Note added in proof: Since this manuscript was submitted, Kallos and Rizok[12] have obtained direct chemical evidence indicating that the pipsyl as well as the tosyl group is covalently bonded to a serine residue of γCHT.

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SELECTIVE INHIBITION OF SYNTHESIS OF RIBOSOMAL RNA IN ESCHERICHIA COLI BY LEVORPHANOL*

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We have previously reported that levorphanol, a close structural analogue of morphine, inhibits RNA synthesis in *E. coli* to the extent of 80–90 per cent.[1,2]   Since ribosomal RNA comprises 80–85 per cent of the total RNA of the cell, these results indicate that the synthesis of ribosomal RNA must be markedly inhibited by levorphanol.   The present experiments were designed to determine the extent to which levorphanol inhibits the synthesis of messenger and transfer RNA.   Evidence will be presented which demonstrates that levorphanol inhibits predominantly the synthesis of ribosomal RNA.   This is in contrast to the findings with actinomycin D which has been shown to inhibit equally the synthesis of all types of cellular RNA.[3,4]

Materials and Methods.—Bacterial strains and media: *E. coli* K-13, a prototrophic isolate of strain K-12, and Hfr Hayes, a methionine requiring K-12 "relaxed" mutant, were used in the present experiments. Cells were grown in minimal medium buffered by 0.05 M triethanolamine at pH 8.2, supplemented with 0.5% sodium succinate and 0.2% casamino acids as sources of carbon as described previously.[2] For the "step-down" experiment the enriched medium was prepared by adding 1% Difco Bacto-peptone to the minimal medium and readjusting the pH to 8.2.

Materials: O-nitrophenyl β-galactoside (ONPG) and isopropylthiogalactoside (IPTG) were purchased from Mann Laboratories, DNAase from Worthington Chemical Co. Levorphanol tartrate and levallorphan tartrate were made available through the generosity of Hoffmann-La Roche, Inc., Nutley, N.J. C^{14}-uracil was purchased from New England Nuclear Corp.

Methods: Radioactive counting and analytical procedures, except as set forth below, were carried out as described in the previous paper.[2]

Measurement of β-galactosidase induction: IPTG, a good inducer of β-galactosidase, was added to growing cultures of *E. coli* K-13 at a concentration of $1 \times 10^{-3} M$. In the cultures treated with levorphanol the inducer was added 20 min following addition of drug. Aliquots of the cultures were removed at intervals, centrifuged, washed, and suspended in 0.05 *M* phosphate buffer, pH 7.5. Portions of the cell suspensions were shaken at 37° for 20 min with 0.05 ml toluene/ml suspension. Measurement of β-galactosidase activity was carried out by the method of Lederberg.[5] 0.1 ml of toluenized cells was added to 1 ml of $2.5 \times 10^{-3} M$ ONPG in phosphate buffer (0.05 *M*, pH 7.5). The change in optical density at 420 mμ was followed in a Zeiss spectrophotometer. Units of enzyme are defined as mμmoles of o-nitrophenol released per hour. Uracil-C^{14} incorporation was measured in the same cultures. Determinations of optical density and viable cell count were performed on portions of the cell suspension. Protein and radioactivity measurements were carried out after acid precipitation.

Radioactive uracil incorporation in "step-down" and control cultures: To obtain "step-down" cultures, logarithmically growing cells in peptone medium buffered at pH 8.2 were collected and washed twice with minimal medium. The washed cells were resuspended in minimal medium at pH 8.2 and shaken at 37°. Levorphanol was added, followed 5 min later by uracil-C^{14} (0.09 μc and 0.9 μg/ml). Aliquots were precipitated with acid and counted at 5-min intervals. Control (not step-down) cultures were obtained by subjecting cells growing in minimal medium to the same washing procedure and resuspending them in minimal medium.

