Mechanism of Cycloheximide Inhibition of Protein Synthesis in a Cell-free System Prepared from Rat Liver*

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SUMMARY

Sites of cycloheximide action on protein synthesis were examined using a cell-free system prepared from rat liver. If all amino acids or aminoacyl transfer RNA were present at the start of incubation, the system appeared to incorporate 14C-leucine mainly by elongation of peptide chains. Under these conditions, high dose levels of cycloheximide were necessary in order to inhibit incorporation extensively. The inhibition could be prevented by raising the glutathione content of the reaction mixture, and particularly by preliminary incubation of a mixture of transferase I and II with high concentrations of glutathione before adding these enzymes to the system. Other sulfhydryl compounds were also effective in protecting against cycloheximide. It has been concluded that the inhibitory action of cycloheximide on peptide chain elongation involves inactivation of transferase II, an enzyme known to have a sulfhydryl requirement. High concentrations of glutathione were also found to prevent the inhibition of cell-free protein synthesis caused by streptovitacin A, a derivative of cycloheximide, but not inhibition caused by emetine or sparsomycin.

If the protein-synthesizing system was first incubated without amino acids or aminoacyl-tRNA, polysomes present at the start of incubation underwent disaggregation. On addition of amino acids at this point, the polysomes became reaggregated and amino acid incorporation is minimal; if at this point amino acids are added to the incubation medium, the polysomes can be reaggregated and labeled amino acids are once more incorporated into peptide chains. The nature of this action of delayed amino acid supplementation is not fully understood, but may be dependent on chain initiation.

This system has been used by us to study the actions of cycloheximide and some other antibiotics on elongation of peptide chains and on the phenomenon of polysome aggregation in response to delayed amino acid supplementation. With this system, we have now shown that chain elongation and polysome aggregation are inhibited by different concentrations of cycloheximide. The action of the antibiotic on chain elongation appears to be directed against aminoacyltransferase II and can be prevented by high concentrations of sulfhydryl compounds, whereas the inhibitory action of cycloheximide on polysome aggregation due to addition of amino acids cannot be prevented by increasing the glutathione content of the medium. A preliminary report of the action of this antibiotic on chain elongation has appeared (14).

MATERIALS AND METHODS

Reagents and Media—The amino acids used were obtained from Nutritional Biochemicals. Other chemicals were reagent grade. Rat liver tRNA stripped of amino acids was purchased repeatedly confirmed in mammalian cells (2–7). Using cell-free systems, several authors (8–11) have obtained data suggesting that the charging of transfer RNA with amino acids is not influenced by the inhibitor, which thus must act at some subsequent stage in protein synthesis. Recent studies suggest that cycloheximide may affect protein synthesis at more than one point. Lin, Mosteller, and Hardesty (12) obtained evidence from experiments on reticulocytes that the antibiotic inhibits the initiation of new peptide chains and the elongation of nascent peptides on ribosomes by different mechanisms.
from General Biochemicals. Cycloheximide was provided by Aldrich, and puromycin dihydrochloride was obtained from Nutritional Biochemicals. Streptovitacin A was a product of Upjohn, and enetine was provided by the S. B. Penick Company, New York, New York. Sparsomycin was kindly supplied as a gift by Dr. L. H. Goldberg of the Harvard Medical School. The uniformly labeled 
$^{14}$C-L-leucine (specific activity, 250 mCi per pmole) and a mixture of 
$^{14}$C-amino acids (1 µCi per mg of uniformly labeled amino acid mixture) were purchased from New England Nuclear. International Chemical and Nuclear Corporation, Irvine, California, provided ($^3$P$^2$P)-GTP (specific radioactivity, 22 mCi per µmole), which was further purified by chromatography on DEAE cellulose. Most of the media used were made up in TKM buffer (0.05 M Tris-HCl, pH 7.6, 0.025 M KCl, and 0.005 M MgCl₂) as described by Wettstein, Staehelin, and Noll (15).

Preparation of Polysomes and Cell-Sap Enzymes—Polysomes (C-ribosomes) were obtained from the livers of fasting 150-g rats by the procedure described by Baliga, Promnek, and Munro (13). To obtain activating and transferring enzymes, cell sap was prepared and deproteinized, first, of free amino acids by dialysis, and then by the procedure described by Baliga et al. (13). Protein was precipitated from this fraction between 30 and 70% saturation with (NH₄)₂SO₄ to yield a mixture of activating and transferring enzymes with minimal content of free or tRNA-attached amino acids; the preparation was used for the system incorporating free amino acids into peptides. For incorporation of amino acids from aminopterin-tRNA into polysomes, unfractonated aminocyclotransferase free of activating enzymes was purified from the cell sap after treatment with Sephadex G-25. The activating enzymes were removed by the traditional method of precipitation at pH 5, and a mixture of transferses I and II was prepared from the supernatant fraction as described by Gasiòr and Moldave (16). With each batch of reaction mixture, the optimal amounts of transferase fraction and (when required) of transferase and activating enzyme fraction were established.

