# INHIBITION OF PROTEIN SYNTHESIS IN MAMMALIAN CELL-FREE SYSTEMS BY CHLORAMPHENICOL*, ‡

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Although chloramphenicol is known to inhibit microbial protein synthesis both in intact cells (1–3) and in cell-free systems (4–8), similar inhibition of protein synthesis does not occur in analogous mammalian systems unless large amounts of chloramphenicol are employed (9–12). The difference in sensitivity to chloramphenicol is most striking in cell-free systems, where cellular permeability is not a factor. Thus complete inhibition of protein synthesis in *Escherichia coli* cell-free systems can be obtained with 0.15 micromoles of chloramphenicol per ml reaction mixture (7). In contrast, comparatively little inhibition of protein synthesis in mammalian cell-free systems has been obtained with 5 to 10 micromoles of the drug per ml reaction mixture (10, 11). The findings of Djordjevic and Szybalski (13) and of Ambrose and Coons (14) appear to be exceptions to these observations. In these studies inhibition of protein synthesis was obtained in tissue cultures with low levels of chloramphenicol providing the drug was in contact with the cells during periods of active protein synthesis.

Despite the difference in sensitivity to chloramphenicol, the requirements for protein synthesis in both microbial and mammalian cell-free systems are remarkably similar and the components involved are even interchangeable (15, 16). The mechanism by which chloramphenicol inhibits protein synthesis in cell-free systems has been studied intensively. It has been found that chloramphenicol does not affect the activation of amino acids or the formation of amino acyl soluble RNA (*s*-RNA) in either mammalian or bacterial cell-free systems (8, 18). In bacterial cell-free systems, however, it has been demonstrated that chloramphenicol interferes with the transfer of amino acids from soluble RNA to ribosomes (8, 17, 18). Since chloramphenicol does not appear to inhibit this step in mammalian systems, it has been suggested that the difference in sensitivity of microbial and mammalian cell-free systems to chloram-

phenicol may be due to a selective effect of the drug on bacterial ribosomes and that this selective effect may be due to functional differences in the ribosomes. Von Ehrenstein and Lipmann have suggested the possibility that the difference in sensitivity may be due to the presence in bacteria of an extra, drug-sensitive step involved in the transfer of amino acids from soluble RNA to ribosomes (11).

Although there is no recognizable effect of chloramphenicol on protein synthesis by mammalian cells (with the exception of the aforementioned tissue culture experiments), hematopoietic depression attributable to chloramphenicol is a well known phenomenon (19–21). The mechanism by which chloramphenicol exerts a toxic effect on human hematopoietic cells remains unknown but can be correlated with high blood concentrations (23, 24) and impaired clearance of the drug (22). Hematopoietic toxicity is also increased in certain conditions characterized by the presence of immature or proliferating erythrocytes. Saidi, Wallerstein, and Aggeler (25) have shown that administration of chloramphenicol to patients with pernicious anemia in relapse blocks the reticulocyte response to vitamin $B_{12}$. A similar lack of reticulocyte response was observed in patients with iron deficiency anemia receiving chloramphenicol during iron therapy. Since inhibition occurs uniformly during the proliferative phases of erythropoiesis it would appear that primitive erythrocytes are markedly susceptible to chloramphenicol inhibition, whereas cells at a later stage of maturation are relatively resistant.

The observations on the sensitivity of immature erythrocytes to chloramphenicol provide an approach to studying the discrepancy between the effect of chloramphenicol on protein synthesis in mammalian systems as compared to microbial systems. Presumably the initial steps in the maturation of erythrocytes require the formation and deposition of ribosomal template RNA capable of directing the synthesis of hemoglobin and of other proteins necessary for cellular development. In mammalian cells template RNA is stable, remaining firmly bound to the ribosome as long as the cell is capable of protein synthesis and is not replenished (26). The possibility therefore exists that the increased susceptibility of primitive erythrocytes to chloramphenicol might be due to an inhibitory effect of the drug on either the ribosomal deposition or function of template RNA being formed during the initial phases of maturation and that more mature cells are resistant to chloramphenicol because template RNA is already firmly bound to ribosomes. The present experiments were designed to simulate the sequence of events in maturing erythrocytes by stimulating the protein synthesis of reticulocyte ribosomes with added template RNA and then determining whether chloramphenicol could inhibit this RNA-induced protein synthesis. Inhibition of protein synthesis induced by the added template RNA was demonstrable with low concentrations of chloramphenicol whereas there was no significant inhibition of protein synthesis attributable to template RNA previously bound to the ribosome (endogenous protein synthesis). A preliminary report on some aspects of these studies has been published (27).

## Methods

The cell-free system employed was a modification of those described by Matthaei and Nirenberg (6) and by Allen and Schweet (12), and consisted of rabbit reticulocyte ribosomes, a supernatant factor prepared from reticulocytes, an energy generating system, amino acids, and buffer. The incorporation of $U-C^{14}$ L-leucine or $U-C^{14}$ L-phenylalanine into trichloracetic acid (TCA)–precipitable material was used as an index of protein synthesis.

The basic plan of the experiments was to determine the increase in protein synthesis stimulated by added template RNA (synthetic or ribosomal) and then to determine the extent to which chloramphenicol could inhibit this induced protein synthesis. The net gain in $C^{14}$ amino acid incorporation was determined by subtracting the amino acid incorporation obtained in control tubes from that obtained in the presence of stimulatory RNA. The net gain with stimulatory RNA in the presence of chloramphenicol was then determined and the results expressed as per cent inhibition.[1]

*Preparation of Ribosomes.*—Reticulocytosis was produced in white New Zealand rabbits with phenylhydrazine by the method of Borsook *et al.* (28), and ribosomes were prepared by a modification of the method of Allen and Schweet (12). Blood with a reticulocyte count of 85 to 95 per cent was obtained by cardiac puncture and washed with 0.13 M NaCl, 0.005 M KCl, and 0.0075 M $MgCl_2$. The packed cells were lysed with 4 volumes of 0.002 M $MgCl_2$, stirred at 4°C for 10 minutes, after which 1 volume of 1.5 M sucrose containing 0.15 M KCl was added. The mixture was stirred for 10 minutes and centrifuged at 15,000 g for 30 minutes at 4°C. The sediment was discarded and the supernate was centrifuged for 2 hours at 105,000 g in a Spinco model L preparative ultracentrifuge at 4°C. The supernatant fluid was subsequently used to prepare pH 5 enzyme (29). The ribosomal pellet was rinsed and resuspended in 0.002 M $MgCl_2$, 0.0175 M $KHCO_3$, and 0.25 M sucrose to the original volume, and then centrifuged at 4°C for 1 hour at 105,000 g. This supernatant was discarded and the pellet rinsed with 0.25 M sucrose. The ribosomal pellet was then resuspended with 0.25 M sucrose in a loose fitting glass homogenizer and the final volume adjusted so as to be equivalent to the original packed cell volume. The ribosomal suspension was centrifuged at 15,000 g for 5 minutes, the sediment discarded, and aliquots of the supernate, containing 1.5 to 3.0 mg protein per ml, were stored at −65°C. The preparations retained activity for at least 4 weeks.

