Inhibition of Cell Growth and the Synthesis of Ribonucleic Acid and Protein in HeLa Cells by Morphinans and Related Compounds

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SUMMARY

The ability of a group of congeners of morphinan and related compounds to inhibit the growth of HeLa cells has been correlated with their ability to inhibit protein and RNA synthesis in the intact cell. With the exception of thebaine, the inhibition of protein synthesis by these compounds is associated with a dissolution of polysome structure. As shown earlier, the effects on protein synthesis appear to be indirect, since the compounds tested did not inhibit amino acid incorporation when added to a cell-free HeLa system, and there is evidence that these compounds profoundly affect intracellular ATP levels. The structural features required for inhibitory action have been analyzed. One interpretation of our findings points to the existence in HeLa cells of a mechanism for recognizing the C- and D-ring regions of the morphinans and related molecules. In the absence of information on the penetration and transport of these compounds, other mechanistic interpretations must be considered.

INTRODUCTION

Levorphanol and levallorphan, two closely related morphinan derivatives with narcotic and narcotic-antagonizing activities, respectively, have been found to inhibit the synthesis of both RNA and protein in cultured mammalian cells and bacteria (1–3), and to decrease the intracellular concentrations of ATP in both types of cells (4). In HeLa cells, these compounds produce a reversible dissolution of polysome structure and the release of free ribosomes (3, 5). The resulting restriction of protein synthesis leads to inhibition of RNA synthesis (3, 5, 6) and a curtailment of the genetic expression mechanisms of the cell. These effects are apparent only in intact cells, however, suggesting that the inhibition of protein synthesis is an indirect effect of the drugs. The effects of these drugs in bacteria are similar to those found in HeLa cells except that protein synthesis seems to be slightly less sensitive than RNA synthesis to the drugs. In addition, the effect on ATP levels is more marked in bacteria than in mammalian cells (4).

On the premise that a receptor mechanism might operate in this response and that some specific structural features might prove essential for drug activity, a group of related morphinans and morphine alkaloids have been compared for their effects on HeLa cells. Evidence is presented that the C-ring region of the molecules and the substitution and charge of the nitrogen

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of the D-ring play determinant roles in the activities of these compounds. The data suggest the existence of a cellular mechanism for recognizing these regions of the molecules.

METHODS

Culture of HeLa cells. To assay for inhibition of growth by the drugs, HeLa cell monolayer cultures were used. Routinely, cells from an exponentially growing suspension culture (3) were harvested, trypsinized, and resuspended in modified Eagle's medium (7). Approximately 5×10^5 cells in 10 ml of this medium were transferred to flasks, and the cultures were incubated in 5% CO_2 in air at 37° for 24 hr. Sterile aqueous solutions of the drugs, adjusted to pH 6.4, were then added to the flasks, and the incubation was continued for an additional 24 or 48 hr. At the end of the incubation period, the medium was decanted and the cells were removed from the the glass by treating the monolayers with 5 ml of 0.05% trypsin. The action of this solution was stopped after 5 min by adding 5 ml of medium containing 10% beef serum. The cells were suspended by pipetting vigorously, and aliquots were counted in a Coulter counter. In all experiments, both *in vitro* and in culture, before addition of the drugs, the drug solutions were adjusted to a pH as near to that of the incubation medium as possible. The pH was checked with a pH meter before and after drug additions, and no changes in pH due to the drugs were observed.

Measurement of RNA and protein synthesis in living cells. In order to assay for RNA or protein synthesis at various times after treatment of cultures, 10-ml aliquots were removed and incubated with 0.5 μC of guanine-8-^{14}C (10 mC/mmole) or 0.5 μC of DL-leucine-1-^{14}C (22 mC/mmole), respectively, for 10 min at 37°. After centrifugation at $800 \times g$ for 5 min, the supernatant fraction was discarded and the cells were washed three times with 2.5% (v/v) perchloric acid. The acid-insoluble residues were dissolved in formic acid, and the radioactivity of the samples was counted in a liquid scintillation spectrometer.

Sucrose gradient analysis of polysomes. To obtain polysomes, cells were allowed to swell for 10 min in hypotonic buffer containing 0.01 M Tris-HCl (pH 7.6), 0.01 M KCl, and 0.0015 M $MgCl_2$ (3) and were then ruptured with a Dounce homogenizer. The homogenate was centrifuged at $1000 \times g$ for 10 min, and the supernatant fraction from this treatment was then centrifuged at $15{,}000 \times g$ for 15 min. The resulting supernatant was layered over a 25-ml, 10–40% linear sucrose density gradient and centrifuged for 2 hr in an SW 25 rotor at $90{,}000 \times g$. Fractions containing 30 drops were collected from the gradients, and the optical density at 260 mμ was determined on each fraction. It was unnecessary to use deoxycholate treatment in the preparation of these polysomes (8), because in HeLa cells they are unattached to membranes.

