalyzes the phosphorylation by ATP of dHMC-5-P (Fig. 2 *A*). This reaction was

undetectable (<0.5 per cent of the maximal value after infection with T2) in normal cells or in extracts of cells infected with T5 (Fig. 2 *A*), a phage without HMC in its DNA. The maximal level of phosphorylating ("kinase") activity for dHMC-5-P was of the same order as that of the kinases for the other deoxynucleotides in-

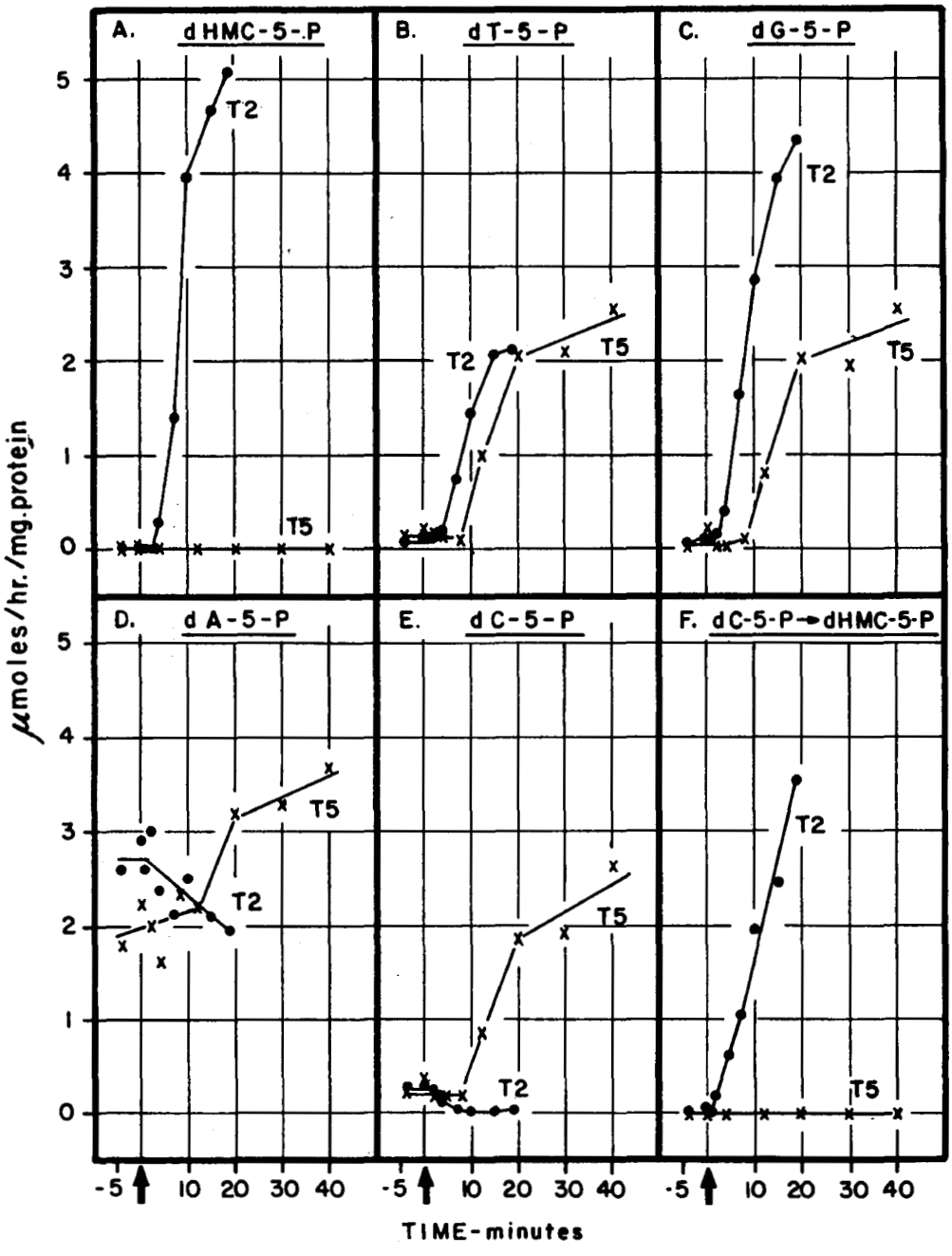

FIG. 2.—Deoxynucleotide-phosphorylating enzymes ("kinases") and hydroxymethylating enzyme levels before and after infection with phage T2 or T5. The arrow indicates the start of infection ("zero minutes," see Method I). Assays were as referred to in Methods.

corporated into viral DNA (see below) and was essentially similar in extracts prepared by either Method I or Method II.