Ribosomes were also obtained from the peripheral blood of patients with sickle cell anemia or from patients with pernicious anemia with reticulocytosis by the same method.

*Preparation of Supernatant Factors.*—A pH 5 enzyme containing soluble RNA, amino acid–activating enzymes, and transfer enzymes was prepared from reticulocytes by a modification of the method of Keller and Zamecnik (29) using the supernatant obtained after centrifuging the lysed reticulocytes for 2 hours at 105,000 g. This supernate contained large amounts of hemoglobin, and the procedure to be described removed all but traces of hemoglobin and other interfering substances. Three volumes of 0.9 M sucrose, 0.004 M $MgCl_2$, and 0.025 M KCl (medium B of Keller and Zamecnik) were added to the reticulocyte supernate and the pH adjusted to 5.2 by slowly adding 1 N acetic acid. All solutions were kept at 4°C during this and subsequent preparative procedures. The precipitate was recovered by centrifugation at 10,000 g for 15 minutes, washed with medium B, and then resuspended by homogenization in glass with 40 to 50 ml of medium B. The protein pellet was again collected by centrifugation at 30,000 g for 10 minutes. The pellet was rinsed with 0.35 M sucrose, 0.035 M $KHCO_3$, 0.004 M $MgCl_2$, and 0.025 M KCl (medium A of Keller and Zamecnik) and then dissolved in this same

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[1] Per cent inhibition =

$$100 - \frac{\text{net gain in amino acid incorporation with chloramphenicol}}{\text{net gain in amino acid incorporation without chloramphenicol}} \times 100.$$

solution by homogenization. The final volume was adjusted to half the original packed cell volume. Insoluble protein was removed by centrifugation at 30,000 g for 10 minutes. The supernatant solution, containing approximately 2 to 3 mg of protein per ml, was stored in aliquots at −65°C and retained activity for more than 4 weeks. Protein determinations were made by the Folin phenol method of Lowry (30).

*Preparation of Ribosomal Template RNA.*—Ribosomal template RNA was isolated from ribosomes which had been stored in 0.25 M sucrose at −65°C by a phenol extraction method using freshly distilled phenol (7, 31). The concentration of the phenol was adjusted to 80 per cent with 0.02 M phosphate buffer, pH 7.2, and the ribosomes were diluted with an equal volume of the same buffer. The ribosomal suspension was then shaken with an equal volume of 80 per cent phenol at room temperature for 15 minutes. The aqueous phase was aspirated from the phenol phase after centrifugation at 3900 g at 0°C for 5 minutes and was then extracted two more times in the same manner, using ½ volume of 80 per cent phenol each time. The final aqueous phase was chilled to 4°C; 0.1 of the volume of 20 per cent sodium acetate and 0.01 of the volume of glacial acetic acid were added and the RNA precipitated with 2 volumes of 95 per cent ethanol at −20°C. The suspension was centrifuged at 20,000 g for 15 minutes and the supernate was discarded. The sediment was suspended by homogenization in glass using a minimal volume of standard buffer (0.01 M tris, 0.01 M magnesium acetate, 0.06 M KCl, pH 7.8). The RNA suspension was then dialyzed for 18 hours at 4°C against 100 volumes of standard buffer with 2 changes of the dialyzing buffer. After dialysis, the RNA solution was centrifuged at 5000 g for 15 minutes. Both the supernate and the sediment (redissolved in standard buffer) were stored at 4°C and retained biologic activity for more than 4 weeks. RNA content was estimated by the orcinol method, using d-ribose as a standard. The concentration of various RNA preparations used in these experiments ranged from 20 to 850 micrograms per ml.

*Reaction Mixtures and Assay Techniques.*—The components of the reaction mixture were similar to those employed by Matthaei and Nirenberg (6). Each reaction mixture contained 100 μmoles of tris (hydroxymethyl) aminomethane, pH 7.8; 10 μmoles magnesium acetate; 50 μmoles KCl; 6 μmoles mercaptoethanol; 0.05 μmole of 19 $C^{12}$ L-amino acids (9); 0.0045 μmole of U-$C^{14}$ L-leucine (approximately 400,000 CPM); 0.025 μmole guanosine triphosphate (GTP); 5 μmoles phosphoenolpyruvate (PEP); 1 μmole K₂ATP; 20 μg of pyruvate kinase; 0.2 to 0.4 mg of pH 5 protein, and 0.15 to 0.25 mg of ribosomal protein.

A balanced amino acid mixture (M-1) containing 19 $C^{12}$ L-amino acids, standard buffer, mercaptoethanol, MgCl₂, KCl, and GTP was prepared so that 0.2 ml of the mixture contained the desired amounts of each compound and was stored at −65°C. Ribosomes, pH 5 enzyme and M-1 were thawed immediately prior to each experiment; the desired amounts of PEP (2.0 mg per each reaction mixture), K₂ATP (0.7 mg per each reaction mixture), and kinase (0.002 μg protein per each reaction mixture) were added. Aliquots were added to tubes containing the desired amounts of chloramphenicol and RNA, and the final volume was adjusted to 1.0 ml with H₂O. All components were added at 4°C and incubation was then carried out in a water bath for 1 hour at 37°C. The reaction was terminated by the addition of 2 ml of 5 per cent TCA. The protein precipitate was collected by centrifugation, washed with 2 ml of 5 per cent TCA, and then extracted with 5 per cent TCA at 90°C for 20 minutes. The precipitate was again washed with 5 per cent TCA, washed twice with ethanol-ether (3:1), and then with anhydrous ether. The residue was dried, dissolved in 1.3 ml of concentrated formic acid, and 1.0 ml aliquots were counted in a liquid scintillation counter (Nuclear-Chicago).

