THE EFFECT OF CHLORAMPHENICOL ON DEOXYRIBONUCLEIC ACID SYNTHESIS AND THE DEVELOPMENT OF RESISTANCE TO ULTRAVIOLET IRRADIATION IN E. COLI INFECTED WITH BACTERIOPHAGE T2

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(Received for publication, July 11, 1955)

In spite of its capital importance, the mechanism of deoxyribonucleic acid (DNA) synthesis remains quite obscure. One of the main reasons for this is the complicated correlations between the synthesis of DNA and other cellular constituents in cells. In phage-infected bacteria the metabolic pathways shunt to phage formation, resulting in production mainly of DNA and protein. Thus the system is considerably simplified, giving a good model for DNA synthesis. Furthermore, the blendor experiment by Hershey and Chase (1) suggested that the parental DNA plays a dominant role in initiating phage infection. An understanding of the mode of action of the injected DNA in the initiation of subsequent synthesis of DNA is strongly to be desired. The present study points to a role for protein synthesis as a prerequisite to DNA synthesis.

Deriving the experimental principle from the report of Wisseman et al. (2) that chloramphenicol selectively inhibits the synthesis of protein without affecting that of DNA in E. coli, use was made of this agent to inhibit protein synthesis in infected cells. At the same time ultraviolet (UV) analysis, one of the most penetrating methods available for following the progress of the infective process, was performed with or without the addition of chloramphenicol.

Materials and Methods

Strains.—E. coli R2 and phage T2, kindly supplied by Dr. A. D. Hershey, were used. The phage was purified according to Herriott and Barlow (3).

Media.—Proteose peptone–yeast extract medium (PY medium) contains per liter 7 gm. bacto-proteose peptone, 3 gm. bacto-yeast extract, 1 gm. glucose, 3 gm. NaCl, 240 mg. MgSO₄·7H₂O, 10 mg. CaCl₂, and 20 mg. Na₂HPO₄·2H₂O, at pH 7.0. Adsorption medium and buffered saline were prepared according to Hershey and Chase (1) and Benzer (4) respectively.

Reagents.—A crystalline sample of chloramphenicol was used. DNA was prepared from phage T2 by the urea method (5). A sample hydrolyzed completely with HCl (6) was mixed with a sample partially hydrolyzed with 1.5 N perchloric acid (7), and neutralized to yield a solution at pH 7.0 containing 3 mg. hydrolyzed DNA per ml.

J. Gen. Physiol., 1956, Vol. 39, No. 4

The Journal of General Physiology
Cultural Conditions.—E. coli cells were grown with aeration at 37° to the concentration of $3 \times 10^8$ cells per ml. in PY medium. Early log phase cells were used throughout. One-step growth experiments were performed as usual except that bacteria were infected in adsorption medium for 10 minutes, sedimented for removal of free phage, resuspended in adsorption medium, and transferred to warm PY medium at time zero. For the determination of intracellular phage, aliquots of culture were dilated with warm PY medium containing 0.01 m KCN or 30 µg. per ml. (9.3 $\times$ $10^{-5}$ M) chloramphenicol. The diluted samples were titrated after incubation for 40 minutes at 37°C. with KCN or 80 minutes at 37°C. with chloramphenicol. Colony counts and plaque counts were performed by the agar layer method (8).

In the experiments for the determination of protein and nucleic acids in growing bacteria, bacterial culture was suitably diluted and distributed into tubes to give the final volume of 10 ml. with or without the addition of chloramphenicol and cultured with aeration. In the experiments to determine the components of phage-infected bacteria, cells were sedimented, suspended in one-fifth the original volume of adsorption medium, and infected with 4 phages per bacterium for 10 minutes at 37°C. The infected cells were transferred at time zero to warm PY medium to give $3 \times 10^8$ cells per ml., distributed into tubes, and incubated. Chloramphenicol was added from a concentrated stock solution.

Chemical Determinations.—Aliquots of whole bacterial culture were precipitated with cold 0.3 M trichloroacetic acid (TCA). The precipitated DNA was estimated by the diphenylamine method (9). Sperm DNA was used as a standard after calibration against DATA of phage T2. The precipitated pentosenucleic acid (PNA) was estimated by the orcinol reaction (10) using D-ribose as a standard.

For the estimation of protein nitrogen, the cold TCA-insoluble portion was extracted twice with hot 0.3 M TCA. After digestion of the hot TCA-insoluble portion with sulfuric acid containing copper selenite and potassium persulfate, ammonium nitrogen was determined by Nessler’s method (11).

