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On the Mode of Action of Levallorphan on *Escherichia coli*: Effects on Cellular Magnesium

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SUMMARY

Inhibition of the growth of *Escherichia coli* by levallorphan increased in effectiveness as the medium concentration of Mg^{++} was decreased. Two molecules of levallorphan were shown to compete with 1 atom of Mg^{++}, presumably on the cell surface. Active transport of Mg^{++} was altered in the presence of the drug, and this effect could be prevented by high concentrations of Ca^{++}. Reduction of cellular Mg^{++} content in the presence of levallorphan is suggested to be one possible cause of growth inhibition.

INTRODUCTION

Morphinan derivatives inhibit the growth of bacteria (1, 2) as well as animal cells (3). They also inhibit replication of RNA phage (4, 5) and virus (6). Among the metabolic processes investigated, ribosomal RNA synthesis was preferentially depressed by the morphinan derivatives (7, 8). This inhibition of ribosomal RNA synthesis apparently reflected a regulatory process triggered by the drug rather than a direct effect on transcription (9, 10). We report here a study of the effect of Mg^{++} on the bacteriostatic effect of levallorphan [D(−)-*N*-allyl-3-hydroxymorphinan] on *Escherichia coli*. It is shown that levallorphan impairs the Mg^{++} transport system. The subsequent depletion of cellular Mg^{++} could be a factor responsible for the limitation of growth.

MATERIALS AND METHODS

E. coli K-12S was grown in a medium previously described (8), containing 2.0 g of $(NH_4)_2SO_4$, 0.0005 g of $FeSO_4 \cdot 7H_2O$, 0.075 g of KCl, 7.5 g of triethanolamine, and 0.138 g of $NaH_2PO_4 \cdot H_2O$ per liter. The medium pH was adjusted to 8.1, followed by addition of 0.246 g of $MgSO_4 \cdot 7H_2O$. Sodium succinate (0.5%) and Difco Casamino acids (0.2%) were added as sources of carbon. Growth was followed by measuring the absorbance at 546 nm in an Eppendorf photometer. Viability of the bacteria was routinely determined by plating suitable dilutions on nutrient agar. Under the conditions used in the present investigation, no loss of viability was observed. The total magnesium content of the organisms was measured by atomic absorption photometry, using a Techtron AA4 instrument, or by radioactivity determinations in the experiments in which ^{28}Mg was used.

Bacteria were filtered through Millipore HA membranes (0.45 μ). The filters were washed with 2 ml of cold buffer (i.e., growth medium with Mg^{++} and carbon sources). For absorption photometry, the bacteria were resuspended in 2 ml of distilled water.

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The precision of the Mg^{++} determination with the Techtron AA4 instrument was ±5%. For determination of ^{28}Mg, the Millipore membranes were dried and counted in toluene scintillation fluid in a Packard Tri-Carb liquid scintillation spectrometer.

Influx of magnesium ions was determined at 23° with logarithmic phase bacteria grown in the presence of 0.15 mM Mg^{++}, after washing and resuspension in an identical medium containing 0.15 mM ^{28}Mg (2.4 mCi/mmole). Samples (1 ml) were removed periodically, filtered through 0.45-μ Millipore HA filters, washed with buffer in order to remove adsorbed Mg^{++} (11), dried, and counted. The loss of intracellular Mg^{++} was determined at 37° in the absence of external Mg^{++} ions. The bacteria, grown in the presence of 0.15 mM $^{28}Mg^{++}$ (2 mCi/mmole) for two generations, were harvested by filtration and resuspended in an Mg^{++}-free growth medium. The binding of $^{45}Ca^{++}$ by exponentially growing organisms was determined in the presence of 0.1 mM Mg^{++}. Aliquots of 1 ml were filtered, washed with 1 ml of cold distilled water (containing 0.6 μM Ca^{++}), and counted. The final concentration of Ca^{++} was 1.1 μM (0.23 mCi/mmole), providing a large excess of the ion in relation to the maximum binding capacity of the cell. $^{45}Ca^{++}$ was counted in the same manner as $^{28}Mg^{++}$.

$^{45}Ca^{++}$ and $^{28}Mg^{++}$ were products of the Commissariat a l'Energie Atomique, Saclay, France. Levallorphan tartrate was a generous gift from Hoffmann–La Roche, Basel, Switzerland.