Preparation of 
$^{14}$C-aminocyclotransferase Protein—The procedure described by Moldave (17) was used. Rat liver cell sap was adjusted to pH 5 to bring down a precipitate containing tRNA and amino-activating enzymes. This precipitate was then redissolved and allowed to react with the 
$^{14}$C-amino acid mixture in the presence of 0.01 M ATP, 0.01 M MgCl₂, 0.01 M GSH, and 0.1 M Tris-HCl (pH 7.6). The aminocyclotransferase was then isolated according to the method of Moldave (17). The final product had a specific activity of 350,000 cpm per mg of RNA.

Incubation—The incubation mixture for incorporation of free amino acids into protein contained 50 mM Tris-HCl buffer (pH 7.6), 1 mM GTP, 80 mM NH₄Cl, 2 mM ATP, 5 mM MgCl₂, 4 mM GSH, 0.5 µCi of uniformly labeled 
$^{14}$C-L-leucine, 500 µg of mixed activating and transferring enzyme protein, and 500 µg of polypseud.polyribosomes in 1 ml of final volume. These amounts of enzymes and polypseudomyclones have been shown to be optimal for promoting incorporation of 
$^{14}$C-leucine into protein (13). Incubations were carried out at 37°C, and radioactivity incorporated into protein was measured with a Nuclear-Chicago gas flow counter on the residue left after treatment with hot trichloroacetic acid (13).

The incubation mixture for transfer of 
$^{14}$C-aminocyclotransferase to polypseudomyclones contained 50 mM Tris-HCl buffer (pH 7.6), either 4 or 20 mM GSH, 0.2 mM GTP, 5 mM MgCl₂, 80 mM NH₄Cl, 100 µg of aminocyclotransferase protein, 500 µg of polysome protein, and 20,000 cpm of 
$^{14}$C-aminocyclotransferase protein in a final volume of 1 ml. Incubations were carried out at 37°C, and radioactivity incorporated into protein was measured as described above.

Hydrolysis of GTP by the protein-synthesizing system was determined by measuring radioactive inorganic phosphorus released from ($^3$P$^2$P)-GTP as described by Conway and Lijmann (18). The reaction mixture was similar to that used for 
$^{14}$C-aminocyclotransferase transfer to peptides, except that only 
$^{14}$C-aminocyclotransferase was present and the GTP was labeled with $^3$P in the terminal phosphate (11,000 cpm per ml of reaction mixture). The reaction mixture was incubated at 37°C for 30 min and the reaction was terminated by adding equal amounts of 0.2 mL dTDP-tungstic acid in 0.02 N HSO₄ and 1 ml of 0.001 M potassium phosphate (pH 6.8) as carrier. The extent of GTP hydrolysis was measured by extraction of the released inorganic phosphate as the phosphomolybdate complex into isobutyl alcohol; the extract was counted for radioactivity using the Nuclear-Chicago gas flow counter.

Polysome Profiles—Incubation mixtures for sedimentation analysis of polysome profiles were first diluted with 0.7 volume of 0.1 M Tris-HCl buffer (pH 7.6), then layered over a linear gradient of 10 to 40% sucrose in TKM buffer. The gradient was centrifuged at 80,000 rpm in the SW-20 rotor of the Spinco model L2 ultracentrifuge for 70 min. The absorbance profile at 260 nm was recorded automatically with a flow cell device in a Gilford model 2000 spectrophotometer. Radioactivity on the gradient was measured on fractions of 12 drops; the protein was precipitated with carrier albumin and the hot trichloroacetic acid-insoluble precipitate was collected on a Millipore filter for counting as described above.

Estimation of Protein—The protein content of the enzymes, polysomes, and gradient fractions was determined by the method of Lowry et al. (19) using bovine serum albumin as the standard.

RESULTS

Effect of Cycloheximide Concentration on Amino Acid Incorporation—The action of cycloheximide was studied in a cell-free system consisting of liver polysomes, activating and transferring enzymes together with cofactors, and 
$^{14}$C-leucine. The system had been prepared depleted of free amino acids (see under "Materials and Methods") and was thus dependent on endogenous amino acids. To one set of tubes, a complete mixture of amino acids was added at the start of incubation; to the other set, no amino acids were added other than 
$^{14}$C-leucine. Fig. 1a shows that, when amino acids were present, increasing levels of cycloheximide caused progressive inhibition of 
$^{14}$C-leucine uptake into protein. Addition of 0.1 µg per ml had a slight action on 
$^{14}$C-leucine incorporation; in order to achieve 75% inhibition, it was necessary to add 1 mg of inhibitor per ml of incubation medium, a level comparable to that used by Wettstein, Noll, and Penman (10) in their cell-free system to retard ribosome movement along the messenger strand. The much smaller residual incorporation of 
$^{14}$C-leucine obtained in the absence of added amino acids (Fig. 1b) was little affected by cycloheximide at any concentration.

