Deoxyribonucleic Acid Metabolism and Virus-Induced Enzyme Synthesis in a Thymine-Requiring Bacterium Infected by a Thymine-Requiring Bacteriophage*

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ABSTRACT: In cultures of *Escherichia coli* infected by T-even bacteriophages, the synthesis of the "early" phage-specific enzymes, required for viral deoxyribonucleic acid (DNA) synthesis, stops at *ca.* 10 min after infection; the production of "late" proteins, including lysozyme and the viral structural proteins, begins at about the same time. Experiments are described which test the suggestion that DNA replication is required for this shift in protein synthesis. The kinetics of phage-induced enzyme formation have been followed in a thymine-requiring host bacterium, *E. coli* B3, infected with T4td8, a thymine-requiring mutant of phage T4, which is unable to initiate the production of the phage-specific thymidylate synthetase. When infection in this system is carried out in the absence of thymine and the presence of 5-fluorodeoxyuridine, which delays the degradation of bacterial DNA, all metabolic routes for the formation of thymidylate are inhibited. Under these conditions the production of phage DNA is extensively but not completely blocked; *ca.* 10 phage equivalents of DNA are synthesized per cell. The formation of mature viral particles is delayed until 60 min after infection, and the burst size, in a singly infected culture, is less than two phage per infected cell.

In the thymine-deprived, FUDR-treated culture, the formation of two early enzymes, deoxycytidylate hydroxymethylase and dihydrofolate reductase, ceases at 10 min as in normal infection. However, the appearance of lysozyme activity is delayed by some 20 min relative to a normal culture. From this experiment it is suggested that DNA synthesis is required in order for the production of late proteins to commence. Apparently the reason that lysozyme formation is only delayed, rather than completely blocked, is due to the incomplete blockage of DNA synthesis. It is estimated that between 5 and 10 phage equivalents of new DNA must be formed per cell in order for the structural gene for lysozyme to be fully expressed.

T-even bacteriophages can direct their own formation in infected cells through a remarkably well-timed sequence of events, which results in the coordinated production of mature viral particles. The operation of the timing mechanism has been the object of numerous studies (Dirksen *et al.*, 1960; Wiberg *et al.*, 1962; Ebisuzaki, 1963, 1965; Epstein *et al.*, 1963; Sekiguchi and Cohen, 1964; Edlin, 1965; see also review by Stent, 1963). A central feature of this mechanism is the cessation, at *ca.* 10 min after infection, of the production of the "early" phage-induced enzymes responsible for viral deoxyribonucleic acid (DNA) synthesis (Flaks *et al.*, 1959), and the onset of synthesis of the viral structural proteins and certain "late" enzymes, such as lysozyme (Koch and Hershey, 1959). Coincident with this shift in protein synthesis is the onset of phage DNA production (Cohen, 1948).

Infection of *Escherichia coli* with T-even phages under conditions in which DNA synthesis is not permitted upsets the timing mechanism. Thus, infection by ultraviolet light-inactivated T2 (Dirksen *et al.*, 1960) or infection of a nonpermissive host by a T4 amber mutant which is blocked in an early stage (Wiberg *et al.*, 1962; Epstein *et al.*, 1963) leads to extensive overproduction of the early enzymes and failure of late stages of development to occur. The suggestion has been advanced (Wiberg *et al.*, 1962) that DNA synthesis is required for cessation of early enzyme synthesis. On the other hand, infection in the presence of 5-fluorodeoxyuridine, a specific inhibitor of DNA synthesis (Cohen *et al.*, 1958), does not significantly affect the timing mechanism (Ebisuzaki, 1963; Sekiguchi and Cohen, 1964; Frey and Melechen, 1965). However, as Sekiguchi and Cohen have pointed out, treatment with FUDR[1] does not completely abolish DNA synthesis, and the possibility has not been ruled out that some event connected with a single DNA replication, such as strand separation, might be responsible for repression.

The thymine-requiring mutants of phage T4 described

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[1] Abbreviations used are: FUDR, 5-fluorodeoxyuridine; BUDR, 5-bromodeoxyuridine; TCA, trichloroacetic acid; dTMP, thymidylate; dCMP, deoxycytidylate; FH_2, dihydrofolate; TPNH, reduced triphosphopyridine nucleotide.

by Simon and Tessman (1963) provide favorable material for studying the relationship between DNA replication and the control of viral protein synthesis. These mutants, which lack the capacity to induce the formation of phage-specific thymidylate synthetase in infected cells (Shapiro *et al.*, 1965) require thymine or thymidine for optimal growth and DNA synthesis (Simon and Tessman, 1963; Mathews, 1965). When one infects *E. coli* B3, a strain which lacks thymidylate synthetase (Barner and Cohen, 1959), with T4td8, one of the thymineless phage mutants, there is no net synthesis of DNA (Mathews, 1965). This paper describes experiments on the synthesis of virus-specific enzymes in the td8–B3 system under various conditions. The results indicate that a small amount of DNA synthesis is required for the transition from early to late protein synthesis.