Effect of levallorphan on the production of bacteriophages T6 and λ: *E. coli* K-13 was grown to a concentration of 2.0×10^8 cells/ml in a medium containing 1% peptone, 5% NaCl, buffered at pH 8.0 with *M*/20 Tris buffer. Levallorphan was added to a final concentration of $1.3 \times 10^{-3} M$. After exposure to the drug for 10 min the cells were infected with T6 or λ particles at a multiplicity of approximately 0.1 particle/bacterium. Infected cells were diluted 10^{-4} into the same medium with and without levallorphan, respectively, and infective centers were counted. One infective center was found for each infected bacterium, indicating that there was no early killing of cells. The infected cultures were then incubated for 1 hr at 37° and lysed with chloroform and lysozyme. Total burst yield of phage per infected cell was determined by plating aliquots of lysate on a lawn of *E. coli* K-13.

Isolation of RNA: Bacteria were harvested by centrifugation and washed with 0.5% KCl-0.5% NaCl solution. The cells were disrupted by grinding the frozen pellet with 2.5 times its weight of alumina A-301. The ground cells were extracted with four 2-ml portions of 0.01 *M* Tris buffer pH 8.0 containing $1 \times 10^{-4} M$ MgCl₂ (TM buffer). The extract was centrifuged in the Lourdes centrifuge at 13,000 rpm for 15 min to remove unbroken cells, cell debris, and alumina. The extract was treated with DNAase (10 μg/ml) at room temperature for 10 min. After addition of sodium lauryl sulfate to a concentration of 0.2%, the solution was extracted with equal volume of redistilled 80% phenol by shaking at room temperature for 1 hr. Layers were separated by centrifugation, and the aqueous layer was re-extracted with half its volume of phenol. This was followed by several ether extractions to remove most of the phenol. RNA was precipitated by the addition of 2 volumes of ethanol and 0.1 volume of 20% potassium acetate, pH 5.0. After standing for at least 1 hr to ensure complete precipitation the pellet was sedimented by centrifugation. The supernatant was discarded, the pellet was redissolved in a small volume of TM buffer, and the optical density of the solution at 260 mμ was determined. Sucrose gradient fractionation was performed on suitable portions of this purified RNA.

Isolation of sRNA from methionine-starved Hfr Hayes: The procedure was identical to that described for the isolation of total RNA, except that the magnesium content of the Tris buffer was $10^{-2} M$ and the buffer extract of broken cells was centrifuged in the Spinco Model L ultracentrifuge for 90 min at 40,000 rpm to remove ribosomes. Sodium lauryl sulfate was omitted. The purified sRNA solution was dialyzed overnight against TM buffer. The optical density of the solution at 260 mμ was determined. The solution of purified "starved" sRNA was given to Dr. Jerard Hurwitz for measuring the extent of methylation.

Sucrose gradient centrifugation: A linear sucrose gradient ranging from 3 to 20% sucrose was prepared using a mixing device similar to that described by Britten and Roberts.[6] The sucrose solutions were prepared in 0.01 *M* Tris buffer, pH 7.4 containing $10^{-4} M$ MgCl₂, $5 \times 10^{-2} M$ KCl,

and 0.001% sodium lauryl sulfate. 0.2 ml of phenol-extracted RNA solution containing an amount of RNA which gave an absorbancy at 260 mμ of 10–12 O.D. units was layered carefully on top of the sucrose gradient (4.4 ml). The tubes were centrifuged in the SW-39 swinging bucket rotor of the Spinco Model L ultracentrifuge for 6 hr at 37,500 rpm at 3°. At the end of this time the centrifuge was allowed to come to a stop without braking, and the rotor was removed to the cold room. Tubes were taken out and pierced at the bottom with a 20-gauge syringe needle. Fractions of 1 drop each were collected. Alternate fractions were diluted with 1 ml of TM buffer and used for determination of optical density at 260 mμ. The other fractions were precipitated by the addition of 2 ml of 0.25 M perchloric acid for membrane filtration and radioactivity measurement as described previously.[2] When a crude TM extract of ground cells containing intact ribosomes was fractionated, centrifugation was carried out for only 90 min. The procedure was otherwise identical.

