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Mode of Action of Tylocrebrine: Effects on Protein and Nucleic Acid Synthesis

MOU-TUAN HUANG AND ARTHUR P. GROLLMAN

Departments of Pharmacology and Medicine and Division of Biology, Department of Molecular Biology, Albert Einstein College of Medicine, Bronx, New York 10461

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SUMMARY

The phenanthrene alkaloid tylocrebrine irreversibly inhibits protein biosynthesis in HeLa cells and rabbit reticulocytes. In HeLa cells, concentrations of tylocrebrine that inhibit protein synthesis by 95% inhibit DNA and RNA synthesis by 75% and 60%, respectively. In contrast to effects on DNA synthesis, inhibition of RNA synthesis is readily reversible. At a concentration of 1 mM, tylocrebrine inhibits RNA polymerase activity in isolated HeLa cell nuclei by 20%.

In reticulocyte lysates, tylocrebrine prevents breakdown of polyribosomes and release of nascent peptide chains. Globin synthesis is inhibited by the drug; this inhibition can be partially overcome by increasing the concentration of mercaptoethanol, glutathione, or dithiothreitol. Tylocrebrine does not prevent synthesis of polyphenylalanin on reticulocyte ribosomes, nor does it affect the activities of guanosine triphosphatase and peptidyltransferase. The principal effect of tylocrebrine on protein biosynthesis is on chain elongation.

INTRODUCTION

Tylocrebrine, a cytotoxic phenanthrene alkaloid isolated from *Tylophora crebriflora*, exhibits potent antitumor activity against Leukemia 1210 in mice (1). The structure of tylocrebrine (Fig. 1) and related phenanthrene alkaloids was established by Gellert *et al.* (2). Tylocrebrine inhibits incorporation of leucine into protein in Ehrlich ascites tumor cells (3) but does not affect synthesis of RNA. In yeast, tylocrebrine affects both cytoplasmic and mitochondrial protein synthesis (4).

The present paper describes the action of tylocrebrine on protein and nucleic acid synthesis in HeLa cells and rabbit reticulocytes. A principal effect of the drug on protein synthesis appears to involve peptide chain elongation. At high concentrations of tylocrebrine, reversible inhibition of RNA synthesis can also be demonstrated.

These investigations were supported in part by American Cancer Society Grant IC-61D. This is publication 283 from the Joan and Lester Avnet Institute of Molecular Biology and the fifth in a series of papers on inhibitors of protein synthesis; the preceding paper is ref. 18. A preliminary report of this work was presented before the American Society of Biological Chemists [*Fed. Proc.* **30**, 627 (1971)].

METHODS

Materials. Tylocrebrine was a gift from Dr. Nathan Belcher of Pfizer Pharmaceuticals. Cycloheximide and emetine were purchased from Sigma Chemical Company; puromycin, from Nutritional Biochemicals; 2-mercaptoethanol (type 1) from Sigma; dithiothreitol, A grade, from Calbiochem; glutathione, from Schwarz BioResearch;

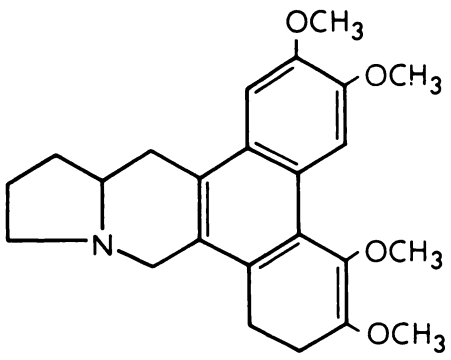

FIG. 1. *Structure of tylocrebrine*

and [^{3}H]GTP(1.3 Ci/mmole), [^{14}C] leucine (200 mCi/mmole), [^{14}C]thymidine (40 mCi/mmole), and [^{14}C]uridine (50 mCi/mmole), from New England Nuclear Corporation. The sources of [^{14}C]phenylalanyl-tRNA and other materials used in these experiments are described elsewhere (5, 6).

Crystallized bovine serum albumin was obtained from Mann Research Laboratories; calf serum and media for tissue culture, from Grand Island Biological Company; calf thymus DNA, from Worthington Biochemical Corporation; Triton X-100, from Rohm and Haas; and *Escherichia coli* RNA polymerase (fraction V), from Miles Laboratories.

Measurement of protein, DNA, and RNA synthesis in HeLa cells. HeLa S_3 cells were grown at 37° in Eagle's minimal essential medium (7) supplemented with 5% calf serum and harvested by centrifugation for 3 min at 1000 × *g*. Cells were resuspended at a concentration of 4 × 10^5/ml in minimal essential medium supplemented with 5% calf serum for measurement of DNA and RNA synthesis, or in leucine-depleted medium supplemented with 5% calf serum for measurement of protein synthesis. [^{14}C] Leucine (200 mCi/mmole, 0.025 μCi), [^{14}C] thymidine (50 mCi/mmole, 0.05 μCi), or [^{14}C]uridine (50 mCi/mmole, 0.025 μCi) was added to 1.5 ml of the cell suspension, and the rates of protein, DNA, and RNA synthesis were estimated by measuring incorporation of their respective precursors into cold trichloracetic acid-precipitable fractions. Cell suspensions were incubated at 37° for 60 min with stirring, and reactions were terminated by adding 2 ml of a cold saline buffer described by Earle (8) (buffer A). Cells were collected by centrifugation at 1000 × *g* for 3 min and lysed in 2 ml of cold distilled water, and 2 ml of 10% trichloracetic acid were added. For measurement of protein synthesis, samples were heated for 15 min at 95°. All solutions were chilled for 10 min; the precipitates were collected on Millipore membrane filters and washed three times with 5% trichloracetic acid, and the radioactivity was determined as described below.