RESULTS

Mg^{++} and growth-inhibitory effect of levallorphan. Levallorphan (0.5 mM) completely inhibited the growth of *E. coli* K-12S in the presence of 0.01 mM Mg^{++}, but the growth rate was only slightly reduced when the Mg^{++} concentration was raised to 10 mM (Fig. 1). Moreover, raising the Mg^{++} concentration subsequent to levallorphan inhibition allowed the culture to resume growth, as long as no loss of viability had taken place (Fig. 2). Thus, in the presence of levallorphan, the growth rate behaved as if limited by the external Mg^{++} ion concentration. Lusk *et al.* (12) found that for *E. coli* K-12 0.001–0.003 mM Mg^{++} was required for half-maximal growth. In the presence of levallorphan, however, the Mg^{++} concentration required was higher and, as shown in Fig. 3, rose with increasing levallorphan concentrations. Thus, levallorphan

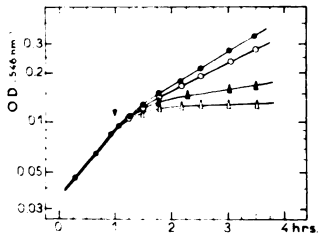

FIG. 1. *Reduction of growth rate of E. coli K-12 by 0.5 mM levallorphan as a function of Mg^{++} concentration*

●——●, 10 mM; ○——○, 1 mM; ▲——▲, 0.1 mM; △——△, 0.01 mM Mg^{++}.

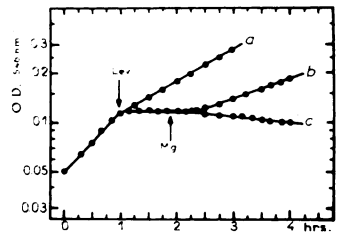

FIG. 2. *Reversal of levallorphan inhibition by addition of Mg^{++}*

Levallorphan, 2.1 mM, was added to a culture containing 10 mM Mg^{++} (*a*) and to two cultures with 1 mM Mg^{++} (*b* and *c*). Culture *b* was later supplemented with Mg^{++} to a final concentration of 10 mM.

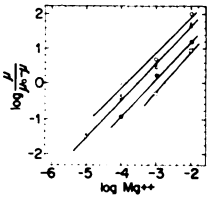

FIG. 3. *Determination of K_m of magnesium for growth process in the presence of increasing concentrations of levallorphan*

μ_0 is the growth rate in control cultures (generations per hour); μ is the growth rate in the presence of the following concentrations of levallorphan: ○——○, 0.4 mM; △——△, 0.5 mM; ●——●, 0.9 mM; □——□, 1.3 mM.

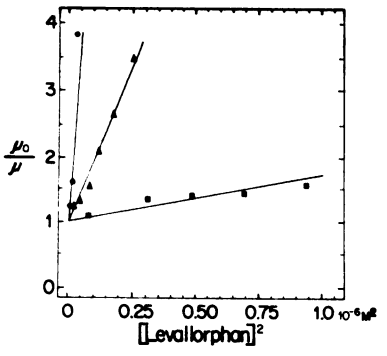

FIG. 4. *Lineweaver-Burk plot for inhibition of E. coli growth rate by levallorphan in the presence of various Mg^{++} concentrations*

μ_0 and μ are defined in the legend to Fig. 3. ●——●, 0.1 mM; ▲——▲, 1 mM; ■——■, 10 mM Mg^{++}.

seemed to limit the availability of Mg^{++} to the bacteria. The possibility that Mg^{++} was removed by chelation to levallorphan was considered, but studies of ultraviolet spectra, NMR, and potentiometric changes showed no evidence of chelation.[2]

The possibility was tested that levallorphan might act as a monovalent cation competing with Mg^{++} for cellular binding sites. If Mg^{++} were considered as a substrate for a putative enzyme system that limits the growth rate, the Michaelis-Menten equation could be formally utilized. This equation was modified, based on the assumption that 2 molecules of levallorphan compete with 1 atom of Mg^{++}. Figure 4 shows a Lineweaver-Burk plot of μ_0/μ (where μ_0 is the growth rate in the absence of levallorphan, μ, in the presence of drug as a function of the square of levallorphan concentration for different Mg^{++} concentrations). The graph shows competition between levallorphan and Mg^{++}. The inhibition constant of levallorphan ($K_i = [X - Lev_2]/[X]\,[Lev]^2$, where X is the divalent binding site) for the "system" was found to be 2×10^8. The apparent K_m for Mg^{++} for growth was calculated to be 4×10^{-5} M.

It was of interest to examine whether other cations could reverse the bacteriostatic action of levallorphan. Ca^{++} was effective, but less so than Mg^{++}, as depicted in Fig. 5. Spermidine (1.5 mM) had a slight effect, but higher concentrations could not be tested since they were inhibitory. Earlier work (9) had shown a more significant reversal by spermidine. This discrepancy may be the result of differences in the strains of *E. coli* employed.