Results.—Evidence for the synthesis of messenger RNA (mRNA) in the presence of levorphanol: An observation reported previously[2] was the persistence of protein synthesis in levorphanol-inhibited *E. coli*. Bacteria producing RNA at only 10–15 per cent of the normal rate continued to make protein at a rate about 50 per cent of that of growing control cultures. This finding can be explained in several ways, one of which is the continuation of mRNA synthesis in the presence of levorphanol.

Further evidence for this possibility could best be obtained by studying the effect of levorphanol on the synthesis of a single functional protein with an unstable mRNA template. Inducible enzymes provide such systems. In fact, the concept of a messenger fraction with a high turnover rate had its origin in the investigations of Jacob and Monod[7] on the induction of β-galactosidase. Figure 1 shows an experiment in which the extent of induction of β-galactosidase in the presence of levorphanol was studied. The inducer (IPTG) was added 20 min after introduction of the drug to ensure completeness of its inhibitory effect. There is no increase in viable cell count in the treated culture during the period of observation. It can be seen that β-galac-

FIG. 1.—Induction of β-galactosidase in the presence of levorphanol. ▲—β-galactosidase activity in control; △—β-galactosidase activity in the presence of 1.35 × 10⁻³ M levorphanol; ●—C¹⁴-uracil incorporation in control; ○—C¹⁴-uracil incorporation in the presence of 1.35 × 10⁻³ M levorphanol.

tosidase induction proceeds for at least 60 min in the presence of levorphanol at about half the rate of the control culture, while during this period only 10–15 per cent of normal RNA synthesis is taking place.

It was shown by Gros *et al.*[8] that very brief pulse-labeling of cells with RNA precursors results in the incorporation of isotope largely into the mRNA fraction. Table 1 shows the results of a pulse-labeling experiment in the presence of levorphanol. The inhibited culture received radioactive uracil 15 min after introduction of levorphanol. Exposure of the cultures to C¹⁴-uracil for intervals of 10 and 20 min resulted in inhibition of RNA synthesis in the normally observed range, whereas only 20–25 per cent inhibition was seen after 30 sec.

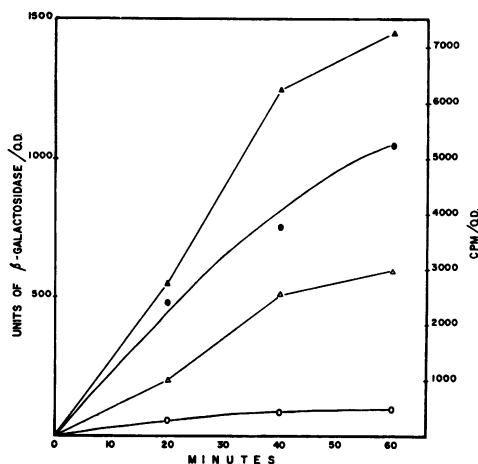Hayashi and Spiegelman[9] have reported that mRNA is virtually the only fraction

TABLE 1

THE EFFECT OF LEVORPHANOL ON PULSE-LABELING OF RNA WITH C[14]-URACIL

Levorphanol conc. ($M \times 10^3$)	30 sec		10 min		20 min	
	Cpm/O.D.	% Incorporation	Cpm/O.D.	% Incorporation	Cpm/O.D.	% Incorporation
0	850	100	9600	100	19,600	100
1.22	635	75	2460	26	3900	20
1.35	610	72	2380	25	5050	25
1.48	690	81	2480	26	4050	21
1.72	610	72	1815	19	3550	18

synthesized for 15–20 min after a culture of *E. coli* is "stepped-down" from rapid growth in rich medium to slow growth in a synthetic medium. Table 2 shows that when levorphanol was added shortly after a step-down, C[14]-uracil incorporation into RNA continued for 20 min at 60–70 per cent of the rate in a step-down culture to which no drug was added. The culture which was transferred from minimal medium to minimal medium showed only 10–13 per cent RNA synthesis in the presence of levorphanol.