Measurement of globin synthesis by reticulocyte lysates. Reticulocytosis was induced in rabbits by daily injection of acetophenylhydrazine (9). Reticulocyte lysates were prepared and the synthesis of globin was measured by a modification of the procedure of Maxwell and Rabinovitz (10), as described in a previous publication (6). The standard reaction mixture contained, in a final volume of 0.5 ml, 10 mM Tris-HCl (pH 7.4), 75 mM KCl, 1 mM ATP, 0.2 mM GTP, 15 mM creatine phosphate, 2 mM magnesium chloride, 2 μM [^{14}C]leucine (200 mCi/mmole), 6 mM 2-mercaptoethanol, a 100 μM mixture of 19 amino acids (9), 0.9 mg/ml of creatine phosphokinase, 60 μM hemin, and 0.10–0.13 ml of lysate. Reactions were terminated by the addition of 5% trichloracetic acid; mixtures then were heated for 15 min at 95° and chilled in an ice bath. Precipitates were collected on Millipore membrane filters, and radioactivity was determined as described below.

Measurement of hemoglobin synthesis in reticulocytes. Hemoglobin synthesis in suspensions of intact reticulocytes was measured by a published modification (9) of the procedures described by Lingrel and Borsook (11) and by Bruns and London (12). Packed cells, 2.5 ml, were resuspended in 25 ml of a complex buffer composed of fresh plasma, 2.5 ml; Eagle's medium lacking leucine, 15 ml; glucose, 0.035%; glycine, 0.12 mg/ml; $NaHCO_3$, 0.8 mg/ml; glutamine, 0.002 M; hemin, 0.12 mg/ml; and [^{14}C]leucine (200 mCi/mmole), 0.2 μM. The reaction mixture (1 ml) was incubated at 37° for 60 min, after which the reaction was terminated by adding a chilled saline solution composed of 0.12 M NaCl, 0.0052 M

KCl, and 0.0075 M magnesium chloride. Cells were collected by centrifugation for 5 min at 3000 × *g*, the supernatant solution was aspirated, and the cells were washed twice with the saline solution described above, containing 0.01 M [^{12}C]leucine. Cells were lysed by adding an equal volume of distilled water and allowed to stand for 5 min at 4°. Cell debris was removed by centrifugation at 12,000 × *g* for 10 min, and hemoglobin was precipitated by adding an equal volume of 10% trichloracetic acid. The solution was heated at 90° for 20 min; precipitates were collected on Millipore filters and washed three times with 5% trichloracetic acid, and the radioactivity was determined as described below. Hemoglobin concentration was estimated spectrophotometrically by its absorption at 450 nm, assuming a hemoglobin concentration of 1 mg/ml in a 1-cm cell to have an optical density of 0.885.

Sucrose density gradient centrifugation. Density gradient centrifugation was performed according to the procedure of Britten and Roberts (13). Samples of 1 ml were layered over 36 ml of 10–25% (w/v) sucrose gradients prepared in a reticulocyte standard buffer (buffer B) (14) composed of 0.01 M Tris-HCl (pH 7.4), 0.01 M KCl, and 1.5 mM magnesium chloride. Gradients were centrifuged at 4° in an SW 27 swinging bucket rotor for 2.5 hr, and the absorbance at 260 nm was determined during collection of the gradient by means of a flow cell (light path, 0.5 cm) attached to a Gilford spectrophotometer. Fractions (1.2 ml) were collected from the bottom of the tube for determination of radioactivity, and sufficient trichloracetic acid was added to each fraction to bring the final concentration to 5%. Fractions were heated at 95° for 15 min, then chilled in an ice water bath. Precipitates were collected on Millipore filters and washed with cold 5% trichloracetic acid, and the radioactivity was determined as described below.

Assay of E. coli RNA polymerase activity. The activity of *E. coli* RNA polymerase was assayed as described by Burgess (15). Reaction mixtures contained, in a final volume of 0.25 ml, 0.04 M Tris-HCl (pH 7.9), 0.01 M magnesium chloride, 0.01 mM EDTA, 0.1 mM dithiothreitol, 0.15 M KCl, 0.5 mg/ml of bovine serum albumin, 0.15 mM concentration each of ATP, UTP, and CTP, 0.15 μM, [^{3}H]GTP (1.3 Ci/mmole), and 0.15 mg/ml of heat-denatured calf thymus DNA. The reaction mixtures were incubated at 37° for 10 min, chilled in an ice bath, then precipitated with 3 ml of 5% trichloracetic acid containing 0.01 M sodium pyrophosphate. After 15 min, precipitates were collected on Millipore filters (0.45-μ pore size), washed four times with 3 ml of 2% trichloracetic acid containing 0.01 M sodium pyrophosphate, and placed in scintillation vials for determination of radioactivity.

Assay of RNA polymerase activity in HeLa cell nuclei. HeLa cells (200 ml, 40 × 10^5 cells/ml) were harvested, washed twice with 50 ml of Earle's buffer, then resuspended in 2

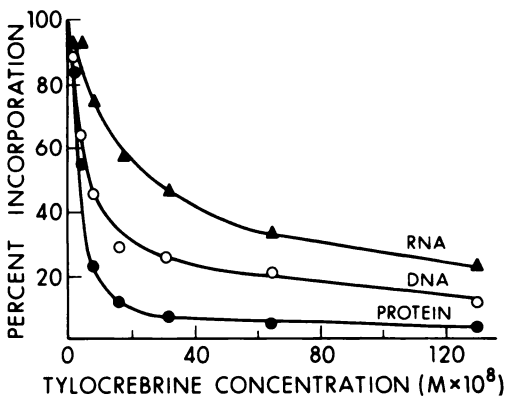

FIG. 2. *Effect of various concentrations of tylocrebrine on biosynthesis of RNA, DNA, and protein in HeLa cells*

Tylocrebrine was added, at the indicated final concentrations, to 1.5 ml of HeLa cells, suspended in leucine-depleted Eagle's medium containing 5% horse serum at a concentration of 4 × 10^5/ml. After 1 min, 1 nmole of [^{14}C]leucine (200 mCi/mmole), 10 nmoles of [^{14}C]thymidine (55 mCi/mmole), or 5 nmoles of [^{14}C]uridine (55 mCi/mmole) were added. The cell suspension was incubated at 37° for 60 min, the reactions were terminated by addition of 3 ml of buffer A, and the rates of RNA, DNA, and protein synthesis were determined as described under METHODS. The percentage incorporation shown is expressed relative to cell cultures to which no inhibitor was added.

