REVERSIBLE INHIBITION OF RNA PHAGE REPLICATION AND MACROMOLECULAR SYNTHESIS BY LEVORPHANOL[1]

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SUMMARY - Reproduction of RNA phages MS2 and Q_β and the synthesis of phage RNA and protein are markedly inhibited (>99%) by levorphanol. Even at concentrations which are virtually without effect on the host, phage yield is decreased 85-90%. Levorphanol is most effective when added before or at the time of infection, but no inhibition is observed when it is added 30 minutes or more after infection. Inhibition of phage reproduction is reversed when, even after exposure to levorphanol for an hour, the infected cells are washed free of drug. An effect of levorphanol on an early event in phage replication is postulated.

INTRODUCTION - We previously reported the selective inhibition of ribosomal RNA synthesis in Escherichia coli by levorphanol and levallorphan, structural analogues of morphine (1,2). During these studies it was found that replication of the RNA bacteriophage f2 was markedly inhibited by levallorphan (Zinder and Simon, unpublished observations). In the present paper we report studies on the inhibition by levorphanol of reproduction and macromolecular synthesis of the closely related phage MS2. Inhibition was shown to be profound, reversible and strongly dependent on time of addition of the drug relative to the time of phage infection. Inhibition of mengo virus RNA synthesis by levallorphan has recently been reported (3).

MATERIALS AND METHODS - Bacteriophages MS2 and Q_β were grown on E. coli Q13 as described by Loeb and Zinder (4). The preparation of phage stocks of $1\text{-}2\times10^{13}$ plaque forming units (pfu) per ml has been described elsewhere (5).

E. coli Q13 was grown in a synthetic medium buffered with either .05M triethanolamine or .05M Tris at pH 7.8, as described by Simon and Van Praag (6). Sodium lactate (0.5%) served as the carbon source and the medium was supplemented with 20 L-amino acids (each 0.1mM) and thiamine hydrochloride (10ug/ml).

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The divalent cation concentration most commonly used was 0.5 mM $CaCl_2$ and 0.5 mM $MgCl_2$.

At a cell density of $2x10^8$ cells/ml the culture was infected with phage at a multiplicity of 10-20 pfu/cell and incubated at 37° for 5 minutes. The cells were centrifuged and washed to remove unadsorbed phage and resuspended in the synthetic medium. Appropriate isotopic precursors and levorphanol were added as indicated in legends. Cultures were incubated for 2 hours at 37° and lysis was completed by the addition of lysozyme (50 μg/ml). After further incubation at 37° for 15 minutes a drop of chloroform was added. The plaque assay was performed by standard techniques (7).

To measure phage RNA and protein synthesis uninfected and infected cultures were treated with rifampicin (8). To the latter rifampicin was added 15 minutes after infection. Incorporation of radioactive precursors was measured by precipitating aliquots (0.5ml) of cultures with 5% trichloroacetic acid, isolating the acid-precipitable fraction by filtration on millipore membranes and counting in a Packard liquid scintillation counter (2).

Viable cell counts were carried out by making suitable dilutions of uninfected cells in calcium-saline (9.0g NaCl and 0.29g $CaCl_2$) and spreading the bacteria on nutrient agar plates.

Levorphanol tartrate was made available for this research by Dr. W. E. Scott, Hoffmann-LaRoche, Inc. Rifampicin was obtained from Calbiochem. ^{14}C-uracil and ^{3}H-leucine were purchased from New England Nuclear Corp.

RESULTS - Inhibition of phage reproduction by levorphanol: When levorphanol, at a concentration which inhibits host growth and RNA synthesis by 60-80 per cent, is added to a culture of *E. coli* Q13 at or before infection with MS2, phage replication is inhibited by more than 99 per cent. Even under conditions at which the effect of levorphanol on the bacteria is minimal virus yield is markedly reduced. Thus, at high divalent cation concentration (5mM $CaCl_2$ and 5mM $MgCl_2$) and lower pH (pH7.0) host growth and RNA synthesis are inhibited only 10-15 per cent by 2.25 mM levorphanol but phage reproduction is reduced 85-90 per cent. Preincu-

TABLE 1

EFFECT OF TIME OF ADDITION ON INHIBITION OF PHAGE YIELD BY LEVORPHANOL

Lev Addition*	pfu/cell	Inhibition
min		%
No drug	1000	---
0	4	99.6
3	20	98
7	100	90
20	350	65
35	1000	0

*Levorphanol (Lev) added at 1.6mM at indicated times after infection

bation of bacteria with levorphanol before infection gives no greater inhibition than addition of drug at time of infection.

