# METABOLIC INSTABILITY OF THE RIBONUCLEIC ACID SYNTHESIZED BY *ESCHERICHIA COLI* IN THE PRESENCE OF CHLOROMYCETIN*

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## INTRODUCTION

In the presence of chloromycetin, cells of many bacteria are unable to synthesize proteins, but a synthesis of nucleic acid continues[1,2]. Previous work with amino acid auxotrophs in this laboratory[3] and elsewhere[4] has demonstrated that individual amino acids play an essential role in nucleic acid synthesis. The experiments which will be reported here were undertaken to study the recovery of cells which have synthesized nucleic acid in the presence of chloromycetin. The results of these experiments indicate that the ribonucleic acid (RNA) synthesized by *Escherichia coli* in the absence of concomitant protein synthesis is extremely unstable *in vivo*.

This result is interesting not only because it stands in sharp contrast to the reported stability of nucleic acid synthesized under normal conditions by *E. coli*[5], but also because it has lead to observations on the action of chloromycetin.

## MATERIALS AND METHODS

### *Organism and media*

Strains of *E. coli* were used in all the experiments. Strain M 83-5 is a phenylalanine-requiring mutant, and strain ML 309-d is a methionine-requiring mutant. Exponential phase cultures were prepared by overnight growth in a basal salt medium, 63 ($KH_2PO_4$, $M/10$; $NH_4Cl$, $M/50$; $MgSO_4$, $M/1000$; $FeSO_4$, $M/100{,}000$; brought to pH 7.0 with KOH), containing 0.2% maltose and supplemented with the appropriate amino acid at a concentration of $5 \cdot 10^{-4} M$, followed by a transfer into fresh medium and a period of new growth. When a medium low in phosphate was desired, the phosphate concentration of 63 was reduced to $10^{-3} M$, and sufficient tris-(hydroxymethyl)-aminomethane buffer, pH 7.5, was added to give a concentration of 0.1 $M$.

### *Measurement of growth*

Growth was measured in terms of the optical density (O.D.) of the cells in a Beckman DU Universal Spectrophotometer at a wavelength of 420 m$\mu$, with a 10 mm light path.

### *Radioactive chemicals*

Adenine-8-$^{14}C$ was obtained from the Schwarz Laboratories, Mount Vernon, New York. $^{32}P$-phosphoric acid was obtained from the Commissariat à l'Energie Atomique, France.

### *Chloromycetin treatment of cells*

Exponential phase cultures growing in either tris or phosphate medium were treated directly

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with chloromycetin in the following manner. At o time sufficient chloromycetin was added to the growing culture to give a final concentration of 20 $\mu$g/ml. The cultures were kept incubating aerobically for 2 hours, during which time the O.D. of the cultures increased about 15%, protein synthesis was inhibited more than 95%, the desoxyribonucleic acid (DNA) of the cells increased 50%, and the RNA of the cells increased 100%. When a specific labeling of the nucleic acid formed during this treatment was desired, adenine-8-$^{14}$C (20,000 c.p.m. per $\mu M$) was added to give a final adenine concentration of $2 \cdot 10^{-4} M$, or, alternatively, $^{32}$P as phosphate ($10^{-3} M$, 10,000 c.p.m. per $\mu M$) was added to cells in the tris medium. These additions were made 2 minutes after the addition of chloromycetin. Following the incubation period, the cells were centrifuged and washed twice in the cold with medium 63 (or tris), and were then ready for study.

*Nucleic acid measurements*

RNA and DNA were determined by colorimetric analyses of the hot 5% trichloracetic acid (TCA)-soluble fraction of the cells. Three-ml samples of the culture were extracted in the cold with 5% TCA, the extract discarded, and then heated with 1.5 ml of 5% TCA at 100° for 30 minutes. 0.3 ml of this fraction was analyzed for ribose by means of the orcinol procedure[6] using either ribose or a suspension of sodium ribonucleate as a standard, and 1.0 ml was analyzed for desoxyribose by a modification of the Dische diphenylamine procedure[7] using calf thymus DNA as a standard.