UV Analysis.—Log phase cells were sedimented and infected with $5 \times 10^{-3}$ phage per bacterium in adsorption medium for 10 minutes at 37°C. The infected cells were resedimented, suspended in adsorption medium, and transferred to PY medium at time zero. At the times specified, aliquots of the culture were diluted 100-fold in ice cold buffered saline and UV analysis was performed according to Benzer (4), using a 5 watt Sankyo germicidal lamp. Preliminary starvation of bacteria, which showed little effect in our system, was omitted. To test the effect of chloramphenicol on the development of UV resistance, infected cells in warm PY medium were added to an equal volume of the same medium containing 60 µg. per ml. of chloramphenicol, further incubated for the time specified, then diluted in ice cold buffered saline before irradiation.

EXPERIMENTAL RESULTS

Effect of Chloramphenicol on Bacterial Growth

Wisseman et al. (2) found that addition of chloramphenicol to growing cultures of E. coli stopped protein synthesis without affecting the rate of synthesis of nucleic acids. Their cultures were observed for only 30 minutes at 25°C.
In our experiments, illustrated in Figs. 1 and 2, the results are complicated by rapid growth in the control cultures, which, of course, does not occur in the presence of chloramphenicol (2). We wish to know how the rate of nucleic acid synthesis per cell is affected by chloramphenicol.

In the control cultures, cell components increased threefold in 40 minutes (see legend, Fig. 2), which corresponds to a generation time of 25 minutes, or an exponential increase of 2.75 per cent per minute. In cultures containing sufficient chloramphenicol to inhibit protein synthesis, the increase in nucleic acids was about 45 per cent of that in controls, or 90 per cent of the initial amount, at the end of 40 minutes. Since nucleic acid synthesis is more or less linear under these conditions (Fig. 1), the rate of increase is about 90/40 or 2.3 per cent per minute. Within the limits of experimental error (compare Figs. 1 and 2), the rate of nucleic acid synthesis per cell is little affected by chloramphenicol over a 40 minute period at 37°C.
Thus, as Wisseman et al. (2) found, chloramphenicol is a specific inhibitor of protein synthesis, and is eminently suited for use in the experiments that follow.

![Graph showing the effect of chloramphenicol on the synthesis of protein, DNA, and PNA by growing bacteria.](image)

Fig. 2. Effect of chloramphenicol on the synthesis of protein, DNA, and PNA by growing bacteria. Cultures similar to those of Fig. 1 but using varying concentrations of chloramphenicol. The cultures were analyzed 40 minutes after addition of chloramphenicol. During this time the amounts of protein, DNA, and PNA in the control culture increased 3.2, 2.8, and 3.0 times the original amount, respectively. Percentage increments are expressed taking those of the control culture as 100 per cent.

**Lysis of Infected Cells by Chloramphenicol**

Results presented in Fig. 3 show that chloramphenicol added to infected cultures during the second half of the latent period causes lysis with release of intracellular phage. In this respect it mimics the action of cyanide and 5-methyltryptophane (12). The amounts of intracellular phage released by 30 µg. per ml. of chloramphenicol added at a given time correspond to the amounts released by cyanide added about 2 minutes later; when 8 µg. per ml. of chloramphenicol is used, the displacement with respect to the cyanide curve is about
3 minutes. This indicates a lag of at least 2 or 3 minutes between time of addition of chloramphenicol and cessation of phage growth.

**Fig. 3.** One-step growth curve and premature lysis curves with cyanide and chloramphenicol. Concentrated log phase cells were infected with one phage per 10 bacteria in adsorption medium. Unadsorbed phage was removed by centrifugation. PY medium was added at time zero. Diluted samples were lysed by cyanide or chloramphenicol at the times specified, at concentrations of 0.01 µ and 30 µg per ml., respectively.

**Effect of Chloramphenicol on DNA Synthesis in Infected Bacteria**

To a series of identical cultures of bacteria infected at time zero, 30 µg. per ml. chloramphenicol was added at various times. Cultures without chloramphenicol served as controls. DNA was estimated at intervals with results shown in Fig. 4. DNA synthesis in the control cultures was rapid, reaching 2.5 to 3 times the original amount after 20 minutes. The increase of DNA was completely suppressed by the addition of chloramphenicol at zero to 2 minutes. Addition at later times showed progressively less effect until at 12 minutes, the rate of DNA synthesis was almost equal to that in the controls. However, protein synthesis and phage formation ceased promptly after chloramphenicol
was added. In some other experiments chloramphenicol added at the 10th minute did not show any inhibitory effect on DNA synthesis. After the addition of the antibiotic, DNA increased almost linearly. Hydrolysate of phage DNA added at a concentration of 0.03 ml. per ml. did not suppress the action of chloramphenicol added at the time of infection. The presence of 5-hydroxymethylcytosine in DNA synthesized in the presence of chloramphenicol was revealed by two-dimensional paper chromatography (6).

