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EFFECTS OF CHLORAMPHENICOL ON RIBONUCLEIC ACID METABOLISM IN T2-INFECTED *ESCHERICHIA COLI*

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SUMMARY

The effect of chloramphenicol on RNA turnover in T2-infected *E. coli* was studied with the aid of ^{32}P-labeled orthophosphate. When chloramphenicol is added *before* infection, the subsequent distribution of radioactivity among RNA mononucleotides is different from the distribution found in control infected cells and resembles the distribution found in uninfected cells. When chloramphenicol is added *after* infection, the ^{32}P distribution in RNA is that typical of infected cells.

If the infected cells are inhibited before or *shortly* after infection, RNA turnover and DNA synthesis are inhibited. When chloramphenicol is added 9 min after infection, conditions that allow DNA synthesis, the rate of ^{32}P incorporation into RNA is increased and turnover of RNA now occurs in the presence of chloramphenicol. Thus RNA turnover is blocked under conditions that prevent DNA synthesis, but RNA turnover is not inhibited when DNA synthesis can proceed.

These observations are in agreement with the concept that some protein synthesis must precede or accompany formation of an RNA peculiar to phage-infected bacteria. It is also suggested that in the presence of inhibitor, RNA is synthesized by reversible reactions from precursor material also used for DNA synthesis.

INTRODUCTION

The ability of chloramphenicol (CP) to inhibit bacterial protein synthesis is well documented[1,2]. Chloramphenicol may also inhibit deoxyribonucleic acid (DNA) synthesis in bacteria infected with certain bacteriophages[3]. If chloramphenicol is added shortly after infection, complete inhibition of DNA synthesis is observed. With increased delay between infection and time of chloramphenicol addition, DNA syn-

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thesis increases so that chloramphenicol added 8 or more min after infection causes no inhibition of DNA synthesis[4]. This behavior has been interpreted to indicate that synthesis of some protein must precede phage DNA synthesis and that once this protein is formed, DNA synthesis can proceed in the absence of concomitant protein synthesis[4,5].

In previous work, we demonstrated that a rapid turnover of ribonucleic acid (RNA) phosphorus occurs in bacteriophage-infected bacteria[6,7]. In these experiments, where ^{32}P-labeled orthophosphate was incorporated into RNA, we noted that the RNA mononucleotides (isolated subsequent to alkaline hydrolysis) did not all have equal specific radioactivities. With T2-infected bacteria, adenylic and uridylic acids had equal specific activities nearly twice that found in guanylic and cytidylic acids. In similar experiments with uninfected bacteria, all RNA mononucleotides attained the same specific radioactivity[8]. These data suggest that bacteriophage infection induces bacteria to synthesize a new kind of RNA peculiar to T2-infected bacteria[6]. Since chloramphenicol can inhibit DNA synthesis by phage-infected bacteria, it is of interest to determine whether it has any effect on RNA turnover or on the pattern of isotope distribution among RNA mononucleotides*. The study of RNA metabolism in the presence of chloramphenicol is also of interest because of the often-observed correlation between RNA and protein synthesis[9].

In the experiments reported here, the turnover of RNA and the synthesis of DNA were measured in phage-infected bacteria inhibited by chloramphenicol before or at various times after infection. Some of these data have been presented in preliminary form[10].

MATERIALS AND METHODS

All experiments were carried out in low-phosphate peptone broth[8]. Occasionally, peptone broth[12] and synthetic medium[6] were used.