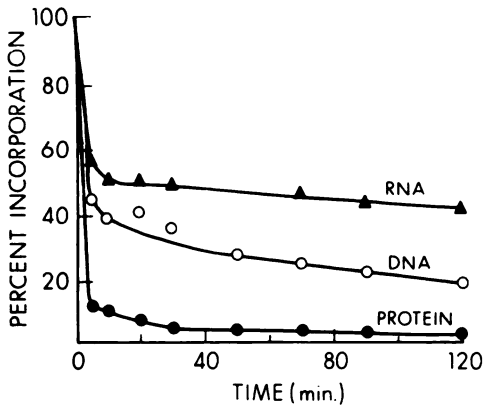

FIG. 3. *Effect of tylocrebrine on synthesis of RNA, DNA, and protein in HeLa cells at various times following exposure to antibiotic*

HeLa cells (4×10^5/ml), suspended in complete Eagle's medium supplemented by 5% horse serum, were divided into two portions of 1.5 ml, and tylocrebrine was added to one at a final concentration of 0.5 μM. Then 1 nmole of [^{14}C]leucine (200 mCi/mmole), 10 nmoles of [^{14}C]thymidine (55 mCi/mmole), or 5 nmoles [^{14}C]uridine (55 mCi/mmole) were added to both cultures. The cells were incubated at 37° for various times, as indicated in the figure; the reaction was terminated and the rates of RNA, DNA, and protein synthesis were determined as described under METHODS.

ml of a buffer composed of 1 mM dithiothreitol, 6 mM KCl, 5 mM magnesium chloride, and 10 mM Tris-HCl, pH 7.9. After 7 min, cells were broken with 15 strokes of a Dounce homogenizer, as previously described (9). Nuclei were recovered by centrifugation at 1000 × *g* for 3 min and washed three times with a buffer containing 0.01 M potassium phosphate (pH 7.7), 2 mM magnesium chloride, 1 mM dithiothreitol, and 15 mg/ml of bovine serum albumin (16). Nuclei were resuspended in 1 ml of the same buffer, and enzyme activity was determined by a modification of the procedure described by Zylber and Penman (17). Reaction mixtures contained, in a final volume of 0.1 ml, 5 mM magnesium chloride, 1.6 mM $MnCl_2$, 6 mM KCl, 50 mM Tris-HCl (pH 7.9), 1 mM dithiothreitol, 10 μM concentration each of ATP, GTP, and CTP, 1 μM [^{3}H]TTP (13 Ci/mmole), 50 mM $(NH_4)_2SO_4$, and 10 μl of a suspension of nuclei. Reaction mixtures were incubated at 30° for 16 min, after which reactions were terminated by addition of 4 ml of cold 5% trichloracetic acid containing 0.01 M sodium pyrophosphate. After 15 min, precipitates were collected on Millipore filters and washed four times with 3 ml of cold 2% trichloracetic acid containing 0.01 M sodium pyrophosphate.

Determination of radioactivity. Radioactive precipitates were collected on Millipore filters, dried, and counted on planchets in a gas-flow counter or in scintillation vials containing 5 ml of a solution composed of 330 ml of Triton X-100 and 35 ml of Liquifluor per liter of toluene.

RESULTS

Effects of tylocrebrine on biosynthesis of protein, DNA, and RNA in HeLa cells. The effect of various concentrations of tylocrebrine on macromolecular synthesis is shown in Fig. 2. During the first hour after addition of the alkaloid, the average rate of protein synthesis is reduced by 50% and 95% at concentrations of 50 and 300 nM, respec-

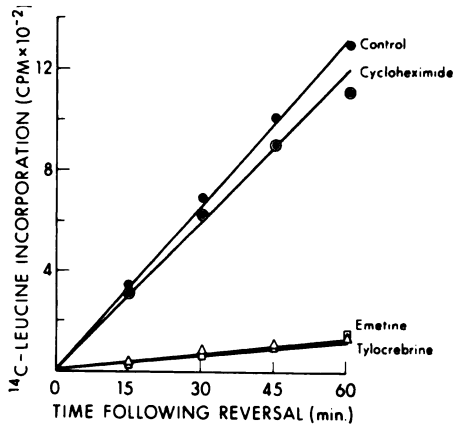

FIG. 4. *Reversal of inhibition of protein synthesis induced by tylocrebrine and other antibiotics*

HeLa cells (5×10^5/ml), suspended in leucine-depleted Eagle's medium containing 5% horse serum, were incubated for 3 min in the presence of the following antibiotics: 1 μM tylocrebrine, 10 μM cycloheximide, and 1 μM emetine. The control culture contained no antibiotics. All cultures, including the control, were washed three times with Earle's buffer at 37° and resuspended in fresh leucine-depleted medium. One nmole of [^{14}C]leucine (200 mCi/mmole) was added, and the rate of protein synthesis was measured as described under METHODS.

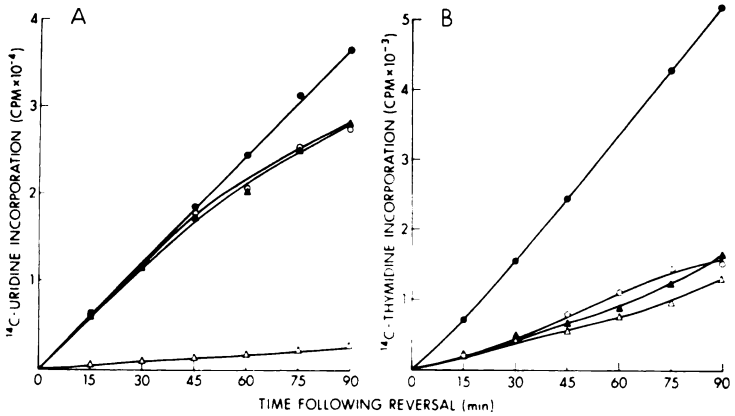

FIG. 5. *Reversal of the inhibition of RNA and DNA synthesis induced by tylocrebrine*

HeLa cells (5×10^5/ml), suspended in complete Eagle's medium containing 5% horse serum, were incubated with 10 μM tylocrebrine for 3 min. (○——○) or for 60 min. (▲——▲). The control culture (●——●) contained no antibiotic. All cultures, including the control, were washed four times with Earle's buffer and resuspended in complete Eagle's medium containing 5% horse serum. To one culture (△——△), 10 μM tylocrebrine was added after washing. A. [^{14}C]Uridine (5 nmoles, 55 mCi/mmole) was added to each culture after the final resuspension. B. [^{14}C]Thymidine (10 nmoles, 50 mCi/mmole) was added to each culture after resuspension. The cell cultures were incubated at 37° for 60 min, and the rates of RNA and DNA synthesis were determined as described under METHODS.

