Inhibition of Protein and RNA Synthesis in HeLa Cells by Levallorphan and Levorphanol

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SUMMARY

The effects of levallorphan, *N*-allyl-3-hydroxymorphinan, on biosynthetic functions of intact HeLa cells and subcellular fractions were examined. When cell cultures were treated *in vivo* with the agent, DNA and lipid synthesis were unaffected; the incorporation of guanine into ribosomal, rapidly labeling, and soluble RNA was reduced; and the RNA polymerase activity of isolated nuclei was depressed. The inhibitory action on RNA synthetic functions seemed to result from a reduction in the protein synthesizing capacity of the cell, which was correlated with dissociation of the polysomes and a reduced ability of subcellular fractions from treated cells to support the incorporation of amino acids. When added directly, levallorphan did not inhibit the synthesis of protein and RNA by *in vitro* systems. The data presented support the conclusions that the primary effect of levallorphan is the inhibition of protein synthesis, brought about by interference with the utilization of messenger RNA. The results confirm the strong dependence of RNA synthesis in HeLa cells on synthesis of protein.

INTRODUCTION

The inhibitory action of morphinan analogs on RNA and protein biosynthesis was first shown by Simon and Van Praag (1, 2). They reported that levorphanol (*N*-methyl-3-hydroxymorphinan) preferentially inhibited synthesis of ribosomal RNA in *Escherichia coli*, while depressing the synthesis of protein only moderately and leaving DNA synthesis unaffected during a single generation. They also reported that levallorphan (*N*-allyl-3-hydroxymorphinan) produced a similar reduction of RNA synthesis.

The present paper describes the action of levallorphan and levorphanol in HeLa cells. Concentrations of levallorphan which had very little effect on DNA and phospholipid synthesis rapidly depressed the synthesis of both RNA and protein. Levorphanol affected RNA and protein synthesis similarly. The inhibition of protein synthesis by these agents was correlated with a breakdown of polysomes *in vivo*. Treatment of cells with levallorphan reduced the capacity of microsomal fractions to incorporate amino acids when directed only by endogenous messenger RNA. Adding polyuridylic acid to the microsomes restored the incorporation activity, revealing an apparent deficiency of messenger RNA in the levallorphan-treated cells.

The transfer of treated cells to drug-free medium readily reversed all of the observed inhibitory effects of levallorphan. Since protein synthesis resumed in such cells, even when new RNA synthesis was prevented by actinomycin D, it is concluded that the inhibition of protein synthesis did not result from a primary inhibition of RNA synthesis. It is suggested instead that levallorphan affects the utilization of existing messenger RNA,

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thereby reducing protein synthesis, and that inhibition of RNA synthesis is a secondary effect.

METHODS

Culture of HeLa cells. HeLa cells were grown in suspension culture at 37° in a modified Eagle's medium (BEHM) (3). Exponentially growing cells were used except for one experiment in which the cells were first grown to a stationary phase. All compounds added to the cultures were first dissolved in double distilled water or in BEHM. When a medium change was necessary to remove the drug, the cultures were centrifuged at 800 *g* for 5 min at room temperature and the cells were resuspended in fresh, prewarmed BEHM or in BEHM obtained by centrifuging a portion of the control culture at 800 *g* for 5 min.

Unless specified otherwise, all centrifugations in the next three sections were made in centrifuges refrigerated to 4°, the washing and extracting solutions were used and kept at 4°, and cells and cell extracts were placed in ice baths between each step.