Conditions of infection and chemical techniques have been described in detail[6,8] but are summarized here for convenience. Log-phase cells of *Escherichia coli B* were infected with a 10- to 15-fold multiplicity (phage/bacteria) of T2r+ bacteriophage. ^{32}P (obtained from the Oak Ridge National Laboratory in the form of inorganic phosphate) was neutralized and added to the culture of phage-infected bacteria at various times after infection. Chloramphenicol, when used, was always added before ^{32}P; in some experiments, even before infection. Detailed experimental manipulations are described in figure and table legends. Aliquots taken at various times were fractionated to isolate acid-soluble materials, lipids, RNA, DNA, and proteins[6]. The RNA mononucleotides produced by alkaline hydrolysis were separated by ion-exchange chromatography[6]. Previously described methods were used to measure ultraviolet light absorption, radioactivity[6], and phosphorus content[13]. In the fractionation procedure, DNA was ultimately obtained in a solution of 2 to 3 N HCl. To measure hydroxymethylcytosine (HMC), the solution was taken to dryness under partial vacuum with nitrogen as the gas phase. After formic acid hydrolysis[14], hydroxymethylcytosine was isolated by paper chromatography[14,15].

* In preliminary experiments HERSHEY, SIMINOVITCH AND GRAHAM found that "chloramphenicol does not block the incorporation of labeled phosphorus into RNA" [of T2-infected cells] "but seems to block the flow out of it"[11]. These results, communicated to us by Dr. HERSHEY prior to publication, provided the impetus for this work.

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In some of the experiments, infected cells in the presence of chloramphenicol were allowed to incorporate isotope and then washed before further incubation. Details of the washing procedure are presented here. The incubation mixture (480 ml) was quickly chilled by rapid addition of 200 ml of crushed ice prepared from frozen peptone broth containing 45 μg of chloramphenicol/ml. After centrifugation for 15 min at 1500 $\times$ g in the cold, the cells were washed by resuspension in 150 ml of cold peptone broth containing 45 μg of chloramphenicol/ml, followed by recentrifugation as before. The washed cells were resuspended in 48 ml of cold peptone broth (no chloramphenicol). Thirty-six ml of the cold suspension was added to 330 ml of peptone broth previously warmed to 37°. The remaining 12 ml of cold cencentrated cells was added to 110 ml of warm peptone broth containing sufficient chloramphenicol to make the final concentration 45 μg/ml. With the bacteria thus returned to the same cell density present before washing, aeration and incubation at 37° were resumed. The orthophosphate concentration was 8.3 μg/ml in low-phosphate broth and 93 μg/ml in peptone broth.

RESULTS

Chloramphenicol has a marked effect on RNA metabolism in phage-infected bacteria, the nature and extent of the effect depending on the time of addition of the inhibitor. When chloramphenicol is added after infection, the initial rate of ^{32}P incorporation into RNA [Fig. 1: CP(A)] is the same as in infected but uninhibited cultures (CON). The RNA of the controls, however, quickly reaches and maintains* a maximum amount of isotope, whereas that of infected and inhibited cells maintains a steady

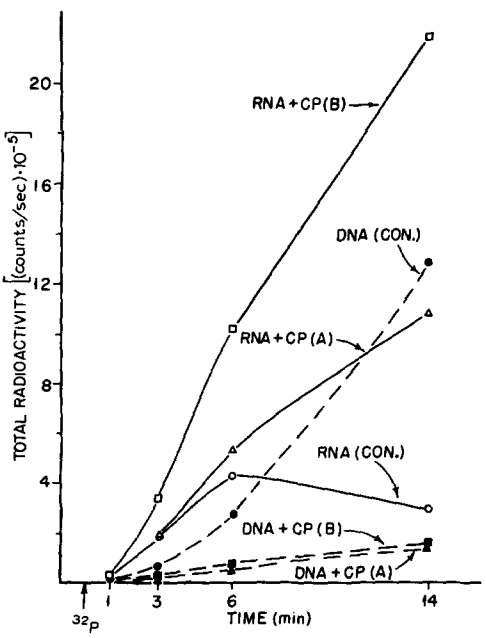

Fig. 1. Effects of chloramphenicol on total ^{32}P uptake by RNA and DNA (low-phosphate broth). Three parallel samples of infected cells were used. ^{32}P was added to all samples 6 min after infection. Control (CON), no further addition. CP(A), chloramphenicol (final concn. 30 μg/ml) added 3 min after infection. CP(B), chloramphenicol (same concn.) added 3 min before infection.