The product of the dHMC-5-P kinase action (with the presumed participation of nucleoside diphosphate kinase in the preparation) was shown to be the triphosphate. Using a 20-fold purified kinase preparation,[19] 25 μmoles of dHMC-TP were prepared and isolated by ion-exchange chromatography, a yield of 92 per cent based on the starting dHMC-5-P. Theoretical specific radioactivity values were found in the isolated dHMC-TP when P^{32}- or C^{14}-labeled dHMC-5-P was the

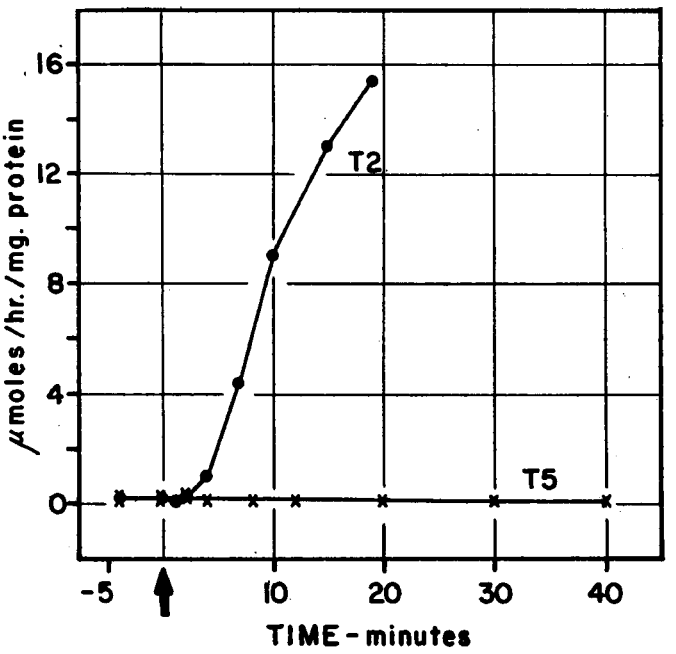

FIG. 3.—dCTPase levels before and after infection with phage T2 or T5. The arrow indicates the start of infection ("zero minutes," see Method I). The incubation mixtures (0.25 ml) contained: 6.0 mμmoles of dCTP labeled with P^{32} in the terminal pyrophosphate group (10^3 cpm per mμmole), glycine buffer, pH 9.2 (0.04 *M*), $MgCl_2$ (0.008 *M*), 2-mercaptoethanol (0.01 *M*) and a quantity of extract containing about 0.3 μg of protein. After 20 min of incubation at 37°, the reaction was terminated by adding 0.5 ml of 0.1 *N* HCl, followed by 0.2 ml of a mixture (containing per ml: 5 mg of crystalline bovine serum albumin, 25 μmoles of sodium pyrophosphate and 25 μmoles of potassium phosphate, pH 7), and 0.1 ml of a Norit suspension (20 per cent packed volume). The Norit was removed by centrifugation and the supernatant fluid was assayed for radioactivity.

starting material. After concentration with Norit, analysis revealed a ratio of HMC: total P: acid-labile P of 1.0: 3.2: 2.2, using an E_m value of 13.5×10^3 at 284 mμ at pH 1.

Kinase Levels for the Other Deoxynucleotides and Levels of the Hydroxymethylating Enzyme.—It is noteworthy that after T2 infection, the kinase levels for dT-5-P and dG-5-P were increased approximately 20 and 45 times, respectively, while that for dA-5-P was essentially unaltered (Fig. 2 *B*, *C*, *D*). Bessman has made similar observations independently.[21] As mentioned above, the kinase levels for

each of the four deoxynucleotides incorporated into phage T2 DNA reach values of about the same magnitude. By contrast, only traces of dC-5-P kinase were detected (Fig. 2 *E*). Furthermore, extracts of T2-infected cells were found to inhibit the dC-5-P kinase activity of normal cell extracts when equal amounts of infected and non-infected extracts were mixed. As will be described below, this inhibitory effect is due to an enzyme (dCTPase) which splits dCTP. By use of fluoride (8×10^{-3} *M*), which inhibits dCTPase more than 98 per cent, but the dC-5-P kinase by 15 per cent or less, it was possible to show that there was actually little or no change in the dC-5-P kinase levels upon infection. Extracts prepared by Method II showed the same kinase patterns after T2 infection.