When the RNA and DNA made in the presence of chloromycetin were labeled with adenine-8-$^{14}$C, a more rapid and accurate method was used for the measurement of the *total nucleic acid* of the culture. One-ml samples were acidified with TCA to a final concentration of 5%, kept in the cold for at least 1 hour, then filtered through membrane filters (Millipore Filter Corp., Watertown, Mass.). The cells were then washed directly on the filters with 5 ml of 5% TCA and twice with 5 ml of distilled water. The filters were then dried and their radioactivity measured by means of an end-window Geiger counter. This method gave results comparable to those obtained by the more laborious procedure involving extraction of the nucleic acids with hot TCA and plating the fraction on planchets for counting. When the nucleic acids had been labeled with radioactive phosphorus, however, the latter procedure had to be employed because of a significant amount of phosphorus that appeared in the hot TCA-insoluble fraction of the cells.

When circumstances required the estimating of radioactive DNA in cells containing adenine-8-$^{14}$C labeled nucleic acids, a chemical separation of DNA and RNA by a modified Schmidt-Thannhauser procedure was carried out. The cells in a 4-ml suspension were extracted with cold 5% TCA, washed, then placed in 1 $N$ NaOH at 37° overnight. The NaOH was then carefully neutralized with HCl, and TCA added to give a final concentration of 5%. The precipitated DNA was then washed with cold 5% TCA and extracted in 1 ml of 5% TCA by heating at 100° for 30 minutes. The TCA of the extracts was then removed by fractionation with ether and 0.3 ml of the extracts were plated on metal planchets for counting.

## RESULTS

### *RNA destruction during growth after chloromycetin treatment*

In a preliminary experiment, cells of a methionine-less mutant, ML 309d, were treated with chloromycetin as described under MATERIALS AND METHODS. As shown by Table I, the amount of RNA was increased 70% and the amount of DNA 50% by this treatment. The cells were centrifuged and washed twice with cold medium to remove the chloromycetin, then resuspended in fresh maltose-minimal medium supplemented with methionine. Upon incubation at 37°, there ensued a 40-minute lag, after which the O.D. began to increase. During the lag period there was a decrease of about 22% in the amount of RNA per ml of culture. No such decrease in the amount of DNA was observed. Owing to renewed growth and RNA synthesis in an experiment of this kind, it is difficult to study the behavior of the RNA fraction synthesized during the chloromycetin treatment. In subsequent experiments, therefore, this RNA fraction was differentiated from the "normal" fraction by labeling with adenine-8-$^{14}$C, and the renewal of growth and RNA synthesis in the "recovery" period was prevented by omitting an essential amino acid from the medium.

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TABLE I

RNA AND DNA COMPOSITION OF CELLS RECOVERING FROM CHLOROMYCETIN TREATMENT

| Time minutes | O.D. | RNA* μg ml culture | DNA** μg ml culture | $\frac{RNA}{O.D.}$ | $\frac{DNA}{O.D.}$ |
|---|---|---|---|---|---|
| Before treatment | 0.497 | 29 | 3.6 | 52 | 7.2 |
| Recovery period | | | | | |
| 0 | 0.544 | 46 | 5.4 | 85 | 9.9 |
| 20 | 0.544 | 43 | 5.5 | 79 | 10.0 |
| 40 | 0.544 | 40 | 5.5 | 74 | 10.0 |
| 75 | 0.664 | 36 | 5.6 | 56 | 8.7 |
| 95 | 0.788 | 40 | 5.8 | 51 | 7.4 |
| 115 | 0.965 | 55 | 6.6 | 57 | 6.8 |
| 145 | 1.320 | 72 | 10.0 | 54 | 7.6 |

* Measured colorimetrically by the orcinol method.
** Measured colorimetrically by the diphenylamine method.

*RNA destruction in resting cells*

Fig. 1 illustrates the results of an experiment in which exponential-phase cells of a phenylalanine-less strain, M 83-5, had been allowed to accumulate nucleic acid in the presence of chloromycetin, phenylalanine, and adenine-8-$^{14}$C. The presence of radioactive adenine caused a specific labeling of the nucleic acid formed during this 2-hour period. The cells were then washed twice in the cold to remove the chloromycetin, the adenine, and the phenylalanine, and resuspended at 37° in maltose-containing basal medium. There ensued an immediate, rapid decrease in the radioactivity and the ribose content of the nucleic acid fraction of the cells. This decrease continued until approximately 50% of the nucleic acid formed in the presence of chloromycetin had been degraded. Analyses of the radioactive DNA formed during the chloromycetin treatment were carried out by its chemical separation from the RNA. The results (Fig. 1) indicated that the DNA was quite stable upon removal of chloromycetin. The behavior of the RNA was so unlike that of the RNA formed under normal conditions by *E. coli* that a further study was made of its degradation.

