Inhibitors of Protein Biosynthesis

V. EFFECTS OF EMETINE ON PROTEIN AND NUCLEIC ACID BIOSYNTHESIS IN HE LA CELLS*

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SUMMARY

Emetine is rapidly concentrated from the media by suspension cultures of HeLa cells, and it inhibits protein biosynthesis by 50% at a concentration in the media of $4 \times 10^{-8}$ M. The synthesis of DNA is partially inhibited by emetine, but the primary effect of the alkaloid in HeLa cells appears to be on the biosynthesis of protein. In contrast to the effects of cycloheximide, the effects of emetine on DNA and protein synthesis are irreversible. Synthesis of host cell RNA is unaffected by emetine, but viral RNA synthesis in poliovirus-infected HeLa cells is inhibited by this drug. Emetine prevents the breakdown of polyribosomes induced by puromycin but does not affect the puromycin-induced release of nascent peptide. The observations reported in this paper provide a biochemical basis for the toxic and therapeutic properties of emetine.

METHODS

Growth of Cells—HeLa S2 cells were grown at 37° in suspension culture in Eagle’s minimum essential medium (6) supplemented with 5% horse serum as previously described (7). For individual experiments, aliquots of the cell suspension were equilibrated with $\frac{70}{3}$ CO$_2$ and incubated at 37° with constant stirring.

Preparation of Cytoplasmic Extracts—Cytoplasmic extracts were prepared by allowing the cells to swell for 7 min in reticulocyte standard buffer (8). Then they were ruptured with 16 strokes of a Dounce homogenizer, and the nuclei and unbroken cells were removed by centrifuging for 10 min at 800 × g. Sodium deoxycholate was added to a final concentration of 0.5% in cytoplasmic extracts subjected to sucrose density gradient centrifugation.

Measurement of RNA, DNA, and Protein Synthesis—Methods for the measurement of protein, RNA, and DNA synthesis in intact HeLa cells have been described in detail in a prior publication (7). DNA polymerase activity in extracts of HeLa cells was measured by the method of Jungwirth and Joklik (9), and thymidine kinase activity was measured by the method of McAusland and Joklik (10). Infection of actinomycin-treated HeLa cells with poliovirus and subsequent measurement of $^{14}$C-uridine incorporation into viral RNA were carried out as described by Summers, Maize®, and Darnell (11).

Measurement of Radioactivity—Radioactivity in $^{14}$C-containing samples was determined in a low background counter with an efficiency of 21%. Aqueous samples containing tritium were counted, at an efficiency of 16%, in 10 ml of the scintillation mixture described by Bray (12). TCA- insoluble samples contained.

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† Career Investigator of the Health Research Council of the City of New York.

The alkaloids of Ipecacuanha (2) have been widely used for medicinal purposes since their introduction into European medicine 300 years ago (3). Although it has long been known (4) that these compounds inhibit the growth of Entamoeba histolytica, the causative agent of amebic dysentery, their mode of action remained obscure until the recent report (5) that emetine (Fig. 1, I), a biologically inactive isomer (4, 5), serves as a control for structural specificity. The studies to be described provide a biochemical basis for most of the known toxic and therapeutic properties of emetine.

The present paper describes the inhibitory effects of emetine on the synthesis of certain macromolecules in HeLa cells. Iso-emetine (Fig. 1, II), a biologically inactive isomer (4, 5), serves as a control for structural specificity. The studies to be described provide a biochemical basis for most of the known toxic and therapeutic properties of emetine.

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suspended at a concentration of 4
hour, respectively. The percentage of incorporation shown is
ml per hour, 985 cpm per ml per hour, and 850 cpm per ml per
DNA synthesis which, in the control reaction, was 4,820 cpm per
The incorporation of radioactivity into TCA-insoluble ma-
ments.

duplicate aliquots of 0.5 ml were transferred to tubes containing
tioned to 3-ml aliquots. After 2 min, 1 mpmole of 14C-leucine
40% horse serum. Metamine was added at the indicated final concen-

FIG. 1. Structural formulae of emetine (I) and isoemetine (II)