Measurement of RNA, protein, DNA, and lipid synthesis. At various times after treatment with 0.001 or 0.002 M levallorphan or levorphanol, 10-ml aliquots of the cultures were incubated with guanine-8-^{14}C or L-leucine-U-^{3}H or DL-leucine-U-^{14}C for 10 min at 37°. After centrifugation of the samples at 800 *g* for 5 min, the supernatant was discarded and the cells were washed three times in either 2.5% perchloric acid or 5% trichloroacetic acid. The residues were dissolved in formic acid and the radioactivity of the samples was counted in a liquid scintillation spectrometer. In certain cases the residues were suspended in 1.0 ml of 0.06 M $KHCO_3$ and the samples were incubated at 37° with 20 μg of ribonuclease to determine the RNase-releasable radioactivity in the acid-insoluble residue. To measure DNA synthesis, aliquots of cells were incubated in the same way for 30 min with thymidine-^{14}C and processed as just described. To determine the incorporation of ^{32}P into lipid, carrier-free orthophosphate-^{32}P (1 μC/ml medium) was added to the cultures. Aliquots were withdrawn periodically, centrifuged, and washed three times with 2.5% perchloric acid. The residues were extracted with successive 5-ml washes of 95% ethanol, ethanol–chloroform (1:2), ether, and ether. The extracts were pooled, evaporated to dryness, and dissolved in formic acid and their radioactivity was determined.

Isolation of RNA. Cultures pretreated for 1 hr with 0.001 *M* levallorphan were incubated at 37° with guanine-8-^{14}C for 1.5 hr before the RNA was isolated. The cultures were harvested by centrifugation at 800 *g* for 3 min. The cells were resuspended in 0.5 M NaCl and 0.02 M sodium citrate. Sodium dodecyl sulfate was added to a final concentration of 0.2%, and the cells were lysed by agitating them at room temperature for 5 mins. The lysate was extracted three times at room temperature with water-saturated phenol. The water-soluble phase was extracted six times with ether to remove the phenol and was dialyzed overnight at 4° against buffer containing 0.01 M Tris, pH 7.4, and 0.001 M $MgCl_2$. Aliquots of this solution corresponding to 2.5 optical density units (260 mμ) were layered onto 5–30% sucrose density gradients and centrifuged for 3 hr at 39,000 rpm, in a SW 39 rotor. Fifteen-drop fractions were collected and 1 ml of water was added to each fraction. The optical density at 260 mμ was read, and the radioactivity in 0.5-ml aliquots was determined.

In vitro protein synthesis. The amino acid-incorporating system previously described by Summers *et al.* (4) was modified slightly for the assay of protein synthesis in cytoplasmic fractions of HeLa cells. Cells were harvested, washed once with 0.154 M NaCl, and centrifuged at 800 *g* for 5 min. They were resuspended to a cell concentration of 4×10^7 per milliliter in a hypotonic buffer containing 0.01 M Tris, pH 7.6, 0.01 M KCl, 0.0015 M $MgCl_2$, and 0.006 M 2-mercaptoethanol. The cells were allowed to swell for 10 min and then were broken with 20 strokes of a

Dounce homogenizer. Microsomes were prepared by first centrifuging them at 1000 *g* for 10 min; the resulting supernatant was centrifuged at 15,000 *g* for 15 min, and the precipitate was discarded. The microsomal fraction was sedimented by centrifugation at 105,000 *g* for 2 hr and suspended in hypotonic buffer at a concentration equivalent to 10^8 cells/ml. The microsomes were assayed with the supernatant obtained by centrifuging at 105,000 *g* or with a pH 5 fraction from this supernatant.

The standard reaction mixture for studying the incorporation of amino acids contained the following materials 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; L-leucine-U-^{14}C, 1.0 μC, 240 μC/μmole, or L-phenylalanine-U-^{14}C, 1.0 μC, 360 μC/μmole; supernatant or pH 5 enzyme, 0.3 ml; microsomes, 0.15 ml. In some cases the reaction mixture also contained 50 μg polyuridylic acid (poly U). The reaction mixtures were incubated at 37°, and at various times 0.1-ml aliquots were transferred to filter paper disks and processed by a slight modification of the method of Mans and Novelli (5). The radioactivity of the disks was measured in a liquid scintillation spectrometer.

Assay of RNA polymerase. The isolation of nuclei from HeLa cells and the method of assaying nuclear RNA polymerase activity was described in a previous paper (6).

Gradient analysis of polysomes. For analysis of polysomic distribution, cells were harvested, washed once in hypotonic buffer at a concentration of 2.5×10^7/ml, allowed to swell for 10 min, and ruptured with 20 strokes of a Dounce homogenizer. The suspension was centrifuged at 15000 *g* for 15 min to remove nuclei and mitochondria. Sodium deoxycholate (final concentration 1%) was added to the supernatant, which was layered onto replicate 10–40% linear sucrose density gradients. These were centrifuged in a Spinco model L for 2 hr at 25,000 rpm. The brake was not used to decelerate the rotor.