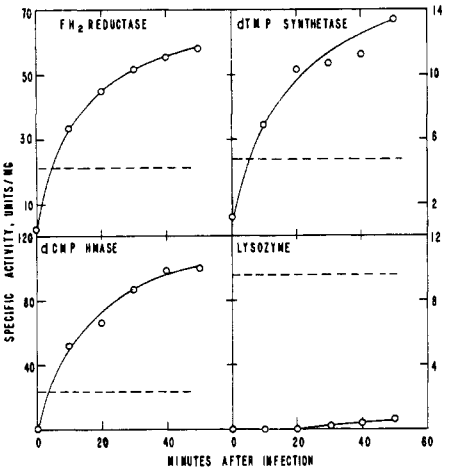

FIGURE 1: Enzyme synthesis in *E. coli* B infected with ultraviolet-irradiated T2. Cells were grown and infected as described in the Experimental Section with T2 which had been irradiated with an average dose of 30 lethal hits/particle. The dashed lines represent the activities of an extract which had been prepared from cells infected with unirradiated T2 for 60 min.

Experimental Section

Phage Strains. T4td8, a thymine-requiring mutant, was kindly supplied by Dr. I. Tessman, along with the parent strain, $T4BO_1$. This latter strain was used as the wild-type control in all experiments and is referred to as td^+ or am^+, depending upon the context of the experiment. T4 amber mutants N82 and N122 were generously provided by Dr. R. S. Edgar. T2 and T6 were obtained in the laboratory of Dr. S. S. Cohen. All phage strains were grown and assayed by standard methods (Adams, 1959).

Bacterial Strains. Escherichia coli, strains B and B3, were obtained in the laboratory of Dr. S. S. Cohen. *E. coli* S26R1E, a derivative of K10 which is permissive for growth of amber mutants, was provided by Dr. R. Wilhelm. The amber strains N82 and N122 were routinely grown and assayed with this strain, while all other phage strains were grown in *E. coli* B.

Growth and Infection of Bacteria. For experiments on the kinetics of enzyme synthesis, cells were grown under forced aeration in M9 medium (Adams, 1959) to an optical density of 80 Klett units. This corresponds to a viable count of *ca.* 2.5×10^8 ml^{-1}. The cells were centrifuged and washed as described in the individual figure legends and resuspended at the same concentration. DL-Tryptophan was added as adsorption cofactor (50 μg/ml), and the cells were infected at a multiplicity of 4. At various times after infection, 200-ml aliquots of the culture were rapidly removed to chilled centrifuge bottles by siphoning under pressure. This permitted the withdrawal of a 200-ml sample in 10 sec. The samples were centrifuged at 5000 rpm for 10 min in a Servall refrigerated centrifuge, and each pellet was resuspended in 3 ml of 0.1 M Tris–HCl buffer, pH 7.5. Extracts were prepared by sonic oscillation, followed by centrifugation for 20 min at 13,000 rpm to remove bacterial debris. The same media and conditions were used in the small-scale experiments on DNA and protein synthesis, with the modifications described in individual figure legends.

Enzyme Assays. Thymidylate synthetase and dihydrofolate reductase were assayed as previously described (Mathews and Sutherland, 1965). Deoxycytidylate hydroxymethylase activity was determined as described by Pizer and Cohen (1962). Lysozyme was assayed by the method of Sekiguchi and Cohen (1964), except that the reactions were carried out at 37 instead of 25°. The decrease in turbidity of a suspension of chloroform-treated cells was monitored continuously with a Beckman Model DB spectrophotometer, and reaction rates were calculated from the initial linear phase of the reaction. All enzyme activities are listed as units/mg of protein.

Chemicals. [2-^{14}C]Uracil, [2-^{14}C]thymine, and [^{14}C]-formaldehyde were purchased from Volk Radiochemical Co. [^{14}C]Leucine was obtained from New England Nuclear. Deoxycytidylate, deoxyuridylate, and TPNH were obtained from the Sigma Chemical Co. FUDR was a gift of Dr. Robert Duschinsky, Hoffman-La-Roche, Inc. 5-Bromodeoxyuridine was a product of Calbiochem.

Other Methods. DNA was extracted and determined in bacterial cultures as previously described (Mathews, 1965), and protein was assayed by the biuret method (Layne, 1957). Incorporation of [^{14}C[uracil into DNA was measured with the use of Millipore filters, as described by Stern *et al.* (1964).

Ultraviolet irradiation of bacteriophages was carried out with the sample in M9 medium lacking glucose in a Petri plate at a distance of 15 cm from a Mineralight UVS11 lamp. All work with ultraviolet-inactivated phages was carried out in a darkened room to prevent photoreactivation.

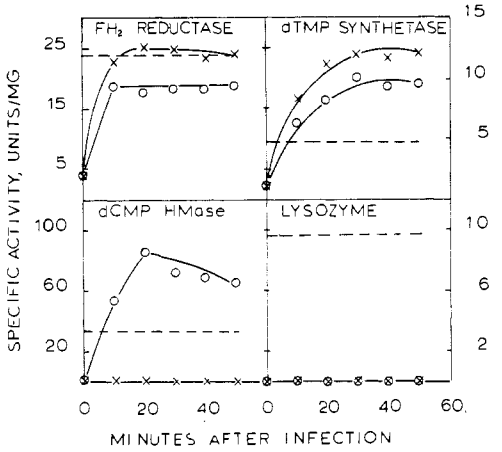

FIGURE 2: Enzyme synthesis in *E. coli* B infected by T4 amber mutants. o- - -o, N82; x- - -x, N122. The dashed line represents the activity of a simultaneously prepared extract of cells infected with T4 am$^+$ for 60 min.