Infection with phage T5, which contains cytosine rather than HMC, showed increased kinase activities for the four deoxynucleotides which are present in its DNA (Fig. 2 *B, C, D, E*). The 10-fold increase in dC-5-P kinase activity in the extracts of T5-infected cells may be contrasted with the absence of any increase in this activity in the extracts of T2-infected cells.

Hydroxymethylating activity was first detected at 4 min after T2 infection, and, as predicted from the results of Flaks and Cohen,[7] was absent from T5-infected cell extracts (Fig. 2 *F*).

An Enzyme which Destroys Deoxycytidine Triphosphate (dCTPase).—The inhibitor of dC-5-P kinase that develops upon T2 infection has been identified as an enzyme which splits dCTP by removal of the terminal pyrophosphate group (Fig. 3). Extracts of uninfected cells have 1 per cent or less of the dCTPase activity observed in extracts of T2-infected cells; the level of dCTPase activity of T5-infected cells was the same as in normal cells. The relative insensitivity to fluoride of the dCTPase activity in normal cells makes it doubtful that it represents the same enzyme found in the T2-infected cells.

After partial purification of the dCTPase,[19] which reduced the level of an inorganic pyrophosphatase to 2 per cent of the dCTPase, it was demonstrated that the complete splitting of 0.92 μmole of dCTP was accompanied by the appearance of 0.74 μmole of inorganic pyrophosphate identified as Norit-nonadsorbable, acid-labile P and 0.15 μmole of orthophosphate. With the purified enzyme preparation, the rates of cleavage of cytidine triphosphate and ATP were less than 1 per cent of that of dCTP; the splitting of dHMC-TP was 1–2 per cent of that of dCTP. The K_m of dCTP for the enzyme is in the region of 10^{-6} *M*. In view of this high affinity for dCTP and of the high level of enzyme activity compared to the dC-5-P kinase in the extract (cf. Fig. 2 *E* and Fig. 3), it is reasonable to suppose that the dCTP concentrations in the infected cell are reduced to an extremely low level.

An Enzyme which Glucosylates the HMC of DNA.—An enzyme which transfers glucose from UDPG to DNA containing HMC was observed in extracts of T2-infected but not T5-infected or normal cells (Fig. 4). With the partially purified enzyme[19] UDPG could not be replaced by glucose, glucose 1-phosphate or glucose 6-phosphate (Table I); similar results were also obtained with the crude extracts. The reaction requires HMC-containing DNA, which for these experiments was enzymatically synthesized from dHMC-TP, dATP, dGTP, dTTP, purified polymerase, and primer DNA derived from any one of several sources (calf thymus, *E. coli*, phage T2, phage ϕX174[22]). When DNA enzymatically synthesized with dCTP in place of dHMC-TP, or when the glucosylated DNA from phage T2 itself

were used, no glucose fixation in DNA was detectable (Table 1). dHMC-5-P and dHMC-TP failed to substitute for HMC-containing DNA as glucose acceptors. For example, in an incubation mixture containing these three types of HMC compounds with 30-fold purified enzyme, the HMC-DNA fixed 46 per cent of the glucose of the UDPG, while dHMC-5-P and dHMC-TP fixed none (<0.5 per cent).

