Inhibition of Protein Synthesis in Intact HeLa Cells

JOAN S. TSCHERNE* AND SIDNEY PESTKA*
Roche Institute of Molecular Biology, Nutley, New Jersey 07110

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Polysome analysis has proved to be a sensitive probe for the mode of action of inhibitors of protein synthesis in intact HeLa cells. To classify the active compounds as inhibitors of initiation, elongation, or termination, their effects on the cellular polyribosome pattern were compared under three conditions. These conditions tested (i) their direct effect on the polyribosome profile; (ii) their effect on ribosome run-off produced by hypertonicity; and (iii) their effects on recovery from hypertonicity. Using this technique, diacetoxyscirpenol, 2-(4-methyl-2,6-dinitroanilino)-N-methylpropionamide, and three alkaloids, harringtonine, isoharringtonine, and homoharringtonine, were found to be inhibitors of initiation. Polysome analysis indicated that in HeLa cells 7.8 \times 10^{-7} \text{M} \text{pactamycin}, which inhibited protein synthesis 94\%, interfered with elongation as well as initiation under these conditions. Emetine, anisomycin, cycloheximide, and trichodermin each gave polysome patterns consistent with inhibition of elongation. Fusidic acid and aurintricarboxylic acid inhibited incorporation of [\text{14C}]leucine into intact HeLa cells, but polysome analysis did not localize any specific inhibitory effects to the initiation, elongation, or termination steps of protein synthesis. The use of specific inhibitors of initiation of protein synthesis has indicated that most, if not all, mammalian messenger ribonucleic acids contain a single initiation site.

It is often assumed that the action of various compounds on protein synthesis in cell-free systems can be extrapolated to explain effects on the intact cell. However, there is considerable evidence which indicates that caution must be observed in making such extrapolations (3-5, 7, 8, 17-19). Therefore, it was useful to undertake a detailed study of the effect of some inhibitors on intact cells to ascertain whether the conclusions from cell-free systems were valid when applied to the whole cell. Impermeability of cells to compounds, of course, can account for differences in effects on cell-free assays and on intact cells. Frequently, however, subtle and unexpected factors may account for these differences (7, 8, 17-19). These include, for example, the possibility that, although an antibiotic may inhibit peptide bond formation on a free ribosome, once that ribosome becomes part of a polysome array the antibiotic binding site is obscured and inhibition is no longer observed.

Comparison of the effects of inhibitors of protein synthesis under three sets of conditions has permitted classification of a number of inhibitors (for reviews see 16, 34) in the intact cell. This was done by incubating intact HeLa cells with the compound directly, with the compound followed by an increase in medium hypertonicity, and with the compound during the period of recovery from hypertonicity. The effects of inhibitors of eukaryotic protein synthesis on polyribosome metabolism of the cells were evaluated. The results, reported in this communication, allowed us to localize the site of action of certain inhibitors with respect to initiation, elongation, and termination.

MATERIALS AND METHODS

All experiments were performed using HeLa S1 cells. The cells were grown in Joklik-modified minimal essential medium for spinner cultures (6) supplemented with 5% fetal calf serum (Gibco). The following compounds, without any further purification, were used in these experiments: emetine (Hoffmann-La Roche, Inc.); pactamycin (Upjohn); anisomycin (Pfizer); fusidic acid (Leo Pharmaceutical Products); aurintricarboxylic acid (Eastman-Kodak Organic Chemicals); cycloheximide (Upjohn); 2-(4-methyl-2,6-dinitroanilino)-N-methylpropionamide (MDMP) (from D. Weeks); diacetoxyscirpenol (Calbiochem); trichodermin (Leo Pharmaceutical Products); and the alkaloids harringtonine, isoharringtonine, homoharringtonine, and cephalotaxine (all gifts from R. Powell). [\text{14C}]leucine was purchased from New England Nuclear at specific activities of 305 and

\footnote{Present address: The Greater New York Blood Center, New York, N.Y. 10021.}

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280 mCi/mmole and from Amersham-Searle at a specific activity of 348 mCi/mmole. [35S]methionine was obtained from New England Nuclear at a specific activity of 200 Ci/mmole. Nonidet P-40 was purchased from the Shell Chemical Company.