It has previously been shown by us (13) with such a system that, after 20 min of incubation without amino acids, incorporation of 
$^{14}$C-leucine ceases altogether but can be restarted by a complete mixture of amino acids. Fig. 1c shows the effect of two levels of cycloheximide on this response to delayed amino
FIG. 1. Effect of various concentrations of cycloheximide on amino acid incorporation. Liver polysomes were incubated with \(^{14}C\)-leucine in an amino acid-dependent protein-synthesizing system for 40 min and incorporation into peptide was measured in the presence of different concentrations of cycloheximide (Cyclo.). a, with all amino acids (AA) present throughout incubation; b, without addition of other amino acids to the medium; c, when the medium was supplemented with all amino acids and with cycloheximide after 20-min incubation. The data are the mean results from two to four experiments.

Acid supplementation. In this case, 0.1 \(\mu\)g of inhibitor per ml reduced the extra incorporation after amino acid addition by 75%, and 1 \(\mu\)g per ml reduced it by 95%. Consequently, sensitivity to cycloheximide is much greater when the amino acids are added 20 min after the start of incubation than when the amino acids are present throughout incubation (Fig. 1a).

Effect of Cycloheximide on Polysome Aggregation—When the cell-free protein-synthesizing system is incubated for 20 min in the absence of amino acids as described above, the polysome pattern shows considerable loss of large aggregates and an accumulation of monosomes and other oligosomes. We have previously shown that the polysome aggregates can be regenerated within 2 min after adding a complete amino acid mixture to the cell-free system (13). This coincides with the increased incorporation of \(^{14}C\)-leucine shown in Fig. 1c. When cycloheximide was added to the medium along with the supplementary amino acids, it prevented this reaggregation at all dose levels from 0.1 \(\mu\)g to 1 \(\mu\)g per ml (Fig. 2). Thus, polysome regeneration and the increased uptake of \(^{14}C\)-leucine in response to delayed amino acid supplementation each show a similar degree of sensitivity to cycloheximide.

When incubation of the reaggregated polysomes is continued in the presence of amino acids, there is subsequent breakdown of polysomes (Fig. 3a). As shown in our earlier paper (13), the stimulus of delayed amino acid addition of \(^{14}C\)-leucine incorporation is not, in fact, linear (as shown in Fig. 1c), but causes an initial rapid burst of uptake, followed by diminishing increments as time of incubation progresses. Consequently, the detailed picture for \(^{14}C\)-leucine uptake after amino acid addition coincides closely with the aggregation and then disaggregation of the polysomes. Since it has been established that high concentrations of cycloheximide can stabilize polysomes \(in vitro\) (10), we examined the effect of various dose levels of cycloheximide on the subsequent disaggregation of polysomes that had been reaggregated by amino acid addition. The polysome system was first incubated for 20 min without exogenous amino acids. Amino acids were then added, followed 2 min later by various levels of cycloheximide, and incubation was terminated at 3 min after addition of the inhibitor. Fig. 3b shows that the polysome...
aggregate was well preserved in the presence of 1 mg per ml of cycloheximide, but at lower concentrations breakdown was considerable. In the samples incubated without addition of cycloheximide (Fig. 3a), the ratio of polysomes to monosomes was 6.7 at 2 min after addition of amino acids, and 3.4 at 5 min after addition, which is compatible with disaggregation seen in the figure. For samples receiving the inhibitor 2 min after the amino acids (Fig. 3b), the ratio of polysomes to monosomes at 5 min of incubation is 4.1 for 1 \( \mu \)g of cycloheximide, 5.0 for 100 \( \mu \)g, and 6.2 for 1000 \( \mu \)g, the latter figure being close to the 2 min figure. Thus, the level of cycloheximide needed to prevent reaggregation of polysomes after amino acid addition (0.1 \( \mu \)g per ml) is much lower than the level necessary to stabilize the polysome aggregates so formed (1 mg per ml).

**Effect of Cycloheximide on Transfer of Amino Acids from tRNA to Polysomes**

It has been reported that cycloheximide has no effect on amino acid activation and binding to tRNA (8). In order to simplify the protein-synthesizing system, we therefore added aminoacyl-tRNA charged with a mixture of \(^{14}C\)-amino acids and added it to an incorporation system consisting of liver polysomes, partly purified transferases, GTP, and GSH. Such a system incorporated the labeled amino acids efficiently into protein.