*Effect of chloromycetin readdition to resting cells*

Since the removal of chloromycetin caused a rapid destruction of the RNA formed in its presence, an experiment was performed to discover whether the re-addition of chloromycetin could halt the degradative process. The experiment was performed with the same strain (M 83-5) and with the same conditions as before. It was found (Fig. 2) that re-addition of chloromycetin at various times during and after the degradation not only halted the net decrease of the RNA, but also initiated an almost complete reincorporation into nucleic acid of the labeled fragments resulting from the RNA destruction. This resynthesis of RNA occurred in the absence of exogenous phenylalanine, and only after a starvation period of 2 hours was the rate of RNA resynthesis accelerated by the addition of this amino acid. It is paradoxical that the absence of exogenous phenylalanine is sufficient to halt normal protein and RNA synthesis, but that re-addition of chloromycetin to the same system can initiate a process known to be dependent on phenylalanine.

*References p. 520.*

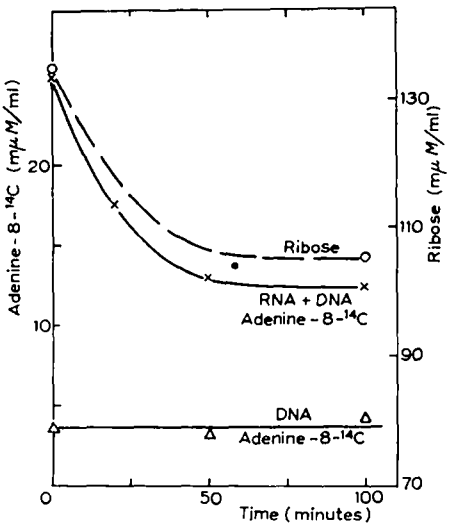


Fig. 1. Degradation in resting cells of the RNA produced during chloromycetin inhibition. Exponential-phase cells of *E. coli* strain M 83–5 were treated with chloromycetin in the presence of labeled adenine. The cells were washed twice in the cold with medium 63, then resuspended in 63 containing 0.2% maltose and incubated at 37°. At various times aliquots of the cells were removed to determine the total radioactive adenine of the cold TCA-insoluble fraction, the ribose content of the hot TCA-soluble fraction, and the adenine-8-$^{14}$C in the DNA fraction. These procedures are described in detail under MATERIALS and METHODS. The curve O---O shows the amount of ribose in the hot TCA fraction of 1 ml of cell suspension. The curve marked ×——× shows the amount of radioactive adenine in the cold TCA-insoluble fraction (RNA + DNA) of 1 ml of cell suspension. The curve marked △——△ shows the amount of adenine-8-$^{14}$C in the DNA fraction of the cells. The O.D. of the cell suspension was 0.90.

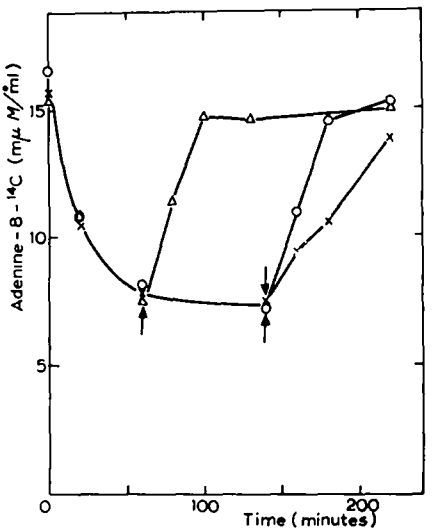


Fig. 2. Effect of the re-addition of chloromycetin to resting cells on the RNA produced during chloromycetin inhibition. The bacterial strain and experimental conditions were identical to those described for Fig. 1, except that, after washing, the cells were resuspended in 3 separate flasks containing maltose-basal medium. At 60 minutes chloromycetin (20 μg/ml) was added to 1 flask (shown by △——△) and at 140 minutes it was added to a second (shown by ×——×). At 140 minutes chloromycetin (20 μg/ml) plus phenylalanine ($5 \cdot 10^{-4} M$) were added to the third flask (shown by O——O). The O.D. of each cell suspension was 0.55.