When polyuridylic acid (poly U) was used to stimulate protein synthesis, the reaction mixtures and assay techniques were identical with those employed above except that U-$C^{14}$ L-phenylalanine incorporation was used as an index of protein synthesis instead of $C^{14}$ L-leucine.

## Materials

2-Phosphoenolpyruvic acid (crystalline tricyclohexylammonium salt), pyruvate kinase (muscle) A grade, specific activity 168 to 254 enzyme units/mg protein, and GTP were obtained from the California Corporation for Biochemical Research, Inc., Los Angeles. ATP (dipotassium salt) was obtained from the Nutritional Biochemicals Corporation, Cleveland. Uniformly labeled $C^{14}$ L-leucine (specific activity 50 to 112 mc/mmole) and U-$C^{14}$-L-phenyl-alanine (specific activity 141 $\mu$c/$\mu$mole) was obtained from Schwarz BioResearch, Inc., Orangeburg, New York. Crystalline chloramphenicol was supplied through the courtesy of Parke, Davis and Company, Detroit. The glucuronide of chloramphenicol was isolated from urine by Dr. Robert Greenway of Highland View Hospital, Cleveland. [$CH_3S$] and [$CH_3SO_2$] analogs of chloramphenicol were obtained through the courtesy of Dr. C. M. Suter, Sterling-Winthrop Research Institute, Rensselaer, New York. The aceto analog of chloramphenicol was supplied by Warner-Lambert Research Institute, Morris Plains, New Jersey. Strepto-mycin was obtained from Nutritional Biochemicals Corporation. Erythromycin base was furnished by Lilly Laboratory for Clinical Research, Indianapolis. Crystalline tetracycline was furnished by American Cyanide Company, Pearl River, New York. Polyuridylic acid was purchased from Miles Chemical Company, Elkhart, Indiana.

### RESULTS

*Characteristics of the Cell-Free System.*—The requirements for protein synthesis by reticulocyte ribosomes were similar to those previously reported in analagous systems (6, 11, 12). The amount of protein synthesis was directly proportional to the amount of ribosomes added to each reaction mixture. Omission of any component such as the energy generating system, superna-tant factors, or ribosomes resulted in absent or markedly reduced incorpora-tion of radioactive amino acids into TCA-precipitable protein. Since $C^{14}$ amino acids of high specific activity were employed in these experiments, it was found that optimum protein synthesis was obtained if the amino acid mixtures were not made deficient in the corresponding $C^{12}$ amino acid.

*Stimulation of Protein Synthesis by Addition of Template RNA Derived from Ribosomes.*—Addition of ribosomal template RNA to the cell-free system re-sulted in a marked increase in the incorporation of $C^{14}$ leucine into TCA-precipi-table material, the increase in protein synthesis being directly proportional to the amount of RNA added to each reaction mixture. In a typical experiment 377 CPM (0.05 m$\mu$mole) of $C^{14}$ leucine were incorporated into protein per 0.15 mg of ribosomal protein in the absence of stimulatory RNA. With the addition of increasing amounts of template RNA to the system there was a progressive increase in the incorporation of $C^{14}$ leucine. The protein synthesis induced by adding 348 $\mu$g of template RNA resulted in the incorporation of 3748 CPM (0.46 m$\mu$mole) of $C^{14}$ leucine per 0.15 mg of ribosomal protein (Fig. 1). In other experiments, the maximum $C^{14}$ leucine incorporation induced by added tem-plate RNA amounted to as much as 14,454 CPM (1.8 m$\mu$moles) per 0.2 mg ribo-somal protein with 348 $\mu$g of RNA compared to 870 CPM (0.11 m$\mu$mole) of $C^{14}$ leucine incorporation obtained with unstimulated ribosomes.

The stimulatory activity of ribosomal template RNA varied with different preparations and was also dependent upon the state of the ribosomes. In various experiments, the net gain in $C^{14}$ leucine incorporation ranged up to 3200 CPM per $\mu$g of added template RNA per mg ribosomal protein. The amount of $C^{14}$ leucine bound by the added RNA in cell-free systems devoid of ribosomes was
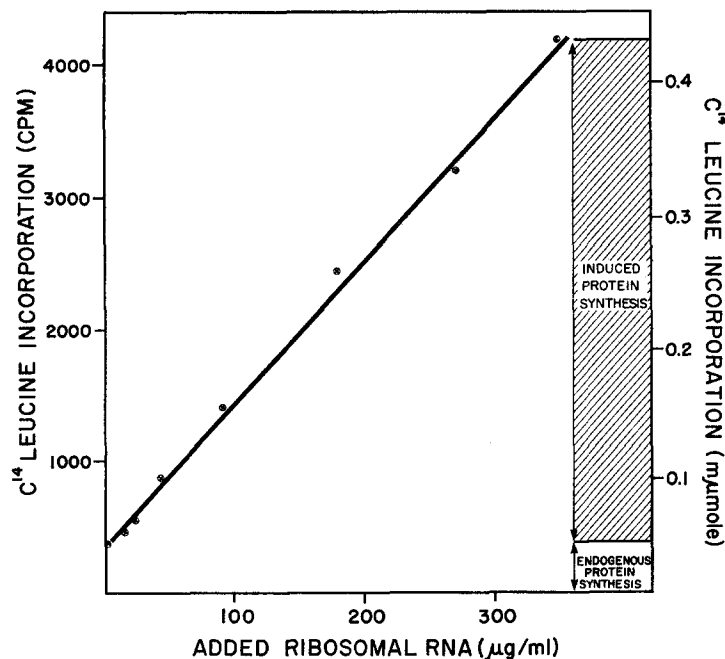


Fig. 1. Stimulation of reticulocyte ribosomal protein synthesis by template RNA isolated from ribosomes. Addition of template RNA to the cell-free systems resulted in an increased incorporation of $C^{14}$ leucine into protein (induced protein synthesis), the increase being proportional to the amount of RNA added. The incorporation of $C^{14}$ leucine into protein is expressed both as CPM and m$\mu$mole per 0.15 mg of ribosomal protein. Approximately 10 CPM (1 $\mu\mu$mole of $C^{14}$ leucine were incorporated into protein per $\mu$g of added RNA. In other experiments as much as 800 CPM of $C^{14}$ leucine were incorporated per $\mu$g of added RNA using an equivalent amount of ribosomes.

negligible. No attempt was made in any of these experiments to separate active components from the template RNA.