TABLE 1

Effect of tylocrebrine on biosynthesis of hemoglobin in rabbit reticulocytes

Hemoglobin synthesis in reticulocytes was measured as described under METHODS. Tylocrebrine was added prior to [^{14}C]leucine.

Tylocrebrine	Incorporation	Inhibition
μM	*cpm/mg*	%
0	8389	
0.01	8192	2
0.1	7899	6
1.0	1893	77
10.0	338	96

tively. Inhibition can be increased to 99% by increasing the concentration of drug to 10 μM. Parallel, but less complete, inhibitory effects are also observed on the rate of synthesis of DNA and RNA, which are inhibited by 50% at concentrations of 60 and 300 nM, respectively.

Inhibition of protein, DNA, and RNA synthesis occurs within two min after addition of tylocrebrine to the medium (Fig. 3). At the concentration of drug used in this experiment (500 nM), the effect is essentially complete after 10 min. DNA synthesis is inhibited by less than 60%, and RNA synthesis by less than 55%, at the concentration of drug used.

Reversibility. Reversibility of the inhibitory effects of tylocrebrine in HeLa cells was measured in the experiment shown in Fig. 4. After exposure to the drug for 3 min, the cells were washed three times and resuspended in fresh medium, and the rates of protein, RNA, and DNA synthesis were determined. Cycloheximide and emetine (18), established reversible and irreversible inhibitors of protein synthesis, respectively, were used in this experiment as controls. The concentration of each antibiotic used in this experiment was determined from preliminary experiments as the one that would inhibit protein synthesis in HeLa cells by 95% for at least 1 hr.

The effects of tylocrebrine on DNA synthesis are irreversible under the conditions used (Fig. 5B), but the effects of the alkaloid on RNA synthesis are reversible (Fig. 5A). Reversal of inhibition of RNA synthesis is complete even after cells have been exposed to tylocrebrine for 1 hr. The inhibitory effect of tylocrebrine on hemoglobin synthesis in intact reticulocytes is also irreversible.

Effect of tylocrebrine on rate of synthesis of

globin. When tylocrebrine is incubated with intact reticulocytes for 1 hr at concentrations of 0.1, 1, and 10 μM, the average rate of hemoglobin synthesis is reduced by 6%, 77%, and 96%, respectively (Table 1). Rabbit reticulocyte lysates were utilized as a cell-free system in which new peptide (globin) chains are initiated. If such lysates are fortified with hemin, [^{14}C]leucine is incorporated into globin at a linear rate for more than 15 min. The effect of tylocrebrine on the rate of globin synthesis in reticulocyte lysates is shown in Fig. 6. At concentrations of 10 μM, the alkaloid inhibits globin synthesis by more than 95% (Fig. 6A); inhibition occurs immediately upon addition of the drug to the reaction (Fig. 6B).

Effect of tylocrebrine on polyribosome structure. Addition of tylocrebrine to a crude reticulocyte lysate decreases the number of single ribosomes, with a concomitant increase in polyribosomes (Fig. 7C) compared to the uninhibited control (Fig. 7A). When the alkaloid is added immediately following a 1-min pulse of [^{14}C]leucine, radioactivity remains in the polysome area (Fig. 7C). If tylocrebrine is added prior to the addition of [^{14}C]leucine, no polysome-bound radioactivity is found (Fig. 7E).

Addition of puromycin to the uninhibited control results in complete breakdown of polysomes to monosomes, and nascent peptide chains are released to the top of the sucrose gradient (Fig. 7B). If puromycin is added to the reaction after incubation with tylocrebrine, 90% of the radioactivity is still released to the top of the gradient, but dissociation of polyribosomes is not observed (Fig. 7D).

Effect of tylocrebrine on assembly of polyribosomes. When rabbit reticulocytes are incubated in the presence of 0.1 M sodium fluoride, polyribosomes dissociate to single ribosomes (Fig. 8A). If these cells are washed and resuspended in a buffer containing the components required for hemoglobin synthesis, polyribosomes re-form (Fig. 8B). In the presence of tylocrebrine (Fig. 8C), complete reassembly of polysomes does not occur; however, dimeric, trimeric, and tetrameric ribosomes are formed. Cycloheximide (Fig. 8D) has similar effects.

Effect of tylocrebrine on synthesis of poly-

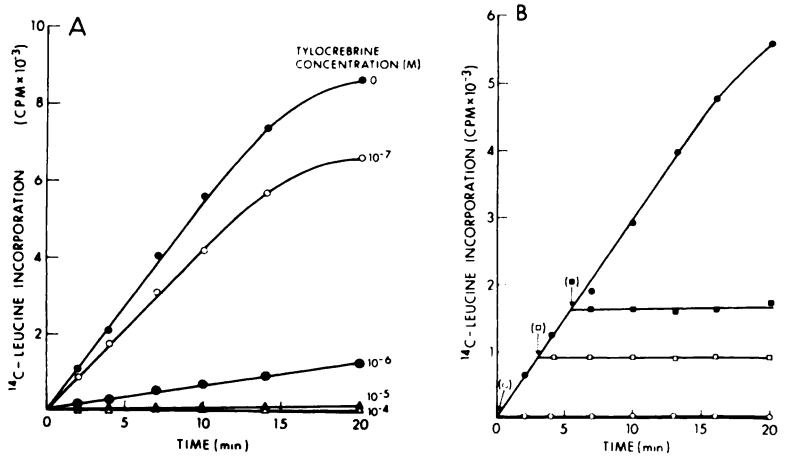

FIG. 6. *Effect of tylocrebrine on the synthesis of globin by reticulocyte lysates*

A. The standard reaction mixture (0.5 ml), described under METHODS, was incubated at 33° in the presence of various concentrations of tylocrebrine as indicated in the figure. At the times indicated, 0.05 ml aliquots were removed and 5% trichloracetic acid was added. Globin synthesis was measured as described under METHODS.