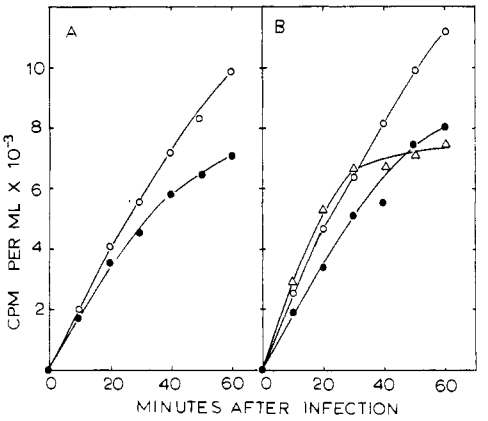

FIGURE 3. Protein synthesis in *E. coli* B infected with ultraviolet-irradiated phage and an amber mutant. A, T2-infected cells; B, T4-infected cells. Cells were grown in the presence of 2×10^{-4} M leucine and infected (10-ml cultures) at a cell concentration of 2.5×10^8 ml^{-1}. Immediately after infection 0.5 μc of [^{14}C]-leucine was added to each culture. Samples of 0.5 ml were removed to 0.5-ml aliquots of 10% TCA at the indicated times. The samples were heated at 90° for 15 min, then chilled, and passed through Millipore filters. The filters were washed with cold 5% TCA, dried, and counted. Ultraviolet phage were irradiated with an average dose of 24 lethal hits for T4 and 30 lethal hits for T2. Open circles, unirradiated T4 am$^+$; closed circles, ultraviolet phage; triangles, T4 am N122.

Results

Effects of Ultraviolet Irradiation and of Infection with Amber Mutants on Phage-Specific Protein Synthesis. In order to establish guidelines for "normal" and "abnormal" kinetics of virus-induced enzyme formation, experiments were carried out with cultures of *E. coli* B infected by ultraviolet light-irradiated T2 and by amber mutants of T4 which are blocked in DNA synthesis. Figure 1 illustrates the pattern of synthesis in cells infected by ultraviolet-inactivated T2. The results substantially confirm earlier observations that infection under these conditions leads to overproduction of early enzymes (Dirksen *et al.*, 1960) and inhibition of lysozyme formation (Sekiguchi and Cohen, 1964). Similar experiments with two T4 amber mutants, N82 and N122, which cannot initiate phage DNA synthesis in *E. coli* B, are also in agreement with previous work (Wiberg *et al.*, 1962; Epstein *et al.*, 1963), including the observed failure of N122 to initiate the synthesis of dCMP hydroxymethylase (Figure 2).

There are some interesting differences in the observed patterns of enzyme synthesis between the cultures infected with ultraviolet-inactivated T2 and the amber mutants; *e.g.*, the early enzyme dihydrofolate reductase (Mathews and Cohen, 1963a) is not produced in abnormal amounts in the experiments with the amber mutants, while infection with ultraviolet-irradiated T2 leads to extensive overproduction of this enzyme. Moreover, the production of lysozyme is completely blocked in the amber-infected cultures but not in the ultraviolet phage-infected culture. This difference may be due to the fact that DNA synthesis is not completely blocked in cells infected by ultraviolet-treated phage, probably owing to multiplicity reactivation, while no detectable formation of DNA takes place in the amber phage-infected cultures (*cf.* below).

A further difference between the two sets of experimental conditions is that, whereas in the amber mutant-infected cells the levels of early enzymes do not increase after 30 min, in the ultraviolet phage-infected cells early enzyme synthesis continues for at least 50 min. A similar difference is seen in the pattern of over-all protein synthesis, as shown in Figure 3. This Figure shows the incorporation of [^{14}C]leucine into protein under various conditions. Infection with ultraviolet-inactivated T2 or T4 leads to a somewhat decreased rate of protein synthesis throughout the whole period examined relative to cells infected with normal phage. On the other hand, a culture infected with N122 shows a nearly equal rate of protein synthesis at first, followed by a sharp drop at 30 min. The reason for this difference is not known. However, it may be partly due to the fact that the small amount of DNA synthesis in ultraviolet phage-infected cells (see below) permits the continued formation of some late proteins. This, however, does not explain the difference in early enzyme synthesis.