This led us to study the effect of levallorphan on the Mg^{++} transport system. In the presence of levallorphan, the rate of labeling of cells by ^{28}Mg was reduced to a greater extent than the growth rate (Fig. 6); thus, a decrease in the cellular magnesium content was anticipated, unless the reduction in influx was compensated for by a decrease in efflux.

Ca^{++} (10 mM) restored ^{28}Mg exchange and the growth rate nearly to normal. In the presence of 1 mM KCN, no ^{28}Mg exchange

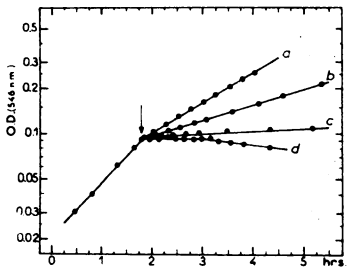

FIG. 5. *Protective effect of Mg^{++}, Ca^{++}, and spermidine on reduction of growth rate by levallorphan*

Levallorphan (2.2 mM) was added as indicated by the arrow, in the presence of 10 mM Mg^{++} (*a*), 10 mM Ca^{++} (*b*), 1.5 mM spermidine (*c*), or no addition (*d*).

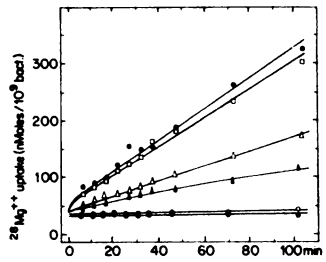

FIG. 6. *Cellular uptake of ^{28}Mg*

Levallorphan, KCN, and/or Ca^{++} were added 15 min before the addition of ^{28}Mg. ●——●, control; △——△, 0.6 mM levallorphan; ▲——▲, 1.2 mM levallorphan; ○——○, 1 mM KCN; ◐——◐, 1 mM KCN + 1.2 mM levallorphan; □——□, 10 mM $CaCl_2$ + 1.2 mM levallorphan.

[2] M. A. Devynck, P. L. Boquet, P. Fromageot, and E. J. Simon, unpublished observations.

occurred, in agreement with the report by Silver (13). This inhibition of exchange by KCN was not reversed by the simultaneous addition of levallorphan.

The reduced rate of ^{28}Mg influx in the presence of levallorphan could be accompanied by a decrease either in Mg^{++} efflux or in the intracellular magnesium pool. To investigate these alternatives, ^{28}Mg efflux was measured under conditions such that the influx was negligible, i.e., with bacteria previously labeled with ^{28}Mg and then resuspended in Mg^{++}-free medium. Figure 7 shows that control organisms lost about 23% of their magnesium, which was then reabsorbed as reported by Webb (14). In the presence of levallorphan, this loss of cellular magnesium reached 50% in 45 min. The initial rate of Mg^{++} efflux was the same with and without levallorphan, but was notably reduced in the presence of cyanide. Thus, this efflux was energy-dependent and not a diffusion process as postulated by Webb (14). Little, if any, of the magnesium released was reabsorbed in the presence of levallorphan, although, as shown in Fig. 6, the Mg^{++} pump was still operating but at a slower rate. As a control, toluene treatment was shown to promote the loss of about 85% of the initial Mg^{++} content.

The addition of Ca^{++} with levallorphan did not prevent the initial loss of ^{28}Mg, but allowed the cell pump to function near normal capacity and thereby to regain Mg^{++} lost while growth continued.

Alteration of cellular magnesium content by levallorphan. The decrease of the cellular Mg^{++} content in the presence of levallorphan is shown in Fig. 8. Under normal conditions the cell content of magnesium, as well as the amount of calcium bound, increased exponentially, as did the cell population. After addition of levallorphan, however, the binding of calcium remained proportional to the absorbance of the culture but the magnesium content decreased immediately. It was therefore of interest to compare the cellular content of magnesium in organisms whose growth rate was either halted by magnesium starvation or reduced by levallorphan (50% of the initial rate). In both cases no loss of viability was observed,

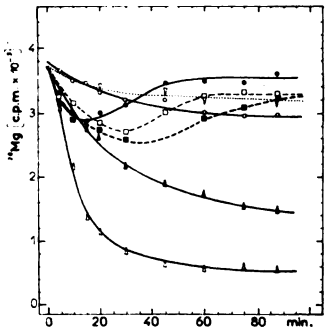

FIG. 7. *Efflux of ^{28}Mg from E. coli cells*
Bacteria grown in the presence of ^{28}Mg were filtered and resuspended in an Mg^{++}-free medium. Additions of levallorphan, $CaCl_2$, toluene, and KCN were made at zero time. ●——●, control; ▲——▲, 1.2 mM levallorphan; ○——○, 1 mM KCN; ▽····▽, 1.2 mM levallorphan + 1 mM KCN; ■- - -■, 10 mM $CaCl_2$ + 1.2 mM levallorphan; □- - -□, 10 mM $CaCl_2$; △——△, 1% toluene.