Assay of protein synthesis in intact cells. HeLa cells were grown as described above; they were collected by centrifugation at 2,800 × g for 2 min at 2 C (IEC-PR 6 centrifuge; rotor no. 253; 2,000 rpm) and washed once by suspension in 10 ml of cold Earle balanced salt solution for suspension cultures (6). They were collected and the cell pellet was resuspended at a concentration of 6 × 10⁶ cells/ml in minimal essential medium for spinner cultures without leucine or methionine and to which 25 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid buffer, pH 7.2, was added. The samples were warmed at 37 C for at least 10 min and then incubated for 5 min with the compound to be tested. After this 5-min incubation, radioactive amino acid was then added and the cells were incubated for an additional 10 min. Portions were withdrawn from each sample, made 0.33 N in sodium hydroxide, and incubated for 20 min at 37 C to hydrolyze any aminoacyl- or peptidyl-transfer ribonucleic acid (tRNA). After neutralization with HCl, radioactivity incorporated into protein was precipitated with 2 ml of cold 10% trichloroacetic acid. After standing at 0 C for at least 10 min, the precipitates were collected on 25-mm diameter membrane filters (Millipore Corp., HAWP); tubes and filters were then washed three times with cold 5% trichloroacetic acid. The filters were dried and radioactivity was determined as previously reported (19).

Polyribosome assay. As was mentioned above, the effect of each compound on the polysome pattern was tested under three conditions: direct effect of the compound on the polysome pattern; effect on ribosome run-off produced by medium hypertonicity (23, 24, 35); and effect on recovery from hypertonicity. Appropriate medium hypertonicity has been shown to block initiation with subsequent run-off of ribosomes from polysomes. After restoration of medium isotonicity, polysomes reform within 15 min to approximately 90% of control values. In polio virus-infected HeLa cells, it has been shown that the reinitiation is synchronous and occurs at the proper site on the messenger RNA (mRNA) (24). An inhibitor of initiation would be expected to cause clearing of the mRNA and to enhance or have no effect on polyribosome run-off due to medium hypertonicity. Presence of an initiation inhibitor during the recovery period should hinder polysome reformation since formation of an active initiation complex would be blocked. On the other hand, an elongation inhibitor should stabilize the polysome pattern and prevent run-off due to medium hypertonicity. However, in the presence of an elongation inhibitor, polyribosomes should not reform readily after restoration of medium isotonicity. Lastly, inhibition of termination would be expected to favor a shift to larger polysomes relative to the control under all three conditions of testing. Thus, in principle, it should be possible to distinguish between initiation, elongation, or termination inhibitors on the basis of their effects on the polyribosome pattern of cells incubated under the three different conditions.

When the effect of a compound on the polysome pattern was tested, HeLa cells were grown and collected by centrifugation as described above. The cell pellet was resuspended at a concentration of 4.3 × 10⁶ cells/ml in the same medium (conditioned medium) in which they had been grown but to which 50 mM N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid, pH 7.2, was added.

In those experiments (panels A, D, and G of Fig. 1–3) where the direct effect of a compound was
FIG. 2. Effect of diacetoxyscirpenol, pactamycin, and emetine on the polysome patterns of normal and NaCl-treated (hypertonic shock) HeLa cells. The data shown in this figure are a continuation of the experiment shown in Fig. 1. All conditions and experimental details are the same as in Fig. 1. (A, D, and G) Effect of incubating the cells with the inhibitors alone; (B, E, and H) effect of a 5-min incubation with the inhibitors followed by medium hypertonicity; (C, F, and I) effect of the inhibitors when they are present during the recovery from medium hypertonicity. The left hand ordinate represents the absorbance at 260 nm (\(A_{260}\)) for the subunit and monosome region of the gradient. The right hand ordinate represents the \(A_{260}\) for the polysome region beginning at the point where the pattern is discontinuous.

examined, 2.5 ml of concentrated cell suspension was incubated at 37 C with the compound at the concentration indicated in Table 1 and 0.20 ml of conditioned medium. The volumes of all samples were brought to 3.0 ml with water and the final cell concentration was 3.6 \(\times\) 10^6 cells/ml. The incubation was continued for 5 min and the reaction was stopped by pouring the entire sample over frozen crushed Earle balanced salt solution. From these samples, lysates and polysomes were prepared as described below.