Failure to obtain stimulation of protein synthesis with added RNA was more often due to inadequate ribosomal activity rather than to inactivity of the added RNA. Preincubation of the ribosomes was usually necessary to obtain significant stimulation by the added RNA. Preincubation of the cell-free system was carried out for 30 minutes at 37°C in the absence of $C^{14}$ leucine. After this initial incubation period, $C^{14}$ leucine was added, and aliquots of the

completed cell-free system were pipetted into tubes containing the desired amounts of RNA and buffer. Incubation was then carried out for an additional 60 minutes before terminating the experiment by the addition of TCA. It was not necessary to replenish the energy-generating system or to add additional pH 5 enzyme during this second period of incubation. The net gain in $C^{14}$
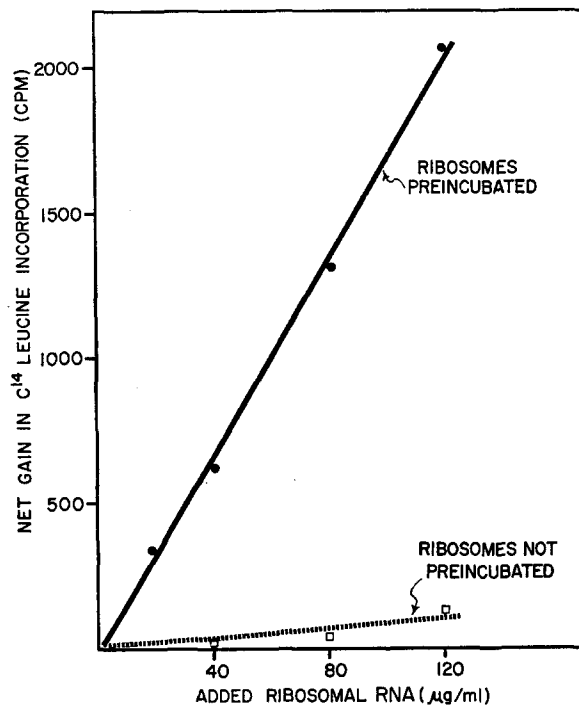


Fig. 2. Stimulation of reticulocyte ribosomal protein synthesis by added template RNA. The amount of RNA-induced protein synthesis obtained with preincubated ribosomes is compared with that obtained with ribosomes not preincubated. The net gain in $C^{14}$ leucine incorporation into protein with preincubated ribosomes is much greater than that obtained with the unmodified ribosomes. The net gain in $C^{14}$ leucine incorporation is expressed in CPM per 0.2 mg ribosomal protein.

leucine incorporation into protein was much greater with ribosomes which were preincubated (Fig. 2). Ribosomes stored for 4 weeks or longer at $-65°C$ in the absence of magnesium or subjected to low magnesium concentrations for 1 hour at room temperature after thawing could be stimulated without preincubation. Optimum results were, however, obtained by preincubation of the ribosomes as described above and were uniformly employed in the experiments reported.

*Chloramphenicol Inhibition of Protein Synthesis Induced by Added Template RNA.*—Chloramphenicol inhibited the protein synthesis induced by added

TABLE I

*Inhibition of Induced Ribosomal Protein Synthesis by Chloramphenicol*

|  |  | Net gain | Inhibition |
| --- | --- | --- | --- |
|  | CPM | CPM | *per cent* |
| Complete system...................................... | 790* | — | — |
| Complete system + 1.0 μmole chloramphenicol............ | 720 | — | 9 |
| Complete system + 2 μg template RNA................... | 1537 | 747§ | 0 |
| Complete system + 2 μg template RNA + 0.0001 μmole chloramphenicol...................................... | 1438 | 648 | 13‡ |
| Complete system + 2 μg template RNA + 0.001 μmole chloramphenicol...................................... | 1305 | 515 | 30 |
| Complete system + 2 μg template RNA + 0.01 μmole chloramphenicol...................................... | 1007 | 217 | 71 |
| Complete system + 2 μg template RNA + 0.1 μmole chloramphenicol...................................... | 831 | 41 | 95 |

* $C^{14}$ leucine incorporation expressed in CPM per 0.2 mg ribosomal protein.

‡ Per cent inhibition $= 100 - \left( \dfrac{\text{Net gain in CPM}}{747} \times 100 \right)$.

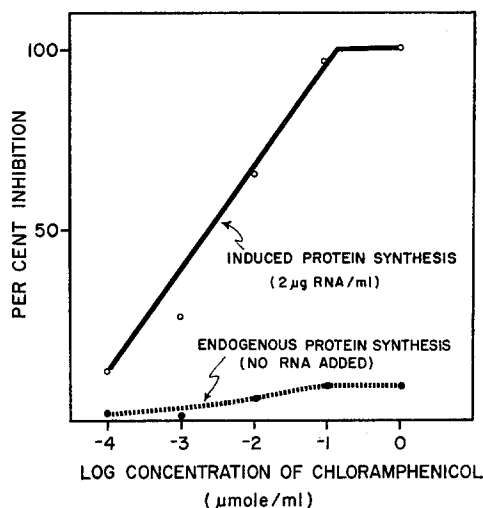§ Equivalent to 0.095 mμmole of additional $C^{14}$ leucine incorporation.



FIG. 3. Chloramphenicol inhibition of ribosomal protein synthesis induced by added template RNA. Protein synthesis occurring without added template RNA (endogenous) was not significantly inhibited by chloramphenicol. In contrast, the additional protein synthesis induced by added template RNA was completely inhibited by chloramphenicol. Beginning inhibition was observed with 0.0001 micromole of chloramphenicol (0.03 microgram per ml reaction mixture), and almost complete inhibition was obtained with 0.1 micromole (32 micrograms). Reaction mixtures were added directly to the tubes containing 2 micrograms of RNA and serial dilutions of chloramphenicol.

template RNA but had comparatively little inhibitory effect on the unstimulated (endogenous) protein synthesis of ribosomes. For example, chloramphenicol decreased endogenous ribosomal protein synthesis by less than 10 per cent when present in concentrations up to 1.0 micromole per ml. In contrast, beginning inhibition of the protein synthesis induced by added RNA was usually obtained with 0.001 micromole of chloramphenicol per ml but could be obtained with as little as 0.0001 micromole of chloramphenicol per ml (Table I, Fig. 3). Almost complete inhibition of the induced protein synthesis was usually obtained with 0.1 micromole of chloramphenicol per ml.