* In Fig. 1, control RNA suffers a definite drop in radioactivity from the earlier maximum. In most experiments this drop is not observed.

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rate of isotope incorporation for a longer period. (It is not known whether an experiment of longer duration would reveal a maximum for ^{32}P incorporation into RNA of inhibited cells.)

A more pronounced effect of chloramphenicol is observed if the inhibitor is added before infection [Fig. 1: CP(B)]. Again the initial rate of isotope incorporation into RNA is maintained throughout the experimental time, but this initial rate is greater than in the control culture, with the result that, 14 min after the addition of ^{32}P, the RNA of the inhibited cells has 4 to 8 times the radioactivity of the control RNA. The effect of chloramphenicol on DNA synthesis is presented in the figure for comparison with RNA.

TABLE I

EFFECTS OF CHLORAMPHENICOL ON RELATIVE SPECIFIC ACTIVITIES OF RNA MONONUCLEOTIDES

Data is from experiment of Fig. 1. RNA mononucleotides, released by alkaline hydrolysis of RNA, were separated by ion-exchange procedures. Recorded are specific activities relative to that of guanylic acid.

Mononucleotide	*Specific activity (counts/sec/μg of P) of mononucleotides under indicated conditions*		
	No CP	*CP added 3 min after infection*	*CP added 3 min before infection*
Cytidylic acid	1.2	1.1	1.0
Adenylic acid	1.8	2.1	1.0
Uridylic acid	1.9	2.1	1.2
Guanylic acid	1.0	1.0	1.0

Addition of chloramphenicol before infection alters the pattern of ^{32}P incorporation into RNA. Normally, with infected but uninhibited cells, the RNA mononucleotides do not have uniform specific activities, in that adenylic and uridylic acids have nearly twice the specific radioactivity of cytidylic and guanylic acids (Table I, column 2; see also reference [6]). This distribution of isotope among the mononucleotides is unchanged by chloramphenicol added after infection (column 3), but when cells are inhibited before infection, the isotope distribution is changed so that all four mononucleotides have nearly equal specific activities (column 4). Since, under similar conditions, the RNA mononucleotides of uninfected bacteria also have equal specific radioactivity[8], addition of chloramphenicol before infection seems to prevent the formation of an RNA peculiar to T2-infected bacteria.

Although addition of chloramphenicol after infection does not change the isotope distribution among RNA mononucleotides from the pattern of uninhibited infected cells, it does have a marked effect on RNA turnover. In the absence of inhibitor, a major portion of radioactive RNA phosphorus is replaced with unlabeled phosphorus when isotope is removed from the medium or diluted with unlabeled orthophosphate[7]. This turnover does not occur if chloramphenicol, added shortly after infection, remains present after removal of isotope (Fig. 2: +CP). Under the same conditions that prevent RNA turnover, DNA synthesis does not occur. When chloramphenicol is removed, however, the infected cells quickly return to their normal metabolic patterns, with loss of isotope from RNA and incorporation of it into DNA (Fig. 2: —CP). It should be noted that the isotope incorporation into DNA represents syn-

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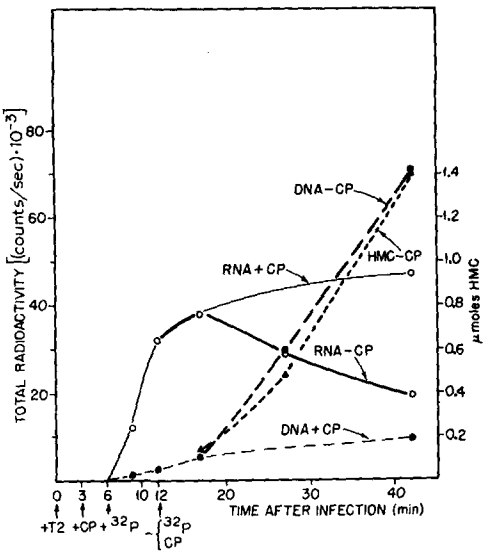