In the experiments (panels B, E, and H of Fig. 1-3) investigating the effect of a compound on the run-off of ribosomes produced by medium hypertonicity, 2.5 ml of cell suspension was incubated at 37 C for 5 min with 1.1 times the final concentration of compound used above (Table 1). Then to make the medium hypertonic, 0.2 ml of 1.5 M NaCl and sufficient water were added for a final volume of 3.0 ml. The incubation in the hypertonic medium with the compound at the concentration indicated (Table 1) was continued at 37 C for an additional 15 min. The reaction was stopped as above; lysates and polysomes were prepared as described below.

The third part of the experiment (panels C, F, and I of Fig. 1-3) measured the effect of the compound on recovery after restoration of the medium to isotonicity. In this case 2.5 ml of the cell suspension was treated with 0.2 ml of 1.5 M NaCl and the volume was brought to 3.0 ml with the conditioned medium used in the cell resuspension. The samples were incubated for 15 min at 37 C in this hypertonic medium before introduction of the compounds to be tested. These
TABLE 1. Summary of effects of protein synthesis inhibitors on normal and NaCl-treated HeLa cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Conc (M)</th>
<th>% Inhibition of protein synthesis</th>
<th>Effect on polysome pattern directly</th>
<th>Effect on polysome run-off during NaCl treatment</th>
<th>Effect on the recovery from NaCl treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibiotic</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Harringtonine</td>
<td>$3.3 \times 10^{-4}$</td>
<td>97.8</td>
<td>Complete run-off</td>
<td>No effect</td>
<td>Slight recovery</td>
</tr>
<tr>
<td>Isoharringtonine</td>
<td>$3.3 \times 10^{-4}$</td>
<td>96.4</td>
<td>Nearly complete run-off</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Homoharringtonine</td>
<td>$3.3 \times 10^{-4}$</td>
<td>98.1</td>
<td>Nearly complete run-off</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDMP</td>
<td>$1 \times 10^{-4}$</td>
<td>62.6</td>
<td>Incomplete run-off</td>
<td>No effect</td>
<td>Limited recovery</td>
</tr>
<tr>
<td>Diacetoxyispcinol</td>
<td>$1 \times 10^{-4}$</td>
<td>95</td>
<td>Incomplete run-off</td>
<td>No effect</td>
<td>Slight recovery</td>
</tr>
<tr>
<td>Pectamycin</td>
<td>$7.8 \times 10^{-5}$</td>
<td>94.3</td>
<td>Incomplete run-off</td>
<td>Incomplete run-off</td>
<td>Limited recovery</td>
</tr>
<tr>
<td>Emetine</td>
<td>$1 \times 10^{-3}$</td>
<td>95.2</td>
<td>Stabilizes</td>
<td>Stabilizes</td>
<td>63%*</td>
</tr>
<tr>
<td>Anisomycin</td>
<td>$6.8 \times 10^{-4}$</td>
<td>96.7</td>
<td>Stabilizes</td>
<td>Stabilizes</td>
<td>48%*</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>$1 \times 10^{-4}$</td>
<td>95.6</td>
<td>Stabilizes</td>
<td>Stabilizes</td>
<td>61.3%*</td>
</tr>
<tr>
<td>Trichodermin</td>
<td>$1 \times 10^{-4}$</td>
<td>94.9</td>
<td>Stabilizes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inhibitor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initiation</td>
<td></td>
<td></td>
<td>Run-off</td>
<td>No effect</td>
<td>Limited recovery</td>
</tr>
<tr>
<td>Elongation</td>
<td></td>
<td></td>
<td>Stabilizes</td>
<td>Stabilizes</td>
<td>Inhibits recovery</td>
</tr>
<tr>
<td>Termination</td>
<td></td>
<td></td>
<td>Stabilizes</td>
<td>Stabilizes</td>
<td>Good recovery</td>
</tr>
</tbody>
</table>

* Recovery calculated according to the following equation: $(A - B)/(C - D) \times 100$, where: $A$ is the area of the polysome region when the polysomes recover from medium hypertonicity for 15 min in the presence of antibiotic divided by the total area of the gradient profile; $B$ is the area of the polysome region when the cells are subjected to medium hypertonicity divided by the total area of the gradient profile; and $C$ is the area of the polysome region when the polysomes recover from medium hypertonicity for 15 min in the absence of antibiotic divided by the total area of the gradient profile.