*Extracellular localization of the fragments*

The experiment just described was repeated to determine whether the fragments resulting from RNA destruction had remained inside the cells. Measurements of radioactivity were made on (a) the whole cells, (b) the cells following extraction with cold 5% TCA, and (c) the culture fluid after removal of the cells by centrifugation. The results (Table II) indicated that during the breakdown of the RNA the fragments left the cells and could be found in the medium. Readdition of chloromycetin initiated a re-entry and reincorporation into nucleic acid of 85% of these extracellular fragments.

*Conditions necessary for the destruction and the resynthesis of RNA*

Experiments were performed to define the conditions necessary for the destruction of the RNA synthesized in the presence of chloromycetin and for its resynthesis from the same fragments. Figs. 3 and 4 illustrate these experiments, the results of which

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TABLE II

ACCUMULATION AND DISAPPEARANCE OF EXTRACELLULAR FRAGMENTS RESULTING FROM RNA BREAKDOWN

| Time* minutes | Adenine-8-$^{14}C$ (mμM, ml culture) | | | |
|---|---|---|---|---|
| | In cells | | In medium | Total Cells + Medium |
| | TCA-insoluble fraction | TCA-soluble fraction | | |
| 0 | 16.2 | 2.4 | 0 | 18.6 |
| 20 | 13.1 | 2.3 | 2.2 | 17.6 |
| 60 | 8.9 | 0.9 | 9.0 | 18.8 |
| 130 | 14.0 | 1.9 | 1.6 | 17.5 |

* At 0 time the cells were suspended in chloromycetin-free medium. At 60 minutes chloromycetin was re-added. See the text for further details.

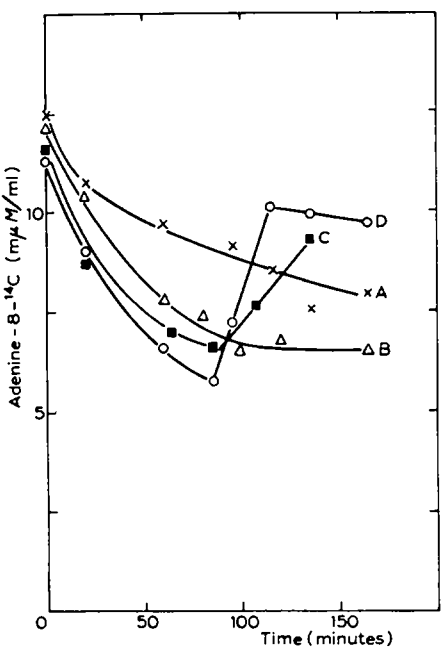


Fig. 3. Effect of various conditions on the destruction and resynthesis of the RNA produced by chloromycetin-inhibited *E. coli*. Experimental conditions were the same as for Fig. 1. The various media is which the cells were suspended after washing twice with distilled water were: medium 63 (O ——O), physiological-saline with 0.1 *M* phosphate buffer (pH 7.5) (■——■), physiological-saline (△----△), and distilled water (×· —×). At 85 minutes, chloromycetin (20 μg/ml), phenylalanine ($5 \cdot 10^{-4}$ *M*) and maltose (0.2%) were added to all the flasks. The O.D. of each cell suspension was close to 0.43.

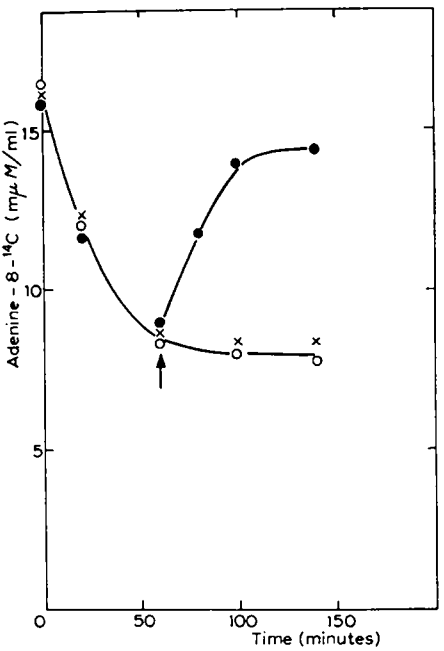


Fig. 4. Effect of DNP on the degradation and resynthesis of the RNA produced by *E. coli* during chloromycetin inhibition. Experimental conditions were the same as for Fig. 1. Three suspensions of cells in medium 63 with 0.2% maltose were treated as follows. One flask contained just basal medium + maltose (●——●), and at 60 minutes chloromycetin was re added. A second flask contained just basal medium + maltose (O----O), and at 60 minutes chloromycetin and DNP ($5 \cdot 10^{-3}$ *M*) were added together. The third flask contained basal medium + maltose (×----×) with chloromycetin and DNP present from 0 time. The O.D. of each cell suspension was close to 0.54.