RESULTS

In Vivo Inhibition of RNA and Protein Synthesis

Levallorphan and levorphanol rapidly inhibited both RNA and protein synthesis in HeLa cells growing in suspension culture. Figure 1 shows the incorporation of guanine-8-^{14}C into RNA and leucine-^{3}H into protein during 10-min intervals at various times following treatment with 0.002 M levallorphan. The inhibition approached the maximum within 45 min. Transfer of the cells to drug-free medium caused an immediate and rapid acceleration of both protein and RNA synthesis; these returned to the control level within 2 hr. Both drugs inhibited to the same degree at this concentration and were about one-half as effective at 0.001 M. In view of the similarity of the cellular responses to the two drugs, levallorphan was used in most of the experiments.

In contrast to the striking inhibition of RNA and protein synthesis, the syntheses of DNA and lipid were relatively insensitive to 0.001 M levallorphan during the first 2 hr after treatment (Table 1). Prolonged exposure to the agent depressed DNA synthesis, in accord with the demonstrated requirement for the synthesis of new RNA and protein in this process (3, 7).

Figure 2 compares the sedimentation character of RNA from treated and control cells in a sucrose gradient. The cells were treated with levallorphan or control solutions for 1 hour before incubation with guanine-8-^{14}C for 1.5 hr and isolation of the RNA. While there were no discernible differences in the type of RNA synthesized, levallorphan depressed the incorporation of radioactivity into all classes of RNA (rapidly labeling fraction > 30 S, 28 S, 18 S, 4 S).

In view of the effect of levallorphan on RNA synthesis, its effect on the activity of RNA polymerase was examined. RNA polymerase activity of nuclei isolated from

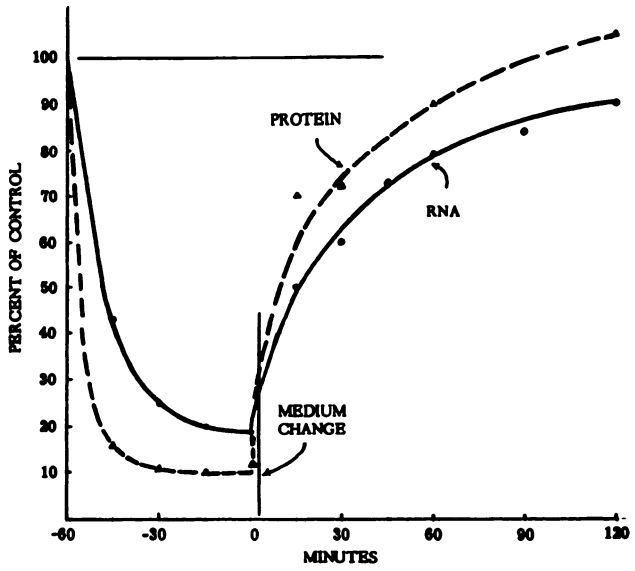

FIG. 1. *Effect of levallorphan on RNA and protein synthesis.*

Cultures containing 3×10^5 cells/ml were treated with 0.002 *M* levallorphan. Ten milliliter aliquots were removed and incubated with guanine-8-^{14}C (0.5 μC, 10 mC/mmole) or L-leucine-^{3}H (4.0 μC, 5 C/mmole) at 37° for 10 min.

HeLa was assayed by measuring the *in vitro* incorporation of cytidine triphosphate-^{3}H (CTP-^{3}H) into RNA (6). Pretreatment of cells with 0.001 M levallorphan reduced the RNA polymerase activity of the isolated nuclei, but the drug had no effect when added directly to the polymerase assay system (Fig. 3). The reduction in RNA polymerase activity in nuclei from cells treated with levallorphan was similar to that observed when cells were pretreated with cycloheximide or puromycin to limit protein synthesis (6). In the latter case, it appeared that the level of RNA polymerase activity was related to a role of protein synthesis in the regulation of RNA synthesis. Thus, it seemed possible that the reduction of polymerase activity by levallorphan might reflect the action of the drug against protein synthesis.