RESULTS

Inhibition of $^{14}$C-labeled amino acid incorporation into protein of intact cells. The effects of various compounds on incorporation of $^{14}$C-labeled amino acid into protein of intact cells are summarized in Table 2. Most compounds tested were good inhibitors of protein synthesis with 50% inhibition occurring at less than $10^{-4}$ M. Exceptions were MDMP, fusidic acid, and aurintricarboxylic acid, which produced 50% inhibitions at $2.8 \times 10^{-5}$ M, $4.8 \times 10^{-4}$ M, and $>1 \times 10^{-3}$ M, respectively. MDMP, though a potent herbicide, may not be as effective in animal cells. Fusidic acid and aurintricarboxylic acid (ATA) produced reasonably good inhibitions of $^{14}$C-labeled amino acid incorporation. Analyses by the polysome assay (data not shown), however, were inconsistent with their known mechanisms of action as inhibitors of elongation and initiation, respectively, in cell-free extracts (12, 30; see below). Although approximately equal amounts of anisomycin and cycloheximide were required for 50% inhibition of protein synthesis, the concentrations of these inhibitors necessary to achieve 95% inhibition differed by an order of magnitude.

Of the four alkaloids, cephalotaxine, harringtonine, isoharringtonine, and homoharringtonine (11, 22), homoharringtonine seemed to be the most effective, although harringtonine and isoharringtonine were nearly as effective. The
latter three alkaloids have been shown to have antitumor activity (22); however, cephalotaxine, the parent compound, which had no antitumor activity, was least effective in inhibiting protein synthesis.

Inhibition of protein synthesis as measured by the polysome assay. As described above, the effect of each inhibitor on the polysome pattern was examined under three distinct conditions. The resultant polysome patterns are shown in Fig. 1 to 3 and the data are summarized in Table 1.

Analyses of polysomes of HeLa cells after exposure to harringtonine, isoharringtonine, or homoharringtonine showed little material remaining in the polysome region. Only the data for harringtonine is shown (Fig. 1D) for it was essentially identical to that for iso- and homoharringtonine. Clearing of ribosomes from internal positions on mRNA, observed as a loss of polysomes, would be expected for initiation inhibitors. Such compounds permit run-off of ribosomes, which cannot reinitiate their progress along mRNA. To further confirm that these alkaloids were inhibitors of initiation, cells incubated with harringtonine were subjected to hypertonicity. Medium hypertonicity (23, 24, 35), itself an effective inhibitor of initiation (Fig. 1B), caused essentially complete run-off of ribosomes from RNA. As expected for an inhibitor of initiation, prior incubation with harringtonine had no effect on the run-off of ribosomes resulting from incubation with high NaCl alone (compare Fig. 1B and E). The effect of harringtonine on the reformation of polysomes after restoration of isotonicity was also consistent with interference at an initiation step of protein synthesis. Because mRNA's are cleared of ribosomes after the medium is made hypertonic (35), an initiation inhibitor would be expected to allow very little recovery of polysomes. Comparison of the pattern after restoration of isotonicity with that observed when harringtonine was added before the recovery period (compare Fig. 1C and F) demonstrated that only minimal recovery of polysomes occurred in the presence of harringtonine. These results are consistent with the concept that harringtonine is an inhibitor of initiation.

The effects of MDMP and diacetoxyscirpenol on polysome patterns of intact HeLa cells were investigated (see Fig. 1 and 2). Some polysomes remained after 5 min of treatment with the inhibitors (Fig. 1G, 2A). However, since ribosome run-off was unaffected by these inhibitors (Fig. 1H and 2B), it was unlikely they hindered elongation or termination. Thus, their effects were consistent with inhibition of initiation.

Some polysomes remained after a 5-min treatment of the cells with 7.8 x 10^-7 M pactamycin (Fig. 2D). When cells treated with pactamycin were subjected to hypertonicity (Fig. 2E), the run-off appeared to have been incomplete. Recovery from hypertonicity occurred to a small extent in the presence of pactamycin (Fig. 2F). These results seemed to indicate that the drug partially inhibited elongation as well as initiation. These effects of pactamycin are compared below with those in other eukaryotic systems (12, 13).