B. Standard reaction mixtures (0.5 ml) were prepared as described under METHODS and incubated at 33°. At 0, 3 and 5 min., tylocrebrine was added at a final concentration of 100 μM. At the times indicated in the figure, 0.05-ml aliquots were pipetted into 5% trichloracetic acid and globin synthesis was measured as described under METHODS.

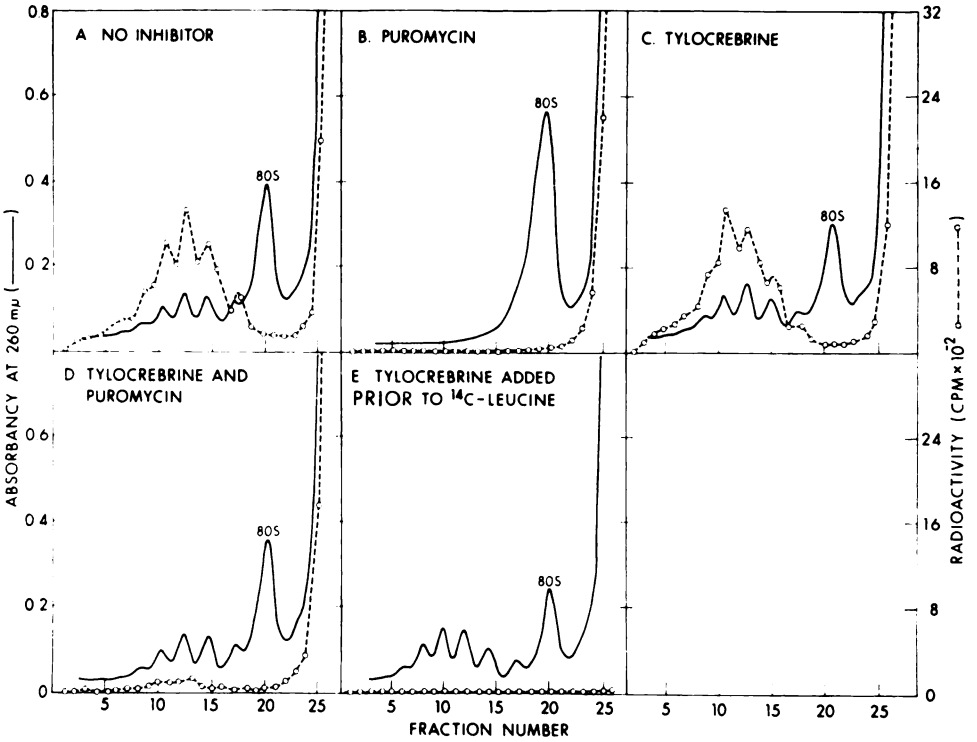

FIG. 7. *Effect of tylocrebrine on attachment of nascent peptides to polyribosomes in the presence and absence of puromycin*

Five standard reaction mixtures (0.24 ml), each containing 5 nmoles of [^{14}C]leucine (300 mCi/mmole), were prepared, incubated at 33° for 1 min, and then treated as follows. A. Reaction terminated immediately. B. Puromycin added to a final concentration at 0.2 mM and the reaction incubated 3 min longer. C. Tylocrebrine added to a final concentration of 0.1 mM and the reaction incubated 3 min longer. D. Tylocrebrine (0.1 mM) added, mixture incubated 3 min longer, then puromycin (0.2 mM) added and the mixture again incubated for 3 min. E. Tylocrebrine (0.1 mM) added prior to the 1 min. pulse with [^{14}C] leucine. All reactions were terminated by addition of 0.8 ml of cold reticulocyte standard buffer and layered on 10–25% sucrose gradients in the same buffer. Gradients were centrifuged as described under METHODS.

phenylalanine and on binding of phenylalanyl-tRNA to ribosomes. As shown in Table 2, 1.2 mM tylocrebrine does not affect the rate of synthesis of polyphenylalanine. Anisomycin, an antibiotic that is known to inhibit peptide bond formation (19), served as control. Tylocrebrine does not inhibit poly U-dependent enzymatic binding of phenylalanyl-tRNA or binding of [^{3}H]poly U or [^{3}H]poly C to washed reticulocyte ribosomes (data not shown).

Effect of tylocrebrine on translocation and peptide bond formation. Several enzymes, including EF-I,[1] EF-II, and peptidyl-transferase, are required for binding of aminoacyl-tRNA to the ribosome, subsequent translocation of peptidyl-tRNA, and formation of peptide bonds, respectively. Activity of the binding enzyme, EF-I, is unaffected by tylocrebrine at a concentration of 0.1 mM (data not shown). The effect of antibiotics on translocation and peptide bond formation was determined by a two-step method, originally described by McKeehan and Hardesty (20). In this assay, translocation of nascent globin peptides is allowed to take place during the first incubation in the presence of EF-II; labeled aminoacyl-tRNA, EF-I, and sodium fluoride are then added, and peptide bond formation is permitted to occur during a second incubation. The assay can thereby distinguish

[1] The abbreviations used are: EF-I (elongation factor I), aminoacyl transfer ribonucleic acid-binding enzyme; EF-II (elongation factor II), aminoacyltransferase II.

inhibitors of translocation and peptide bond formation from these compounds that affect neither or both. The data shown in Table 3 indicate that tylocrebrine inhibits translocation. Cycloheximide, an inhibitor of translocation (20, 21), and anisomycin, an inhibitor of peptide bond formation (19. 22), served as controls for this experiment.