Measurement of protein synthesis in vitro. Protein synthesis was determined by measuring the incorporation of radioactive amino acids into protein by a cell-free system utilizing a $15{,}000 \times g$ supernatant fraction prepared by the method given in the preceding section. The standard reaction mixture contained the following, in a volume of 1.0 ml: Tris-HCl, pH 7.6, 100 μmoles; KCl, 50 μmoles; $MgCl_2$, 14 μmoles; 2-mercaptoethanol, 6 μmoles; ATP, 1.0 μmole; GTP, 0.025 μmole; sodium phosphoenolpyruvate, 10.0 μmoles; pyruvate kinase, 25 μg; 19 unlabeled amino acids, 0.1 μmole each; uniformly labeled L-leucine-^{14}C, 1.0 μC, 240 μC/μmole (1); and $15{,}000 \times g$ supernatant, 0.1 ml. The reaction mixture was incubated at 37°, and at various times 0.1-ml aliquots were transferred to filter paper discs and dropped into cold 10% trichloracetic acid. After 1 hr, the samples were transferred to 5% trichloracetic acid and heated to 90° for 20 min. After cooling, the samples were washed twice with cold 5% trichloracetic acid, transferred to a mixture of 100% ethanol and ether (1:1), and heated in a water bath at 37° for 15 min. Then the samples were washed twice in ether and dried. The radioactivity of

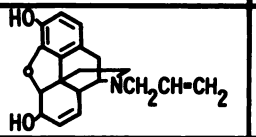
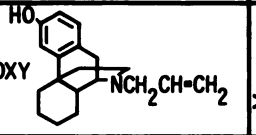
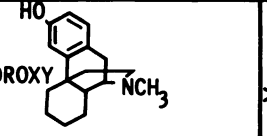
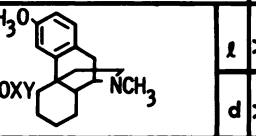
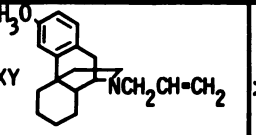
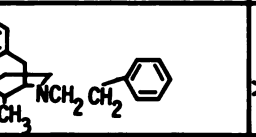
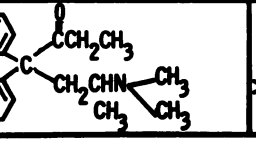
TABLE 1

Inhibition of HeLa cell growth after 48 hr of drug treatment

HeLa monolayers were treated with drugs and incubated in 5% CO_2 and air at 37°. After 48 hr the cells were trypsinized and counted. Thebaine methiodide was not tested for inhibition of cell growth. In calculating the percentage inhibition of cell growth, the baseline was taken as the number of cells per monolayer at the time of addition of the compound under study. The percentage inhibition at 48 hr is then equal to

$$\frac{\text{control cells} - \text{treated cells}}{\text{control cells} - \text{baseline cells}} \times 100$$

Thus, when the cells were killed, the percentage inhibition was greater than 100 and is so indicated in the table. An almost complete kill of cells occurred with drugs II–VIII at 1 mM by 48 hr.

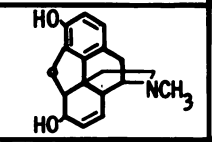
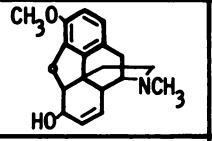
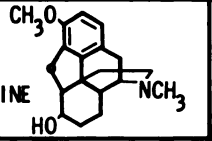
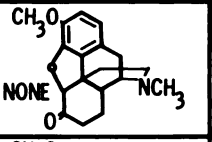
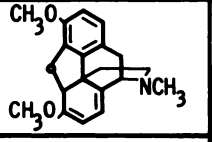
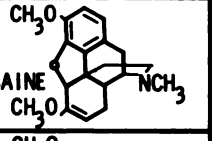
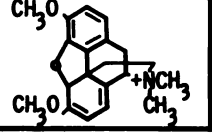
	COMPOUND		MOLAR CONCENTRATION		
			1×10^{-3}	1×10^{-4}	1×10^{-5}
			% Inhibition of growth		
I	N-ALLYL NORMORPHINE 		57	0	0
II	N-ALLYL-3-HYDROXY MORPHINAN (LEVALLORPHAN) 		>100	30	0
III	N-METHYL-3-HYDROXY MORPHINAN LEVORPHANOL 		>100	16	0
IV	N-METHYL 3 METHOXY MORPHINAN 	l	>100	13	0
V		d	>100	13	0
VI	N-ALLYL 3 METHOXY MORPHINAN 		>100	35	8
VII	PHENAZOCINE 		>100	100	10
VIII	d, l, METHADONE 		>100	40	0

the discs was measured in a liquid scintillation spectrometer.