The anticipated effects on the polysome patterns, of incubating intact HeLa cells with elongation inhibitors, were shown by emetine, anisomycin, cycloheximide, and trichodermin

<table>
<thead>
<tr>
<th>Compound</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50% (M)</td>
</tr>
<tr>
<td>Pactamycin</td>
<td>2.7 x 10^-7</td>
</tr>
<tr>
<td>MDMP</td>
<td>2.8 x 10^-8</td>
</tr>
<tr>
<td>Diacetoxyscirpenol</td>
<td>1.6 x 10^-7</td>
</tr>
<tr>
<td>Harringtonine</td>
<td>2.1 x 10^-7</td>
</tr>
<tr>
<td>Isoharringtonine</td>
<td>2.2 x 10^-4</td>
</tr>
<tr>
<td>Homoharringtonine</td>
<td>1.4 x 10^-4</td>
</tr>
<tr>
<td>Cephalotaxine</td>
<td>9.8 x 10^-3a (22.4%)</td>
</tr>
<tr>
<td>Emetine</td>
<td>1.2 x 10^-8</td>
</tr>
<tr>
<td>Anisomycin</td>
<td>4.3 x 10^-7</td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>6.8 x 10^-7</td>
</tr>
<tr>
<td>Trichodermin</td>
<td>1.1 x 10^-4</td>
</tr>
<tr>
<td>Fusidic Acid</td>
<td>4.8 x 10^-4</td>
</tr>
<tr>
<td>Aurintricarboxylic acid</td>
<td>1 x 10^-3a (44%)</td>
</tr>
</tbody>
</table>

*a Highest concentration tested.
*b This sample contained 1% ethanol which inhibited protein synthesis approximately 8%.
(Fig. 2 and 3). Because they inhibit peptide bond formation and/or translocation, these agents stabilize the polyribosomes. The pattern observed on incubation of cells with each of these compounds alone was similar to that of control samples (Fig. 1A) where no inhibitor had been added. Since run-off of ribosomes during medium hypertonicity presumably occurs by completion of nascent chains already on the ribosome (24), the disappearance of polysomes during hypertonicity, therefore, required protein synthesis. Inhibitors of elongation would be expected to prevent this run-off. As shown in Fig. 2 and 3, emetine, anisomycin, cycloheximide, and trichodermin prevented run-off. If elongation inhibitors completely blocked peptide bond formation or translocation in the presence of such agents no recovery of polysomes would be expected during the recovery period. These four elongation inhibitors, however, allowed 48 to 63% restoration of the polysomes during the 15-min recovery period.

DISCUSSION

Examination of inhibitors of protein synthesis for their effects on HeLa cell polysomes under three conditions (directly, during hypertonicity, and during recovery from hypertonicity) provides a method for localizing their site of action in the intact cell. It also provides a convenient means of differentiating between initiation, elongation, and termination inhibitors. Treatment of cells with harringtonine, MDMP, and diacetoxyscirpenol resulted in either few or no polysomes under all three test conditions consistent with inhibition of initiation in intact HeLa cells. By this analysis, emetine, anisomycin, cycloheximide, and trichodermin were shown to be elongation inhibitors in agreement with the results of a number of other studies (9, 10, 14, 31). Elongation inhibitors, whether used directly or during hypertonicity, cause stabilization of the polysomes in a pattern similar to the control sample (Fig. 1A). Only small polysomes were observed on addition of the inhibitors during the recovery period. On the other hand, a specific inhibitor of termination would be expected to cause an accumulation of heavy polysomes relative to the control under all three conditions. In HeLa cells, we found no evidence that any of the compounds tested were acting specifically and only at the termination step of protein synthesis.

Trichodermin was reported to be a termination inhibitor (33; B. S. Hansen and M. H. Vaughan, Fed. Proc. 32:494, 1973). Using a temperature-sensitive mutant of yeast whose ribosomes run-off but reinitiate very slowly when the temperature is raised to the restrictive temperature, Stafford and McLaughlin (27) compared the rate of polysome reformation and the specific activity of ribosome-bound nascent peptides in the presence of trichodermin or cycloheximide during recovery. They suggested that in yeast trichodermin acts as an inhibitor of termination. In addition, Wei et al. (33) have reported trichodermin to be a preferential inhibitor of termination in intact HeLa cells. Their data (Table 2 in reference 33), like ours (Fig. 3, Table 1), indicate that neither trichodermin nor cycloheximide block reformation of polyribosomes and that reformation of polysomes occurred to a similar extent with both antibiotics.