TABLE II

*Inhibition of Induced Ribosomal Protein Synthesis by Chloramphenicol in Heterologous Systems*

|  | Net gain | Inhibition |
|---|---|---|
| *Rabbit reticulocyte ribosomes (0.29 mg protein) + rabbit liver ribosomal template RNA* | | |
| | CPM | CPM | *per cent* |
| Complete system.......................................... | 1606 | — | — |
| Complete system + 97 μg RNA........................... | 2920 | 1314 | — |
| Complete system + 97 μg RNA + 0.1 μmole chloramphenicol....................................... | 1839 | 233 | 82 |
| *Rabbit reticulocyte ribosomes (0.25 mg protein) + dog liver ribosomal template RNA* | | |
| Complete system.......................................... | 267 | — | — |
| Complete system + 38 μg RNA........................... | 583 | 276 | — |
| Complete system + 38 μg RNA + 0.1 μmole chloramphenicol....................................... | 351 | 84 | 70 |
| *Human reticulocyte ribosomes (0.15 mg protein) + rabbit reticulocyte ribosomal template RNA* | | |
| Complete system.......................................... | 410 | — | — |
| Complete system + 2 μg RNA............................ | 7190 | 6780 | — |
| Complete system + 2μg RNA + 0.1 μmole chloramphenicol .. | 480 | 70 | 99 |

Protein synthesis induced by template RNA derived from other sources was also inhibited by the addition of chloramphenicol. Increased incorporation of $C^{14}$ leucine into TCA-precipitable material was obtained when rabbit reticulocyte ribosomes were incubated with template RNA isolated from either rabbit liver ribosomes or dog liver ribosomes. In all instances almost complete inhibition of the induced protein synthesis was observed when chloramphenicol was added in a concentration of 0.1 micromole per ml (Table II). Similarly, addition of rabbit reticulocyte template RNA to *human reticulocyte ribosomes* resulted in an increase in protein synthesis which was completely inhibited by adding chloramphenicol in a concentration of 0.1 micromole per ml (Table II).

The effect of chloramphenicol in blocking induced protein synthesis could

not be overcome by adding increasing amounts of template RNA to the reaction mixture (Fig. 4). Essentially the same percentage of inhibition was obtained with a given concentration of chloramphenicol in cell-free systems stimulated with 2 $\mu$g of template RNA per ml as was obtained when 348 $\mu$g of RNA per ml was added.
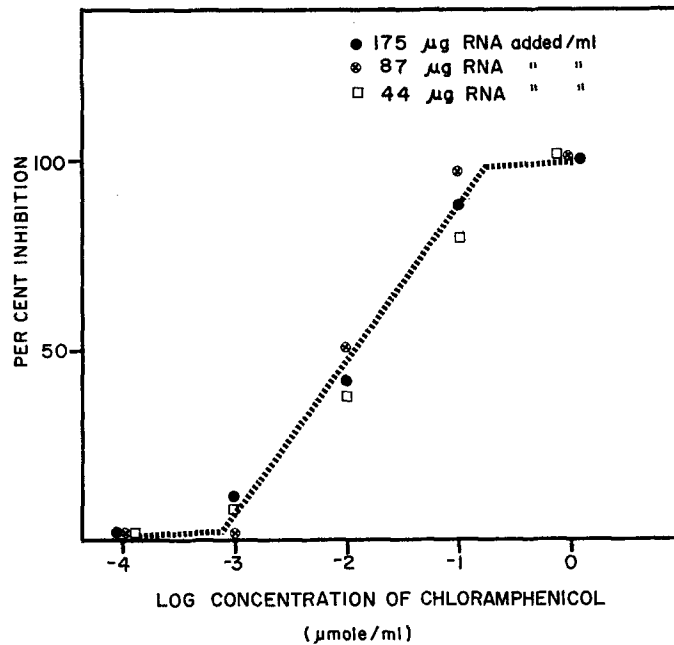


FIG. 4. The effect of increasing amounts of added template RNA on chloramphenicol inhibition of induced protein synthesis. Chloramphenicol inhibition of induced protein synthesis could not be abolished by adding increasing amounts of template RNA. In this experiment essentially the same percentage of inhibition was obtained with a given concentration of chloramphenicol whether the cell-free system was stimulated with 44 $\mu$g of RNA or 175 $\mu$g of RNA. In other experiments similar results were obtained with RNA concentrations ranging from 2 to 348 $\mu$g per ml reaction mixture.

The effect of chloramphenicol on the binding of added template RNA by ribosomes was studied in the following manner. P[32]-labeled template RNA was prepared from E. coli K12 $\lambda$ ribosomes and added to rabbit reticulocyte ribosomes previously preincubated in a complete system. After 10 minutes' further incubation, the mixture was chilled to 0°C and the ribosomes separated by centrifugation at 105,000 g for 60 minutes at 4°C, washed with 0.25 M sucrose, and dissolved in concentrated formic acid. Similar preparations were made in which chloramphenicol was added to the system together with the P[32] RNA

after preincubation of the ribosomes. There was a progressive decrease in the amount of $P^{32}$-labeled RNA bound to the ribosomes with increasing concentrations of chloramphenicol, the maximum decrease being 50 per cent in the presence of 0.1 $\mu$mole of chloramphenicol per ml (Table III).

Chloramphenicol inhibited induced protein synthesis with equal effectiveness if added simultaneously with the stimulatory RNA or if present in the cell-free system prior to the addition of the RNA. There was a progressive decrease in the inhibitory effect of chloramphenicol if it was added after incubation with template RNA was begun, so that no significant inhibition was observed when chloramphenicol was added 40 minutes after the RNA.

*Inhibition of RNA-Induced Protein Synthesis by Chloramphenicol Analogs*

TABLE III

*The Effect of Chloramphenicol on Ribosomal Binding of $P^{32}$-Labeled Template RNA*

|  | Ribosomal binding of $P^{32}$ RNA* | |
| --- | --- | --- |
|  | cpm‡ | *per cent* |
| Ribosomes + $P^{32}$ RNA | 2090 | 100 |
| Ribosomes + 0.001 $\mu$mole chloramphenicol + $P^{32}$ RNA | 1600 | 79 |
| Ribosomes + 0.01 $\mu$mole chloramphenicol + $P^{32}$ RNA | 1550 | 69 |
| Ribosomes + 0.1 $\mu$mole chloramphenicol + $P^{32}$ RNA | 983 | 47 |

\* 15 $\mu$g $P^{32}$ RNA/ml.