The infection of *E. coli* with the DNA containing bacteriophage T2 was shown, by the classical experiments of Volkin and Astrachan,[10] to result in the formation of an unstable RNA fraction with a base composition which reflects that of the phage DNA. It is now generally accepted that this is the mRNA which carries the genetic information for the production of phage-specific proteins. The effect of levallorphan[10a] on the production of phages λ and T6 in *E. coli* K-13 was tested. In the presence of 1.3×10^{-3} M levallorphan the yield of T6 particles per infected cell was 100, as compared to 130 in the absence of the drug. The corresponding values for the yield of λ were 11 and 14, respectively. Thus, levallorphan, at a concentration at which we found it to inhibit RNA synthesis profoundly, had no significant effect on the production of DNA bacteriophages and therefore on the synthesis of their messenger RNA.

Evidence for the synthesis of transfer RNA (sRNA) in the presence of levorphanol: Figure 2 shows the results of an experiment in which *E. coli* K-13 was incubated with C[14]-uracil in the presence and absence of levorphanol for 60 min followed by a 15-min exposure to a large excess of C[12]-uracil. This chasing procedure results in the labeling of ribosomal and transfer RNA with little radioactivity in the rapidly turning-over messenger fraction. RNA isolated from these cultures by the phenol procedure was fractionated in a sucrose gradient. It can be seen that C[14]-uracil incorporation into the 23S and 16S ribosomal RNA peaks is inhibited to a much greater extent than incorporation into the 4S transfer RNA peak. From the areas under the peaks it was

TABLE 2

EFFECT OF LEVORPHANOL ON C[14]-URACIL INCORPORATION AFTER STEP-DOWN

	5 min		10 min		15 min		20 min	
	Cpm/O.D.	% of control	Cpm/O.D.	% of control	Cpm/O.D.	% of control	Cpm/O.D.	% of control
Peptone → Minimal								
Control	8,700	100	12,200	100	16,400	100	17,500	100
1.2×10^{-3} M levorphanol	5,150	59	8,200	65	12,400	76	12,700	72
Minimal → Minimal								
Control	13,400	100	23,000	100	31,000	100	38,000	100
1.2×10^{-3} M levorphanol	1,800	13	3,200	14	3,500	12	3,400	9

FIG. 2.—Sucrose gradient fractionation of RNA isolated from cultures of *E. coli* K-13 labeled with .C^{14}-uracil in the presence and absence of levorphanol. Two 500-ml cultures of *E. coli* growing in minimal medium, one of which was treated with 1.35×10^{-3} M levorphanol, were incubated with C^{14}-uracil (0.0052 μc and 4 μg/ml for control, 0.012 μc and 4 μg/ml for inhibited culture) for 60 min followed by exposure to 200 μg/ml of C^{12}-uracil for 15 min. The higher specific activity of the uracil used in the treated culture served to ensure sufficient radioactivity in the RNA. Isolation and fractionation of RNA was performed as described in *Methods*. The upper portion of the figure represents the RNA profile derived from the control culture, the lower portion that of the levorphanol-treated culture. Areas under peaks were corrected for the difference in specific activity of the C^{14}-uracil.
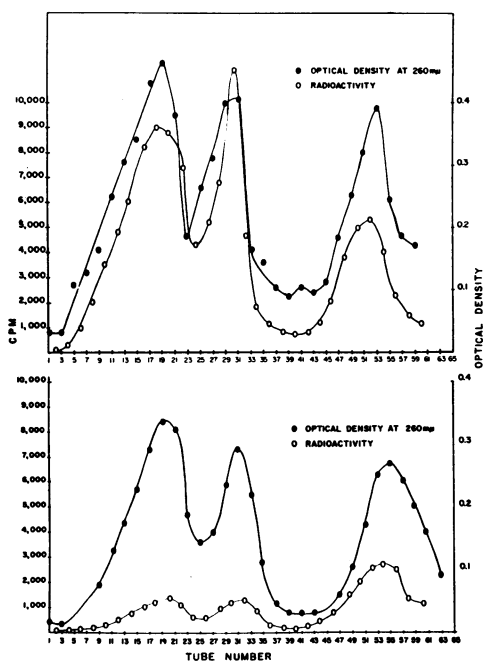
calculated that the entry of isotope into the ribosomal RNA in the presence of levorphanol is only 6–8 per cent of control, whereas 35–40 per cent of control radioactivity is found in sRNA. Incorporation into total RNA was 12–14 per cent of that in the control culture. Comparable results were obtained in similar experiments in which intact ribosomes were separated from sRNA by sedimentation at $105,000 \times g$ or by sucrose gradient centrifugation.