TABLE 1

Effect of levallorphan on the synthesis of DNA and phospholipid

Treatment		DNA[a]	Phospholipid[b]	RNA[b]
Levallorphan 0.001 M	Hours	Thymidine-^{14}C (cpm)[c]	Orthophosphate-^{32}P (cpm)[d]	
−	1	1320	10250	32500
+	1	1040	11300	16900
−	2	1670	28200	91000
+	2	1440	23400	38700
−	3	1800	53200	135000
+	3	1430	38500	54000

[a] Thirty-minute period of labeling with thymidine-^{14}C.

[b] Accumulated label, ^{32}P present from zero time.

[c] Radioactivity incorporated by 9×10^6 cells.

[d] Radioactivity incorporated by 15×10^6 cells.

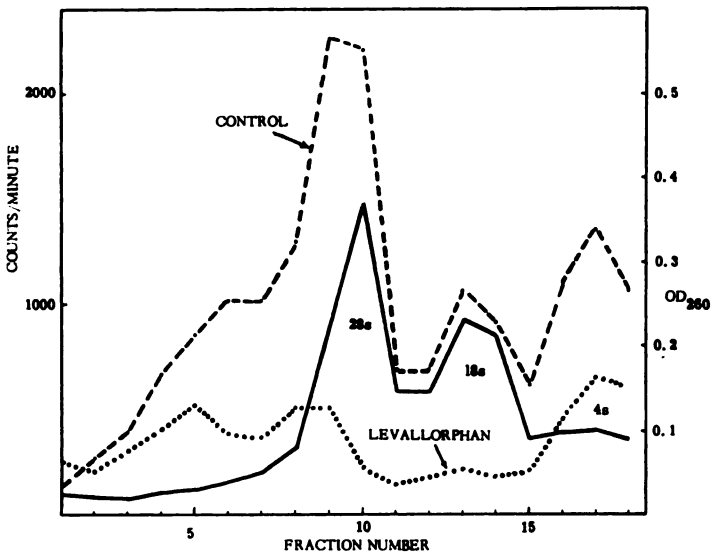

FIG. 2. *Effect of levallorphan on the synthesis of various types of RNA*

Cells treated for 60 min with 0.001 M levallorphan. Guanine-8-^{14}C (0.1 μC/ml medium, 10 mC/mmole) added for 90 min. RNA extracted with phenol; 2.5 optical density units ($O.D._{260}$) layered on gradient. ——$O.D._{260}$ of treated and control cells; ---- radioactivity of control cells; ····· radioactivity of levallorphan-treated cells.

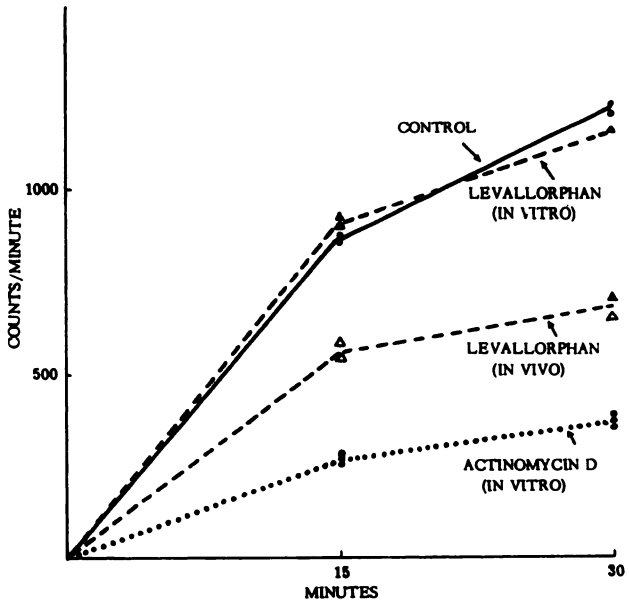

FIG. 3. *Effect of levallorphan on RNA polymerase activity*

Cells incubated for 60 min with 0.001 M levallorphan *in vivo* or 0.001 M levallorphan added *in vitro*. Each point represents the incorporation of CTP-^{3}H (1 μC, 1.25 C/mmole) by nuclei from 2×10^{6} cells.