‡ cpm per 0.2 mg ribosomal protein.

*and Other Antibiotics.*—Inhibition of the increased protein synthesis induced by added template RNA was also observed with analogs of chloramphenicol in which the $NO_2$ group is replaced by $[CH_3SO_2]$, $[CH_3S]$ or aceto groups. These compounds have antimicrobial activity comparable to that of chloramphenicol and were as effective as chloramphenicol in inhibiting induced protein synthesis (Table IV). In contrast, the glucuronide detoxification product of chloramphenicol which has no antibiotic activity was completely lacking in an inhibitory effect.

The effect of streptomycin, tetracycline, erythromycin, and penicillin on RNA-induced protein synthesis was also determined. None of these antibiotics inhibited protein synthesis by ribosomes to any significant extent in the absence of stimulatory RNA (endogenous protein synthesis). When protein synthesis was stimulated with added template RNA, a 27 per cent inhibition of the induced protein synthesis was obtained with streptomycin (0.01 $\mu$mole per ml) compared to a 73 per cent inhibition obtained with chloramphenicol (0.01 $\mu$mole per ml) under identical conditions. In the same experiment, addition of erythromycin (0.01 $\mu$mole per ml) resulted in 100 per cent inhibition, and addition of tetracycline (0.01 $\mu$mole per ml) resulted in 75 per cent inhibition

whereas penicillin (1000 units per ml) was completely devoid of any inhibitory activity (Table IV). The inhibitory activity of streptomycin did not increase with greater concentrations of the drug, essentially the same percentage of inhibition being obtained with 0.1 $\mu$mole as was obtained with 0.001 $\mu$mole per ml. Inhibition of induced protein synthesis by both erythromycin and tetracycline was linear with respect to concentration.

*Chloramphenicol Inhibition of Protein Synthesis Induced by Poly U.*—Addition of poly U to a cell-free system also resulted in a marked increase in protein synthesis as measured by incorporation of $C^{14}$ phenylalanine into TCA-precipitable material (Fig. 5). Manipulation of the ribosomes was not necessary to

TABLE IV

*Inhibitory Effect of Other Antibiotics on Induced Protein Synthesis*

| | Inhibition of induced protein synthesis |
|---|---|
| | *per cent* |
| Chloramphenicol, 0.01 $\mu$mole/ml | 73 |
| Chloramphenicol analogs | |
|     $CH_3SO_2$ analog, 0.01 $\mu$mole/ml | 78 |
|     $CH_3S$ analog, 0.01 $\mu$mole/ml | 68 |
|     Aceto analog, 0.01 $\mu$mole/ml | 71 |
| Chloramphenicol glucuronide, 0.01 $\mu$mole/ml | 0 |
| Streptomycin, 0.01 $\mu$mole/ml | 27 |
| Erythromycin, 0.01 $\mu$mole/ml | 100 |
| Tetracycline, 0.01 $\mu$mole/ml | 75 |
| Penicillin, 1000 units/ml | 0 |

obtain poly U stimulation; essentially the same amount of $C^{14}$ phenylalanine was incorporated when preincubated or non-preincubated ribosomes were used in the reaction mixtures. The maximum amount of stimulation was obtained when 75 $\mu$g per ml of poly U were added to the reaction mixture, the addition of larger amounts did not result in more incorporation of $C^{14}$ phenylalanine. Approximately 20 $\mu\mu$moles of phenylalanine were incorporated per $\mu$g of poly U added when 0.25 mg of ribosomes were present in the system.

Inhibition of the protein synthesis induced by added poly U was obtained with chloramphenicol but could be overcome by adding increasing amounts of poly U. Complete inhibition was obtained with 0.1 $\mu$mole of chloramphenicol per ml reaction mixture when poly U was added in amounts up to 2.5 $\mu$g per ml (Table V). A progressive decrease in the amount of inhibition obtained with 0.1 $\mu$mole of chloramphenicol was observed when the amount of poly U added was increased, no chloramphenicol inhibition being obtained when 25 $\mu$g of poly U were present in the reaction mixture.

Chloramphenicol inhibition occurred only if it was present in the reaction
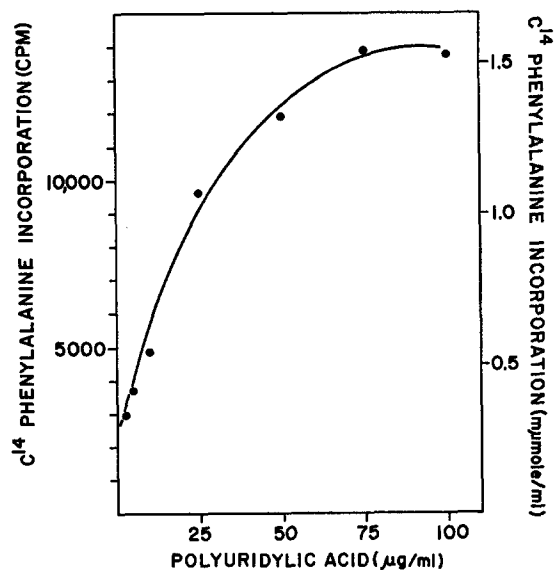
Fig. 5. Stimulation of protein synthesis induced by polyuridylic acid (poly U). Addition of increasing amounts of poly U resulted in an increased incorporation of $C^{14}$ phenylalanine into TCA-precipitable material. The maximum incorporation in this system was obtained with 75 $\mu$g of poly U per ml reaction mixture. Each reaction mixture contained 0.0014 $\mu$mole of $C^{14}$ phenylalanine (approximately 420,000 CPM), 0.05 $\mu$mole of $C^{12}$ phenylalanine, and 0.25 mg of ribosomal protein per ml. Other components of the reaction mixture are as given in Methods. Approximately 200 CPM of $C^{14}$ phenylalanine (25 $\mu\mu$moles of phenylalanine) were incorporated into protein per $\mu$g of poly U added, when 0.25 mg of ribosomes were present in the reaction mixture.