Mandel and Borek[11] found that sRNA formed during methionine starvation of a relaxed (RC^{rel}) methionine requiring *E. coli* mutant was devoid of methylated bases. The extent to which sRNA isolated from such a culture can be methylated is a measure of the amount of new sRNA formed during methionine starvation. This can be determined by incubating sRNA with one of the methylating enzymes described by Gold *et al.*[12] and measuring the amount of radioactivity incorporated when C^{14}-methyl-S-adenosylmethionine is the methyl donor. These enzymes exhibit a high degree of specificity for sRNA.

An experiment was carried out in which cultures of a relaxed methionine-requiring mutant were starved of methionine in the presence and absence of levorphanol. The extent of methylation was determined on sRNA isolated from these cultures. Aliquots of the same culture were used for measuring C^{14}-uracil incorporation into total RNA. Table 3 shows the results of this experiment which indicate that production of sRNA is affected much less by levorphanol than over-all RNA synthesis. Thus, when uracil incorporation proceeded at only 15 per cent of the control rate, methylation of sRNA occurred at 62 per cent of control.

Discussion.—The evidence presented indicates that a concentration of levorphanol which inhibits ribosomal RNA synthesis by 90–95 per cent allows continued synthesis of the messenger and transfer fractions at about half the rate of growing control cultures. There are reasons for suggesting that the actual effect of levorphanol on

TABLE 3

EFFECT OF LEVORPHANOL ON METHYLATION OF "STARVED" sRNA

Levorphanol (M)	Uracil incorporation (cpm)	% of control	Methylation of sRNA (mμm)*	% of control
0	20,600	100	0.241	100
1.1 × 10^{-3}	9,800	47	0.204	85
1.8 × 10^{-3}	3.100	15	0.148	62

* mμmoles of methyl-C^{14} incorporated in 60 min when sRNA (500 mμm of nucleotides) was incubated with C^{14}-methyl-S-adenosylmethionine and the enzyme which converts uracil to thymine in sRNA.

the synthesis of mRNA and sRNA may be even less than the twofold inhibition observed. For example, in the sucrose fractionation experiment a relatively small breakdown of ribosomal RNA by nucleases would result in apparent inhibition of sRNA synthesis, because a given percentage of ribosomal RNA added to the 4S peak would provide a much larger increment of radioactivity in the control than in the inhibited culture. In the pulse-labeling and step-down experiments, some of the apparent inhibition of mRNA synthesis may result from the possibility that these procedures are not completely selective for the synthesis of mRNA alone. A particularly interesting situation exists in the experiments in which β-galactosidase induction was measured in the presence of levorphanol. Mandelstam[13] has shown that the presence of a carbon source in a nongrowing culture leads to virtually complete catabolite repression of β-galactosidase induction. In our experiments there was induction at about half the normal rate in the presence of a carbon source in cultures not growing because of treatment with levorphanol. Even if levorphanol has no direct effect on β-galactosidase synthesis, the experiments of Mandelstam lead to the prediction that little or no induction should occur under these conditions because of catabolite repression. Our finding may be explained by the continued synthesis of DNA and protein which may result in the removal of catabolites responsible for repression. The recent report of Paigen[14] demonstrating that amino acids are potent repressors of β-galactosidase synthesis supports this view. The decrease in rate of induction seen in our experiments may be a direct effect of the drug, or may be at least partially explainable by residual catabolite repression. The enzyme induction seen at concentrations of levorphanol which profoundly inhibit RNA synthesis gains added significance in the light of reports that other agents known to affect the synthesis of nucleic acids or proteins, such as actinomycin D,[15] mitomycin,[16] puromycin,[16] chloramphenicol,[14, 16] and streptomycin,[16] inhibit enzyme induction markedly at concentrations below those at which they exhibit measurable effects on macromolecular synthesis.