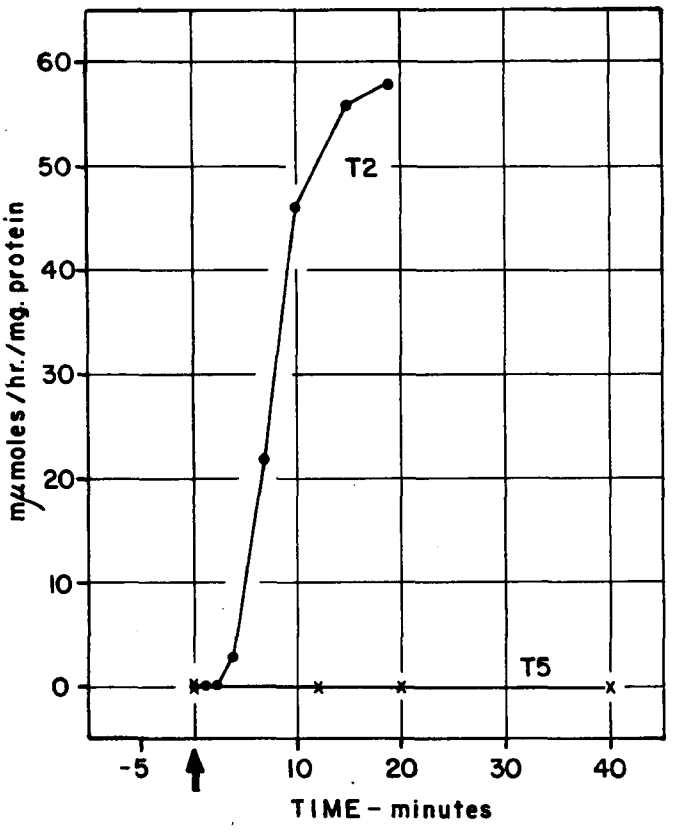

FIG. 4.—DNA-glucosylating enzyme levels before and after infection with phage T2 or T5. The arrow indicates the start of infection ("zero minutes," see Method I). The incubation mixtures (0.20 ml) contained: 10 mμmoles of UDPG labeled uniformly with C^{14} in the glucose residue (2×10^3 cpm per mμmole), Tris buffer, pH 7.5 (0.1 *M*), glutathione (0.02 *M*), DNA synthesized enzymatically (using thymus or phage T2 DNA as primer) and containing about 1 mμmole of HMC, and extract containing 10–50 μg of protein. After 15 min of incubation at 30°, the mixture was treated as in the "polymerase" assay of incorporation of a labeled deoxynucleotide into an acid-insoluble product (see Methods).

In the presence of an excess of HMC-DNA, the glucose of UDPG is transferred completely to the DNA. With an excess of UDPG and enzyme, the fixation of glucose in DNA reaches a limiting value, which is a function of the HMC-DNA present (Fig. 5). The number of glucose residues fixed in this experiment was approximately 60 per cent of the number of HMC residues in the added DNA. At this point it may be premature to regard the glucosylation limit observed with

TABLE 1

SPECIFICITY OF THE ENZYME WHICH GLUCOSYLATES DNA

Expt. No.	Conditions	Glucose fixed in DNA, mμmoles
1	Complete system (10 mμmoles of C^{14}-UDPG)	0.69
	Add C^{12}-UDPG (10 mμmoles)	0.65*
	Add C^{12}-glucose (200 mμmoles)	0.53
	Add C^{12}-glucose 1-P (200 mμmoles)	0.59
	Add C^{12}-glucose 6-P (250 mμmoles)	0.59
	Replace UDPG with C^{14}-glucose (10 mμmoles)	0.00
	Replace UDPG with C^{14}-glucose 6-P (12 mμmoles)	0.00
2	Complete system (DNA containing 0.46 mμmole of HMC)	0.28
	Replace HMC-DNA with cytosine-DNA (containing 0.75 mμmole cytosine)	0.00
	Replace HMC-DNA with T2 DNA (containing 2 mμmoles HMC)	0.00

The complete system had the composition and was treated as described in Figure 4, using a 30-fold purified enzyme, 0.3 μg in Expt. 1 and 1.5 μg in Expt. 2. In each case the DNA was glucosylated to its limit (see Fig. 5).

* The glucose fixed is calculated on the basis of the specific radioactivity of the UDPG after dilution of the C^{14} sample with C^{12}-UDPG.