TABLE V

*Inhibition of Protein Synthesis Induced by Polyuridylic Acid*

| Amount of polyuridylic acid | Inhibition obtained with chloramphenicol (0.1 $\mu$mole/ml) |
|---|---|
| *$\mu$g/ml* | *per cent* |
| 1.0 | 100 |
| 2.5 | 100 |
| 5.0 | 62 |
| 10.0 | 17 |
| 25.0 | 0 |

mixture prior to the addition of poly U or if added simultaneously with poly U. No inhibition was demonstrable if chloramphenicol was added after poly U.

The [$CH_3SO_2$], [$CH_3S$] and aceto analogs of chloramphenicol were as effective as chloramphenicol in decreasing protein synthesis induced by poly U.

DISCUSSION

These experiments demonstrate that under certain conditions chlorampheni-col can inhibit protein synthesis in mammalian cell-free systems as effectively as it inhibits protein synthesis in analogous microbial systems. Significant inhibition in mammalian systems was obtained only when protein synthesis was stimulated by the addition of template RNA to the system, there being comparatively little inhibition of protein synthesis in the absence of stimula-tory RNA. Current evidence indicates that protein synthesis in bacteria is mediated by a continual supply of rapidly turning over RNA (32, 33) whereas in mammalian cells template RNA is comparatively stable, remaining attached to the ribosomes as long as the cell is capable of protein synthesis (26). Since chloramphenicol inhibits protein synthesis in mammalian cell-free systems only during the phase of addition of template RNA, the apparent discrepancy in the sensitivity of mammalian and microbial cells to chloramphenicol may be related to the known difference in rates of turnover or stability of template RNA, rather than to intrinsic differences in the mechanism of protein synthesis. These observations are in accord with the hypothesis that protein synthesis in intact mammalian cells may be susceptible to chloramphenicol inhibition only at the time that new messenger or template RNA is being formed. More mature cells may be resistant to chloramphenicol because informational RNA already deposited on ribosomes is not accessible to chloramphenicol inhibition.

There is now considerable evidence that chloramphenicol may inhibit pro-tein synthesis in mammalian cells either by interfering with the function of messenger RNA or by blocking its attachment to ribosomes. Ambrose and Coons (14) recently suggested that chloramphenicol may inhibit protein syn-thesis in mammalian cells by interfering with messenger RNA function. This suggestion was based on the ability of low levels of chloramphenicol to inhibit antibody synthesis in lymph node tissue cultures which were stimulated by the addition of antigen. Inhibition of antibody synthesis was obtained if chloramphenicol (0.15 μmole per ml) was present in the culture medium for at least 6 days following the introduction of antigen. It was suggested that the continuous presence of chloramphenicol in the culture media inhibited antibody synthesis by interfering with the function of new messenger RNA formed in response to the antigenic stimulus. The inhibition of protein synthesis in human bone marrow cultures exposed to prolonged low levels of chloramphenicol (0.12 μmole per ml) described by Djordjevic and Zybalski (13) may also be due to a similar mechanism.

The present experiments offer more direct evidence that chloramphenicol can inhibit the function of messenger RNA. The results are in accord with the hypothesis that chloramphenicol inhibits the function of template RNA by blocking its attachment to ribosomes rather than by direct inactivation of RNA. Addition of increasing amounts of template RNA to the cell-free system

did not alter or overcome the amount of inhibition attributable to a given concentration of chloramphenicol. If chloramphenicol exerted its inhibitory effect by combining with template RNA to inactivate it, the addition of increasing amounts of RNA should have resulted in a progressive decrease in chloramphenicol inhibition. Kúcan and Lipmann (47) have also noted that chloramphenicol inhibition is independent of the amount of E. coli m-RNA added to stimulate protein synthesis in microbial cell-free systems. Since chloramepheni-
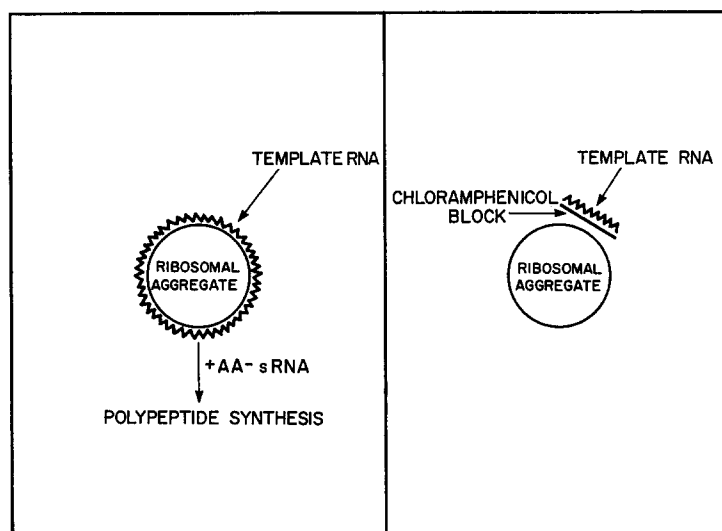


Fig. 6. Hypothesis on mechanism of action of chloramphenicol. The results of these studies suggest that chloramphenicol may inhibit protein synthesis in both bacterial and mammalian cells by interfering with the function of newly formed messenger or template RNA. Chloramphenicol may interfere with the function of informational RNA by successfully competing for ribosomal binding sites and thereby preventing its attachment to ribosomal subunits. (AA, amino acid.)

col does not inhibit protein synthesis in the absence of stimulatory RNA and since chloramphenicol does not directly inactivate template RNA, it is concluded that chloramphenicol may exert an inhibitory effect by interfering with the attachment of template RNA to ribosomes (Fig. 6). The decrease in ribosomal binding of $P^{32}$ RNA in the presence of chloramphenicol as demonstrated in the present report also suggests that this may be an important mechanism of action of chloramphenicol. Jardetzky and Julian (46) have recently demonstrated by ultracentrifugation studies employing sucrose gradients, that chloramphenicol does prevent the binding of $C^{14}$ poly U to E. coli ribosomes, and we have confirmed these findings with labeled RNA and reticulocyte ribosomes (34). Rendi and Ochoa (8) have previously suggested that chloram-

phenicol might interfere with the attachment of messenger RNA to ribosomes basing their suggestion on the accumulation of messenger-like RNA in bacteria treated with chloramphenicol (35–38).