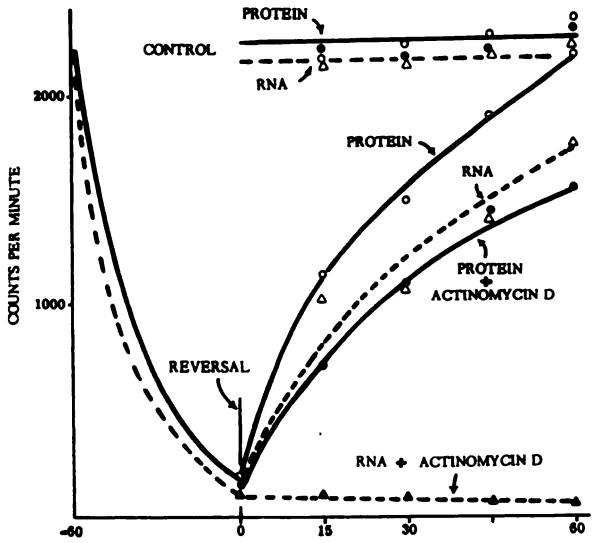

FIG. 4. *Reversal of inhibition of protein synthesis in the presence of actinomycin D*

Cultures containing 3×10^5 cells/ml treated for 1 hr with 0.002 M levallorphan. Medium changed to drug-free medium or to medium containing 1 μg/ml actinomycin D at reversal. Ten-milliliter aliquots removed and incubated with guanine-8-^{14}C (0.05 μC/ml medium, 10 mC/mmole) or DL-leucine-^{14}C (0.2 μC/ml, 10 mC/mmole) at 37° for 10 min.

Relationship between RNA Synthesis and the Effect of Levallorphan on Protein Synthesis

The resumption of both RNA and protein synthesis following removal of the cells from medium containing levallorphan provided a means of testing whether the recovery of protein synthesis depended on the synthesis of new RNA. Accordingly, cells which had been treated with levallorphan for 1 hr were transferred to medium containing actinomycin D but no levallorphan, and their recovery was studied. As Fig. 4 shows, such cells resumed protein synthesis, even though actinomycin D prevented the synthesis of new RNA. The recovery closely paralleled that in levallorphan-treated cells transferred to drug-free medium. Since protein synthesis could be restored without the synthesis of additional RNA, it appeared probable that the inhibition of protein synthesis did not result from a primary inhibiton of RNA synthesis by levallorphan. The results are more compatible with a primary action of the drug on some step affecting the utilization of RNA in the process of protein synthesis.

Further evidence supporting this thesis was obtained by comparing the distribution of polysomes from levallorphan-treated cells with those of cells treated with other known inhibitors of protein synthesis and RNA synthesis. Figure 5 shows distribution patterns obtained by centrifuging deoxycholate-treated cytoplasmic fractions from HeLa cells through 10 to 40% sucrose density gradients. Levallorphan treatment rapidly reduced the amount of polysomes and concomitantly increased the level of free ribosomes. The end result is like that produced by puromycin, which has been shown to terminate growing peptide chains (8) and to cause the release of ribosomes from the polysomic structure (9). The observation that the dissociation of HeLa polysomes occurred much more rapidly under the influence of levallorphan than with actinomycin D (10) supports further an action of levallorphan which is *proximal* to protein synthesis rather than RNA synthesis.