Table I shows the mean results obtained from three such studies with this system. Addition of large amounts of cycloheximide at the beginning of incubation caused a 50\% inhibition of this transfer of label (tube 1 versus 2). It will be noted that, in order to achieve this degree of inhibition, a concentration of 1 mg of cycloheximide per ml was necessary. This lack of sensitivity is comparable to that found above for \(^{14}C\)-leucine incorporation in the presence of abundant free amino acids in the system (Fig. 1a). The inhibitory action of the antibiotic could be enhanced if the fraction containing the transferases was incubated beforehand with cycloheximide (tube 3 versus 4). It has been suggested that cycloheximide inhibits transferase II (11), and this enzyme is known to be sulfhydryl-dependent (20). Consequently, it occurred to us that cycloheximide may retard peptide elongation by inactivating the sulfhydryl groups of this enzyme and that high concentrations of sulfhydryl compounds might compete effectively with the inhibitor and prevent its action. Accordingly, the GSH content of the medium during incubation was raised from 4 \( \mu \)moles to 20 \( \mu \)moles. Table 1 shows partial protection against inhibitory action of the previously added cycloheximide (tube 4 versus 5). The action of cycloheximide was also diminished by adding 4 \( \mu \)moles of GSH during preliminary incubation (tube 4 versus 6), but no better protective effect was obtained if 20 \( \mu \)moles were added during prior incubation along with cycloheximide than if the GSH was added later during incubation (tube 5 versus 7). A series of incubations was therefore carried out in which the transferase fraction was incubated with various sulfhydryl compounds before addition of cycloheximide (tubes 9 to 13). Increasing the glu-

<table>
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<tr>
<th>Tube</th>
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<tr>
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<td>13</td>
<td>+</td>
<td>20 Mercaptoethanol</td>
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* DTT, dithiothreitol.

The incubation mixture for transfer of \(^{14}C\)-aminoacyl-tRNA to polysomes contained 50 mM Tris-HCl buffer (pH 7.6), 0.2 mM GTP, 5 mM MgCl\(_2\), 80 mM NH\(_4\)Cl, 100 \( \mu \)g of aminoacyltransferase protein, 500 \( \mu \)g of polysome protein, and 20,000 cpm of \(^{14}C\)-aminoacyl-tRNA in a final volume of 1 ml. The amounts of sulfhydryl compounds present in the incubation medium are as shown in the table. Incubations were carried out at 27\(\circ\) for 25 min. In most experiments, as indicated, the transferases were incubated at 37\(\circ\) for 5 min in 0.5 ml of buffer before adding the polysomes, GTP, and \(^{14}C\)-aminoacyl-tRNA. Glutathione, dithiothreitol, mercaptoethanol, and cycloheximide (1 mg per tube) were added either during preliminary incubation or during incubation, as indicated in the table. The data shown are the average of three experiments.

**Table I**

Effect of preliminary incubation of transferases with sulfhydryl compounds on inhibitory action of cycloheximide on protein synthesis in cell-free system.
tathione level to 20 μmoles did not affect incorporation by the system in the absence of the inhibitor (tube 8 versus 9), but prior incubation with this level of glutathione greatly reduced the inhibitory action of cycloheximide added subsequently (tube 10 versus 11). Even more effective protection against cycloheximide was obtained by adding dithiothreitol or mercaptoethanol to the enzyme during previous incubation (tubes 12 and 13). The effects of preliminary incubation of the transferase preparation with various levels of GSH, dithiothreitol, or mercaptoethanol are shown in Fig. 4. This shows that transfer of 14C-amino acids in the absence of cycloheximide was not significantly influenced by different levels of the sulfhydryl compounds. However, with increasing concentration of these sulfhydryl agents the inhibitory action of cycloheximide diminished. It is interesting to note that dithiothreitol, which has two sulfhydryl groups, was maximally effective at a level of 10 μmoles per tube, whereas a similar degree of protection against inhibition by the other two compounds required 20 μmoles.

Since Sutter and Moldave (20) have shown that prior incubation of transferase II with glutathione accelerates particularly the initial rate of 14C-aminoacyl transfer to ribosomal peptide, we examined the effect of GSH level on both the initial transfer rate and the final plateau attained in the presence of cycloheximide. Fig. 5 shows that, in absence of the inhibitor, previous incubation of the transferase fraction with 20 μmoles of GSH resulted in a marginally greater initial incorporation rate and also a slightly higher final plateau than were obtained in the presence of 4 μmoles of GSH. When cycloheximide was added to the reaction mixture following prior incubation with 4 μmoles of GSH, it had an extensive inhibitory action both on the initial rate of reaction and on the final plateau attained. Preliminary incubation with 20 μmoles of GSH protected against cyclohexi-

![Fig. 4. The effect of cycloheximide on aminocyl transferases previously incubated with -SH compounds. The effect of cycloheximide on transfer of 14C-amino acids from aminoacyl-tRNA to peptides was examined using a system consisting of liver polysomes, GTP, Mg++, 14C-aminoacyl-tRNA, transferase enzymes, and buffer. The transferase fraction in this system was incubated at 37°C for 5 min with various amounts of glutathione (- - - -), mercaptoethanol (- - -), or dithiothreitol (-----) in a total volume of 0.5 ml of buffer. The other constituents of the reaction were then added to give a final volume of 1 ml and incubation was continued for 30 min at 37°. Cycloheximide (1 mg) was added to half of the samples at the end of preliminary incubation. The figure represents the mean values obtained in three experiments.](image)