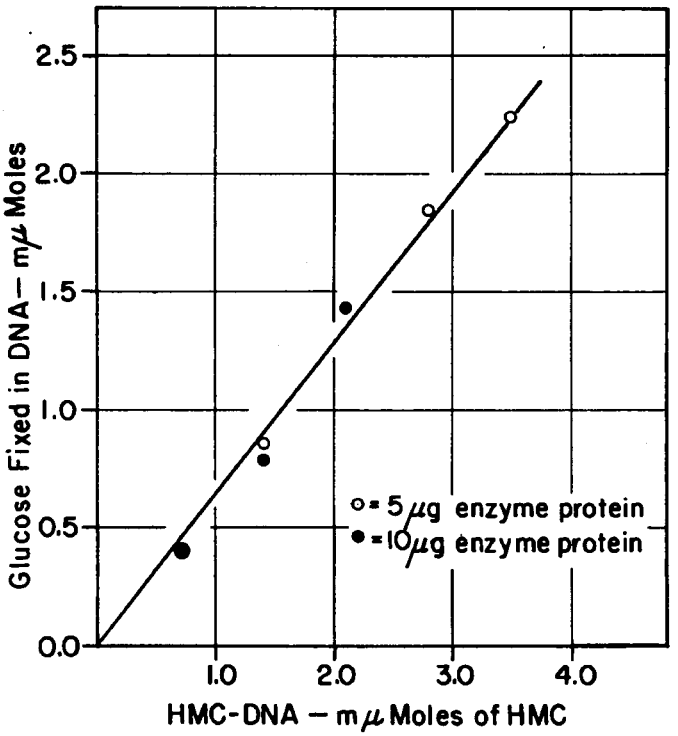

FIG. 5. — Limit of glucose fixation in DNA as a function of the amount of DNA added. The experimental details were as in Fig. 4, using the partially purified enzyme. The HMC-DNA was prepared with thymus DNA as primer.

a given sample of HMC-DNA as distinctive for the type of primer used in the enzymatic synthesis of the HMC-DNA. Further studies are required to determine how conditions of enzymatic polymerization, as well as the isolation of the DNA, may influence the glucose/HMC ratios obtained.

The C^{14}-glucose fixed in DNA was rendered acid-soluble by crystalline pancreatic deoxyribonuclease. When 52 per cent of the nucleotides were no longer acid-precipitable, 88 per cent of the glucose had been made acid-soluble. When the C^{14}-glucosylated HMC-DNA was digested to completion under conditions which Lehman has found[23] to yield a quantitative conversion of phage T2 DNA to 5′-mononucleotides, over 95 per cent of the radioactivity was found in the HMC deoxynucleotide fractions of the ion-exchange chromatogram.

Increase Rate of DNA Synthesis upon Infection.—DNA synthesis, measured by the standard assay,[2] but with dHMC-TP instead of dCTP, is increased 12-fold in extracts prepared 19 min after infection with T2 (Fig. 6). Little or no DNA synthesis can be measured in these extracts when dCTP replaces dHMCTP. However,

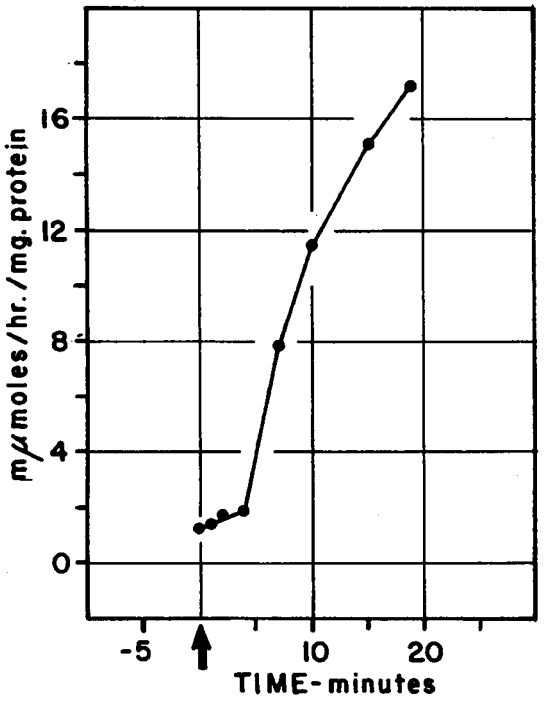

FIG. 6—DNA "polymerase" levels before and after infection with phage T2 with dHMC-TP as substrate in place of dCTP. The arrow indicates the start of infection ("zero minutes," see Method I). The incubation mixtures (0.30 ml) contained: 10 mμmoles each of dGTP, dATP, dTTP and dHMC-TP (C^{14}, 1×10^3 cpm per mμmole), Tris buffer, pH 7.5 (0.07 *M*), $MgCl_2$ (0.007 *M*), 2-mercaptoethanol (0.001 *M*), 0.04 ml. of "heated DNA" and sonic extract containing 10–50 μg of protein. The "heated DNA" was prepared by heating at 100° for 5 min, at pH 9.2, an extract of T2-infected cells; this preparation could be purified without loss of activity by treatment with ribonuclease, Norit, dialysis and precipitation with acid; it was inactivated by treatment with deoxyribonuclease. Further details of procedure were as referred to in Methods.

using 0.005 *M* F^- to inhibit dCTPase, DNA synthesis was elevated to levels near those observed in assays with dHMC-TP.