These results confirm the impression gained from experiments with uninfected bacteria, that the rate of DNA synthesis, once under way, is not affected by chloramphenicol.
Effect of Chloramphenicol on the Development of Resistance to UV Irradiation

Benzer (4) carefully studied the effects of irradiation at various times during the development of T2r in infected bacteria. For each time, a survival curve was constructed, showing the logarithm of the fraction of bacteria still able to yield virus as a function of dose of UV. Such curves show, in general, two features:

![Graph showing survival curves](image)

**Fig. 5.** Survival curves of phage bacterium complexes irradiated after different times of development. Concentrated log phase cells were infected with 5 phage particles per thousand bacteria in adsorption medium, and unadsorbed phage was removed by centrifugation. PY medium was added to start phage growth at time zero. At the times specified on the curves samples of the culture were diluted 100-fold with ice cold buffered saline and the resistance to UV was determined.

an initial plateau indicative of the number of radiation-sensitive targets per bacterium, and an ultimate linear slope measuring the radiation sensitivity of individual targets. Because of various complications, these curves cannot be interpreted too literally: they do, however, show characteristic features at various times, and thus reflect the evolution of the infectious process. Our results for cultures not containing chloramphenicol, shown in Fig. 5, confirm
those obtained by Benzer. During the first 8 minutes, the curves remain essentially linear, but show progressive changes in slope, as if each cell contained a single UV-sensitive target that was acquiring greater resistance during this period. At the 9th to 11th minute, the curves change shape, acquiring an initial plateau which indicates multiple targets. From this time on, the curves retain a multiple target character.

![Graph showing survival of defective centers against UV dose in minutes]

**Fig. 6.** Inhibition of the development of UV resistance by chloramphenicol. Cultures similar to those of Fig. 5 were mixed 5 minutes after the start of phage growth with an equal volume of warm PY medium containing 60 μg. per ml. chloramphenicol, and further incubated. Samples were diluted and irradiated at the times specified after the addition of chloramphenicol.

To ascertain the effect of chloramphenicol, experiments were performed in several different ways. Fig. 6 presents one in which infected cells were transferred at the 5th minute to a medium containing 30 μg. per ml. of chloramphenicol, samples of which were then irradiated after the additional times indicated. The UV resistance of the cells 5 minutes after the addition of chloramphenicol (10 minutes after infection) was similar to that of a control irradiated at 6 or 7 minutes after infection. Prolonged incubation with chloramphenicol did not cause further changes in spite of considerable increase of DNA during
the time. In the absence of irradiation, infected bacteria survived chloramphenicol well for 20 minutes, then decreased slightly. The radiation survival was corrected for the observed spontaneous losses. The yields of phage obtained by diluting out the antibiotic were normal up to 20 minutes' exposure, but decreased about one-third after 30 minutes.

![Survival curves of phage bacterium complexes](image)

**Fig. 7.** Survival curves of phage bacterium complexes irradiated 10 minutes after the addition of chloramphenicol at different times of development. Cultures were started in a manner similar to those of Fig. 5. At the times indicated, a culture was diluted with an equal volume of warm PY medium containing 60 μg. per ml. chloramphenicol, and further incubated for 10 minutes. Then a sample was diluted and irradiated.

For the experiment reported in Fig. 7, infected cells were transferred to medium containing chloramphenicol at various times following infection and subsequently incubated for 10 minutes. The curves corresponding to transfer at 6 minutes or less show single target shape. From 7 minutes on the curves show a definite plateau. These results show once more that chloramphenicol stops the intracellular development of UV resistance within about 2 minutes of the time of its addition.
The inhibition of the development of UV resistance by chloramphenicol was reversed after diluting the inhibitory agent. This result is presented in Fig. 8. Hydrolysate of phage DNA added at a concentration of 0.03 ml. per ml. did not show any effect on the development of UV resistance or on its inhibition by chloramphenicol.