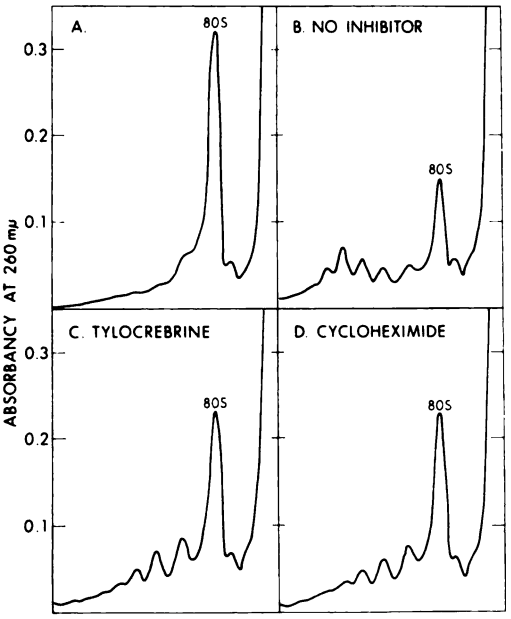

FIG. 8. *Effect of tylocrebrine on reassembly of polyribosomes*

Intact reticulocytes were suspended in a complex medium composed of fresh plasma (20 ml), leucine-deficient Eagle's medium (33 ml), NCl medium (Grand Island Biological Company) (33 ml), an amino acid mixture (9) (3.75 ml), 8% glucose (0.25 ml), 0.02 M glycine (1.0 ml), 1.2 M $NaHCO_3$ (1.0 ml), and 3.3 mM hemin (3.0 ml). Sodium fluoride (final concentration, 10 mM) was added, and the cells were incubated for 15 min at 37°. Cells were washed three times with cold buffer, resuspended in the original medium, and treated as follows. A. Unincubated control. B. Incubated at 37° for 8 min. C. Incubated at 37° for 8 min in the presence of 100 μM tylocrebrine. D. Incubated at 37° for 8 min in the presence of 100 μM cycloheximide. Cells were collected by centrifugation at 3000 × *g* for 5 min in a Sorvall SS-34 rotor. The supernatant solution was decanted, and the cells were lysed by adding 2 volumes of distilled water. Cell debris was removed by centrifugation at 10,000 × *g* for 10 min. The supernatant fluid (0.1 ml) was diluted by addition of 0.6 ml of reticulocyte standard buffer, then layered on 15–30% sucrose gradients in reticulocyte standard buffer and centrifuged in an SW 41 rotor at 41,000 rpm for 75 min.

TABLE 2

Effect of tylocrebrine on synthesis of polyphenylalanine

The reaction mixture (0.125 ml) contained 0.025 M Tris-HCl (pH 7.4), 0.06 M KCl, 0.008 M magnesium chloride, 0.006 M dithiothreitol, 0.008 M GTP, 25 μg of poly U, 28 μg of *E. coli* [^{14}C]phenylalanyl-tRNA, and 100 μg of washed ribosomes (6). Inhibitors were added prior to the addition of poly U and ribosomes. Reactions were incubated at 37° for 10 min, then stopped by the addition of 5% trichloracetic acid. Hot acid-insoluble radioactivity was determined as described under METHODS

Inhibitor added	Concentration	[^{14}C]Phe-tRNA incorporation	Inhibition
	mM	*cpm*	%
None		1116	
Anisomycin	0.3	46	96
	0.01	381	66
Tylocrebrine	1.2	1016	9

Effect of tylocrebrine on activity of guanosine triphosphatase. Tylocrebrine does not inhibit the activity of GTPase associated with washed ribosomes or with EF-II (Table 4). Cycloheximide, an inhibitor of translocation, also has no effect on the activity of GTPase (20) (Table 4). Fusidic acid is known to inhibit GTP hydrolysis (23), and served as control for this experiment.

Effect of tylocrebrine on globin synthesis in the presence of sulfhydryl compounds. EF-II and ribosomes require the presence of sulfhydryl compounds for optimal activity (Fig. 9). Added mercaptoethanol is maximally effective in stimulating protein synthesis at a concentration of 3 mM. At higher concentrations, this effect is somewhat less marked; however, antagonism to the inhibitory effect of tylocrebrine occurs as the concentration of mercaptoethanol is increased from 3 to 8 mM. The effects of tylocrebrine on globin synthesis in the presence of various concentrations of sulfhydryl compound are also shown in Fig. 9. In the absence of added sulfhydryl agents, the inhibitory effect of tylocrebrine is maximal. Under these conditions, 0.4 μM tylocrebrine inhibits protein synthesis by 95%.

Reticulocyte lysates contain significant

TABLE 3

Effect of tylocrebrine on translocation and peptide bond formation

The first mixture (0.25 ml), containing 0.06 M Tris-HCl (pH 7.5), 0.07 M KCl, 0.004 M magnesium chloride, 0.01 M dithiothreitol, 0.2 mM GTP, and 500 μg of ammonium chloride-washed ribosomes, was incubated at 37° for 15 min, and the reaction was stopped by chilling at 0°. [^{14}C]-Leucyl-tRNA (15 μg, 9000 cpm), EF-I (40 μg), NaF (8 μmoles), and the indicated inhibitor were then added. Sufficient Tris, KCl, magnesium chloride, GTP, and dithiothreitol were added to maintain their original concentrations in the reaction mixture, the final volume being 0.5 ml. The second reaction mixture was incubated at 37° for 20 min, and incorporation of [^{14}C]leucine into hot trichloracetic acid-insoluble peptide was measured as described under METHODS. EF-II (40 μg) was added to the first or second incubation as indicated in the table.

Inhibitor added	After translocation (second incubation)[a]		Before translocation (first incubation)[b]	
	Incorporation	Inhibition	Incorporation	Inhibition
	cpm	%	*cpm*	%
None	940		940	
Cycloheximide (1 mM)	1062	0	513	45
Anisomycin (0.1 mM)	110	88	73	92
Tylocrebrine (0.1 mM)	961	0	434	54

[a] Inhibitor added after incubation with EF-II.
[b] Inhibitor added before incubation with EF-II.

amounts of endogenous glutathione. Using the method of Zehavi-Willner *et al.* (24), glutathione was determined to be 2 mM in those experiments in which no sulfhydryl compounds were added. If 25 mM glutathione or dithiothreitol is added, the inhibitory effect of the alkaloid is decreased to less than 20%.