FIG. 2. Effect of varying concentrations of emetine on the synthesis of protein, RNA, and DNA in HeLa cells. HeLa cells were suspended at a concentration of 4 × 10^6 cells per ml in leucine-deficient Eagle's medium containing 0.04 ml% leucine and 5% horse serum. Emetine was added at the indicated final concentrations to 3-ml aliquots. After 2 min, 1 mpmole of 14C-leucine (100,000 cpm), 10 mpmoles of 14C-thymidine (100,000 cpm), or 5 mpmoles of 14C-uridine (60,000 cpm) were added. After 60 min, duplicate aliquots of 0.5 ml were transferred to tubes containing cold Earle's solution (15), and the cells were prepared for the determination of radioactivity as described under "Methods." The incorporation of radioactivity into TCA-insoluble material was used to calculate the linear rate of protein, RNA, and DNA synthesis which, in the control reaction, was 4,620 cpm per ml per hour, 985 cpm per ml per hour, and 850 cpm per ml per hour, respectively. The percentage of incorporation shown is expressed relative to these control values. The experiment illustrated in this figure is representative of 10 separate experiments.

taining tritium were collected on Millipore membrane filters and
treated with 0.6 ml of NH_4OH for 15 min. The filters were then
dissolved in 10 ml of the scintillation mixture and counted at an

Density Gradient Centrifugation—Density gradient centrifugation was performed as previously described (1, 7) according to the procedure of Britten and Roberts (13). Samples of 1 ml were layered over 26 ml of 15 to 30% (w/v) or 5 to 20% (w/v) gradients of sucrose dissolved in reticulocyte standard buffer. Gradients were centrifuged at 4° in the SW-25.1 swinging bucket rotor or, in some cases, the No. 30 fixed angle rotor of a Spinco centrifuge. Optical density at 260 nm was determined during
collection of the gradients by means of a flow cell with a light path of 0.5 cm attached to a Gilford spectrophotometer. Fractions (1 ml each) were collected, an equal volume of 10% TCA was added to each fraction, and the precipitates were collected on Millipore filters for the determination of radioactivity.

Materials

Emetine was obtained from S. B. Penick and Company, New York, and recrystallized three times. Isoemetine was a gift from Dr. H. T. Openshaw, tubulosine was provided by Dr. A. Popelak, anisomycin was donated by Dr. K. Butler, streptovitacin A was supplied by Dr. J. T. Correll of the Upjohn Company, Kalamazoo, Michigan, and cycloheximide and puromycin were purchased from Nutritional Biochemicals. 3H-Emetine (700 mC per millmole) and 3H-isometine (700 mC per mmole) were prepared according to the method of Openshaw and Whittaker (14) by the catalytic reduction of (+)-O-methylpsychotrine with 3H_2O. After crystallization, both alkaloids contained less

than 1% of the isomeric compound as determined by thin layer chromatography on Silica Gel G in a solvent system composed of chloroform-methanol (85:15). Phosphoehymyruvate and crystalline pyruvate kinase were purchased from C. F. Boehringer und Sohne; calf thymus DNA from Worthington; serum and Eagle's minimum essential medium from Grand Island Biological Company; ATP and GTP from P-L Biochemicals, Milwaukee, Wisconsin; dATP, dGTP, and dCTP from Schwarz BioResearch; and phosphors from Packard. 14C-thymidine with a specific activity of 25 mC per mmole, 14C-uridine with a specific activity of 26 mC per mmole, 14C-phenylalanine tRNA with a specific activity of 320 mC per mmole, and 13C-thymidine 5'-triphosphate with a specific activity of 40 mC per mmole were purchased from New England Nuclear. Purified type 1 poliovirus, actinomycin, and deoxyuridine were kindly provided by Dr. Donald Summers.

Results

Effects of Emetine on RNA, DNA, and Protein Synthesis—The effects of emetine on nucleic acid and protein synthesis in nonsynchronized suspension cultures of HeLa cells is shown in Fig. 2. The rate of protein synthesis was inhibited by 50% at an emetine concentration of 4 × 10^{-4} M, by 95% at a concentration of 4 × 10^{-3} M, and by 99% at a concentration of 1 × 10^{-3} M. There is a parallel effect on the synthesis of DNA, but, at maximal inhibition, the rate of DNA synthesis remained 20% of control values. RNA synthesis was slightly stimulated at low concentrations of emetine and was inhibited 20% by high concentrations of the alkaloid.