It should be emphasized that these measurements of rates of DNA synthesis with dHMC-TP were made with heated DNA as primer; when unheated DNA was used, there was no demonstrable increase in rate in the infected cell extracts. Heated DNA of phage T2 or calf thymus origin served as well as that used in Figure 6. The basis for this requirement for heated DNA with infected cell extracts requires further investigation.

DISCUSSION

In addition to the interest inherent in understanding the nature of viral infection of a cell, studies of the pathway of DNA synthesis in infected cells provide a means of testing and expanding our conceptions about the mechanism of DNA replication in normal cells.

Control of DNA Synthesis at the Level of Deoxynucleotide Phosphorylation.—It appears from studies with various analogues of the naturally occurring purines and pyrimidines[3] that an analogue in the form of a deoxynucleoside triphosphate is incorporated into DNA when its structure has the hydrogen-bonding properties of the base it replaces. For example, uracil and 5-bromouracil are both effective substitutes for thymine. Yet, uracil is never found in DNA[24] while bromouracil, when supplied to the cell, readily replaces thymine in the DNA.[25] The reason appears to be the absence of any mechanism for phosphorylating deoxyuridylate as contrasted with the availability of an enzyme for phosphorylating 5-bromodeoxyuridylate or 5-methyldeoxyuridylate (thymidylate).[3]

The present studies of phage-infected cells provide additional examples of control at the phosphorylation level. Earlier observations with 5-methyldeoxycytidylate[3] had suggested the lack of an enzyme for phosphorylation of a 5-substituted cytosine deoxynucleotide, and posed the problem of how 5-hydroxymethylcytosine deoxynucleotide becomes a substrate for phage T2 DNA synthesis. This problem seems to be solved by the development of a new enzyme after phage T2 infection of the cell. According to our studies, and those of Flaks and Cohen,[7] the synthesis of a compound novel for the cell is carried out by a new enzyme, the formation of which is presumably induced by the phage DNA. Another example of control at the phosphorylation level is provided by the device which appears to be responsible for the absence of cytosine in T2 DNA. For lack of a mechanism to eliminate the dCTP-synthesizing enzyme system, the evolution of an enzyme to destroy the dCTP provides an effective alternative.

Direct Substitution on DNA.—Genetic studies indicate that the glucose contents of DNA of phages T2, T4, and T6, and strains derived from them by recombination, are a fixed property resembling other phenotypic characters of these phages.[9, 10] It appears plausible, therefore, that the precise arrangement of these glucosylated HMC residues in the DNA may be a source of genetic information, and insight into the nature of the replication of these types of phage DNA would clearly be of considerable value.

The incorporation of a fixed proportion of non-, mono-, and polyglucosylated derivatives of dHMC-TP during the polymerization of the DNA chain is difficult to conceive because it is likely that these derivatives would all behave similarly in hydrogen-bonding to guanine. It becomes even more difficult to conceive the incorporation of these various HMC residues in a precise sequence in DNA on the basis of selection of the triphosphate derivatives. The existence of an enzymatic system for direct glucosylation of DNA offers an alternative which seems to circumvent these difficulties. At this stage, the information is still too fragmentary to determine whether this glucosylating enzyme, and perhaps an additional one for polyglucosylation, will be sufficient to explain the replication of various phage DNA's. It is apparent that further studies with T4- and T6-, as well as T2-infected cell enzyme systems are essential.

Superficially analogous to the partial glucosylation of the HMC residues in phage DNA is the partial methylation of the cytosine residues in certain plant and animal DNA's, such as wheat germ and calf thymus.[24] In the light of our findings, it would be interesting to look for an enzymatic mechanism for direct methylation of DNA in these tissues.

Kinetics of Enzyme Development and Enzyme Reactions.—The temporal pattern of development of all the enzymes studied in this report that were either "new," or the levels of which were significantly raised, was similar. The first traces of change were apparent at four minutes after T2 infection and about ten minutes after T5 infection. While the precision of measurement of these time intervals is not great, it is clear that there is a time lag before significant levels of these enzyme activities appear. Several groups of investigators[26, 27, 28] have shown that 5-methyltryptophan and chloramphenicol administered to cells at levels sufficient to inhibit protein synthesis also blocked DNA synthesis when given before or up to about 5 min after infection. When given 10 min or later following T2 infection, the inhibition of protein synthesis had only a small or even no effect on the rate of DNA synthesis. It seems reasonable to consider that the time lag we have observed may be related to the chloramphenicol-sensitive interval required for development of the enzymatic machinery for DNA synthesis, as well as other protein components vital to virus information. There have been indications of non-phage protein synthesis immediately upon infection[29] and it remains to be determined what fraction of this protein can be identified with the enzymes described here.