Preparation of dihydrothebaine. Dihydrothebaine was prepared by hydrogenation of thebaine over palladous chloride. The method of Small *et al.* (9) was slightly modified for this purpose. Thebaine, 5 g, was dissolved in 10 ml of 3 M acetic acid to which were added 0.27 ml of concentrated HCl, 0.27 ml of 1% palladous chloride, and 7 mg of gum arabic. The mixture was hydrogenated at a starting pressure of 46.5 psi. In 4 hr the uptake of the hydrogen corresponded to 1.6 moles per mole of thebaine. The mixture was filtered to remove the catalyst. Ether, 10 ml, was added to the filtrate; the dihydrothebaine was precipitated by adding 2 N NaOH and recrystallized from ethyl acetate. The yield was 1.65 g of dihydrothebaine (33%). The product melted at 162–163° and the specific rotation was −262° ($c = 1.067$ in benzene).

Preparation of thebaine methiodide. Thebaine, 300 mg, was dissolved in 100 ml of anhydrous ethyl ether. Iodomethane,

TABLE 1 (*Continued*)

	COMPOUND	MOLAR CONCENTRATION		
		1×10^{-3}	1×10^{-4}	1×10^{-5}
		% inhibition of growth		
IX	MORPHINE 	13	0	0
X	CODEINE 	5	5	0
XI	DIHYDROCODEINE 	5	0	0
XII	DIHYDROCODEINONE 	5	5	0
XIII	THEBAINE 	100	20	0
XIV	DIHYDROTHEBAINE 	20	0	0
XV	THEBAINE METHIODIDE 	–	–	–

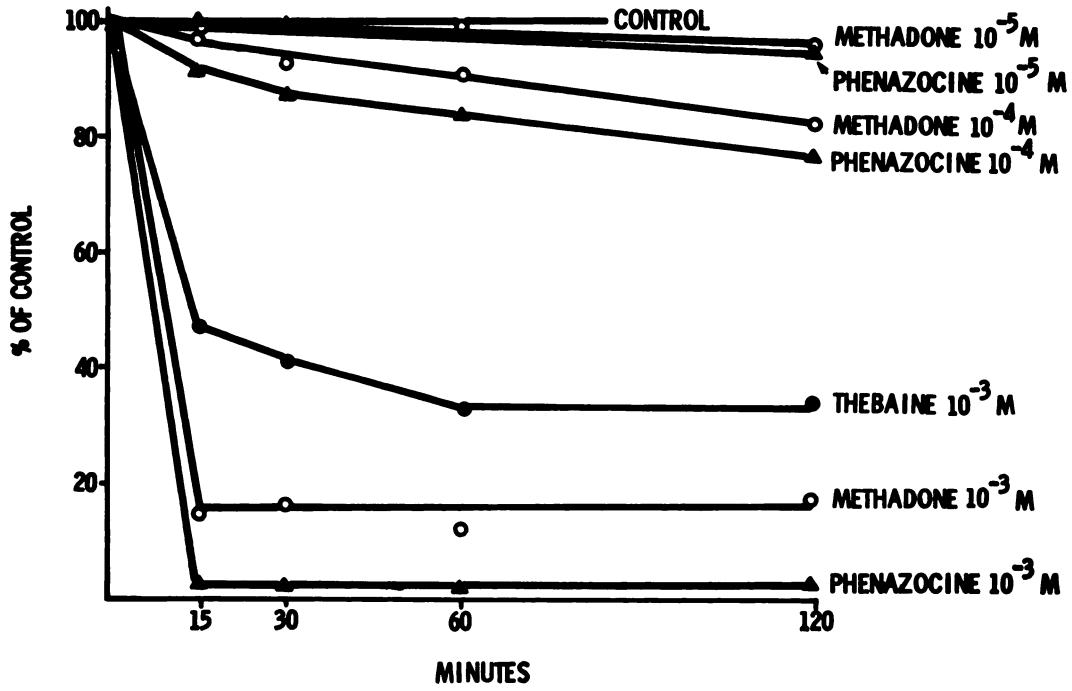


FIG. 1. *Inhibition of protein synthesis at various times following drug addition*

Cultures of 330,000 cells/ml were treated at zero time, and 5-ml aliquots were removed and incubated with DL-leucine-1-^{14}C as described in METHODS and Table 2. The radioactivity of the treated samples is expressed as a percentage of the radioactivity of the control. The control cells incorporated 3300 ± 150 cpm/ 1.65×10^6 cells.