In contrast to levallorphan, cycloheximide promoted the buildup of polysomes. This finding is in accord with evidence that cycloheximide inhibits the

breakdown of ribosomal aggregates *in vitro* (11). When levallorphan was added to cells previously treated with cycloheximide, it decreased the amount of polysomes and increased the number of free ribosomes as compared to cycloheximide controls. Conversely, cells which were pretreated with levallorphan and subsequently exposed to cycloheximide

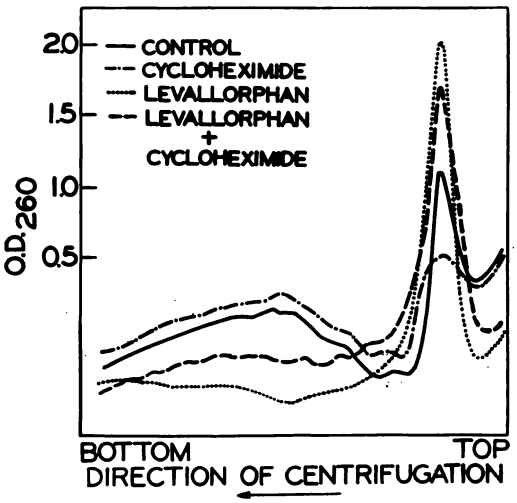

FIG. 5. *Effect of levallorphan or cycloheximide on ribosome and polysome content of HeLa cells*

Cultures containing 3×10^5 cells/ml were treated for 60 min with 0.002 *M* levallorphan or 10 μg/ml cycloheximide or both. Aliquots containing the cytoplasmic fraction from 37.5×10^6 cells were layered on 10–40% sucrose density gradients and were centrifuged for 2 hr at 25,000 rpm.

contained more polysomes than the controls treated only with levallorphan. thus, these two agents appear antagonistic with respect to the maintenance of polysome structures in the intact cells, even thought both inhibit protein synthesis.

When cells were subjected to a 1- or 2-min pulse of leucine-^{14}C, a significant amount of radioactivity in the form of recently synthesized, nascent protein was associated with the polysomes (Fig. 6). Pretreatment of the cells with cycloheximide (10 μg/ml), levallorphan (0.002 M), or levorphanol (0.002 M) for 30 min almost completely prevented the appearance of radioactivity in the polysome structures. While actinomycin D (0.4 μg/ml) and NaF (0.002 M) also reduced this incorporation significantly, neither was as effective as levallorphan or levorphanol.

The Effect of Levallorphan Treatment on Protein Synthesis in Cell-Free Systems

To determine more clearly what element in the process of protein synthesis levallorphan affected, the cytoplasm of treated and control cells was fractionated and studied for its ability to support amino acid incorporation. Levallorphan had no effect when added directly to these systems. The soluble protein fraction (105,000 *g* supernatant) or the pH 5.0 enzyme fraction derived either from control or levallorphan-treated cells supported equally well the incorporation of phenylalanine by microsomes from control cells (Table 2). On the

TABLE 2

Effect of levallorphan on ability of microsomes to incorporate phenylalanine

Microsomes	Supernatant	Prior treatment of cells[b] (+ or − 0.002 M levallorphan)	pH 5 Fraction	Incorporation[a] − Poly U (cpm)	Incorporation[a] + Poly U (cpm)
−	−			560	2050
−	+			500	1410
−			−	1130	2940
−			+	960	2890
+	−			49	1670
+	+			49	2070
+			−	110	3790
+			+	81	4660

[a] Incorporation of L-phenylalanine-^{14}C, 1μC/ml, 360 mC/mmole by 1.5×10^6 cell equivalents in 60 min.

[b] Cells (4×10^5/ml) treated for 1 hr.

other hand, the microsome fraction from levallorphan-treated cells had a very limited capacity to incorporate amino acids. This inactivity appeared to account for the restricted protein synthesis of intact, treated cells. Although inactive when isolated, these microsomes incorporated phenylalanine when polyuridylic acid was added as an exogenous messenger; in fact, microsomes from levallorphan-treated cells