Effect of tylocrebrine on binding of [^{14}C] *tRNA to ribosomes.* The effect of tylocrebrine on binding of uncharged *E. coli* tRNA to reticulocyte ribosomes was compared with that of cycloheximide, a compound that blocks this reaction. Tylocrebrine markedly enhanced the binding of tRNA to ribosomes at a concentration of 1 mM (Table 5). Under the same experimental conditions, both aurintricarboxylic acid and cycloheximide inhibit this reaction.

Effects of tylocrebrine on activity of RNA polymerase. At concentrations of 100 and 10 μM, tylocrebrine inhibits the activity of *E. coli* RNA polymerase by 52% and 39%, respectively. Proflavin inhibits this enzyme to a similar degree at these concentrations (Table 6). However, tylocrebrine only inhibits activity of RNA polymerase in HeLa cell nuclei by 16% at a concentration of 1 mM, while proflavin inhibits by 69% at 0.1 mM.

DISCUSSION

In rabbit reticulocytes, tylocrebrine can be assumed to act primarily on protein synthesis, as these cells neither retain nor synthesize sufficient RNA and DNA to account for the observed effect on the rate of globin synthesis. In HeLa cells, tylocrebrine also affects RNA and DNA synthesis to varying degrees, but the predominant effect is again exerted on protein synthesis. Inhibition of RNA synthesis in HeLa cells can readily be reversed by suspending the cells in fresh medium, but effects of the drug on protein and DNA synthesis appear to be irreversible.

The rapid partial inhibition of DNA synthesis in HeLa cells by tylocrebrine probably results from a primary effect on protein synthesis, as the latter process is required for concurrent synthesis of DNA in animal cells (25–28). Anisomycin (9), cycloheximide (29), emetine (18), pactamycin (30), and puromycin (25), all of which inhibit protein synthesis in animal cells, simultaneously inhibit DNA synthesis (9, 18; see ref. 31 for other references).

Tylocrebrine rapidly inhibits the rate of RNA synthesis, but the mechanism of this inhibitory effect is unclear. Phenanthrene compounds are known to bind to DNA (32), and tylocrebrine inhibits activity of the *E. coli* DNA-dependent RNA polymerase to the same extent as proflavin. Thus tylocrebrine may inhibit RNA synthesis by affecting the activity of RNA polymerase. No cell-free preparation of RNA polymerase from HeLa cells is presently available in

TABLE 4

Effect of tylocrebrine on GTPase activity

All reaction mixtures contained, in a final volume of 0.125 ml, 0.05 M Tris-HCl (pH 7.4), 0.08 M KCl, 8 mM magnesium chloride, 8 mM dithiothreitol, and 4 μM [^{32}P]GTP (168 Ci/mole). Ribosomes (200 μg), EF-I (50 μg), EF-II (70 μg), poly U (25 μg), and phenylalanyl-tRNA (30 μg) were present as indicated in the table. Reaction mixtures were incubated at 37°, and reactions were terminated by addition of 2.5 ml of 0.02 M silicotungstic acid dissolved in 0.02 M H_2SO_4. $^{32}P_i$, liberated by hydrolysis during the first 10 min of incubation, was determined as the phosphomolybdate complex following extraction into 2.5 ml of a 1:1 mixture of isobutyl alcohol–benzene (w/v). An aliquot of the organic phase was evaporated to dryness, and the radioactivity was determined.

Components present	Inhibitor added	$^{32}P_i$ released	Inhibition
		cpm	%
EF-II	None	3,301	
	Cycloheximide (1 mM)	3,216	3
	Tylocrebrine (0.3 mM)	3,198	4
	Fusidic acid (1 mM)	1,954	41
Ribosomes and EF-II	None	4,805	
	Cycloheximide (1 mM)	4,474	7
	Tylocrebrine (0.3 mM)	4,747	2
	Fusidic acid (1 mM)	3,177	34
Ribosomes, EF-I, EF-II, poly U, and phenylalanyl-tRNA[a]	None	13,163	
	Cycloheximide (1 mM)	13,402	0
	Tylocrebrine (0.3 mM)	14,621	0
	Fusidic acid (1 mM)	7,382	44

[a] Components required for amino acid polymerization.

which initiation of new RNA chains occurs in the presence of exogenous template. Incorporation of labeled triphosphates into a crude nuclear preparation of RNA polymerase, an assay that measures chain elongation in the presence of endogenous template, was only slightly affected by the drug.

After adding tylocrebrine to a reticulocyte lysate, inhibition of protein synthesis occurs within a few seconds, suggesting that the functional effect of the drug is on elongation of peptide chains. In similar preparations, inhibitors of chain initiation, such as aurintricarboxylic acid, sodium fluoride, or pactamycin, only act following a delay of 1–2 min. In addition, inhibitors of initiation induce breakdown of polyribosomes to single ribosomes and ribosomal subunits and result in release of nascent peptides. Tylocrebrine prevents breakdown of polyribosomes and inhibits release of nascent peptides. These observations suggest that the principal effect of tylocrebrine on protein synthesis is exerted on the elongation of peptide chains.

Two of the enzymatic reactions required for chain elongation involve translocation of peptidyl-tRNA and formation of the peptide bond. Puromycin reacts directly with peptidyl-tRNA located in the peptidyl (donor) site on the ribosome, causing premature termination and release of peptide chains from the polyribosome (33). This reaction, which does not require supernatant factors or GTP, has been used to examine the action of inhibitors of chain elongation. Established inhibitors of peptide bond formation, such as anisomycin[2] and sparsomycin (34), inhibit puromycin-induced release of peptidyl-tRNA from polysomes. Tylocrebrine does not prevent this reaction, suggesting that the drug does not affect peptide bond formation. Further support for this conclusion is provided by the observa-

[2] M.-T. Huang and A. P. Grollman, unpublished results.

tion of Battaner and Vazquez (35) that tylocrebrine does not affect the "fragment reaction."