5 ml, was added, and the mixture was stirred in a closed vessel for 3 hr at room temperature. The thebaine methodide precipitate was filtered and washed with anhydrous ether. The melting point as determined by the heat of fusion method was 253°.

Source of materials. The drugs used in this study were obtained as follows: *N*-methyl-3-hydroxymorphinan (levorphanol), *N*-allyl-3-hydroxymorphinan (levallorphan), and 3-methoxymorphinan were gifts from Hoffmann-La Roche; thebaine, morphine, codeine, dihydrocodeine, dihydrocodeinone, and 6-dimethylamino-4,4-diphenyl-3-heptanone (methadone) were obtained from S. B. Penick and Company; 2′-hydroxy-2-(*N*-β-phenethyl)-5,9-dimethyl-6,7-benzomorphan (phenazocine) was a gift from Smith Kline & French; and *N*-allylnormorphine (nalorphine) was obtained from Merck and Company.

RESULTS

Inhibition of HeLa cell growth. The abilities of the different morphinan drugs to inhibit the growth of HeLa cell monolayer cultures are compared in Table 1. Among the five morphinan compounds (II–VI), it is apparent that the *N*-allyl derivatives (narcotic antagonists) were slightly more active than the *N*-methyl narcotic analogues; however, all inhibited HeLa cell growth significantly. This effect of the allyl group is seen also in *N*-allylnormorphine (compound I).

Masking the phenolic function with a methoxy group in either levorphanol or levallorphan did not affect the activity. This may indicate that the phenolic group is not involved in the inhibitory action of these compounds. Equally surprising was the observation that the *d* and *l* isomers of *N*-methyl-3-methoxymorphinan (IV and

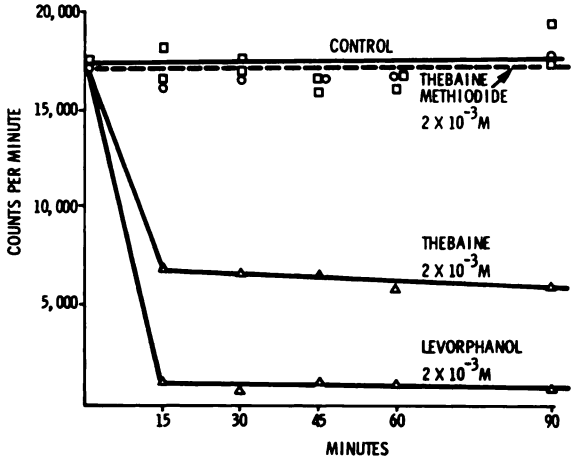


FIG. 2. *Protein synthesis in HeLa cells following treatment with thebaine, thebaine methiodide, and levorphanol*
Cultures containing 350,000 cells/ml were treated with a 2 mM concentration of each drug tested, and protein synthesis was assayed periodically as described in Table 2.