The multiplicity of enzyme changes described in this study along with the findings on the hydroxymethylating[7] and deoxyuridylate-methylating[30] enzymes, are all directly related to DNA synthesis. Enzyme measurements related to other metabolic pathways in infected cells have as yet disclosed few significant changes.[31] It would be surprising if further exploration of the phage-infected cell failed to reveal additional examples of new or augmented enzyme activities related to the requirements imposed by rapid phage synthesis.

In view of the pitfalls inherent in assaying the level of an enzyme activity in a cell extract, let alone in the cell itself, a detailed evaluation of the various enzyme values in terms of virus DNA synthesis does not seem warranted. However, it is interesting to note that the rates of the kinases and the glucosylating enzyme are all greater than that found for the DNA polymerizing activity and, further, that the increase in the latter activity over levels found in uninfected cell extracts is about the same as the increase in DNA synthesis in whole cells following T2 infection. Also it is remarkable that the dA-5-P kinase activity (very likely identical to adenylate kinase) is about 10 times that of the other deoxynucleotide kinases in the uninfected cell extract and does not change upon T2 infection, while after infection the other kinases and hydroxymethylase reach levels comparable to the dA-5-P kinase. Finally, it is noteworthy that the dC-5-P kinase activity does not increase upon T2 infection but remains at the relatively low level observed in the uninfected cell, whereas it increases about 10-fold upon T5 infection.

References to increases in level of a preexisting enzyme carry no implication that the additional enzyme activity is identical to the old or even that more enzyme has been synthesized "*de novo.*" To resolve this important point, it will be necessary to characterize isolated preparations of the normal- and infected-cell enzymes and to establish by tracer techniques that the enzymes developed after infection have, like induced enzymes,[32] been synthesized from the amino acid pool.

Control of DNA Synthesis by the Nature of the DNA Primer.—Perhaps the most

interesting question regarding T2 infection of *E. coli* is how the T2 DNA seems to preempt from the host DNA the primer function for the polymerase system. The present studies can explain why DNA synthesized by the phage T2-*E. coli* system contains hydroxymethylcytosine rather than cytosine, but not why the base composition is that of phage rather than that of host DNA. An observation reported in this paper which may bear on this question is the dependence of the augmented polymerase activity of the infected cell extracts on a physically altered DNA primer. When "native" calf thymus or phage T2 DNA was used, the polymerase values of infected and normal cell extracts were similar. However, with DNA samples heated at pH 9, the values for dHMC-TP incorporation with infected cell extracts were increased 12-fold, while the normal cell values were unaffected or even depressed. It is not clear as yet whether infection has resulted in formation of a new type of polymerase or in an increase of the normal polymerase and new factors which modify it. Further studies to clarify this question may lead to a better insight into how the injected phage DNA proves to be the chosen primer for this system.

SUMMARY

1. Extracts of *E. coli* infected with bacteriophage T2 have been shown to contain three enzymes which are undetectable in extracts of uninfected or in T5-infected cells. These are: (*a*) an enzyme which phosphorylates hydroxymethyldeoxycytidine 5-phosphate, leading to the synthesis of the triphosphate, (*b*) an enzyme which removes the terminal pyrophosphate group specifically from deoxycytidine triphosphate, and (*c*) an enzyme which transfers glucose from uridine diphosphate glucose directly to the hydroxymethylcytosine of certain DNA's.

2. These new enzymes can account for (*a*) the availability of the triphosphate of hydroxymethyldeoxycytidine for the enzymatic synthesis of T2 DNA, (*b*) the absence of deoxycytidylate from T2 DNA, and (*c*) the presence of glucose on a fixed fraction of the hydroxymethylcytosine residues in DNA.