Virus-Specific Protein Synthesis in Thymine-Deprived Cultures. Figure 4 shows the kinetics of phage-induced enzyme formation in *E. coli* B3 infected with the thymine-requiring T4 mutant, td8, under various conditions. Production of early enzymes is shut off after 10 min either in the presence or the absence of thymine, and no significant difference is observed in the rates of lysozyme formation. No net DNA synthesis occurs in

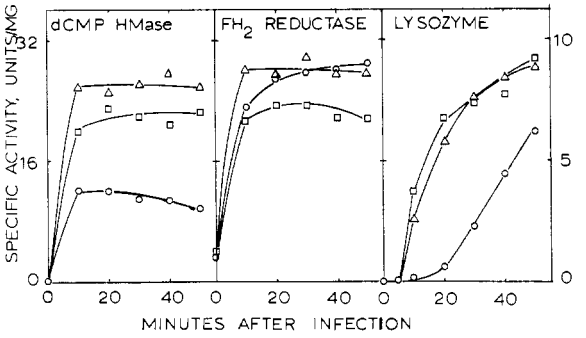

FIGURE 4: Enzyme synthesis in *E. coli* B3 infected with T4td8. Cells were grown in 2-l. cultures, centrifuged, and washed as described in the Experimental Section, and resuspended in the presence of 10 μg/ml of thymine (triangles), in the absence of added thymine (squares), and in the absence of thymine plus the presence of 30 μg/ml of FUDR (circles). Extracts prepared from cells removed at various times after infection were assayed for dCMP hydroxymethylase, FH_2 reductase, and lysozyme. The left-hand ordinate gives the specific activities of FH_2 reductase.

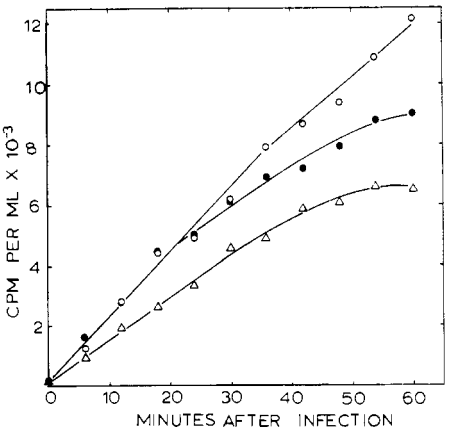

FIGURE 5: Protein synthesis in *E. coli* B3 infected with T4td8. Cells were grown in 2×10^{-4} M nonradioactive leucine as described in the legend to Figure 3, centrifuged, washed, and resuspended in the same medium containing 10 μg/ml of thymine (open circles), no thymine (closed circles), and 30 μg/ml of FUDR without thymine (triangles). After 10 min of further incubation to deplete intracellular thymine pools, phage was added at a multiplicity of 4. Immediately after infection [^{14}C]-leucine was added and incorporation into protein followed as described in the legend to Figure 3.

the td8–B3 system in the absence of added thymine (Mathews, 1965). Thus this experiment demonstrates that an increase in the level of intracellular DNA is not a prerequisite for the shift from early to late protein synthesis. However, significant virus DNA synthesis can take place in the thymine-starved cells, with thymidylate being provided *via* the breakdown of bacterial DNA (Simon and Tessman, 1963). Accordingly a method was sought whereby DNA synthesis could be further limited, primarily by inhibition of cellular DNA breakdown. It appeared that this objective could be accomplished by addition to a thymine-starved culture of 5-fluorodeoxyuridine, for the following reasons: (1) FUDR has been reported to inhibit the breakdown of bacterial DNA by some as yet unknown mechanism (Volkin and Ruffilli, 1962) and (2) FUDR is converted *in vivo* to the corresponding 5′-mononucleotide, which can inhibit any residual thymidylate synthetase activity resulting from the "leakiness" of either the phage or the bacterial mutant (Cohen *et al.*, 1958; Barner and Cohen, 1959).

In a thymine-starved, FUDR-treated culture, as shown in Figure 4, the formation of the two early enzymes examined is shut off after 10–15 min as in normal infection. However, the production of lysozyme is delayed by 15–20 min. Thus it would appear that under these conditions DNA synthesis has been reduced to the point that operation of the early-to-late switching mechanism is at least partially interfered with. Further experiments bearing on this point are described below.

In the presence of FUDR the activity of dCMP hydroxymethylase was somewhat lower than that observed in normal infection. This may be due to inhibition of the enzyme by a nucleotide of FUDR. When

TABLE I: dCMP Hydroxymethylase Activities of Cells Infected in the Presence and Absence of FUDR.[a]

Phage	Hydroxymethylase Act. (units/mg) −FUDR	+FUDR
T2	33.2	22.2
T4td$^+$	21.1	13.3
T4td8	20.6	14.7
T6	24.9	27.8

[a] Cultures (200 ml) of *E. coli* B were infected for 20 min in the presence and absence of 30 μg/ml of FUDR.

E. coli B was infected with T2 or T4, addition of FUDR led to decreased activities of the hydroxymethylase, as shown in Table I, while the activity of the T6 enzyme was not significantly affected (*cf.* Sekiguchi and Cohen, 1964). This is reminiscent of the finding of Mathews and Cohen (1963b) that the T2- and T6-specific thymidylate synthetases differ in their sensitivity to inhibition by 5-fluorodeoxyuridylate. However, the apparent inhibitory effect of FUDR on hydroxymethylase formation should not obscure the fact that the kinetics of production of this enzyme are the same as those observed in normal infection, *i.e.*, cessation of enzyme synthesis occurs after 10 min.