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can be summarized as follows. In the absence of chloromycetin, the rate of degradation of RNA is maximal in basal medium or in saline plus phosphate. The rate is almost maximal in saline alone, but is very much reduced when the cells are placed in distilled water. If 2,4-dinitrophenol (DNP) ($10^{-3}M$) is added to the cells after chloromycetin treatment, RNA destruction occurs at the same rate in the presence and absence of chloromycetin. Thus, the mere *presence* of chloromycetin is insufficient to stabilize the RNA made in its presence. The resynthesis of RNA from the labeled fragments resulting from its destruction occurs only in the presence of chloromycetin, phosphate, and an energy source, and does not occur in the presence of DNP.

*Results with $^{32}PO_4$ labeling*

When radioactive phosphorus was employed instead of adenine-8-$^{14}$C to label the nucleic acids formed in the presence of chloromycetin, the subsequent degradation of RNA in resting cells was accompanied by a parallel decrease in the radioactive phosphorus content of the hot TCA-soluble fraction (Fig. 5). The resynthesis of RNA that occurred upon readdition of chloromycetin, however, was not accompanied by a reincorporation of labeled phosphorus. This result has been interpreted as indicating that the degradation of RNA proceeds at least as far as the nucleoside stage, and that resynthesis entails new phosphorylation of the fragments.

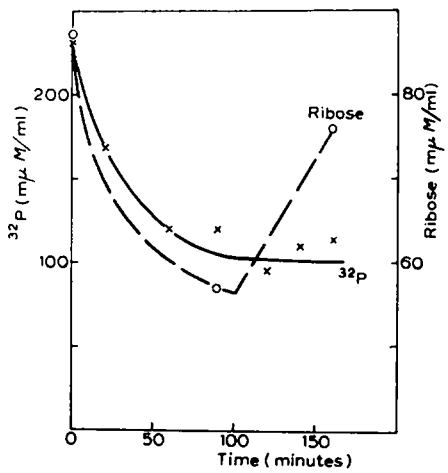


Fig. 5. Degradation in resting cells of $^{32}$P-labeled RNA produced during chloromycetin inhibition, followed by resynthesis of non-labeled RNA upon chloromycetin addition. The conditions were the same as in Fig. 1 except that the tris medium was used in place of medium 63, and radioactive phosphorus as phosphate ($10^{-3}M$, 10,000 c.p.m./$\mu M$) was used in place of adenine-8-$^{14}$C. Chloromycetin was re-added at 100 minutes. The O.D. of the cell suspension was 0.64.

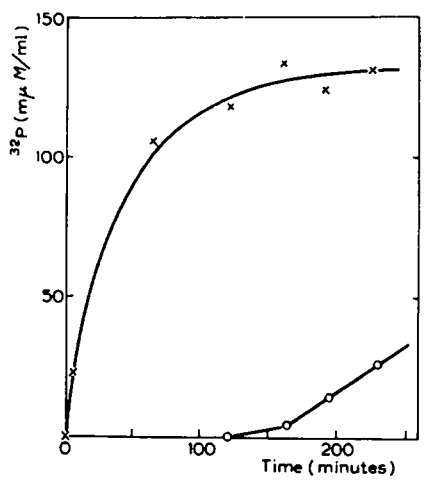


Fig. 6. Incorporation of $^{32}$P into nucleic acid in the presence of chloromycetin. Flask A (×——×) received chloromycetin and carrier-free $^{32}$P as phosphate (final concentration 0.1 $\mu$c/ml) at 0 minutes. Flask B (○——○) received chloromycetin at 0 minutes and $^{32}$P at 120 minutes. The O.D. of each cell suspension was close to 0.49.

*$^{32}P$ incorporation into RNA in the presence of chloromycetin*

The results with $^{32}$P labeling indicated that this isotope might be useful in detecting any renewal ("turnover") of RNA in the presence of chloromycetin. An exponential-

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phase culture of strain M 83-5 in tris medium containing $10^{-3}$ phosphate was divided between two flasks. Both flasks received chloromycetin (20 $\mu$g/ml) at 0 time, and both were incubated aerobically. Carrier free $^{32}$P-phosphate was added to flask A at 0 time and to flask B at 120 minutes. Samples were removed at various times and the $^{32}$P content of the nucleic acid fraction was determined. The results (Fig. 4) indicate that there is at most an extremely slow incorporation of phosphate into nucleic acid after the maximum amount has been synthesized in the presence of chloromycetin.