The question may be raised whether the isotope incorporation into the 4S peak which persists in the presence of levorphanol may result from the fact that the enzyme which attaches the terminal trinucleotide (ApCpC-) to sRNA[17, 18] is not inhibited. This has been found to be the explanation for the apparent resistance of sRNA in HeLa cells to actinomycin D.[19] In the present experiments this possibility appears to be excluded. Since none of the three terminal bases contain methyl groups, the occurrence of considerable methylation (60–85% of control) of sRNA made in the presence of levorphanol during methionine starvation indicates that *de novo* synthesis of sRNA molecules has taken place. Moreover, paper electrophoresis of alkaline hydrolysates of sRNA isolated from cells labeled with C^{14}-uracil in the presence of levorphanol showed that more than 50 per cent of the radioactivity was present in uracil.

The present experiments do not provide an understanding of the mechanism by which levorphanol exerts its selective effect.   Inhibition of an enzyme involved in the synthesis of ribosomal RNA would furnish an explanation, provided that different classes of RNA are produced by divergent synthetic pathways.   However, other possibilities would have to be invoked if all types of RNA are made by a single synthetic pathway as is suggested by current results from a number of laboratories.   Evidence that DNA serves as template for the synthesis not only of mRNA but of ribosomal RNA and sRNA as well comes from hybridization experiments[20-22] and from the observation that synthesis of all cellular RNA is blocked by actinomycin D.[3, 4]   Preliminary results are also available indicating that all classes of RNA may be made by the enzyme RNA polymerase, originally regarded as the synthetic device for mRNA only.   Weiss[23] has reported evidence for the formation of ribosomal RNA by RNA polymerase *in vitro*.   Hurwitz *et al.*[24] have found that products of RNA polymerase can be methylated by enzymes which exhibit a high degree of specificity for sRNA.

If a cell makes all its RNA by a single enzymatic process, it would seem to require control mechanisms to enable it to vary separately the rate of synthesis of different types of RNA.   Levorphanol might then exert its effect primarily on the mechanism which regulates the rate of formation of ribosomal RNA.

The possibility must also be considered that levorphanol may be able to exert a selective effect on the particular portion of the DNA molecule which codes for ribosomal RNA.

Several laboratories[25-27] have reported recently that ultraviolet irradiation of cells causes differential inhibition of synthesis of various types of RNA.   This system differs from ours in that profound inhibition of DNA synthesis and cell viability is observed at doses of UV below those at which any effect on RNA synthesis is noted.

Summary.—Evidence is presented for the selective inhibition of the synthesis of ribosomal RNA by levorphanol in *Escherichia coli*.   A concentration of levorphanol which exerts 90–95 per cent inhibition of synthesis of ribosomal RNA allows continued synthesis of messenger and transfer RNA fractions at about half the rate of a growing control culture.

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*EFFECT OF CORTISONE ON GENETIC RESISTANCE TO MOUSE HEPATITIS VIRUS IN VIVO AND IN VITRO**

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It is known that the genetic resistance of C3H mice to mouse hepatitis virus (MHV) is phenotypically manifested in the macrophage system when tested *in vitro*,[1, 2] and that macrophages obtained from such resistant mice may be converted to temporary susceptibility if exposed to extracts of cells from a susceptible (PRI) strain of mice.[3] The established role of cortisone in altering the susceptibility of various animals to infectious diseases has led us to test the effect of this hormone on the genetic resistance of C3H mice. We have found that C3H mice become highly susceptible to mouse hepatitis virus after administration of cortisone, and that cells from C3H mice also become susceptible *in vitro* if cultures are treated with cortisone. This latter is demonstrable in macrophage cultures obtained from resistant mice, and in cultures of liver from eight-day-old mice, in which liver parenchymal cells as well as macrophages are destroyed.

Materials and Methods.—Inbred strains of C3H (resistant) and PRI (susceptible) mice were used.[2] Mouse hepatitis virus was originally obtained from Dr. J. B. Nelson of The Rockefeller Institute and has been maintained by serial passage in an inbred strain of PRI mice. Frozen stocks were kept at $-30°C$.