The effects on over-all protein synthesis of thymine

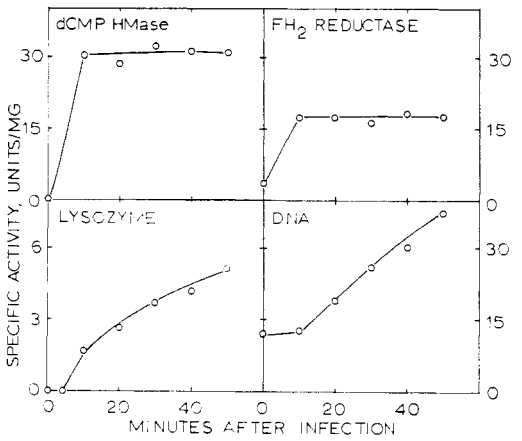

FIGURE 6: Phage-specific enzyme synthesis in the presence of 5-bromodeoxyuridine. Experimental details were the same as those of Figure 4, except that 10 μg/ml of BUDR was added just prior to infection. No thymine was present throughout the course of infection.

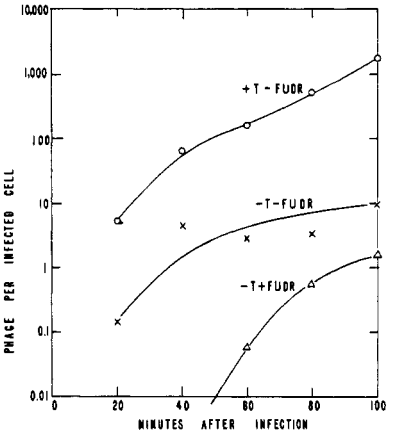

FIGURE 7: Growth of td8 in *E. coli* B3. Bacteria were grown in M9 plus 3 μg/ml of thymine to a Klett reading of 80. They were centrifuged, washed, and resuspended in 0.25 the original volume of M9 containing 50 μg/ml of DL-tryptophan and no thymine. They were incubated for 20 min, and then subdivided into three 10-ml portions. Thymine (10 μg/ml) was added to one portion and FUDR (30 μg/ml) to another, as shown in the figure. Each culture was infected with td8 at a multiplicity of 0.1. Three minutes after infection each culture was diluted 10-fold into the same medium. At 7 min, samples were removed for determination of infective centers and unadsorbed phage. At the indicated times further samples were removed and lysed with chloroform for assay of infectious particles.

deprivation and FUDR treatment are shown in Figure 5. In this experiment the incorporation of [^{14}C]leucine into protein was followed, as described earlier (Figure 3). The thymine-starved culture synthesizes protein as rapidly as the control culture up to *ca.* 30 min, with a decrease occurring after that time. The rate of protein synthesis drops at about the same time in the thymine-deprived, FUDR-treated culture, but the rate is lower than that of the control throughout the entire period examined. Frey and Melechen (1965) have described a similar drop in the rate of protein synthesis at *ca.* 30 min which occurs in T2-infected *E. coli* B treated with FUDR. These results, together with those of the experiments on DNA synthesis (see below) suggest a need for continued DNA synthesis in order for sustained protein synthesis to occur.

Effect of BUDR Incorporation on Phage-Induced Enzyme Formation. Kamiya *et al.* (1965) have shown that in animal cells infected with pseudorabies virus, incorporation of 5-bromodeoxyuridine into viral DNA prolongs the synthesis of early virus-specific proteins and inhibits late stages of development. They have proposed that progeny DNA regulates some aspect of the infective process and that the defective DNA made in the presence of BUDR cannot exert the normal control. This proposal has been tested in the T4 phage system by following viral enzyme synthesis in a thymine-deficient culture of *E. coli* B3 infected with td8 in the presence of BUDR. As shown in Figure 6, the incorporation of this analog into phage DNA does not alter the normal pattern of synthesis of early and late enzymes.

DNA Metabolism in Thymine-Deprived Cultures. Because lysozyme formation was delayed in the thymine-starved culture which had been treated with FUDR, it became necessary to examine DNA metabolism in *E. coli* B3 infected with td8, in order to see whether phage DNA synthesis was completely or only partially inhibited. Figure 7 shows the effects of thymine starvation and FUDR treatment upon the growth of infectious viral particles. Infection in the absence of thymine gives rise to a burst size of *ca.* 10 plaque-forming particles/infected cell, which is similar to the value reported by Simon and Tessman (1963). The addition of FUDR to such a culture delays phage production until *ca.* 60 min after infection and reduces the burst size considerably, in this experiment to a value of 1.6. Since the cells were singly infected (average multiplicity of infection, 0.1), less than one new phage particle had been produced per infected cell, on the average, at the conclusion of the experiment. It should be pointed out that in the experiment of Figure 7, the high burst size observed in cells infected in the presence of thymine is probably due to secondary infection occurring after the first cycle of growth.