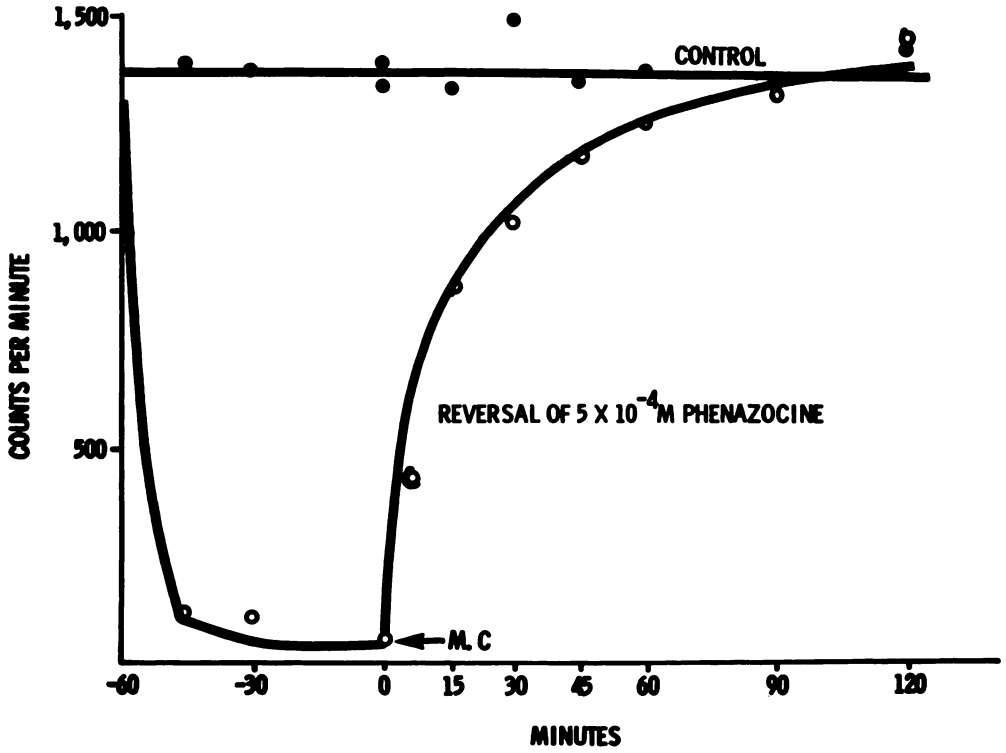


FIG. 3. *Inhibition of protein synthesis by phenazocine, and reversal of this effect by changing the medium*
The determination of protein synthesis has been given in METHODS and Table 2. Cell cultures containing 250,000 cells/ml were treated with 5×10^{-4} M phenazocine, and 5-ml aliquots were removed for assay of protein synthesis. After 60 min, cells were harvested and resuspended in fresh medium (M.C), and protein synthesis was assayed for the next 2 hr. Data are expressed as counts per minute per aliquot of cells.

V) had the same activity in this test system.

In contrast, the C-ring appears to play a more important role in the inhibition mechanism, since morphine (IX), codeine (X), dihydrocodeine (XI), and dihydrocodeinone (XII), which have substitutions on the C-ring, were strikingly less effective than compounds I–VI. However, methoxylation of the hydroxyl function and introduction of conjugated unsaturation in the C-ring to form thebaine (XIII), a nonnarcotic alkaloid, yielded high inhibitory activity. This suggests that the ether bridge between rings A and C, as well as the methoxy function at position 6 in ring C, do not play an active role in the inhibition, but can be tolerated as long as the ring is maintained planar by the unsaturation. When thebaine was reduced to dihydrothebaine (XIV), a loss of inhibitory activity was apparent which points out the importance of unsaturation of the C-ring. In addition to the shape of the D-ring, the charge on the nitrogen appears to be critical because conversion of the tertiary nitrogen of thebaine to a quaternary state to form thebaine methiodide (XV) resulted in complete loss of inhibitory activity.

In the course of these studies two nonmorphinan narcotics were tested: phenazocine (VII), which has no C-ring, and methadone (VIII), which can be considered to have an open D-ring. Both compounds were highly inhibitory to HeLa cell growth (Table 1). In this case the high activity of these two compounds may reflect an advantage of flexibility in the regions between rings C and D. Conversely, the coupling of a C-ring to the D-ring containing the tertiary nitrogen may impose certain conformational constraints on this region of the molecule in fitting the hypothetical receptor.

Effects of morphinans and morphine alkaloids on RNA and protein synthesis. In general, the relative activity of the compounds in the growth assay could be correlated with their ability to inhibit the synthesis of RNA and protein. These data are presented in Table 2. In the case of protein synthesis, the action of the compounds was rapid and reached nearly maximal effectiveness within 15 min (Figs. 1 and 2). Once maximal inhibition was achieved for a given drug concentration, this inhibition remained relatively constant for as long as 2 hr. This suggests that the degree of inhibition results from the establishment of some equilibrium state.