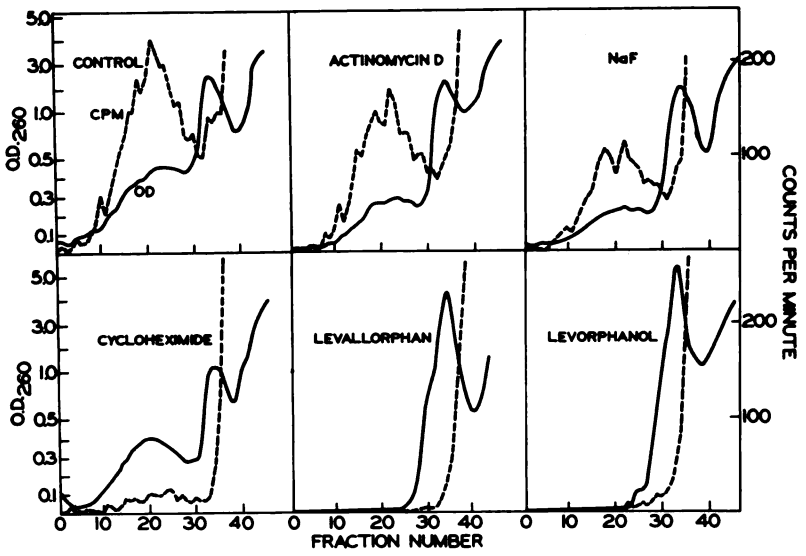

FIG. 6. *Effect of various agents on the ability of polysomes to incorporate amino acid*

Cultures containing 7.5×10^7 cells at a cell concentration of 3×10^5/ml were treated with the drugs for 30 min. Drug concentrations were: levallorphan, levorphanol, and sodium fluoride 0.002 *M*; cycloheximide, 10 μg/ml; actinomycin D, 0.4 μg/ml. The cells were harvested and resuspended in 25 ml BEHM without serum containing one-fortieth the normal concentration of amino acid and the same concentration of drug. Leucine-^{14}C (5.0 μC/μmole) was added for 2.5 min. The cells were poured over crushed ice and processed as described in the Methods section. Each tube contained the cytoplasmic fraction from 62.5×10^6 cells. The solid line represents optical density at 260 mμ. The broken line represents radioactivity.

incorporated more radioactivity than those of control cells. It thus appears that the microsome fraction of levallorphan-treated cells is deficient in endogenous messenger RNA; but is otherwise competent for protein synthesis. The observation that recovery of protein synthesis *in vivo* was independent of new RNA synthesis (Fig. 4) suggests strongly that the drug affects the availability or utility of existing messenger RNA in addition to inhibiting the synthesis of RNA.

DISCUSSION

Although the effects of levorphanol on HeLa cells have not been investigated in the same detail as those of levallorphan, it seems likely that both drugs act in a similar manner. Their action involves a rapid inhibition of RNA and protein synthesis without appreciable effect on DNA and phospholipid synthesis. The selectivity of the response makes it unlikely that they act as dinitrophenol to limit energy metabolism (12). Instead the rapidity of the inhibition of protein synthesis and the recovery of this process in the absence of RNA synthesis argue for a primary action of levallorphan against some step in protein biosynthesis.

While the *in vivo* effects mimic in many ways the action of puromycin, levallorphan and levorphanol are inactive when added directly to the cell-free amino acid incorporating system. *In vivo* treatment of the cells, however, produces cell-free systems which are inactive for amino acid incorporation. The primary deficiency of the incorporating system appears to be a lack of usable messenger RNA; a deficiency which could be compensated for by the addition of polyuridylic acid. While levallorphan inhibits the synthesis of RNA in living cells, it appears unlikely that the restricted synthesis of new RNA is the actual cause of the deficiency in the amino acid incorporating system. It appears instead, that levallorphan treatment may

modify existing RNA so as to decrease its effectiveness as a messenger for the amino acid incorporating system. It is, however, possible that the agent affects the ability of amino acid incorporating systems to use native messenger RNA without interfering with the ability of the system to use polyuridylic acid as the messenger. In either case it is conceivable that these morphinan compounds will help in the study of translational control in protein synthesis.

The inhibition of RNA synthesis by levallorphan is not explained by these studies, although it is similar to that observed when protein synthesis is depressed by puromycin or cycloheximide (6). Those observations and the present ones are consistent with a concept of RNA synthesis requiring the concomitant synthesis and utilization of critical proteins (13). The depression of RNA polymerase activity in levallorphan-treated cells is also like that produced by puromycin and cycloheximide. While the inhibition of RNA synthesis appears secondary to the inhibition of protein synthesis, it remains possible that a single process affecting the metabolism of RNA could account for the restriction of both RNA and protein synthesis.

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