In earlier experiments (Mathews, 1965) DNA synthesis in cells infected with td8 was followed by the diphenylamine reaction. From this assay we cannot ascertain whether phage DNA is being synthesized from the pool of deoxyribonucleotides released by the breakdown of bacterial DNA. However, if a radioactive DNA precursor is added at the time of infection, and if infection is carried out at a sufficiently high multiplicity to essentially abolish bacterial DNA syn-

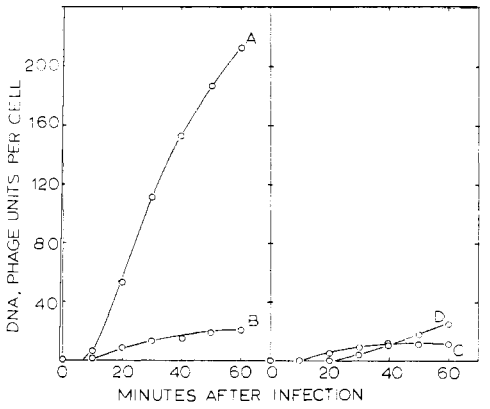

FIGURE 8: Incorporation of [2-^{14}C]uracil into phage DNA. A 50-ml culture of *E. coli* B3 was grown in the presence of 3 μg/ml of thymine and 20 μg/ml of uracil. When the culture reached a Klett reading of 80, it was centrifuged, washed twice in M9, and resuspended in 50 ml of M9 containing 20 μg/ml of uracil and 50 μg/ml of DL-tryptophan. The culture was divided into four 12-ml portions. To the first (A) was added 10 μg/ml of thymine; no addition was made to the second (B); 30 μg/ml of FUDR was added to the third (C); and 10 μg/ml of thymine was added to the fourth (D). Infection was carried out with td8 at a multiplicity of 8; in the fourth culture only, this phage had been irradiated with ultraviolet light (24 lethal hits/particle). Immediately after infection, 0.4 μc of [2-^{14}C]uracil was added to each culture. At the indicated times, 1.0-ml samples were removed and pipetted into 1.0 ml of 10% TCA. DNA was isolated from the TCA precipitates and counted on Millipore filters as described by Stern *et al.* (1964).

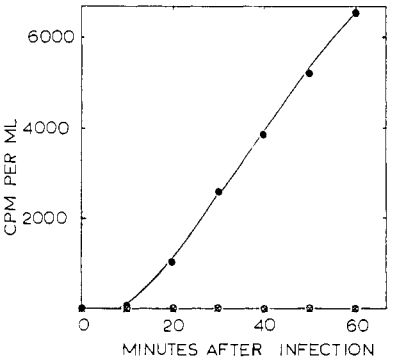

FIGURE 9: Incorporation of [2-^{14}C]uracil into the DNA of T4 and two amber mutants. *E. coli* B was grown in the presence of 20 μg/ml of uracil to a Klett reading of 80. 50 μg/ml of DL-tryptophan was added, and the culture was divided into four 12-ml portions. The three cultures were infected at a multiplicity of 8 with T4am$^+$ (closed circles), N82 (open circles), and N122 (crosses), respectively. Immediately after infection 0.4 μc of [2-^{14}C]uracil was added to each culture. Samples were removed and incorporation of label into DNA determined as described in the legend to Figure 10.

thesis by uninfected cells, then one can specifically follow the formation of viral DNA. Such experiments have been carried out by Sekiguchi and Cohen (1964), who followed the incorporation of [2-^{14}C]uracil into phage DNA, using a polyauxotrophic host organism which required uracil for growth.

Incorporation of labeled uracil into DNA has been used in the present investigation to provide an index of phage DNA synthesis. Since the bacterial strains used in this study are not uracil auxotrophs, cells were grown and infected in the presence of 20 μg/ml of nonradioactive uracil, a concentration sufficiently high to repress the formation of the pyrimidine biosynthetic enzymes (L. Pizer, personal communication). Immediately after infection [2-^{14}C]uracil was added to each culture. Samples were removed at various times, and incorporation of labeled uracil into DNA was determined by the Millipore filtration method of Stern *et al.* (1964).

Figure 8 shows the results of an experiment in which phage DNA synthesis was followed in the td8–B3 system in the presence and absence of thymidine, and in the presence of FUDR without thymine. Phage equivalents of DNA synthesized were calculated by incubating a portion of the thymine-containing culture to lysis, purifying the phage by differential centrifugation and deoxyribonuclease treatment, and determining the amount of radioactivity per infective unit. It should be noted that under the conditions of this experiment, the only phage DNA component to become significantly labeled is hydroxymethylcytosine, since the genetic blocks prevent the incorporation of uracil into thymidylate.

The experiment of Figure 8 shows that significant synthesis took place in all three cultures. *Ca.* 20 phage units of DNA per cell were formed in the thymine-starved culture, with the thymine presumably supplied from bacterial DNA. Addition of FUDR reduced new DNA synthesis in a thymine-starved culture to *ca.* 10 phage units per cell. In a parallel experiment with ultraviolet-irradiated td8 in the presence of thymine, DNA synthesis, which was completely blocked at first, commenced after 20 min, presumably owing to multiplicity reactivation, since infection was carried out in the dark to prevent photoreactivation.