In all cases, changing the medium by removal of the drug led to reversal of the inhibitory action. As an example of this phenomenon, the reversal of the effect of phenazocine on protein synthesis is shown in Fig. 3. As cited previously for levallorphan, the recovery of protein synthesis

TABLE 2

Inhibition of protein and RNA synthesis in HeLa cells after 60 min of drug treatment

Experimental procedures are given in METHODS. Suspension cultures containing approximately 300,000 cells/ml were treated with drugs, and 5-ml aliquots were periodically removed and incubated for 10 min with 0.5 μC of DL-leucine-1-^{14}C or guanine-8-^{14}C. The samples were processed, and the radioactivity was determined. The data are expressed as the percentage inhibition of incorporation of radioactivity into treated cells compared with untreated cells. The dashes indicate that drugs were not tested at that concentration. Control cells incorporated approximately 2000 cpm of leucine-^{14}C into protein and 7000 cpm of guanine-^{14}C into RNA per 1.5×10^6 cells.

Compound	Inhibition of protein synthesis		Inhibition of RNA synthesis	
	1 mM drug	2 mM drug	1 mM drug	2 mM drug
	%	%	%	%
Codeine (X)	—	11	—	19
Dihydrocodeine (XI)	—	4	—	8
Dihydrocodeinone (XII)	—	15	—	28
Thebaine (XIII)	36	81	25	58
Dihydrothebaine (XIV)	23	49	—	—
Thebaine methiodide (XV)	—	0	—	—
N-Allylnormorphine (I)	—	36	—	26
Levallorphan (II)	44	90	40	80
Levorphanol (III)	45	97	50	90
Levomethorphan (IV)	80	—	—	—
Dextromethorphan (V)	75	95	—	—
Phenazocine (VII)	97	98	—	98
dl-Methadone (VIII)	87	98	—	97

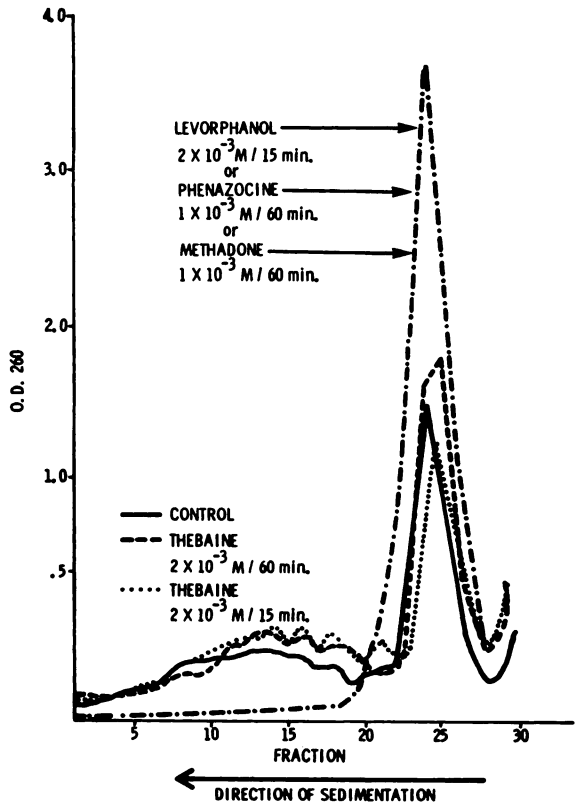


FIG. 4. *Effect of treatment with levorphanol, phenazocine, methadone, and thebaine on polysome distribution in HeLa cells*

Cultures were treated with the drugs tested for 15 or 60 min as indicated. Cells were harvested and polysomes prepared as described under METHODS. Polysomes from 40 × 10^6 cells were centrifuged through 10–40% linear sucrose density gradients. The ordinate represents optical density at 260 mμ.

occurred even when RNA synthesis was blocked by actinomycin D (3). This is true also for thebaine and phenazocine.

As reported previously, the inhibition of protein synthesis in living HeLa cells by levorphanol and levallorphan is attended by a breakdown of the polysome structure and the release of free ribosomes (3). In the present study, inhibited cells were also analyzed for their polysome and free ribosome content. As shown in the sedimentation patterns (Figs. 4 and 5), phenazocine, methadone, and methorphan (both the *d* and *l* isomers) caused the disruption of polysomes. Surprisingly, the polysome structure remained intact for considerable periods during thebaine inhibition of protein synthesis.

In a previous paper it was shown that the *in vivo* breakdown of polysomes induced by levorphanol and levallorphan could be prevented with levels of cycloheximide which inhibit protein synthesis (3). Figure 6 shows that levels of thebaine which inhibit protein synthesis were unable to mimic this action of cycloheximide. These data suggest that the action of thebaine is slightly different from that of the morphinan derivatives and synthetic narcotics tested, in that it inhibits protein synthesis without disrupting polysome structure. Perhaps this difference derives from the nature of the C-ring in thebaine.