![Fig. 5. The effect of glutathione level during preliminary incubation on the subsequent action of cycloheximide during subsequent incubation. Transfer of 14C-amino acids from aminoacyl-tRNA to peptides was examined during different times of incubation in the presence or absence of cycloheximide. The system consisted of liver polysomes, GTP, Mg++, 14C-aminoacyl-tRNA, transferase enzymes, and buffer. The transferase fraction was previously incubated at 37° for 5 min with either GSH, dithiothreitol, or mercaptoethanol. The transferase fraction was then incubated for 30 min at 37° with either 4 or 20 μmoles of GSH, dithiothreitol, or mercaptoethanol. The figure shows incorporation of 14C-amino acids in the absence of cycloheximide was not significantly influenced by different levels of the sulfhydryl compounds. However, with increasing concentration of these sulfhydryl agents the inhibitory action of cycloheximide diminished. It is interesting to note that dithiothreitol, which has two sulfhydryl groups, was maximally effective at a level of 10 μmoles per tube, whereas a similar degree of protection against inhibition by the other two compounds required 20 μmoles. The data shown are the average of two experiments.](image)

**Table II**

<table>
<thead>
<tr>
<th>Tube</th>
<th>Polysomes</th>
<th>Glutathione</th>
<th>Cycloheximide (1 mg/tube)</th>
<th>GTP, 14C-aminoacyl-tRNA, transferases</th>
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mide at all stages of the reaction. This eliminates the possibility that the inhibitory action of cycloheximide is directed only against the initial rate of aminocyl transfer, which is particularly subject to GSH stimulation according to Sutter and Mol-dave (20).

In addition, experiments were performed in which the polysomes instead of the transferases were previously incubated with cycloheximide or GSH. Table II shows that prior incubation of the polysomes with cycloheximide potentiated the action of the inhibitor very little, and preliminary incubation of the polysomes with 20 pmoles of GSH did not confer any additional protective effect against cycloheximide over and above that obtained by adding the same amount of GSH during incubation. It is to be noted that the polysomes used in these studies were washed with NH₄Cl and would thus not be expected to carry aminoacyl-

transferase II (21). From these various experiments, it can be concluded that the maximal protective effect of GSH against cycloheximide is obtained when the transferase preparation is previously incubated with the sulfhydryl compounds and the cycloheximide is added subsequently.

Since maximal protection by sulfhydryl compounds is obtained by prior incubation with transferases, an experiment was carried out in which 20 pmoles of GSH were added to 0.5 ml of transferase solution, and they were incubated together for 10 min. Then the GSH concentration was lowered by dilution to 4 mm before adding the rest of the system for transferring amino acids from aminopentyl-tRNA. Controls prepared previously incubated with 4 pmoles of GSH were diluted similarly, but enough GSH was then added to maintain the final concentration at 4 mm. Cycloheximide was added to some of the tubes at the beginning of incubation. Fig. 6 shows that enzyme previously incubated with 20 pmoles of GSH was much less inhibited by cycloheximide than was enzyme previously incubated with 4 pmoles. Thus, prior treatment with a high level of GSH has a protective action against the inhibitor.

Effect of Cycloheximide on Release of Peptides by Puromycin—Puromycin releases incomplete peptide chains from ribosomes by substituting for aminocyl-tRNA at the acceptor site on the ribosome and subsequently reacting to form a peptide bond (22-24). It has been shown that cycloheximide retards this release of action of puromycin (11). Accordingly, the capacity of GSH to reverse this effect of cycloheximide was evaluated. Polysomes were incubated for 5 min with 14C-aminopentyl-tRNA, GSH, GTP, and the transferase fraction. The polysomes were then harvested on a sucrose gradient, and the incorporation of 14C activity into peptides was examined at various points on the gradient (Fig. 7a). Most of the incorporated radioactivity was

Fig. 6. The effect of preliminary incubation of aminocyltransferases with cycloheximide and glutathione on initial rate of amino acid transfer. The system used transferred 14C-aminopentyl-tRNA to peptides and consisted of a mixed transferase fraction, liver polysomes, GTP, Mg++, GSH, 14C-aminopentyl-tRNA, and buffer. The transferase fraction was incubated at 37°C for 10 min with 4 or 20 pmoles of GSH in 0.5 ml of buffer. It was then adjusted by dilution to provide the same amount of enzyme and 4 pmoles of GSH in all samples. The other constituents of the reaction were then added to give a final volume of 2 ml. Cycloheximide (1 mg) was added at this point to some of the tubes. Samples were taken at different time intervals for assay of incorporation into peptides. The data are the average of two experiments.