That the observed radioactivity was due entirely to the incorporation of uracil into phage DNA was shown by viable count of all of the cultures both before and after infection. Since <0.1% of the cells remain uninfected in each culture, the synthesis of bacterial DNA was negligible. Moreover, a control experiment in which uracil incorporation was followed in *E. coli* B infected with wild-type T4 and with the DNA-blocked amber mutants N82 and N122, showed no detectable DNA synthesis in cells infected with the mutants (Figure 9).

FUDR has been reported to block the breakdown of bacterial DNA in T2-infected cells (Volkin and

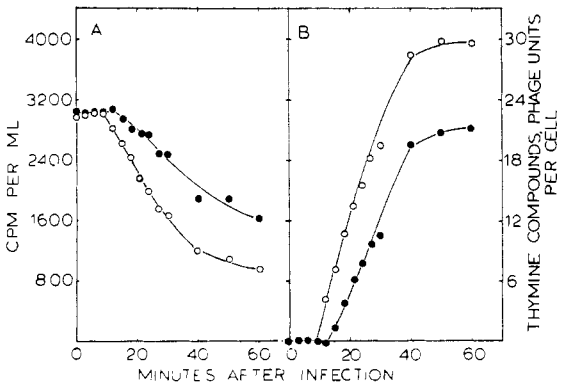

FIGURE 10: Degradation of DNA of *E. coli* B3 following infection by T4 am N122. A 25-ml culture was grown to a concentration of 2.5×10^8 bacteria/ml in the presence of 2 μg/ml of thymine containing 1 μc of [2-^{14}C]thymine. The cells were centrifuged, washed twice, and resuspended in 25 ml of medium containing 50 μg/ml of DL-tryptophan and no thymine. They were then divided into two 12-ml portions. To one was added 30 μg/ml of FUDR (closed circles); no further additions were made to the other (open circles). Infection was carried out at a multiplicity of 4. At the indicated times 0.5-ml samples were withdrawn and added to 0.5-ml aliquots of 10% TCA. Experiment A shows the decrease in acid-insoluble radioactivity. The TCA-containing samples were filtered through Millipore filters, and the filters were washed with cold 5% TCA, dried, and counted. In a similar experiment, shown in B, the TCA-containing samples were centrifuged and aliquots of the supernatant plated on stainless steel planchets and counted to give a measure of acid-soluble radioactivity.

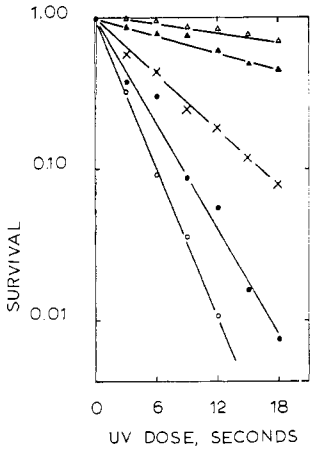

FIGURE 11: Development of resistance to ultraviolet in *E. coli* B3 infected with td8. Bacteria (150 ml) were grown to a Klett reading of 80, harvested, washed twice, and resuspended in 20 ml of M9 without glucose. The cells were incubated for 1 hr to exhaust nutrient reserves. DL-Tryptophan was added to 50 μg/ml, followed by td8 at a multiplicity of 0.002 phage/bacterium. After a 10-min adsorption period, the cells were centrifuged and washed in M9 to remove unadsorbed phage. A sample was diluted into chilled M9 for subsequent determination of ultraviolet sensitivity of the infective centers at time zero. Phage development was started by pipetting 7 ml portions of the culture into tubes containing glucose (final concentration, 3 mg/ml). In addition one of the tubes contained thymine (final concentration, 10 μg/ml), and another contained FUDR (final concentration, 30 μg/ml). The cultures were incubated at 37° for 10 min, and then samples were diluted into chilled M9 for subsequent determination of ultraviolet resistance. O——O, survival curve of free td8; ●——●, infective centers at time zero; x——x, culture containing FUDR; ▲——▲, culture with no additions; △——△, culture containing thymine.

Ruffilli, 1962). However, inhibition was obviously not complete in the present system, since sufficient thymidylate was released in the presence of FUDR to allow the synthesis of 10 phage units of DNA per cell. Accordingly, studies were made of the effect of FUDR alone. In early experiments the DNA of *E. coli* B3 was labeled before infection by growth in radioactive thymine. Infection was carried out in nonradioactive medium, and bacterial DNA degradation was followed by measuring the decrease in acid-insoluble radioactivity as a function of time after infection. However, such experiments were misleading, since the reincorporation of the released acid-soluble material into phage DNA was not completely prevented. In order to remove this complication, the effect of FUDR was studied in *E. coli* B infected with the T4 amber mutant N122. In this system released nucleotides are not incorporated into DNA since, as shown above, no DNA synthesis takes place.