The effects of 10^{-4} M concentrations of emetine on DNA, RNA, and protein synthesis in HeLa cells were observed within several minutes after exposure to the alkaloid (Fig. 3). The inhibitory effect on DNA synthesis was slightly less rapid than the inhibition of protein synthesis. The rate of RNA synthesis slowly decreased with time but continued at greater than half the control rate for 5 hours after complete inhibition of protein synthesis. As incorporation of 3H-thymidine into DNA was inhibited by emetine, the effects of the alkaloid on some intermediate steps in DNA synthesis, catalyzed by cell-free extracts, were examined.

I am grateful to Drs. Norman Whittaker and W. G. Duncombe for carrying out this reduction and isolating the reaction products in purified form.

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Emetine failed to inhibit the phosphorylation of thymidine (Table I) or the incorporation of TTP into DNA (Table II).

**Uptake of Emetine and Isoemetine**—The uptake of ^3H-emetine and ^3H-isometine by intact cells is dependent on time of incubation and on temperature (Fig. 4). The uptake is proportional to the concentration of alkaloid in the media at concentrations of less than 5 x 10^-5 M (Fig. 5). No differences in uptake between emetine and the biologically inactive ellemer were detected.

**Effect of Emetine on Attachment of Nascent Peptide to Polyribosomes and on Subsequent Release by Puromycin**—Exposure of growing cultures of HeLa cells to emetine for 2 min resulted in a decrease in the number of single ribosomes and a concomitant increase in polyribosomes (Fig. 6C). In cells exposed to labeled amino acids for 2 min, nascent peptide was detected in the polyribosome region (Fig. 6A) and, if emetine was added 2 min after

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**Table I**

**Effect of emetine on thymidine kinase activity in cytoplasmic extracts**

Reaction mixtures contained, in a final volume of 80 μl, 10 μmoles of Tris HCl, pH 7.8; 1 μmole of magnesium chloride; 2 μmoles of ATP; 25 μl of cytoplasmic extract; emetine at the indicated concentration; and 10 μmoles of ^14C-thymidine (100,000 cpm). At the indicated intervals, 15-μl aliquots were applied to the origin of Whatman No. 3 filter paper and subjected to high voltage electrophoresis for 45 min at 5000 volts in a solvent system composed of pyridine-acetic acid-water (1:10:69). The paper was dried, and its radioactivity was estimated by scanning the paper chromatogram and determining the area under the appropriate peak by planimetry.

<table>
<thead>
<tr>
<th>Additions</th>
<th>Time (min)</th>
<th>TMP (cpm)</th>
<th>TDP (cpm)</th>
<th>TTP (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>20</td>
<td>2000</td>
<td>1900</td>
<td>3500</td>
</tr>
<tr>
<td>None</td>
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<td>2100</td>
<td>3800</td>
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<tr>
<td>Emetine, 10^-3 M</td>
<td>60</td>
<td>0</td>
<td>0</td>
<td>7600</td>
</tr>
</tbody>
</table>

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**Table II**

**Effect of emetine on DNA polymerase activity in cytoplasmic extracts**

The complete reaction mixture, in a final volume of 0.3 ml, contained 20 μmoles of Tris HCl, 2 μmoles of MgCl₂, 0.7 μmoles of 2-mercaptoethanol, 20 μmoles each of dATP, dGTP, dCTP, and ^3H-dTTP (150,000 cpm), 50 μg of heated calf thymus DNA, 50 μl of cytoplasmic extract containing 100 μg of protein, and emetine at the indicated concentration. After incubating for 30 min at 37°, the reaction was terminated by the addition of an equal volume of 1 N perchloric acid. After standing in the cold for 30 min, the precipitate was collected on Millipore filters, washed with cold 0.2 m perchloric acid, and prepared for the determination of radioactivity as described under "Methods."

<table>
<thead>
<tr>
<th>System</th>
<th>Radioactivity incorporated (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>4276</td>
</tr>
<tr>
<td>Minus thymus DNA</td>
<td>10</td>
</tr>
<tr>
<td>Plus emetine, 10^-3 M</td>
<td>4130</td>
</tr>
<tr>
<td>Plus emetine, 10^-4 M</td>
<td>4194</td>
</tr>
</tbody>
</table>
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Fig. 5. Uptake of $^3$H-emetine and $^3$H-isoemetine as a function of the concentration of alkaloid in the media. HeLa cells were suspended at a concentration of $4 \times 10^5$ cells per ml. Varying amounts of $^3$H-emetine and $^3$H-isoemetine were added to individual cultures. The cells were incubated for 5 min at $37^\circ$, washed, and collected by filtration as described in the legend to Fig. 4, and the radioactivity was determined as described under "Methods." - - - - - , emetine-treated; O-O-O, isoemetine-treated.