3. The DNA-polymerizing enzyme assayed with hydroxymethyldeoxycytidine triphosphate in place of deoxycytidine triphosphate reveals about a 12-fold increase in activity after T2 infection. Increases, after T2 infection, in the levels of thymine and guanine deoxynucleotide phosphorylating enzymes (by 20–45 fold) bring their activities up to the level of the adenine deoxynucleotide phosphorylating enzyme which is unchanged; the level of the cytosine deoxynucleotide phosphorylating enzyme remains at a low level.

4. After T5 infection, levels of the thymine, guanine, and cytosine deoxynucleotide phosphorylating enzymes increase by 10–40 fold, bringing their activities up to the level of the adenine deoxynucleotide phosphorylating enzyme, which increases about 2-fold.

5. The new enzymes and the increases in level of the enzymes occurring in normal cells are first detectable about 4 min after infection with phage T2 and about 10 min after infection with phage T5. These results are consistent with previously published studies which have indicated with the use of inhibitors of protein synthesis that viral DNA synthesis requires a preliminary period of protein synthesis.

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‡ Fellow of the National Foundation.

[1] The abbreviations used in this report are: cpm, counts per minute; ATP, adenosine triphosphate; dA-5-P, deoxyadenosine 5′-phosphate; dC-5-P, deoxycytidine 5′-phosphate; dG-5-P, deoxyguanosine 5′-phosphate; dHMC-5-P, hydroxymethyldeoxycytidine 5′-phosphate; dT-5-P, deoxythymidine 5′-phosphate; dATP, deoxyadenosine triphosphate; dCTP, deoxycytidine triphosphate; dGTP, deoxyguanosine triphosphate; dHMC-TP, hydroxymethyldeoxycytidine triphosphate; dTTP, deoxythymidine triphosphate; DNA, deoxyribonucleic acid; HMC, 5-hydroxymethylcytosine; UDPG, uridine diphosphate glucose; Tris, tris-(hydroxymethyl)aminomethane; KPO_4, potassium phosphate buffer; PRPP, 5-phosphoryl α-D-ribofuranose 1-pyrophosphate; E_m, molar extinction coefficient.

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[18] When fluoride was present in the kinase assays, the nucleotides were adsorbed to and eluted from Norit before phosphatase treatment in Stage II of the assay.

[19] For large-scale enzyme preparations, 50-liter cultures at the late exponential phase (2×10^9 cells/ml), in modified M-9 medium (see Methods) but lacking $CaCl_2$, were treated with 3–4 $T2r^+$ per cell and 10 min later with 50 γ of chloramphenicol per ml. The culture was then chilled to 0° with ice over a 10-min period and harvested by centrifugation. The initial steps in the purification of the dHMC-5-P kinase, dCTPase, glucosylating enzyme, and hydroxymethylating enzyme were the same. Sonic extracts, prepared in 5 volumes of 0.05 *M* glycylglycine buffer, pH 7.0, were centrifuged and the supernatant fluid diluted with buffer to contain 10 mg of protein per ml. Streptomycin sulfate (5 per cent solution), equal to 0.3 volume of the diluted extract, was added; the supernatant fluid collected after centrifugation contained 3 to 4 mg of protein per ml. It was adjusted to pH 8 with KOH and applied to a diethylaminoethylcellulose (Brown Co.) column [Peterson, E. A., and H. A. Sober, *J. Am. Chem. Soc.*, **78**, 751 (1956)], equi-

librated with 0.02 *M* KPO_4, pH 8.0, containing 0.01 *M* 2-mercaptoethanol. Linear gradients were applied and the purifications obtained are expressed relative to the supernatant fluid of the sonic extract; all solutions contained 0.01 *M* 2-mercaptoethanol. Glucosylating enzyme: gradient of 0.08 to 0.32 *M* NaCl in 0.02 *M* KPO_4, pH 8.0–30× purification; dCTPase: gradient of 0.08 *M* to 0.32 *M* NaCl in 0.02 *M* KPO_4, pH 8.0–24× purification, rechromatographed with a gradient of 0.06–0.32 *M* KPO_4, pH 6.5–85× purification; dHMC-5-P kinase: gradient of 0.06 to 0.32 *M* KPO_4, pH 6.5–20× purification; hydroxymethylating enzyme: gradient of 0.3 *M* KPO_4, pH 6.5 to 1.0 *M* KPO_4, pH 6.1–230× purification. Fractions containing 2-mercaptoethanol were not frozen. Active fractions were concentrated by precipitation with solid $(NH_4)_2SO_4$. Further details of the purification procedures will be published elsewhere.

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