Chloramphenicol at a final concentration of 8 μg. per ml. slowed but failed to stop the evolution of response to UV. This means that the concentration of 8 μg. per ml. is not sufficient for the complete inhibition of some chloramphenicol-sensitive process other than nucleic acid synthesis, and lower concentrations (18) might be expected to yield confusing results.
DISCUSSION

Cohen (13) found that protein synthesis was not interrupted by infection. DNA synthesis stopped for 7 to 10 minutes, then started up again. 5-methyltryptophane added at the time of infection prevented the resumption of DNA synthesis. His data suggested the requirement of protein synthesis for DNA synthesis in infected cells. Our results on infected cells extend his findings by showing that protein synthesis is prerequisite to the initiation of new DNA synthesis, but is not necessary for its continuation. In uninfected cells, the situation may be comparable, but is not subject to equivalent analysis. Only after phage infection can one observe the synthesis of a new kind of DNA getting under way.

Benzer (4) pointed out that the development of resistance to UV of the phage-producing capacity of the infected bacterium could be interpreted in the following way. The radiation-sensitive material in the virus particle must be called upon to perform a series of stepwise functions, each characterized by a certain cross-section for inactivation by UV. As each step is passed, the radiation sensitivity decreases correspondingly. In view of Stent's finding (14) that the development of resistance to decay of constituent radiophosphorus in the parental DNA parallels development of resistance to UV, the radiation-sensitive targets must be the parental DNA, at least during the early stages of infection.

We find that the development of UV resistance is blocked by chloramphenicol even when the antibiotic is added at times late enough to permit continued synthesis of DNA. Thus the development of UV resistance seems to depend on protein synthesis, and to parallel the rate of DNA synthesis achieved rather than the amount of DNA accumulated. This seems to show that the new DNA is not itself the radiation-sensitive target in the infected bacterium. Among other more complicated hypotheses, one must consider the possibility that genetic specificity brought in by DNA may be transferred to protein before new DNA synthesis can start.

SUMMARY

To elucidate the role of protein synthesis in DNA formation, E. coli R2 infected with phage T2 was studied as a model, employing chloramphenicol to inhibit protein synthesis. The following results were obtained.

1. Chloramphenicol inhibited protein synthesis but not synthesis of nucleic acids in uninfected bacteria.
2. Studies of the effect of chloramphenicol on phage maturation indicated a delay of 2 minutes between time of addition and cessation of phage growth.
3. The increase of DNA in phage-infected bacteria was completely suppressed by the addition of chloramphenicol within 2 minutes following infec-
EFFECT OF CHLORAMPHENICOL ON NUCLEIC ACID SYNTHESIS

Addition at later times showed progressively less inhibitory action depending upon the time interval, and addition after the 10th or 12th minute showed no appreciable effect on DNA synthesis despite the cessation of intracellular phage formation and protein synthesis.

4. When chloramphenicol was added to infected cells the increase of resistance to UV stopped within 2 minutes, whether or not DNA synthesis continued. Thus evolution of resistance paralleled the rate of DNA synthesis achieved, but not the amount of DNA accumulated.

5. We conclude that in infected bacteria, protein synthesis is necessary to initiate DNA synthesis but is not essential for its continuation. The resistance to UV that characterizes infected cells near the midpoint of the latent period is not due to accumulation of DNA, but depends on some chloramphenicol-sensitive process (probably protein synthesis) completed at about the time the rate of DNA synthesis becomes maximal.

It is a pleasure to express our appreciation to Dr. A. D. Hershey for his advice and encouragement.

Addenda.—After the present experiments were completed, the authors were informed of work along similar lines. Burton (15) and Melechen (16) are independently working on the role of protein synthesis in DNA synthesis of E. coli infected with phage T2: the former using amino acid–requiring mutants and 5-methyltryptophane, and the latter chloramphenicol. Matsushita (17) also stated that DNA synthesis in the course of UV-induced phage formation in E. coli K12 was inhibited by chloramphenicol when added before the onset of increase but not sensitive when added after the synthesis was in progress. On the other hand, Rosenbaum et al. (18) presented a note, describing effects of chloramphenicol on DNA synthesis in E. coli infected with phage T2, which contradict our present conclusion. However, we believe that the low concentration of antibiotic used by these authors was inadequate to inhibit protein synthesis.

REFERENCES
17. Matsushita, H., 1955, Read before the Symposium on Nucleic Acid and Nucleo-protein, held at Tokyo.