The distinction between thebaine and levorphanol is also reflected in the ability of microsomes supported with the soluble

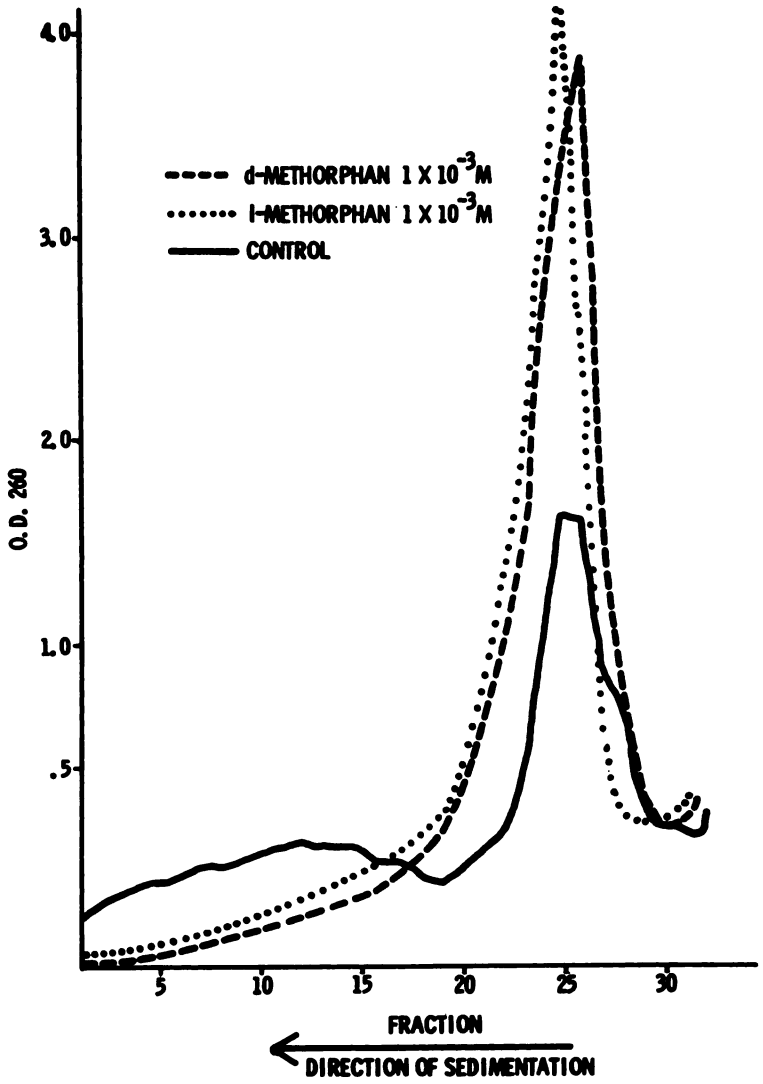


FIG. 5. *Dissociation of polysomes due to treatment of HeLa cells with d- or l-methorphan*

Cultures containing 270,000 cells/ml were treated with 1 mM *d*- or *l*-methorphan for 15 min. Cells were harvested, and polysomes prepared from the cultures were centrifuged through 10–40% sucrose gradients. Each gradient contained polysomes from 40 × 10^6 cells. The ordinate represents optical density at 260 mμ

protein fraction from treated cells to incorporate radioactive leucine. As shown in Table 3, the incorporation of L-leucine-^{14}C by a microsomal system from thebaine-inhibited cells was nearly equivalent to that of a microsomal system from control cells. However, incorporation of radioactive leucine by microsomes of levorphanol-treated cells was depressed by more than 80%. This finding is in accord with the marked dissolution of the polysome structure observed in such cells (Figs. 4 and 5). Again, this action required the intact cell structure, since adding either agent directly to the microsomal system had no effect on the incorporation of leucine. As will be described elsewhere, the free ribosomes from the levorphanol-treated cells were able to utilize polyuridylic acid effectively as a messenger when supported with radioactive