Table 1 illustrates the importance of adding the drug at or shortly after phage infection. As the time elapsed between infection and treatment with levorphanol is lengthened, there is a progressive decrease in the inhibition of plaque formation. When levorphanol is added 30 minutes or more post-infection there is no longer any effect on phage yield. Identical results were obtained with the RNA phage Q_β.

Reversibility of inhibition: In view of recent results indicating that levorphanol has an effect on the bacterial cell membrane it was suspected that the effect might be exerted on the process by which the phages adsorb to the bacterial F pili. It can be seen from Figure 1 that this is not the mode of action of levorphanol. Cells were treated with MS2 at a multiplicity of 10-20 pfu/cell and were incubated for 5 minutes in the presence of levorphanol. The cells were then centrifuged and washed to remove unadsorbed phage particles and reincubated in the presence of the drug. When the drug was removed 30 minutes or 60 minutes after infection and in-

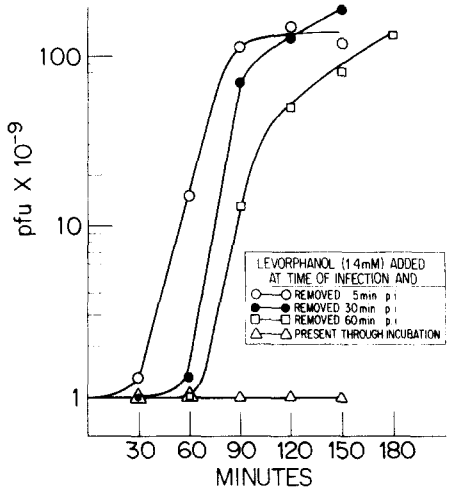

Fig. 1 - Reversibility of inhibitory effect of levorphanol on phage reproduction in MS2-infected E. coli.
Levorphanol used at 1.4mM. Experiments performed as described in Methods.

cubation was continued in drug-free medium, there was a normal yield of phage as well as a normal growth curve. The latter was merely displaced to the right by the amount of time the culture was exposed to levorphanol. The inhibition by levorphanol is thus completely reversible. This, together with the substantial decrease in phage yield seen even when levorphanol is added 7 minutes after the phage, at which time most, if not all, the cells would be infected, makes it highly unlikely that levorphanol affects the infection process.

Effect of levorphanol on phage RNA and protein synthesis: Rifampicin has been found to inhibit host RNA and protein synthesis while allowing considerable phage macromolecular synthesis to occur (8). This effect was not as selective in our hands with MS2 as it was in the experiments with f2 of Fromageot and Zinder. When rifampicin was added 15 minutes after infection with MS2 we obtained the greatest selectivity. Host RNA synthesis was decreased 95% while phage RNA synthesis was reduced 70%.

Using rifampicin to minimize host RNA synthesis we investigated the effect on phage RNA synthesis of addition of levorphanol at various intervals following in-

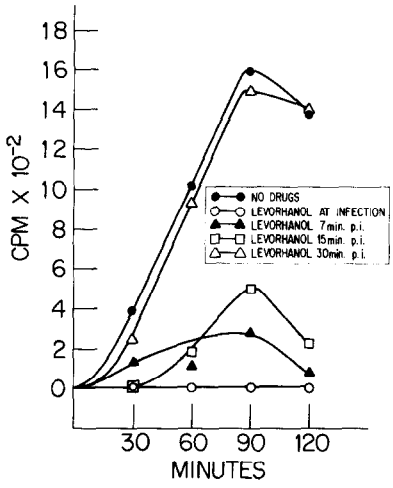

Fig. 2 - Effect of time of levorphanol addition on the inhibition of MS2 RNA synthesis.