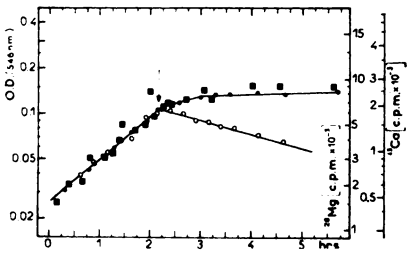

FIG. 8. *^{28}Mg and ^{45}Ca contents of E. coli cells before and after levallorphan treatment*
Levallorphan (1.1 mM) was added as indicated by the arrow to cultures containing ^{28}Mg (50 μM, 2 mCi/mmole) (○——○) or ^{45}Ca (1.1 μM, 0.23 mCi/mmole) (■——■) in the presence of 0.1 mM Mg^{++}. Absorbance of the culture was followed at 546 nm (●——●).

and the Mg^{++} content was equal to 13.6 μatoms/10^{12} cells, as compared to 19.5 μatoms for control cells. Thus, levallorphan shifted the balance between external Mg^{++} and cellular magnesium content.

DISCUSSION

The data presented above establish that the level of *E. coli* growth inhibition by levallorphan is a function of the external Mg^{++} concentration: the lower the Mg^{++} concentration, the more pronounced will be

the inhibition of growth by a given drug concentration.

Mg^{++} is accumulated in *E. coli* by an active transport system, as reported by Silver (13) and Lusk and Kennedy (15), in which the influx and efflux processes are coupled. Levallorphan apparently alters the active transport of Mg^{++} and unbalances influx and efflux. The latter process continues in the presence of levallorphan, while the influx process is impaired and, in contrast to normal organisms, does not operate at very low external Mg^{++} concentrations. It should be pointed out that energy-producing systems are required for stimulation of Mg^{++} efflux by levallorphan.

The concentrations of levallorphan used in the present work do not cause a depletion of the ATP pool,[3] but much higher concentrations can induce other changes leading to a decrease of the ATP pool, as reported by Greene and Magasanik (2).

To understand the mode of action of levallorphan, it is important to note that Ca^{++} partially reverses the effects of levallorphan on Mg^{++} transport, despite the fact that Ca^{++} does not compete with Mg^{++} for cellular Mg^{++} uptake. In addition, Ca^{++} is not concentrated by an active transport system in *E. coli* (16). Calcium and magnesium are bound to the cell wall, and particularly to lipopolysaccharides (17), and play a role in the maintenance of the proper structure of the cell envelope. Thus, it is likely that the antagonism between Ca^{++} and levallorphan takes place at the surface of the cell.

The displacement of external Ca^{++} or Mg^{++} by levallorphan, enhanced by the lipophilic character of the drug, might induce subtle structural changes in the cell membrane, altering some active transport systems without promoting energy-independent permeation. Alterations of other transport systems by morphinan derivatives have been reported. Gale (18) showed that heroin and levallorphan increased the rate of transport into *Staphylococcus aureus* of some amino acids, such as aspartate and alanine, and inhibited that of lysine and proline, with concomitant inhibition of protein synthesis. Simon *et al.* (19), working with *E. coli* and levorphanol, observed an alteration of the permeability of cells to putrescine, amino acids, spermidine, and K^+. Thus, levallorphan may disturb a number of permeation processes, each of which might limit growth, and since it has been shown that bacterial RNA and Mg^{++} contents generally vary together (11), a decrease of the growth rate would generally be accompanied by a reduction in RNA and Mg^{++} contents. However, the regulatory process coupling Mg^{++} and RNA contents cannot account for an alteration of the Mg^{++} transport system.

In the presence of levallorphan, *E. coli* K-12 seems to mimic the *E. coli* mutant isolated by Lusk *et al.* (12), which requires either high Mg^{++} concentrations or simultaneous addition of Ca^{++} for growth.

We are therefore led to suggest that the inhibition of Mg^{++} transport may contribute to the reduction of the growth rate of *E. coli* by levallorphan.

Most of the effects observed in the presence of levallorphan resemble those observed by Silver *et al.* (20) in the presence of various polyamines such as Ihrrediamine. All these effects are thought to be secondary to structural changes in the cell membrane, as suggested by studies on the interaction of levallorphan with *E. coli* spheroplasts (21) and by the altered phospholipid metabolism seen in levorphanol-treated bacteria (22, 23).

[3] P. L. Boquet, M. A. Devynck, and P. Fromageot, manuscript in preparation.

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