Differences in Chloramphenicol Sensitivity of Cell-free Amino Acid Polymerization Systems*

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In contrast to other inhibitors, particularly puromycin, chloramphenicol has a rather variable effect on cell-free systems for protein synthesis of different species. At concentration levels of $10^{-5}$ to low $4 \times 10^{-4}$ M, which are comparable to levels in vivo, bacterial systems are inhibited 75 to 90% whereas those from animal tissue (1, 2) or yeast (3) seem not to be affected. It is reported (4) that protein synthesis in thymus nuclei is inhibited by nearly $10^{-2}$ M chloramphenicol; however, a suspicion that this inhibition may be nonspecific is confirmed by Jardetzky, who finds that with a cell-free system from Ehrlich ascites tumor at $800 \mu g$, not only the bacteriostatically active D-threo isomer but also the inactive L-threo, D-erythro, and L-erythro isomers are equally or more inhibitory. In contrast, leucine incorporation by Nathans et al. (5), who concluded that the chloramphenicol effect precedes that of puromycin in the chain of reactions, both acting after amino acid activation (6). Furthermore, with the Escherichia coli system responded exclusively to the bacteriostatically active isomers. A preliminary localization of chloramphenicol action was obtained with E. coli preparations by Nathans et al. (5), who concluded that the chloramphenicol effect precedes that of puromycin in the chain of reactions, both acting after amino acid activation (6). Furthermore, with the E. coli system, Nirenberg and Matthaei (7) reported a relatively low response of polyuridylic acid-dependent phenylalanine polymerization to chloramphenicol. The present study attempts to correlate the inhibition with the nature of the template on the ribosomes.

EXPERIMENTAL PROCEDURE

Methods

Assay Systems—DNase treatment and preliminary incubation of the $30,000 \times g$ supernatant fraction to reduce the blank were carried out as described by Nirenberg and Matthaei (7) with E. coli B that had been grown, harvested, and washed as described (8). After the addition of 5 $\mu g$ of DNase per ml, the supernatant was centrifuged at $15,000 \times g$ for 10 minutes. To the resulting supernatant, $\beta$-mercaptoethanol was added to a final concentration of 0.005 $M$, and this mixture was centrifuged at $30,000 \times g$ for 30 minutes. The $30,000 \times g$ supernatant fraction (S-30) was incubated at $35^\circ$ for 30 minutes with the components required for amino acid incorporation into protein: 0.003 $M$ ATP, 0.0002 $M$ GTP, 0.01 $M$ phosphoenolpyruvate, 30 $\mu g$ of pyruvate kinase per ml, 0.01 $M$ glutathione, 0.011 $M$ magnesium acetate, 0.03 $M$ KCl, 0.05 $M$ Tris-HCl, pH 7.8, each amino acid at $4 \times 10^{-3}$ $M$, 5 $\mu g$ of DNase per ml, 1 $mg$ of sRNA2 per ml, and a volume of S-30 equal to half the total incubation volume. After overnight dialysis against 0.01 $M$ Tris-HCl, pH 7.8, 0.01 $M$ magnesium acetate, 0.03 $M$ KCl, and 0.005 $M$ $\beta$-mercaptoethanol, the pre-incubated mixture was stored at $-20^\circ$. For some experiments, ribosomes were isolated from the DNase-treated $30,000 \times g$ supernatant fraction by centrifugation for 2 hours at $105,000 \times g$; the ribosomal pellet was rinsed with 0.01 $M$ Tris, pH 7.4, and 0.01 $M$ magnesium acetate, and suspended in the same buffer.

The suspension was centrifuged at $15,000 \times g$ for 10 minutes, and ribosomes were removed from the supernatant fluid by centrifugation at $105,000 \times g$ for 2 hours. This washing was repeated three more times. The ribosomes were finally re-suspended and stored as described previously (8). The assay conditions are outlined under Fig. 1.

RNA Preparations—E. coli sRNA was prepared from fully grown E. coli as described (8). E. coli RNA with stimulatory activity as described in the Nirenberg-Matthaei system (7) was prepared from exponentially grown E. coli by shaking 10 $g$ of paste with 30 ml of 0.5% aqueous solution of reconstituted sodium dodecyl sulfate (9) for 15 minutes in the cold. The suspension was extracted twice with phenol and precipitated with 2 volumes of ethanol at $-20^\circ$ in the presence of 0.1% NaCl. The precipitate was dissolved in 3 ml of 0.001 $M$ sodium acetate buffer, pH 5.1, containing 0.05 $M$ KCl, and was dialyzed overnight against two changes of the same buffer.