The effects of emetine differ from those of puromycin which results in extensive breakdown of polyribosomes to single ribosomes and complete release of labeled polypeptide (Fig. 6B). These effects of puromycin are modified by prior exposure to emetine for 2 min; under these conditions, 85% of the nascent peptide is released from the polyribosomes with little degradation of polyribosomes (Fig. 6C).

Irreversibility of Effects of Emetine and Related Inhibitors of Protein Synthesis. Despite functional and conformational similarities (5), emetine differs from cycloheximide and anisomycin in that its effects on HeLa cells are not reversed by suspending the cells in fresh media (Fig. 7). In this respect its irreversible action is similar to that shown by tubulobin and by glutarimide antibiotics such as streptovitacin A (Fig. 7). The concentration of each antibiotic used in this experiment was determined by preliminary experiments to inhibit protein synthesis by 95% for at least 2 hours.

Inhibition of Viral RNA Synthesis by Emetine in Poliovirus-infected HeLa Cells—In Fig. 8, the effects of emetine on the synthesis of RNA in actinomycin-treated HeLa cells infected with poliovirus are shown. The studies of Summers et al. (11) have established that incorporation of $^{14}$C-uridine under these conditions represents primarily the synthesis of viral RNA. As seen in Fig. 8, viral RNA synthesis is inhibited by emetine.

Binding of $^3$H-Emetine to Ribosomes—The binding of $^3$H-emetine to ribosomes was studied by density gradient techniques and by equilibrium dialysis. When HeLa cells were incubated for 5 min with $^3$H-emetine and then lysed and centrifuged through sucrose density gradients, no significant binding of the alkaloid to single ribosomes or polyribosomes was observed. Similarly, when isolated ribosomes suspended in reticulocyte standard buffer were incubated at $37^\circ$ with $^3$H-emetine $(2 \times 10^{-6} \text{ M})$ or $^3$H-isoemetine $(2 \times 10^{-6} \text{ M})$ and then centrifuged at 100,000 X $g$ at 4$^\circ$, the same amounts of radioactivity were found to be associated with the rinsed pellets. $^3$H-Emetine binding to ribosomes was also studied by equilibrium dialysis. $^3$H-Isoemetine was required as a control for nonspecific binding since the ipecac alkaloids bind to a large variety of different proteins. No significant differences in binding between the active and inactive alkaloids were observed.

Discussion

The studies reported in this paper establish that emetine is concentrated from the media by HeLa cells and that it rapidly inhibits protein biosynthesis. HeLa cells used in these experi-

\[ A. P. Grollman, unpublished observations. \]
The concentration of emetine required to inhibit protein synthesis of intact cells by 50% is $4 \times 10^{-8}$ M, but in cell-free preparations, the concentration of drug required for a similar degree of inhibition is $8 \times 10^{-4}$ M (5). As the degree of concentration only partially accounts for this difference in sensitivity between intact cells and cell-free preparations, compartmentalization in the intact cell and exposure of additional binding sites for the inhibitor in the cell-free preparations may occur. It should also be noted that it is relatively easy to achieve virtually complete inhibition of protein synthesis in intact cells by treatment with emetine, while very high concentrations of the alkaloid are required to achieve complete inhibition in the cell-free preparations. Similar effects of cycloheximide on protein synthesis in L cells (16), rabbit reticulocytes (17), and yeast (18) have been noted.

Emetine can be assumed to act primarily on protein synthesis in reticulocytes, as these cells neither retain nor synthesize sufficient RNA or DNA to account for the rate of hemoglobin synthesis (19, 20). In HeLa cells, emetine inhibits the synthesis of host cell DNA although the activities of thymidine kinase and DNA polymerase are not affected by the drug. The observed inhibition of DNA synthesis is probably secondary to a primary effect on protein synthesis, as the latter process is required for the concurrent synthesis of DNA in animal cells (21-24). Anisomycin, puromycin, cycloheximide, and tubulosine, all of which inhibit protein synthesis in mammalian cells, are also known to inhibit the synthesis of DNA (7, 22, 25, 26).

Protein synthesis is also required for the synthesis of RNA in poliovirus-infected HeLa cells. Accordingly, inhibitors of protein synthesis, such as emetine, would be expected to prevent the synthesis of viral RNA.