The mechanism by which chloramphenicol might prevent the attachment of messenger or template RNA to ribosomes is suggested by the studies of Jardetzky (39). Jardetzky demonstrated on the basis of nuclear magnetic resonance studies that the steric configuration of chloramphenicol bears a striking resemblance to uridine-5'-phosphate. Chloramphenicol may therefore inhibit the binding of new template RNA by competing with uridylic acid residues for ribosomal attachment. Such inhibition of binding between ribosomes and RNA would explain why chloramphenicol decreases protein synthesis induced by added template RNA. Kúcan and Lipmann (47) have recently demonstrated in E. coli cell-free systems that protein synthesis stimulated by synthetic copolynucleotides containing guanylic acid (poly UG) or cytidylic acid (poly UC) can be inhibited by chloramphenicol. These results are consistent with an hypothesis that chloramphenicol inhibits attachment of RNA to E. coli ribosomes by competing with pyrimidine nucleotide residues.

The polyribosomes, structures containing several ribosomal subunits, probably held together by messenger RNA, are the major sites of protein synthesis in reticulocytes (26, 40, 41). These polyribosomes break down to 80S units coincident with hemoglobin synthesis (42). It may be postulated that in the present experiments, preincubation of the ribosomes results in a dissociation of polyribosomes coincident with endogenous protein synthesis and that added template RNA becomes associated with ribosomal subunits resulting in stimulation of protein synthesis. When chloramphenicol is added to the system it inhibits the stimulatory effect of the added template by successfully competing for ribosomal binding sites, thereby preventing the combination of RNA and ribosomal units necessary for the induction of protein synthesis.

Although the stimulation of protein synthesis with added template RNA usually required the use of preincubated ribosomes, poly U stimulation of protein synthesis was readily obtained with ribosomes which were not previously preincubated. Poly U may be more potent than natural polyribonucleotides in stimulating protein synthesis because of differences in binding to ribosomes or because the increased numbers of uridylic acid residues allow for more effective attachment to ribosomes. Similar observations were made by Okamoto and Takanami (43) who demonstrated that poly U was more effective than natural polyribonucleotides in effecting formation of polyribosome complexes. The inability of chloramphenicol to decrease poly U stimulation when added 10 minutes after poly U, also suggests that poly U attachment to ribosomes is either more firm or more rapid than that of added ribosomal template RNA. Template RNA isolated from ribosomes may be less effective than poly U because of the presence of non-specific RNA which may compete for binding sites but which is not effective in stimulating protein synthesis.

The ability of increasing amounts of poly U to overcome chloramphenicol inhibition also suggests that there may be differences in the mode of action or differences in ribosomal binding of poly U as compared to template RNA isolated from ribosomes. It seems unlikely that the inability of added ribosomal template RNA to overcome chloramphenicol inhibition is due to adding inadequate amounts of active template RNA since no decrease in chloramphenicol inhibition was observed over a wide range.

Inhibition of protein synthesis by chloramphenicol derivatives is correlated with the antimicrobial effect. Analogs of chloramphenicol formed by the substitution of the $NO_2$ group by $[CH_3SO_2]$, $[CH_3S]$ or aceto groups are effective antimicrobial agents and were as effective as chloramphenicol in inhibiting protein synthesis induced by added template RNA. In contrast, the glucuronide of chloramphenicol, a detoxification product which has no antibacterial effect (44), was completely ineffective as an inhibitor of protein synthesis. The activity of these derivatives of chloramphenicol in inhibiting induced protein synthesis is in accord with the hypothesis that chloramphenicol inhibition may occur because of its steric resemblance to a pyrimidine nucleotide. The addition of glucuronic acid to the propanediol moiety of chloramphenicol alters the steric similarity to uridylic acid and results in the loss of antimicrobial effect as well as the loss of ability to inhibit protein synthesis. In comparison, substitution of the $NO_2$ group does not alter the steric configuration, and there is no loss of antimicrobial effect or a loss in the ability to inhibit protein synthesis.

Streptomycin, erythromycin, and tetracycline in low concentrations also inhibited protein synthesis induced by added RNA. Both erythromycin and tetracycline were as effective as chloramphenicol in inhibiting the induced protein synthesis. These compounds have previously been shown to have a mode of action similar to that of chloramphenicol in bacterial cell-free systems but were ineffective in mammalian cell-free systems unless excessive amounts of the drugs were employed. There is considerable evidence, however, that cell wall impermeability may exclude these drugs from sensitive sites within the cell and it is unlikely that a ribosomal site of action is an important mechanism with these drugs (45).

The ability of chloramphenicol to inhibit induced protein synthesis both in mammalian cell-free systems and in tissue culture correlates with the observations on the marked sensitivity of immature erythropoietic cells to chloramphenicol. In all of these situations it may be assumed that the function of newly formed messenger RNA is inhibited by chloramphenicol. It is tempting to speculate that similar mechanisms may be involved in the hematopoietic toxicity observed with chloramphenicol therapy under ordinary circumstances. Duration of therapy as well as free chloramphenicol blood levels in relation to cell turnover time may be important factors in the development of blood dyscrasias.

Further evidence that chloramphenicol can inhibit *de novo* protein synthesis

by mammalian cells is presented in the accompanying report on the inhibitory effect of chloramphenicol on antibody synthesis *in vivo*.

## SUMMARY

Chloramphenicol can inhibit protein synthesis in mammalian cell-free systems as effectively as it inhibits protein synthesis in analogous microbial systems. Significant inhibition in mammalian systems was obtained only when protein synthesis was stimulated by the addition of template RNA to the system, there being comparatively little inhibition of protein synthesis by ribosomes in the absence of stimulatory RNA. It is postulated that chloramphenicol may inhibit the function of messenger or template RNA by successfully competing for ribosomal binding sites, thereby preventing the attachment of RNA to ribosomes.

The apparent discrepancy in the sensitivity of mammalian and microbial cells to chloramphenicol may be related to differences in turnover or stability of ribosomal template RNA rather than to intrinsic differences in mechanism of protein synthesis.

These observations are in accord with the suggestion that protein synthesis in intact mammalian cells may be susceptible to chloramphenicol inhibition only at the time that new messenger or template RNA is being deposited on ribosomes and that more mature cells may be resistant because informative RNA already deposited on ribosomes is not accessible to chloramphenicol inhibition. The inhibitory effect of chloramphenicol on protein synthesis in proliferating cells may be an important factor in hematologic toxicity attributable to chloramphenicol.

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