Fig. 2. Effect of chloramphenicol on RNA turnover and DNA synthesis: Chloramphenicol (final concn. 45 μg/ml) added 3 min after infection (low-phosphate broth). After 6 min of ^{32}P incorporation by infected cells in the presence of chloramphenicol, the cells were washed free of isotope and chloramphenicol and reincubated in the presence (+CP), or absence (—CP) of chloramphenicol. Detailed protocol in the methods section. Recorded are the counts incorporated into RNA and DNA and, after choramphenicol was removed, the amount of hydroxymethyl cytosine (HMC) synthesized. Values corrected to constant sample size containing 310 O.D. units (260 mμ) of RNA.

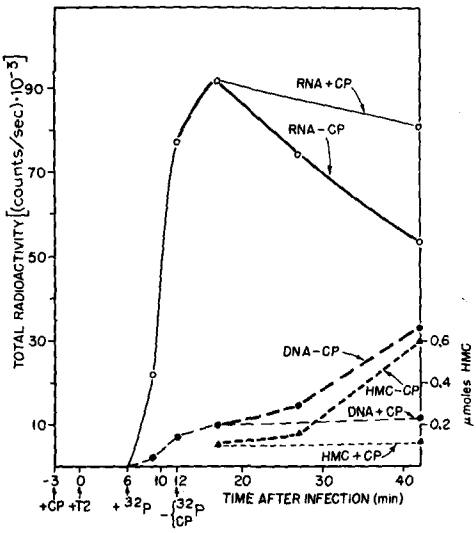

Fig. 3. Effect of chloramphenicol on RNA turnover and DNA synthesis: Chloramphenicol (final conc. 45 μg/ml) added 3 min before infection. Other than time of chloramphenicol addition, same protocol as for Fig. 2. Values corrected to constant sample size containing 350 O.D. units (260 mμ) of RNA.

thesis of phage and not bacterial DNA since the rate of isotope incorporation exactly parallels the appearance of hydroxymethylcytosine.

A similar turnover experiment was performed with cells inhibited before infection (Fig. 3). When isotope is removed from the medium, the continued presence of chloramphenicol prevents synthesis of DNA and loss of isotope from RNA. If chloramphenicol is removed, loss of isotope from RNA and its incorporation into DNA are observed as in cells inhibited after infection.

There is, however, a significant difference between the two inhibited cultures in that, in cells inhibited *after* infection, turnover of RNA and synthesis of DNA start almost simultaneously, whereas in cells inhibited *before* infection, a considerable amount of radioactivity is lost from RNA before synthesis of DNA starts. A possible clue to the reason that RNA turnover precedes DNA synthesis is provided when we examine the distribution of radioactivity lost from RNA as a function of time (Table II). During the period when no DNA synthesis accompanies loss of radioactivity from RNA (17 to 27 min for cells inhibited before infection), the relative losses from mononucleotides are roughly proportional to the base ratios of uninfected bacterial RNA. In the next time interval, when synthesis of DNA has started, these relative losses from RNA mononucleotides no longer reflect the base ratios of uninfected bacteria, but approach the isotope distribution pattern of infected cells. (Note that the con-

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TABLE II

DISTRIBUTION OF THE TOTAL RADIOACTIVITY LOST FROM RNA AFTER REMOVAL OF CHLORAMPHENICOL

Data is from experiments of Figs. 2 and 3. RNA mononucleotides, released by alkaline hydrolysis of RNA were separated by ion-exchange procedures and their total radioactivities were determined. The amount of radioactivity lost from each mononucleotide over the designated time period was calculated.