## DISCUSSION

The work of PARDEE and his collaborators[8] has demonstrated that the RNA formed in the presence of chloromycetin by cells of *Escherichia coli* can be distinguished from "normal" RNA by its electrophoretic mobility, its centrifugability, and by the relative ease with which it can be dissociated from accompanying protein. The results of experiments presented here indicate another, perhaps more striking, difference between normal RNA and the RNA formed during chloromycetin treatment. A major portion of the latter compound has been found to be degraded rapidly *in vivo* upon the removal of chloromycetin.

When cells that have been treated with chloromycetin are washed free of the antibiotic and placed in a medium permitting growth, a portion of their RNA is rapidly degraded. When growth in the recovery period is restricted by the absence of an essential amino acid, the destruction of RNA leads to an accumulation in the medium of the resulting fragments.

The process of degradation is about half-completed in 15 minutes at 37°. It occurs at a much faster rate when the cells are suspended in saline or in phosphate buffer than in distilled water, and it probably does not require energy, since DNP does not inhibit the process. For some reason yet unknown, the degradation reaches completion when 50–60% of the RNA made in the presence of chloromycetin has been degraded.

The re-addition of chloromycetin to cells which have already degraded the RNA made during previous chloromycetin treatment results in an uptake from the medium, and a repolymerization into RNA, of the fragments present from the previous destruction. This phenomenon likewise is a rapid one, requiring only 40 minutes for its completion. Both an energy source and phosphate are necessary for this re-synthesis; DNP blocks it completely.

When radioactive phosphate was used to mark the unstable RNA, it was found that the degradation of RNA was accompanied by a parallel loss from the cells of about 50% of their radioactivity. Re-addition of chloromycetin initiated a resynthesis of RNA which, however, was not accompanied by a concomitant uptake of radioactive phosphate. This isotope had equilibrated with the non-radioactive phosphate present in the medium in a ten-fold excess. This result indicates that the degradation probably proceeds beyond the nucleotide stage*.

The phenomenon of resynthesis emphasizes a peculiar aspect of the action of chloromycetin. Cells that have ceased to make protein and RNA because of a deficit

* This interpretation has been confirmed by more recent work in which uracil and hypoxanthine have been identified as two of the fragments.

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of an essential amino acid (but which have not been starved for a long period) will, upon the addition of chloromycetin, accumulate RNA quite rapidly. Thus, under certain conditions, chloromycetin seems to initiate a reaction which does not proceed in its absence. Two different explanations of this phenomenon suggest themselves: (a) chloromycetin could increase the rate of formation of an inherently unstable RNA, or (b) chloromycetin might block a reaction which destroys RNA, allowing it to accumulate by some independent process.

Theoretically, these two explanations are testable. The former hypothesis requires that there be a continued renewal of RNA in the presence of chloromycetin, while the latter predicts no renewal under these conditions. Attempts have been made to detect such a renewal of RNA using adenine-8-$^{14}$C (results not shown in this paper) and using $^{32}$P-phosphate. In both cases an extremely slow rate of incorporation due to renewal of RNA was observed. The rates, however, were insignificant compared to the rate of degradation of RNA observed when chloromycetin is removed or to the rate of its resynthesis when chloromycetin is readded. These results thus tend to eliminate the possibility that chloromycetin increases the rate of formation of a metabolically unstable RNA. The second hypothesis, that chloromycetin acts by blocking a reaction which destroys RNA, is rendered less likely by the observation that destruction of RNA will occur in the presence of chloromycetin if DNP is added, thus underlining the complexity of the phenomenon. The situation can probably best be verbalized by saying that both chloromycetin and an energy supply seem necessary for the stability of this fraction of RNA.

## SUMMARY

A major portion of the ribonucleic acid formed by *Escherichia coli* in the presence of chloromycetin is rapidly degraded *in vivo* upon removal of the antibiotic. The fragments formed by the degradation accumulate in the medium, and, upon the readdition of chloromycetin, re-enter the cells and are repolymerized into RNA. The bearing of these findings on the action of chloromycetin is discussed.

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