Fig. 7. Effect of cycloheximide and puromycin on distribution of labeled polypeptide on polysome profile. The system used transferred 14C-aminopentyl-tRNA to peptides and consisted of a mixed transferase fraction, liver polysomes, GTP, Mg++, GSH, 14C-aminopentyl-tRNA, and buffer to give a final volume of 1 ml. Following incubation at 37°C, the polysomes were separated on a sucrose gradient and the profile was recorded (---). Fractions were taken from the gradient and 14C-incorporation into peptides was measured (——). In the case of tubes d, e, and f, the transferase fraction in 0.5 ml of buffer was given preliminary incubation for 5 min with the amount of GSH described below. In addition, in some tubes the amount of GSH was increased from 4 to 20 pmoles per tube, and puromycin (200 µg) or cycloheximide (1 mg) or both were added to some samples. The gradients shown represent the following conditions of incubation: a, incubation for 5 min without inhibitors or preliminary incubation; b, similar to preceding conditions with addition of puromycin after 3 min of incubation; c, incubation with cycloheximide for first 3 min of incubation, followed by an additional 2 min with puromycin; d, preliminary incubation of transferases for 5 min with 4 pmoles of GSH, followed by 5-min incubation alone, then incubation with cycloheximide for 5 min, followed by 5 min with puromycin; e, preliminary incubation of transferases with 20 pmoles of GSH, then 5-min incubation alone, then incubation with cycloheximide for 5 min, followed by 5 min with puromycin; f, preliminary incubation of transferases with 20 pmoles of GSH, followed by 10-min incubation, and finally 5 min with puromycin. The experiment was replicated twice.
found on the polysome aggregates, with little release into the supernatant fraction. When puromycin was added during the final 2 min of incubation, the activity in the polysomes was much reduced and the supernatant fraction of the gradient contained most of the radioactivity (Fig. 7b). When cycloheximide was added 3 min before the puromycin, more activity remained on the polysome aggregates and very little was released (Fig. 7c).

Disaggregation of polysomes to monosomes as a result of puromycin action was also reduced by addition of cycloheximide (cf. Fig. 7, b and c). To examine the effect of glutathione on this action of cycloheximide, the transferase fraction was previously incubated in the presence of either 4 or 20 pmoles of GSH for 5 min; the 14C-aminoacyl-tRNA and cofactors were then added, and incubation was continued for 15 min with addition of cycloheximide and then puromycin as indicated in Fig. 7, d, e, and f. Fig. 7d confirms that cycloheximide reduces total incorporation and prevents release of peptide chains by puromycin when the transferase has been previously incubated at the lower GSH level (cf. Fig. 7b). However, when the enzyme was first incubated with 20 pmoles of GSH (Fig. 7c), incorporation of the 14C-amino acids was very extensive in spite of the presence of the cycloheximide, thus confirming protection against inhibition by previous treatment of the transferase fraction with GSH. Furthermore, the puromycin almost completely released the 14C-peptides into the supernatant fraction. It will also be noted that the polysome profile under these conditions showed considerable degradation, notably the presence of a large monosome peak. These actions of puromycin were then similar to its effects in the absence of cycloheximide (Fig. 7f). It will be seen from comparison of Fig. 7f with Fig. 7b that the presence of high concentrations of GSH actually potentiates the releasing action of puromycin.

These experiments confirm that cycloheximide retards protein synthesis without releasing the peptide chains (10). It also prevents release of peptide chains by puromycin, an action of cycloheximide that can be prevented by previous incubation of the transferase enzyme fraction with large amounts of GSH. According to Skogerson and Moldave (25), puromycin reacts more extensively with peptidyl-tRNA when it is at the peptidyl site on the ribosomes, its presence at that site is dependent on transferase II and GTP, thus explaining inhibition of the puromycin reaction by cycloheximide.

Effect of Cycloheximide and Other Inhibitors on GTP Hydrolysis and Amino Acid Incorporation in Presence of Different Concentrations of GSH—From the preceding evidence, the inhibitory action of cycloheximide on chain elongation appears to be directed against transferase II, which is the only sulfhydryl dependent enzyme involved in aminoacyl transfer (20). Nishizuka and Lipmann (26) have found that the GTP hydrolysis reaction required for chain elongation in a bacterial protein-synthesizing system also involves a sulfhydryl-dependent enzyme. This enzyme (translocase) may be identical with transferase II (25). We (14) have recently shown that cycloheximide inhibits GTP hydrolysis in our mammalian system. The effective concentrations of cycloheximide are similar to those required for inhibition of 14C-aminoacyl transfer to peptide chains in the system, and the action of cycloheximide can be similarly prevented by glutathione (14). This confirms the interrelationship between cycloheximide and GSH by means of a separate reaction. In the present series of studies, we have used the hydrolysis of GTP to follow the action of other inhibitors of protein synthesis in order to supplement the information obtained from amino acid incorporation.