For the gift of turnip yellow mosaic virus RNA, we are indebted to Dr. James Ofengand, and for the gift of t2 RNA, to Dr. Norton Zinder.

Preparation of Polymers—Crude polynucleotide phosphorylase was prepared from lyophilized Micrococcus lysodeikticus by the method of Singer and Guss (10). After the first (NH4)2SO4 precipitation, the enzyme was applied to a DEAE-cellulose column and eluted between 0.2 and 0.4 $M$ KCl. Synthetic polynucleotides were prepared as described for poly UG by Chapeville et al. (11) and are listed in Table I. Base ratios of copolymers were determined by the method of Smith and Marshall (12).

14C-Labeled poly UC (1:1 input ratio) was prepared by use of CDP-2-14C with a specific activity of 0.4 $\mu$C per $\mu$moles.

Materials

M. lysodeikticus was obtained from Miles Chemical Company, Clifton, New Jersey. UDP, ADP, GDP, CDP, ATP, and GTP

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(sodium salts) were obtained from Pabst Laboratories. Phosphoenolpyruvate, trisodium salt, and pyruvate kinase were products of C. F. Boehringer und Soehne, Mannheim, Germany. Chloramphenicol was obtained from Parke, Davis and Company. 14C-Amino acids were products of Nuclear-Chicago Corporation, New England Nuclear Corporation, and Schwarz BioResearch, which also provided CDP-2-14C.

**RESULTS**

*Comparison of Inhibition of Background Incorporation to Incorporation Stimulated by Addition of Various RNAs—* When leucine incorporation was tested with freshly prepared *E. coli* 30,000 × g supernatant fraction, it was inhibited by chloramphenicol to approximately 50%, as shown in Fig. 1A. After preliminary incubation of such a preparation, the background incorporation was much lower but remained similarly inhibited (Fig. 1B). Addition of *E. coli* RNA caused considerable stimulation, proportional to the amount added. The stimulated synthesis, however, in contrast to the background, was practically completely suppressed by chloramphenicol and only the background incorporation remained. The degree of inhibition is independent of the amount of RNA added (see upper curve, Fig. 1B). A similarly strong inhibition is seen with 14C-leucine incorporation elicited by turnip yellow mosaic virus (13) or *E. coli* f2 phage RNA (14) (Table II).

In the hope of obtaining a better understanding of the reasons or this variability of response to the inhibitor with endogenous and added messenger RNA, it was decided to explore systematically the response to chloramphenicol of polypeptide synthesis elicited by the addition of polynucleotides.

*Inhibition of Amino Acid Polymerization on Synthetic Polynucleotides—* In confirmation of Nirenberg and Matthaei (7), poly U-dependent phenylalanine incorporation was found to be rather resistant to chloramphenicol. However, when phenylalanine incorporation was studied with mixed polynucleotides, it appeared that polypeptide synthesis with poly UC and poly UG as templates was quite strongly responsive to chloramphenicol as inhibitor. With poly UA as a template, on the other hand,

<table>
<thead>
<tr>
<th>Synthetic polynucleotide preparations</th>
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<tbody>
<tr>
<td>Polymer</td>
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<tr>
<td>-------------</td>
</tr>
<tr>
<td>Poly U</td>
</tr>
<tr>
<td>Poly A</td>
</tr>
<tr>
<td>Poly UC</td>
</tr>
<tr>
<td>Poly UC</td>
</tr>
<tr>
<td>Poly AU</td>
</tr>
<tr>
<td>Poly UG</td>
</tr>
<tr>
<td>14C-Poly UC*</td>
</tr>
</tbody>
</table>

* Cytidylic acid-2-14C.