Mononucleotide	*Total radioactivity lost in indicated periods after infection**			
	CP added 3 min after and removed 12 min after infection		*CP added 3 min before and removed 12 min after infection*	
	17–27 min	*27–42 min*	*17–27 min*	*27–42 min*
Cytidylic acid	13	17	19	24
Adenylic acid	30	33	23	26
Uridylic acid	35	36	25	26
Guanylic acid	22	14	33	24

* The total activity lost by each nucleotide is expressed as a percentage of the total lost by all four mononucleotides.

tribution from guanylic acid is no longer the greatest but is equal to that from cytidylic acid and less than the contribution from adenylic and uridylic acids.) With cells that do not exhibit any lag in DNA synthesis (chloramphenicol either added after infection or never present), the pattern of isotope loss immediately resembles the typical pattern of incorporation by infected cells. The data suggest that in cells inhibited before infection, the loss of radioactivity from RNA, immediately after removal of chloramphenicol, occurs during a period when the metabolic patterns of the cell are changing from those present in uninfected bacteria to those typical of infected cells. When the change is accomplished, as indicated by the altered pattern of RNA turnover, phage DNA synthesis starts. Thus the preliminary loss from this RNA is not directly comparable to RNA turnover by normal infected bacteria but resembles the behavior of uninfected cells inhibited by chloramphenicol. Such cells accumulate excess RNA that is lost after removal of chloramphenicol and before resumption of growth[16,17].

Chloramphenicol inhibits DNA synthesis and RNA turnover only when it is added either before or shortly after infection. If the interval between infection and time of chloramphenicol addition is extended, DNA synthesis and RNA turnover can both occur in the presence of inhibitor. In the experiment recorded by Fig. 4b, chloramphenicol, added 9 min after infection, had no effect on the rate of DNA synthesis. However, even though RNA turnover is observed in this experiment, the inhibitor does have a marked and complex effect on RNA metabolism. Most striking is the increased incorporation of ^{32}P, an increase that varied in several experiments between 2- and 5-fold. This increase over the control is not observed when cells are inhibited 1 min after infection (Fig. 4a). In fact, the level of radioactivity attained in the RNA of late-inhibited cells is similar to that attained when isotope is added early after infection (control and inhibited cells of Fig. 4a). Apparently, in the presence of chloramphenicol, the amount of radioactivity incorporated into RNA does not depend on the time after infection that chloramphenicol and ^{32}P are added. This is in contrast to control cells where the amount of ^{32}P incorporated into RNA decreases

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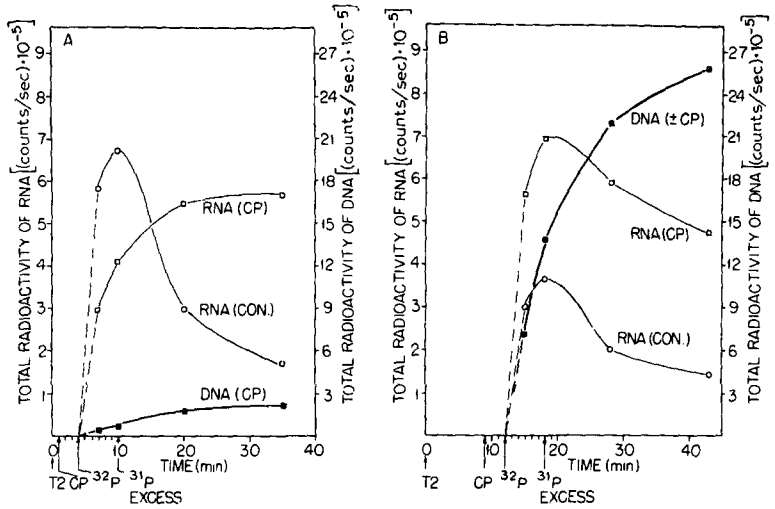