First, the conditions for hydrolysis of (γ-32P)-GTP were examined using the protein-synthesizing system for amino acid transfer from aminoacyl-tRNA to peptide. Fig. 8 shows the amounts of 32P released as inorganic phosphate in this cell-free protein-synthesizing system when all reagents are present and also in the absence of aminoacyl-tRNA or ribosomes. A small release is apparent when either of the latter is deleted from the system, but this reaction terminates after about 5 min, whereas the complete system causes more extensive GTP hydrolysis which proceeds for at least 30 min. Thus, release of 32P in the complete system is largely dependent on amino acid transfer to peptides. The amino acid-dependent hydrolysis of GTP was used to study the action of glutathione on inhibition of protein synthesis by various compounds. Samples were also incubated in the same reaction mixture with 14C-aminoacyl tRNA to allow comparison of their action on amino acid incorporation. Table III shows the effect of preliminary incubation of the transferase enzyme preparation with either 4 or 20 pmoles of glutathione. Following this prior incubation, the incorporation system was completed, using (γ-32P)-GTP or 14C-aminoacyl-tRNA, and inhibitors were added at this point. In confirmation of earlier studies (14), cycloheximide at a concentration of 1 mg per ml extensively inhibited both 32P release and 14C-amino acid transfer to peptide when the transferase fraction had been previously incubated with 4 pmoles of glutathione, but inhibition was slight when the transferase fraction was previously incubated with 20 pmoles of the sulfhydryl reagent. Streptovitacin A, a hydroxylated derivative of cycloheximide, was also added at a concentration of 1 mg per ml, since this is known to inhibit protein synthesis effectively in cell-free systems (4). Table III shows that it behaved like cycloheximide. Grollman (27) has shown that emetine inhibits the amino acid transfer reaction in
cell-free protein synthesizing systems, and suggest that this may occur because of its structural similarity to cycloheximide. We therefore added 100 µg of emetine per ml to our transfer system, a concentration shown by Grollman to be within the effective range, and confirmed that both amino acid incorporation and 32P release were extensively inhibited. However, unlike cycloheximide, neither reaction could be protected against inhibition when the glutathione concentration was raised. Grollman (28) has recently found that emetine differs from cycloheximide in its action on HeLa cells. On suspending the cells in fresh medium, the inhibitory action of cycloheximide could be reversed, whereas that of emetine could not; however, in Grollman's system the action of streptovitacin A was also found to be irreversible. Finally, the action of sparsomycin was studied in our system at a concentration of 2 µg per ml, which is known to inhibit protein synthesis in mammalian cell-free systems (29).

Table III shows that inhibition of amino acid incorporation was largely suppressed by this concentration, and that raising the glutathione concentration did not alleviate this effect. However, inhibition of 32P release by sparsomycin was slight. This result, which implies an unexpected dissociation between the two requirements of peptide bond formation on ribosomes, has been confirmed on several occasions and may indicate uncoupling by sparsomycin of GTP hydrolysis from other events in protein synthesis.

**Effect of Cycloheximide on Polysome Reaggregation in Presence of Extra GSH**—It was demonstrated above that polysome reaggregation caused by delayed addition of amino acids to a medium lacking free amino acids could be inhibited by much lower concentrations of cycloheximide than those necessary to achieve inhibition of chain elongation. Since this suggests a separate action of the inhibitor, we tested the capacity of sulfhydryl compounds to protect against this effect of cycloheximide. Fig. 9 shows polysome reaggregation in response to addition of amino acids when the GSH concentration in the medium was raised from 4 mM to 20 mM. The usual reduction in monosomes and accumulation of polysomes was obtained, and the presence of 0.1 µg of cycloheximide was again sufficient to prevent reaggregation (compare Fig. 2). Thus, GSH does not influence this action of cycloheximide.

In the same experiments, 14C-leucine was present in the medium, and incorporation of radioactivity into protein was examined after 40 min of incubation. When no other amino acids...
were added to the medium, 2270 cpm per tube were incorporated. When the complete amino acid mixture was added after 20 min of incubation, incorporation rose to 6250 cpm per tube. If, however, 0.1 µg of cycloheximide was added along with the amino acid mixture, incorporation was then reduced to 3010 cpm per tube; that is, more than 80% of the stimulant action of the amino acids was eliminated. In these experiments, the concentration of GSH was 20 mM throughout incubation, but the inhibitory effect of cycloheximide was similar in degree to that obtained in the presence of 4 mM GSH (Fig. 1c).

These studies provide further evidence that the action of cycloheximide on reaggregation and on peptide elongation is due to different mechanisms.

**Discussion**

Lin et al. (12) consider that cycloheximide acts separately on chain initiation and on chain elongation. When they incubated intact reticulocytes with NaF and cycloheximide, both compounds inhibited chain initiation, but cycloheximide also affected elongation of nascent peptide chains. Our results provide direct evidence for such a dual action under conditions of cell-free protein synthesis. We have employed a system in which the presence of amino acids at the start of incubation results mainly in elongation of previously existing peptide chains on the ribosomes. On the other hand, preliminary incubation of the system without amino acids results in disaggregation of the polysomes and, when amino acids are subsequently added, the polysomes reaggregate by a process that presumably requires initiation of peptide chains. The concentration of cycloheximide needed for effective inhibition of chain elongation in this system is 10 times greater than that needed to prevent complete polysome reconstitution on adding amino acids. This is the reverse of the finding of Stanners (30), who examined the action of cycloheximide on hamster embryonic cells in tissue culture and found that low doses inhibited movement of ribosomes along the messenger, but had less action on reattachment of free ribosomes to the messenger. Our cell-free system appears to have a limited capacity for polysome reaggregation, and this might well make it more sensitive to inhibition than the same process in the whole cell. The second source of evidence of a dual site of action of cycloheximide is shown by the prevention of its action by sulfhydryl compounds. These compounds can extensively reduce the inhibitory action of cycloheximide on chain elongation but not on amino acid-induced polysome reaggregation, even at the very low concentration of inhibitor needed to prevent reaggregation.