Examination of the effect of MDMP on the polysome pattern (Fig. 1G) indicated that not all of the polysomes had run off the message as would be expected for an inhibitor of initiation. However, MDMP is quite insoluble in water and is therefore prepared as a 0.1 M stock solution in 100% ethanol. To choose a concentration where the ethanol is not inhibitory to protein synthesis, it was necessary to test the effect of MDMP on the polysome pattern at a concentration (10^-4 M) which inhibited protein synthesis only 62%. This low inhibition probably accounts for the incomplete clearing of the mRNA. MDMP did not interfere with run-off due to hypertonic shock, but allowed some recovery from such treatment. This latter effect could have been the result of the incomplete inhibition of protein synthesis. The results are consistent with inhibition of initiation by MDMP as previously reported by Weeks and Baxter (32) in extracts of wheat germ.

Of the six proposed inhibitors of initiation tested, only the results with pactamycin are not consistent with sole inhibition of initiation. A considerable amount of material remained in the polysome region after pactamycin treatment and hypertonic shock. The polysome patterns indicated that at 7.8 x 10^-7 M pactamycin, which produced 94% inhibition of protein synthesis, inhibition of elongation as well as of initiation occurred. These results are in agreement with those of Tai et al. (30) in Escherichia coli. They compared the effects of pactamycin in a cell-free system which carried out only elongation with those in a system which initiated as well as elongated. Their findings indicated that the concentrations of pactamycin which inhibited elongation overlapped extensively with those which inhibited initiation. In contrast, with rabbit reticulocyte
extracts, considerably higher concentrations of pactamycin (1.6 × 10^{-4} M) were required to inhibit elongation than initiation (4.2 × 10^{-7} M; 12). Analysis of polyribosome profiles of those extracts, however, revealed that ribosome run-off was not complete even at 4.2 × 10^{-7} M pactamycin suggesting some overlap of inhibition of initiation and elongation (12). On viral infection of mammalian cells, results with pactamycin are consistent with predominant inhibition of initiation of viral-coded proteins in intact cells (1, 2, 28). Nevertheless, polyribo- some profiles of poliovirus-infected HeLa cells indicated 10^{-7} M pactamycin, which chiefly inhibited initiation, did not produce complete run-off ribosomes (28). In addition, possible concentration of pactamycin by HeLa cells might preclude the fine control of antibiotic concentration possible in cell-free systems. All these studies indicated that effects of pactamycin on initiation and elongation overlapped.

Analyzing the effects of fusidic acid andATA on intact HeLa cells by the methods outlined failed to localize the site of action of these inhibitors. For both, the results of studies under the three sets of conditions for polysome analysis were not consistent with the established mechanism of action of these drugs. Neither fusidic acid nor ATA was a very active inhibitor of protein synthesis in intact HeLa cells. To observe an effect with either of them fairly high concentrations (fusidic acid, 4.8 × 10^{-4} M; ATA, 10^{-4} M) were required. In such large quantities presence of an impurity in the commercial preparations could have exerted a substantial effect on the observed results. Also, the apparent inhibition of protein synthesis at such high concentrations might be due to nonspecific interactions such as detergent effects or other effects leading to cell death. Therefore, we made no conclusions concerning the mechanism of action of these two drugs in intact HeLa cells.