Figure 10 shows the results of two experiments in which the effect of FUDR was examined as described above. One such experiment followed the loss of acid-insoluble material from cells whose DNA was prelabeled by growth in thymine. In a complementary experiment the appearance in the medium of acid-soluble radioactivity was measured. The data of the latter experiment were used, together with the specific radioactivity of the thymine in the medium, to compute the number of phage units of thymine compounds released per cell. Both experiments gave similar results. FUDR does not completely block cellular DNA breakdown. However, it does delay it by several minutes and reduces its final extent. It is of interest that, even in the absence of FUDR, there is no detectable DNA breakdown in the first 10 min, a result in agreement with that of Nomura *et al.* (1962). Comparison of the data of Figures 9 and 10 shows, for the first 30 min, a reasonably good correspondence between the number of phage units per cell of thymine released from bacterial DNA and synthesis of phage DNA in the absence of thymine. The eventual extent of phage DNA breakdown may be greater in the amber N122 system than in the td8-infected cells if phage-specific deoxyribonucleases, like

other early enzymes, are formed in abnormal amounts in cells infected by the amber mutant (Wiberg *et al.*, 1962).

The apparent discrepancy between the present results and those of Volkin and Ruffilli (1962) cannot readily be explained, since the nature of the effect of FUDR on DNA breakdown is not known. However, if the effect of FUDR is due to inhibition of a phage-specific deoxyribonuclease by a nucleotide of FUDR, as seems plausible, then the T2-induced enzyme may be more susceptible to inhibition than the T4-induced enzyme.

Resistance of td8–B3 Complexes to Ultraviolet Light. Measurement of the increase in resistance of phage-infected cells to ultraviolet light inactivation with time after infection, as described by Luria and Latarjet (1947), has been used by many investigators to study the extent to which normal metabolism takes place in an infected cell (*cf.* Benzer, 1952; Stent, 1963). Although the basis for the increase in resistance is not known, it has been considered by some to be akin to multiplicity reactivation, with development of resistance being dependent upon the synthesis of replicas of the parent DNA molecule. A Luria–Latarjet experiment has been performed with *E. coli* B3 infected in the presence and absence of thymine and in a thymine-starved culture to which FUDR was added at the time of infection. Results are shown in Figure 11. The infective centers at zero time are slightly more resistant to ultraviolet light than the free phage, as reported by Benzer (1952) for cells grown in minimal medium. The cultures with thymine present and thymine absent both show considerable increase in resistance by 10 min after infection. The thymine-deprived, FUDR-treated culture shows much less increase but is, nevertheless, more resistant than the zero-time sample. These results are consistent with the experiments on DNA synthesis and viral enzyme synthesis in this system: in the absence of thymine and FUDR, phage DNA synthesis is inhibited, but not to a sufficient extent to significantly affect either the pattern of early and late protein synthesis or the development of resistance to ultraviolet light. Addition of FUDR brings about a reduction of DNA synthesis which, although not yielding a complete block, does perturb the pattern of viral enzyme formation and the development of ultraviolet resistance. In the latter case neither effect is complete, probably because the block in DNA synthesis is not complete.

Discussion

The experiments described here indicate that some DNA replication is required for the activation of late protein synthesis in T4-infected cells. Since blockage of DNA synthesis was not complete in the td8–B3 system, we cannot definitively say how much replication is required, nor can we tell whether DNA replication is required for the shutoff of early protein synthesis. However, the data do permit a rough estimate. In an FUDR-treated, thymine-starved culture, the rate of lysozyme formation approached that of a normal culture at *ca.* 20 min after infection (Figure 4). At this time there were *ca.* five phage units per cell of newly formed DNA (Figure 8). On the other hand, the culture containing neither thymine nor FUDR and which synthesized lysozyme as in normal infection, contained about nine phage equivalents of DNA at 20 min. One might conclude, therefore, that slightly more than five DNA replicas must be formed in the infected cell under these conditions in order for the early-to-late switching operation to occur normally.

That continued DNA synthesis is required for sustained protein synthesis late in infection is indicated by the experiments on [^{14}C]leucine incorporation into protein. In the td8–B3 system very little DNA synthesis takes place after 30 min in the absence of added thymine (Figure 8), either in the presence or absence of FUDR. In both of these cultures the rate of protein synthesis also decreases after 30 min (Figure 5). Similar results, even more sharply defined, are seen in experiments with *E. coli* B infected with amber mutants which are blocked in DNA synthesis throughout the course of infection (Figures 3 and 9). On the other hand, in cells infected with ultraviolet-irradiated phage, DNA is synthesized late in infection, and protein synthesis continues for at least 60 min, although the rate is somewhat diminished late in infection. It is of interest that small amounts of lysozyme are formed late in infection in cells infected by ultraviolet-irradiated T2 (Figure 1) and T4 (not shown), where DNA synthesis is not completely blocked. However, in cultures infected by the DNA-blocked amber mutants, both lysozyme formation and DNA synthesis are below detectable levels. Although this evidence may be circumstantial, it is at least consistent with the idea that some DNA replication is required for late protein synthesis.

In order to more rigorously test the involvement of phage DNA synthesis with the control of viral protein formation, we must have a phage–host system in which DNA synthesis can be completely blocked and restored at will in varying amounts. None of the systems so far studied possesses both of these attributes. Present studies in this laboratory are directed toward finding a temperature-sensitive or amber mutant which meets these requirements.

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