One site of action of cycloheximide on protein synthesis has been provisionally identified with transferase II. Involvement of transferases was originally suggested by Siegel and Sider (8) on the grounds that cycloheximide did not appear to have an effect on amino acid-activating enzymes. Felicetti, Colombo, and Baglioni (11) showed that cycloheximide inhibits release of peptide chains from polysomes by puromycin and concluded that transferase II is the site of action of the inhibitor. On the other hand, two other groups of investigators (23, 31) failed to obtain an inhibitory effect of cycloheximide on puromycin-dependent release of peptides from reticulocyte polysomes. Both of these groups used 20 mM GSH in their incubation systems, whereas Felicetti et al. (11) used 2 mM GSH. We have confirmed the inhibition of puromycin when the GSH concentration of the medium is 4 mM. Our data further demonstrate that this action of cycloheximide can be prevented by prior incubation of the crude transferase fraction with higher levels of GSH (up to 20 µmoles). These observations emphasize the importance of GSH concentration in the medium when studying the action of inhibitors of transferase II. Inhibition of release of peptides by puromycin occurs because, when harvested, most of the peptide is attached to tRNA at the aminoacyl site on the ribosomes (25). Since puromycin must bind to this ribosome site before forming the peptide bond, the puromycin-tRNA has first to be translocated to the peptidyl site. This accounts for the inhibitory action of cycloheximide on puromycin release and for the stimulant effect of GSH.

Our experiments throw some light on the nature of the action of cycloheximide on transferase II. Sutter and Moldave (20) demonstrated with purified preparations of rat liver transferase II that the enzyme gives maximal incorporation when previously incubated with various sulfhydryl compounds, greatest activity being achieved by prior incubation with concentrations of GSH of 20 mM or higher. It appears that purified transferase II is readily inactivated through its sulfhydryl groups and that it can be regenerated by sulfhydryl donors. In the case of the crude transferase preparations used by us, which include both enzymes I and II, it is probable that transferase II is less unstable (20). This is supported by the data in Fig. 4 which show that varying the levels of GSH and other sulfhydryl compounds during preliminary incubation did not appreciably affect transferase activity during subsequent incubation. Nevertheless, although in our unpurified system the enzyme shows no great increase in activity as a result of previous incubation with high levels of GSH, these high concentrations gave added protection against subsequent addition of cycloheximide (Fig. 4). This is not due to the high GSH concentration during subsequent incubation, since the protective effect persists even if the GSH content is lowered before the cycloheximide and other reactants are added at the end of preliminary incubation of the enzyme (Fig. 6). This agrees with the finding of Sutter and Moldave (20) that prior treatment of purified transferase II with high levels of GSH increases subsequent aminoacyl transfer at a lower GSH concentration. Our experiment in which the GSH concentration was lowered before adding cycloheximide (Fig. 6) demonstrates that mercapto and the inhibitor do not undergo direct chemical reaction.

Protection by GSH against inhibition of transferase II allows one to test other antibiotics in order to determine whether they act in the same way as cycloheximide. The action of high GSH concentrations on the inhibition of protein synthesis by streptomycin A is exactly similar to that of cycloheximide, to which it is structurally related (Table III). Emetine was thought by Grollman (27) to be another analogue of cycloheximide, but in our experiments its inhibitory action is not prevented by increasing the GSH concentration in the cell-free system. Grollman (28) found that protein synthesis in HeLa cells was inhibited by cycloheximide, streptomycin, and emetine, but when the cells were suspended in fresh medium the inhibition was removed only in the case of cycloheximide. In contrast, our data show that the inhibitory action of emetine can be distinguished from that of streptomycin by its insensitivity to GSH. This does not, of course, eliminate transferase II as the site of action of emetine. Finally, sparsomycin was examined, since it represents another antibiotic whose precise site of action on peptide bond formation remains uncertain (32). An increase in GSH concentration also
failed to prevent the inhibition caused by this antibiotic. An interesting feature of these inhibitors was that cycloheximide and streptovitacin inhibited GTP hydrolysis to the same extent as amino acid transfer from aminoacyl-tRNA. Emetine also inhibited GTP hydrolysis fairly strongly, but sparsomycin was almost without effect on this reaction while causing extensive inhibition of amino acid incorporation. This demonstrates a considerable uncoupling of GTP hydrolysis from peptide bond formation when this antibiotic is present.

REFERENCES

Mechanism of Cycloheximide Inhibition of Protein Synthesis in a Cell-free System Prepared from Rat Liver
B. S. Baliga, A. W. Pronczuk and H. N. Munro

J. Biol. Chem. 1969, 244:4480-4489.

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