E. coli Q13 cultures infected with phage MS2 were treated with rifampicin (100µg/ml) 15 minutes after infection. The rifampicin was dissolved in 50% dimethylsulfoxide (1mg/ml). An equal volume of the solvent was without effect on control cultures. Levorphanol (1.6mM) added at the times indicated. ^{14}C-uracil (0.1µc/5µg/ml) added simultaneously with rifampicin.

fection. Figure 2 shows that addition of levorphanol simultaneously with phage results in complete inhibition of phage RNA synthesis. This inhibition decreases with increasing intervals between phage and drug addition until at 30 minutes post-infection no decrease of phage RNA synthesis by levorphanol is observed. Similar results were obtained for the effect of levorphanol on phage protein synthesis (not shown).

The decrease in effectiveness of levorphanol with time after infection could be explained by a phage-induced alteration in the bacterial membrane gradually reducing the ability of levorphanol to enter the cell. This possibility was explored by the experiment depicted in Table 2. RNA synthesis in infected and uninfected cells was compared when levorphanol was added at time of infection or 35 minutes later. Rifampicin was not used in this experiment. The effective inhibition of host RNA synthesis when levorphanol was added 35 minutes post-infection constitutes good evidence against a decreased cell permeability towards levorphanol. When levorphanol was added at the time of phage infection incorporation of uracil into RNA was identical in infected and uninfected cultures, confirming our previous finding that phage

TABLE 2

EFFECT OF TIME OF ADDITION OF LEVORPHANOL ON HOST AND PHAGE RNA SYNTHESIS

Cells	Lev	Time of Addition**	^{14}C-uracil* 60 min	90 min	120 min
	mM	min	cpm	cpm	cpm
Uninfected	None	--	9300	37,800	69,100
	1.6	0	3150	8090	14,830
	1.6	35	4250	11,950	17,350
Infected	None	--	9100	39,300	73,300
	1.6	0	2350	8280	15,150
			2300	6950	7300
	1.6	35	4650	15,230	22,450

* ^{14}C-uracil (0.1μc/5μg/ml) added 35 min after infection; incorporation into the acid precipitable fraction measured as described in Methods.

** Figured from time of infection.

RNA synthesis is completely blocked. When levorphanol was added 35 minutes after infection there was a difference in the rate of RNA synthesis from that in infected or uninfected cells treated with levorphanol at zero time. This difference increased with incubation time up to 120 minutes and was of a magnitude expected for phage RNA synthesis from experiments with rifampicin.

DISCUSSION - The results reported here demonstrate that levorphanol is a potent inhibitor of the production of infectious RNA phages. The results are similar for f2, MS2 and Qβ. The inhibition is completely reversible and is most effective when levorphanol is added close to or at the time of infection. The rapid decrease of inhibition of infectious particle formation, as well as of macromolecular synthesis, with time elapsed between infection and drug treatment leads one to suspect that levorphanol exerts its inhibitory effect on an early process in the reproductive cycle of the phage. The site of action of the drug is as yet obscure, but the data allow us to eliminate a number of possibilities. Inhibition cannot be on the synthesis of RNA or protein generally since the major portion of macromolecular synthesis occurs be-

tween 30 minutes and 90 minutes after infection, yet addition of levorphanol 30 minutes post-infection is totally without effect. A similar argument can be applied to an effect of levorphanol on metabolic energy. Greene and Magasanik (9) have reported the disappearance of cellular ATP when cells are treated with high levels of levorphanol and have suggested that this may be the primary site of levorphanol action. It is difficult to see how this could apply to the present results in view of the striking difference between drug action at time of infection and 30 minutes thereafter.

The profound inhibition of phage reproduction and its total reversibility should make levorphanol a very useful drug for further explorations of RNA phages and for the study of host macromolecular synthesis in infected cells. The latter is possible since 90 per cent inhibition of phage production is seen under conditions of minimal or no effect on the host.

The phage-inhibitory action of levorphanol seems to be relatively unique. Both rifampicin and actinomycin D exert stronger inhibition on the host than on the phage while levorphanol exerts a preferential inhibition on the phage. Moreover, the effects of rifampicin and actinomycin are difficult to reverse and do not show the complete dependence on time of addition exhibited by levorphanol.

The question of how levorphanol inhibits reproduction of RNA phage is of considerable interest and should be, it is hoped, more readily accessible to an experimental approach than its effect on ribosomal RNA synthesis in bacteria, the mechaism of which has so far eluded us.

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