Fig. 4. Turnover of ^{32}P RNA and accumulation of ^{32}P DNA as a function of time after infection of isotope addition. Effects of chloramphenicol (low-phosphate broth). To parallel samples of infected cells, ^{32}P labeled orthophosphate was added at designated times after infection. Six min after ^{32}P was added, its specific radioactivity was reduced by adding sufficient pH 7.5 phosphate buffer ^{31}P to increase the orthophosphate concn. from 1.5 μg of P/ml to 225 μg of P/ml. Where chloramphenicol (CP) was used, it was added to a final concn. of 30 μg/ml, 3 min prior to ^{32}P. Control indicates no chloramphenicol added. To correct for slight variations in cell numbers, data was normalized to a constant amount of RNA. RNA scale is magnified 3-fold relative to DNA scale.

sharply with time after infection that ^{32}P is added[8]. Thus the supposed increased in ^{32}P incorporation caused by the late addition of chloramphenicol may be actually a decrease in the control.

The data presented were obtained with cells grown and infected in low-phosphate broth. Experiments described by Figs. 1 and 2 and Table I were also run in synthetic medium and yielded results similar to those reported.

DISCUSSION

Some insight onto the action of chloramphenicol on RNA metabolism might be obtained from comparing effects in uninfected and infected bacteria. Within 2 to 3 min of adding chloramphenicol to a growing bacterial culture, protein synthesis stops, but the synthesis of nucleic acids continues until their amounts are approximately doubled[16]. The additional RNA can be distinguished from normal RNA by physicochemical and metabolic differences. In contrast to normal RNA that is metabolically stable[18], the extra RNA disappears after chloramphenicol is removed[16,17]. At the physiochemical level, the abnormal RNA has a lower affinity for proteins than does normal RNA[19], even though no difference in base composition is observed[20]. There does not seem to be much similarity between infected and uninfected bacteria with regard to the effect of chloramphenicol on RNA metabolism. But this might be expected since even with infected bacteria there is no consistent effect of chloramphenicol.

Although the effects of chloramphenicol on RNA metabolism may be expressed in many ways, one generalization can be made. It is observed that chloramphenicol

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prevents any change from the pattern of isotope distribution among the RNA mononucleotides present at the time inhibitor is added. Thus, when chloramphenicol is added after infection, the subsequent pattern of isotope incorporation is that typical of infected cells; but when chloramphenicol is added before infection, the pattern of uninfected cells remains until removal of the inhibitor. Similarly, the extra RNA formed by uninfected cells in the presence of chloramphenicol has the same base composition as normal RNA. The data indicate that the synthesis of some protein, presumably peculiar to phage infection, must occur before alteration of the isotope incorporation pattern of RNA. Questions about how and why the pattern of isotope incorporation changes after infection, and why the pattern after infection is similar[6,7] to the base composition of bacteriophage DNA[21] are unanswered.

An interesting correlation between RNA turnover and DNA synthesis is observed in phage-infected bacteria. When chloramphenicol is added in time to inhibit DNA synthesis, RNA turnover is also inhibited. If chloramphenicol is added late, both DNA synthesis and RNA turnover occur. Previous experiments[8] indicate that RNA phosphorus can be used for DNA synthesis. We suggest that RNA is in a reversible equilibrium with phosphorus-containing intermediates that are also used in DNA synthesis. When chloramphenicol is added late and rapid synthesis of DNA occurs, the intermediates are irreversibly incorporated into DNA. As a consequence, radioactivity leaves RNA and, in the absence of exogenous ^{32}P, is replaced by unlabeled phosphorus. Correspondingly, when timely addition of chloramphenicol prevents DNA synthesis there would be no drain on RNA radioactivity and no turnover. Apparently, in inhibited but uninfected bacteria also, RNA is synthesized by reversible reactions since, as mentioned, the extra RNA disappears after chloramphenicol is removed.

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