In the presence of emetine, cycloheximide, anisomycin, or trichodermin at concentrations which inhibit ^{14}C-labeled amino acid incorporation into protein 95%, approximately 50% of the polysomes reform within 15 min after the restoration of isotonicity to the medium (Table 1). Experiments have indicated (data not shown) that radioactive amino acids are incorporated into protein on polysomes during reformation of polysomes even when elongation inhibitors are used at concentrations in excess of those necessary to inhibit protein synthesis greater than 99%, as measured by ^{14}C]leucine incorporation. These findings suggest that these inhibitors, thought to block protein synthesis, do in fact allow some movement along the mRNA. This may indicate that ribosomes may move along mRNA without protein synthesis or that peptidyl-tRNA may possibly fall off ribosomes during a block in elongation. The peptidyl-tRNA hydrolase would then hydrolyze this species for recycling. J. R. Menninger (Fed. Proc. 33:1335, 1974) has shown that, indeed, in bacteria some inhibitors which interfere with protein synthesis stimulate premature release of peptidyl-tRNA from cells. Other interpretations are also possible. For example, HeLa cells synthesize protein at a rate of 5.2 amino acids/s per ribosome (36). During the 15-min recovery period, an average mRNA would have ribosomes traversing it at a rate of 15.6 nucleotides/s per ribosome. If the ribosomes were 90 nucleotides apart, every 5.8 s an additional ribosome would begin traversing the mRNA. At 95 and 99% inhibition of protein synthesis, the rate of ribosome movement should be reduced to 0.78 and 0.16 nucleotides/s, respectively; in 15 min, at least 7.8 and 1.6 ribosomes, respectively, should have attached and begun traversing the mRNA (and, perhaps, even more if initiation is not inhibited and ribosomal packing onto mRNA can be increased). This calculation and the results suggest that evaluation of the polysome profile may be a more sensitive method of detecting small degrees of protein synthesis than amino acid incorporation.

It is clear that postulated mechanisms of inhibitor action based on data from cell-free systems may not reflect the actual mechanism in whole cells. For example, trichodermin belongs to a class of compounds called 12,13-epoxytrichothecones which block the puromycin fragment reaction (3). By this assay they would appear to be inhibitors of peptidyl transferase activity. They would be expected to interfere with the elongation or termination step of protein synthesis. Actually, they fall into two categories when tested in whole cells (5): one class which inhibits initiation and another which inhibits elongation. Schindler (25) recently showed that it is possible to distinguish the initiation-type inhibitors from the elongation-type in a cell-free system if the proper assays are employed. The methods described in this communication for localization of inhibitor action in whole cells should also be quite convenient and useful.

Harringtonine was an effective inhibitor of initiation in HeLa cells. Direct treatment of the cells with harringtonine led to essentially complete disappearance of the polyribosomes (see Fig. 1D) and a concomitant appearance of
monosomes and subunits. Treatment during the period of recovery from hypertonicity prevented reformation of the polyribosomes. Preliminary results, both from labeling of mRNA in intact HeLa cells and from experiments with radioactive mRNA in wheat germ extracts, suggested that harringtonine did not interfere with binding of mRNA to monosomes. Therefore, in the presence of harringtonine, ribosomes should be fixed to all initiation sites on mRNA. Thus, messages with one initiation site should appear as monosomes, messages with two as disomes, and so forth. Because only monosomes were found in the presence of harringtonine, that is, no distinct dimers or heavier polysomes, the results are consistent with the presence of only one initiation site and presumably one termination site on each mRNA of HeLa cells. If messages with multiple initiation sites existed, such extensive clearing of the mRNA would not be expected. For example, messages with three initiation sites would yield a trimmer peak in the polysome pattern after harringtonine treatment.

It should be pointed out that there are several underlying assumptions involved in the conclusions drawn above. The conclusions depend on the sedimentation properties of the ribosome-mRNA complexes formed by harringtonine treatment. An mRNA with ribosomes at two initiation sites is expected to sediment as a dimer. On an mRNA strand coding for two proteins of 50,000 molecular weight, the two ribosomes bound to the separate initiation sites would be separated by an RNA of approximately 450,000 daltons. Such a polymer might make a sufficiently large contribution to the frictional coefficient of the mRNA-ribosome complex so that it sediments slower than a dimer. Furthermore, it is also possible that other mechanisms might restrict the potential number of alternatives. For example, if there were more than one initiation site on each mRNA strand, ribosomes might not be able to bind to them independently. Recovery of polysomes after restoration of medium isotonicity in the presence of harringtonine might then yield a pattern of monosomes although multiple initiation sites existed. However, the fact that monosomes are observed almost exclusively when polyribosomes run-off in the presence of harringtonine alone (without medium hypertonicity) would tend to argue against this objection. For an addition of harringtonine, ribosomes most probably are randomly distributed along the entire mRNA strand.

There is strong evidence that at least two cistrons of both Qβ and R17 RNAs have been translated in mammalian cell-free systems (15, 26). This indicates that eukaryotes have the capability of translating mRNA's which have more than one initiation site. However, the data presented in this communication suggest that most, if not all, mRNA's of HeLa cells like those of yeast (21) possess single initiation sites.

ACKNOWLEDGMENTS

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LITERATURE CITED