![Fig. 1](http://www.jbc.org/content/249/4/517/F1.large.jpg)  
Fig. 1. Inhibition of RNA-stimulated amino acid incorporation. Samples of DNase-treated 30,000 × g supernatant fraction of *E. coli (A)*, and of corresponding but preliminarily incubated supernatant fraction (B) containing no RNA (O), 125 μg (△), and 260 μg of *E. coli* RNA (○), were assayed in a system containing, in a final volume of 0.25 ml, 0.75 μmole of ATP, 2.5 μmoles of phosphoenolpyruvate, 7.3 μg of pyruvate kinase, 0.075 μmole of GTP, 2.5 μmoles of GSH, 2.5 mg of magnesium acetate, 7.5 μmoles of KC1, 12.5 μmoles of Tris, pH 7.8, 0.025 μmole of each L-amino acid except leucine, 0.005 μmole of 14C-L-leucine (specific activity, 10 μc per μmole), 0.25 μg of *E. coli* sRNA, and 1.2 mg of protein in corresponding 30,000 × g supernatant fractions. Chloramphenicol in the indicated concentrations was added before the incubation. All tubes were kept in an ice bath, and chloramphenicol was generally added next to last, before the tested RNA. Checks with the poly U-stimulated phenylalanine incorporation showed that identical results were obtained whether the inhibitor was added before or after poly U and 14C-phenylalanine, indicating that the order of addition was unimportant. The samples were incubated for 45 minutes at 37°, precipitated with 5% trichloroacetic acid and extracted at 90° for 15 minutes. Precipitates were washed twice with cold 5% trichloroacetic acid and once with ethanol-ether (1:1), dissolved in concentrated formic acid, plated, and counted in a windowless gas flow counter.
Samples of incubated 30,000 X g supernatant fraction of E. coli prepared as described under "Methods" were incubated under the conditions described for Fig. 1, with the addition of turnip yellow mosaic virus (TYMV) (13), and phage f2 RNA (14) where indicated. Chloramphenicol was added next to last, before the tested RNA and before immersing the tubes for incubation in a bath at 37°C.

Inhibition by chloramphenicol of $^{14}$C-leucine incorporation in E. coli system stimulated by viral RNA

<table>
<thead>
<tr>
<th>Additions</th>
<th>Chloramphenicol concentration (mM)</th>
<th>$^{14}$C-Leucine incorporated (μmoles)</th>
<th>Inhibition (％)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>8.6</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>0.0075</td>
<td>7.0</td>
<td>12</td>
</tr>
<tr>
<td>None</td>
<td>0.06</td>
<td>3.8</td>
<td>56</td>
</tr>
<tr>
<td>TYMV RNA, 3.5 μg.</td>
<td>0</td>
<td>148.0</td>
<td>70</td>
</tr>
<tr>
<td>TYMV RNA, 3.5 μg.</td>
<td>0.0075</td>
<td>44.5</td>
<td>95</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0</td>
<td>3.7</td>
<td>0</td>
</tr>
<tr>
<td>None</td>
<td>0.0075</td>
<td>3.3</td>
<td>11</td>
</tr>
<tr>
<td>None</td>
<td>0.12</td>
<td>2.8</td>
<td>24</td>
</tr>
<tr>
<td>f2 RNA, 50 μg</td>
<td>0</td>
<td>96.0</td>
<td>57</td>
</tr>
<tr>
<td>f2 RNA, 50 μg</td>
<td>0.0075</td>
<td>39.8</td>
<td>58</td>
</tr>
<tr>
<td>f2 RNA, 50 μg</td>
<td>0.03</td>
<td>11.5</td>
<td>88</td>
</tr>
<tr>
<td>f2 RNA, 50 μg</td>
<td>0.12</td>
<td>4.5</td>
<td>96</td>
</tr>
</tbody>
</table>

The inhibition was almost as low as with straight poly U. As seen in Fig. 2, 8 X 10⁻³ m chloramphenicol inhibited phenylalanine incorporation 20％on poly U and 30％on poly UA, but 75％on poly UC and poly UG. This increased response to chloramphenicol with poly UC and poly UG templates was likewise found when other amino acids responding to these nucleotide combinations were used as markers (15, 16). Fig. 3 shows very moderate inhibition for phenylalanine, isoleucine, or tyrosine incorporation on poly UA; in contrast, phenylalanine, leucine, proline, and serine incorporation on poly UC was 2 to 3 times more sensitive to the inhibitor. The dependence of response to chloramphenicol as inhibitor on the cytidylic acid content of the template appears quite clearly in tests for proline incorporation on poly UCs with increasing cytidylic acid content (Fig. 4).

After the discovery by Gardner et al. (17) that poly A is specific for polylysine synthesis, and in view of the relative insensitivity of poly UA as template to chloramphenicol, it appeared desirable to test the effect of the inhibitor on polylysine synthesis. As shown in Fig. 5, the inhibition of lysine polymerization on poly A is intermediate.