The experiment described in Fig. 3 relates the inhibition of protein synthesis by emetine to the well established effects of puromycin. It is generally considered that attachment of an incoming aminoacyl-tRNA or puromycin molecule to the nascent peptide chain requires the movement of ribosomes along the strand of mRNA (8, 27, 28). The attachment of puromycin terminates the chain, and the incomplete peptide is released from the ribosome (29, 30). This process correlates temporally with the breakdown of polyribosomes to single ribosomes. It is surprising, therefore, that 85% of the nascent peptide would be released by puromycin in the emetine-treated cell without polyribosome breakdown. One must tentatively conclude that the integrity of the polyribosome is somehow preserved in the presence of emetine while allowing puromycin attachment and subsequent release of incomplete peptide chains. Similar effects have been noted with cycloheximide (31, 32). The rapid decrease in the number of single ribosomes and the concomitant increase in polyribosomes in emetine-treated cells may be a related process. The latter phenomenon has been observed in

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* E. Robbins, personal communication.

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**Fig. 7.** Reversal of the inhibition of protein synthesis by emetine and related antibiotics. HeLa cells, suspended in leucine-depleted Eagle's medium containing 5% horse sera at a concentration of $4 \times 10^4$ cells per ml, were incubated with the following concentrations of antibiotic for 2 min: cycloheximide, $10^{-5}$ M; streptovitacin A, $10^{-8}$ M; tubulosine, $10^{-4}$ M; or emetine, $10^{-4}$ M. The control culture contained no antibiotic. All cultures, including the control, were reversed by resuspending the cells three times in 3 ml of fresh leucine-depleted medium. After allowing 15 min for equilibration, 1 n mole of $^{14}$C-leucine (100,000 cpm) was added, and aliquots were taken for the measurement of protein synthesis at the indicated intervals as described in the legend to Fig. 2. The data shown are representative of eight separate experiments.

**Fig. 8.** Effect of emetine on the biosynthesis of poliovirus RNA. HeLa cells ($4 \times 10^4$ cells per ml), suspended in complete Eagle's medium without added horse serum, were infected with poliovirus at an input multiplicity of 300 plaque-forming units per cell. Actinomycin (10 \mu g per ml) and guanosine (3 mM) were added at the time of infection, and the cells were allowed to incubate at $37^\circ$ for 2 hours. The cells were washed twice with cold medium and resuspended in Eagle's medium containing 1 \mu C of $^{14}$C-uridine. The addition of radiisotope corresponded to zero time shown in the figure. Incubation was continued at $37^\circ$, and aliquots were taken at the indicated intervals, and the incorporation of $^{14}$C-uridine into RNA was determined, as described in the legend to Fig. 2.
polyribosome profiles of cycloheximide-treated mammalian cells (31-33) and in similar preparations from chloramphenicol-treated bacteria (34). It is also possible that the profile in these antibiotic-treated cells more closely represents the situation in the normal cell and that the untreated cell reflects a differential release of ribosomes due to residual protein synthesis at 0° (35).

In its mode of action, emetine resembles cycloheximide and the related glutarimide antibiotics (cf. Reference 36), anisomycin (7) and tubulosine (26), all of which inhibit protein synthesis in mammalian cells and yield at the level of the transfer reaction. On a molar basis, emetine is a more active inhibitor than cycloheximide in intact HeLa cells and in cell-free preparations from rabbit reticulocytes (5). The irreversibility of its effect allows one to distinguish emetine from anisomycin and cycloheximide on a functional basis. However, irreversibility of the inhibitory activity of other glutarimide antibiotics can be attributed to a binding site which is unrelated to its effects on protein synthesis (5, 17). Accordingly, the effects of acetoxycycloheximide (37), streptovitacin A (38), tubulosine, and emetine on protein biosynthesis appear to be remarkably similar. The conformational similarities previously observed between these chemically unrelated compounds also suggest that their site of action may be the same.

Emetine is widely used as an anabolic agent (39), emet (40) and expectorant. Its toxic effects in man, particularly on the myocardium, are well established (41). There is good evidence that emetine and isoemetine show less than 1% of the activity of emetine. In contrast, the emetic effects of emetine, as tested by Wang and Bhargava, are demonstrable only at very high concentrations of emetine and not structurally specific. The activities of emetine are consistent with inhibition of protein biosynthesis as the primary mode of action of this alkaloid.

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