The effect of inhibitors on translocation of peptidyl-tRNA can conveniently be determined by a two-step assay, originally described by McKeehan and Hardesty (20). This assay is based on the assumption that peptidyl-tRNA will be located at the peptidyl site on the ribosome following their incubation in the presence of EF-II and GTP. Labeled aminoacyl-tRNA can then be bound to the aminoacyl site on the ribosome in the presence of sodium fluoride, an agent that prevents initiation of new globin peptides. Under these experimental conditions, further incorporation of amino acids into peptides is blocked by inhibitors of peptide bond formation but not by inhibitors of translocation. Tylocrebrine has no effect at this stage of the reaction; however, if the drug is present prior to addition of EF-II and GTP, incorporation of amino acids is inhibited by approximately 50%. Assuming that nascent peptide is equally distributed between the aminoacyl and peptidyl sites on the ribosome, this partial inhibition would be anticipated by any agent that blocks translocation.

TABLE 5

Effect of tylocrebrine on binding of [^{14}C]tRNA to ribosomes

The reaction mixture contained, in a volume of 0.125 ml, Tris-HCl, 0.05 M, pH 7.4; $MgCl_2$, 0.008 M; KCl, 0.06 M; *E. coli* [^{14}C]tRNA, 20,000 cpm; ammonium chloride-washed ribosomes, 100 μg; and inhibitors as indicated in the table. The reaction mixture was incubated at 0° for 20 min. Radioactivity bound to ribosomes was collected on Millipore filters and determined as described under METHODS.

Inhibitor added	Concentration	[^{14}C]tRNA bound	Control
	mM	*cpm*	%
None		840	
Aurintricarboxylic acid	1.0	172	21
	0.3	347	41
Cycloheximide	1.0	514	61
	0.3	650	77
Tylocrebrine	1.0	2583	310
	0.4	1181	140

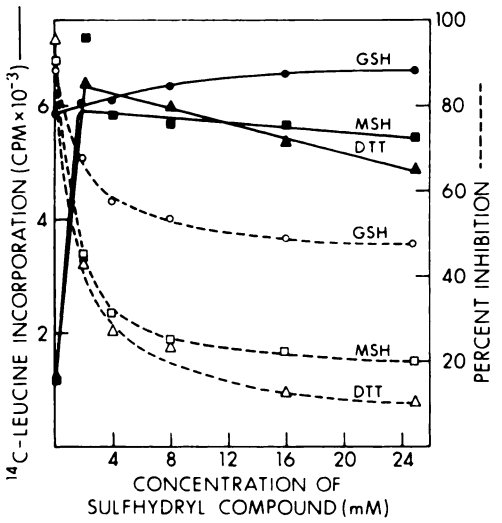

FIG. 9. *Effect of sulfhydryl compounds on tylocrebrine-induced inhibition of globin synthesis*

Two standard reaction mixtures, each containing various concentrations of sulfhydryl compound as indicated in the figure, were prepared. Tylocrebrine was added to one of these at a final concentration of 0.4 μM. Reaction mixtures were incubated for 8 min at 33°, then terminated by adding 4 ml of cold 5% trichloracetic acid. Globin synthesis was measured as described under METHODS. GSH, glutathione; MSH, mercaptoethanol; DTT, dithiothreitol.

Puromycin releases 90% of peptide chains, even in the presence of tylocrebrine. If translocation is completely inhibited by the drug, peptidyl-tRNA should accumulate in the aminoacyl site and thus be unable to react with puromycin. It would appear, therefore, that tylocrebrine somehow affects that aspect of translocation which involves movement of mRNA along the ribosome. Other antibiotics have been reported to have similar effects (18, 36).

The action of tylocrebrine on protein synthesis resembles that of cycloheximide in certain respects (20). Both antibiotics inhibit translocation, when tested by the two-step assay described by McKeehan and Hardesty, but do not affect peptide bond formation or hydrolysis of GTP (20). In intact cells, however, the action of cycloheximide on protein synthesis is reversible, while that of tylocrebrine is not. Such irreversible action may result from binding of the drug to the

TABLE 6

Effect of tylocrebrine on activity of RNA polymerase prepared from E. coli and HeLa cells

The activity of RNA polymerase was determined as described under METHODS. For the assay of enzyme prepared from *E. coli*, the reaction mixture was incubated for 10 min at 37° in the presence of the indicated concentrations of tylocrebrine or proflavin. For the assay of enzyme prepared from HeLa cell nuclei, tylocrebrine or proflavin was added to the reaction mixture at the indicated concentration, the reaction was incubated at 31° for 15 min, and radioactivity was determined as described under METHODS.

Inhibitor added	Concentration	*E. coli* enzyme		HeLa cell enzyme	
		Incorporation	Inhibition	Incorporation	Inhibition
	μM	cpm	%	cpm	%
None		41,000		8,925	
Tylocrebrine	1,000			7,468	16
	100	19,600	52	7,746	13
	10	25,100	39	7,985	11
Proflavin	300			2,939	68
	100	16,900	59	2,749	69
	10	23,000	44	7,191	19

ribosome or from inactivation of EF-II. Cycloheximide, under certain conditions, affects chain initiation, while tylocrebrine does not appear to have this effect. Cycloheximide also inhibits binding of deacylated tRNA to the ribosome (37), while tylocrebrine markedly stimulates the binding reaction. Cycloheximide inhibits polyphenylalanine synthesis on reticulocyte ribosomes (37), but tylocrebrine has no effect on this process.

Ribosomes and EF-II require sulfhydryl compounds for maximal activity. In this regard, sulfhydryl group proteins on the larger subunit appear to be more essential than those of the smaller subunit (38). The presence of mercaptoethanol and other sulfhydryl compounds diminishes the inhibitory effects of tylocrebrine. Sulfhydryl compounds have also been reported to protect against the inhibitory effects of cycloheximide in cell-free systems prepared from rat liver (21). The exact mechanism of this effect remains obscure.

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