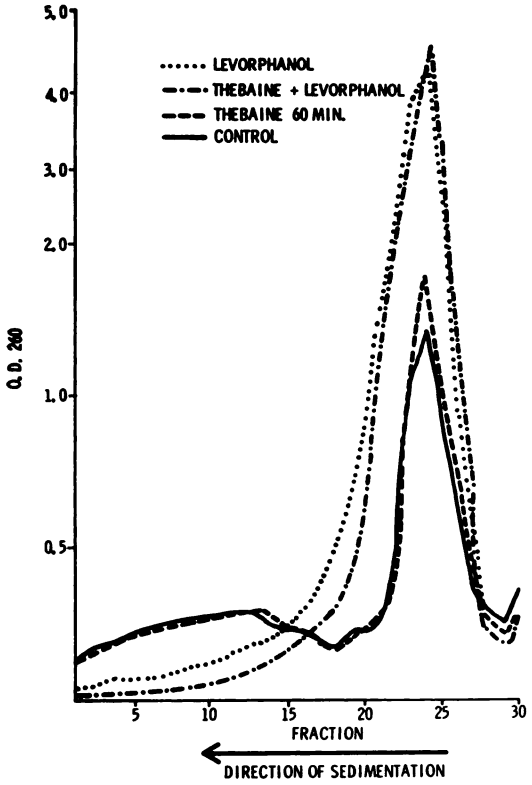


FIG. 6. *Inability of prior treatment with thebaine to prevent levorphanol-induced breakdown of polysomes*

A cell culture containing 400,000 cells/ml was treated with 2 mM thebaine for 30 min before the addition of 2 mM levorphanol for 30 min. Control cultures were treated with thebaine for 60 min or with levorphanol for 30 min. Cultures were harvested and polysomes prepared. Each gradient contained polysomes from 40×10^6 cells. The ordinate represents optical density at 260 mμ.

phenylalanine and the soluble cytoplasmic protein fraction from HeLa cells.

DISCUSSION

As described earlier (3), the inhibitory action of morphinan compounds on the growth of HeLa cells can be correlated with the ability of these compounds to inhibit protein synthesis. This action, however, appears to be indirect, since the agents have little or no effect on the incorporation of amino acids when added directly to the cell-free systems. To explain this situation

TABLE 3

Effect of thebaine and levorphanol on incorporation of leucine-^{14}C into protein by intact cells and by a microsomal system derived from treated cells

Cultures containing 350,000 cells/ml were treated with 2 mM levorphanol or thebaine for 15 min. To determine protein synthesis in intact cells, 5-ml aliquots were removed and incubated with 0.5 μC of DL-leucine-1-^{14}C for 10 min as described in METHODS. The remainder of the cells were harvested, and a $15,000 \times g$ supernatant fraction (microsomal system) was prepared which contained the cytoplasm equivalent to that of 40×10^6 cells/ml. Aliquots (0.1 ml) of this fraction were incubated for 60 min with supporting constituents and 1.0 μC of L-leucine-^{14}C (240 μC/μmole) in a volume of 1.0 ml, as described in METHODS.

Prior treatment	Incorporation of leucine-^{14}C	
	Intact cells[a]	Microsomal system[b]
	cpm/10 min	*cpm/60 min*
Control	817	5837
Thebaine, 2 mM	82	5258
Levorphanol, 2 mM	82	993

[a] Radioactivity incorporated by 1×10^6 cells.

[b] Radioactivity incorporated by supernatant fraction equivalent to 1×10^6 cells.

we have postulated that the morphinans, in the cellular environment, modify some process which alters either the availability of messenger RNA or the state of some constituent of the protein-synthesizing system which is required in the use of natural messenger RNA (3). This action, however, does not affect the ability of the free ribosomes to use the synthetic messenger, polyuridylic acid. It is possible that the decrease in the ATP levels in HeLa cells by levallorphan observed by Greene and Magasanik (4) may be closely related to the inhibition of protein synthesis. While the present study does not identify the particular step or reaction affected *in vivo* by morphinans, it does show that this action is shared by a number of narcotic and related non-narcotic analogues.

In interpreting the data, it must be kept in mind that alterations of the drug molecules may affect the ability of the com-

pounds to enter the cells. At the present time, the permeability properties of the drugs tested have not been determined. This aspect of the problem must be studied in order to evaluate the structure-activity relationships effectively. A comparison of the structural features of the compounds studied suggests that rings C and D may be specifically concerned with the inhibitory action, although the effects of substituents in these regions on the charge and polarity of the compounds cannot be ignored. One interpretation of these data suggests that a receptor may exist which recognizes the C- and D-ring region of the morphinan molecules. If this hypothetical receptor were a component of an enzyme-substrate complex, as suggested by Bloom and Goldman for the catecholamine system (10), one might postulate that the presence of the morphinan compound triggers or directs the action of an enzymatic process against an essential constituent of the protein-synthesizing system. In this case morphinans may prove useful tools in elucidating controls which operate at the translational level of protein synthesis.

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