Lack of Response to Chloramphenicol of Attachment of Poly-nucleotide to Ribosomes—Since incorporation on poly UC-depandent polymerization showed the greatest sensitivity, chloramphenicol was tested for its ability to affect the capacity of ribosomes to bind this polynucleotide. For this purpose, poly UC containing cytidylic acid-2-¹⁴C was prepared as described under "Methods," and attachment of the polynucleotides to ribosomes was assayed as described by Spyrides and Lipmann (18). Sucrose gradient diagrams of charging the ribosomes with poly

Fig. 2. Inhibition by chloramphenicol of E. coli cell-free system stimulated by synthetic polynucleotides. Incubation conditions were the same as in Fig. 1, except that 2.5 μg of synthetic polynucleotides, the indicated concentrations of chloramphenicol, and 1.5 mg of protein in incubated 30,000 X g supernatant fraction were included; $^{14}$C-phenylalanine (specific activity, 4.57 μc per μmole) and complementary mixture of amino acids were used instead of $^{14}$C-leucine. Corresponding blank values, obtained from samples containing the same chloramphenicol concentrations but no polymers, were subtracted for each point, and incorporation was calculated as percentage of the corresponding noninhibited control. One hundred per cent incorporation represents 4.3 μmoles of phenylalanine with poly U, 1.4 μmole with poly UA, 1.5 μmoles with poly UC, and 0.15 μmole with poly UG.

Fig. 3. Inhibition by chloramphenicol of the incorporation of different amino acids stimulated by poly UC and poly UA. Inhibition of phenylalanine, leucine, proline, and serine incorporation in the system stimulated by poly UC (5.7:1), and of phenylalanine, isoleucine, and tyrosine incorporation stimulated by poly UA (8.8:1). Conditions were the same as in Fig. 1, except that 1.25 μg of polynucleotides, indicated concentrations of chloramphenicol, and incubated 30,000 X g supernatant containing 1.5 mg of protein were used. In each case, the $^{14}$C-amino acid was supplemented by the other nonradioactive amino acids. Incorporation was calculated as described for Fig. 2; 100％incorporation represents 2.6 μmoles for phenylalanine, 0.85 μmole for leucine, 0.68 μmole for proline, and 0.20 μmole for serine in the case of poly UC, and 1.64 μmole for phenylalanine, 0.11 μmole for isoleucine, and 0.16 μmole for tyrosine in the case of poly UA.
The experiments indicate that amino acid polymerization on a naturally attached messenger RNA is more resistant to chloramphenicol inhibition than polymerization occurring on a template added in vitro; quite impressive is the nearly complete inhibition of protein synthesis by 0.1 mM chloramphenicol (Table II) when virus RNA and Zinder's T2 phage RNA are added to the ribosomes. At a similar concentration, chloramphenicol inhibits endogenous synthesis only to about 50%. This observation may have a relation to the high resistance of animal-derived systems, which frequently have a stable messenger RNA attached to the ribosomes. However, in experiments conducted with Mr. R. Krug, the polymerization of phenylalanine on reticulocyte ribosomes with added poly U (19) was also found to be resistant to chloramphenicol. This may indicate, as suggested by the resistance of a combination of yeast ribosomes and E. coli supernatant fraction (3), that the site of chloramphenicol action is on the ribosome.

The experiments with various synthetic polynucleotides show decided differences. Poly U- and poly UA-dependent amino acid polymerizations are only slightly inhibited by chloramphenicol, irrespective of the amino acid tested. Poly UC-dependent incorporation is strongly inhibited and is proportional to the cytidylic acid content. Similarly, the presence of guanylic
acid in the polynucleotide intensifies blocking. We are not able to propose an explanation of this differential effect.

SUMMARY

By use of aminoacyl soluble ribonucleic acid (sRNA) as a source of amino acid in the polymerization on Escherichia coli ribosomes, leucine incorporation is found to be more strongly inhibited by chloramphenicol if messenger RNA added in vitro, such as virus RNA, is used than if the background incorporation on the freshly isolated ribosome is followed.

When synthetic polynucleotides are used on E. coli ribosomes, polyuridylic acid- or polyuridylic-adenylic acid-dependent phenylalanine polymerization is inhibited 20 to 30% by 10⁻⁴ M chloramphenicol. The presence of cytidylic or guanylic acid in the polynucleotide increases the effect of chloramphenicol to 75% or more, independent of the amino acid polymerized. The inhibition of polylysine synthesis on polyadenylic acid is between the two extremes.

No effect of chloramphenicol could be detected when polyuridylic-cytidylic acids were attached to the ribosomes.

REFERENCES

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