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Effect of Levorphanol on Putrescine Transport in *Escherichia coli*

ERIC J. SIMON,[1] LIVIU SCHAPIRA, AND NANCY WURSTER

Department of Medicine, New York University Medical Center, New York, New York 10016

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SUMMARY

The disappearance of cellular putrescine observed when *Escherichia coli* cultures were treated with levorphanol was shown to be largely the result of an acceleration of putrescine efflux produced by the drug. The efflux of putrescine from *E. coli* exhibited a stringent requirement for metabolic energy. It was found to be greatly reduced by carbon source starvation, by treatment with metabolic inhibitors, or by low temperature. The efflux of putrescine stimulated by levorphanol was also virtually abolished by such treatments. Levorphanol also inhibited the uptake of putrescine by *E. coli* cells. Evidence is presented which indicates that the inhibition of uptake and the stimulation of efflux represent separate effects of levorphanol. Competition between levorphanol and putrescine for a carrier or binding site was ruled out by kinetic experiments, which showed that inhibition of putrescine uptake by levorphanol was not competitive. The effect of levorphanol on the putrescine pool was readily reversible. Replenishment began without measurable delay upon removal of the drug. Levorphanol also produced reversible alterations in the permeability of *E. coli* to most, if not all, amino acids, spermidine, and K^+. Possible mechanisms of the effects of levorphanol on cell membranes are discussed.

INTRODUCTION

We have previously reported that levorphanol, a synthetic narcotic analgesic closely related to morphine, inhibits bacterial growth (1). A biochemical effect of the drug observed in *Escherichia coli* was a prompt and marked depression of RNA synthesis (2). This inhibition was found to involve predominantly the synthesis of ribosomal RNA (3). In an effort to elucidate the mechanism of action of levorphanol on ribosomal RNA synthesis, the relation between the drug and the polyamines, putrescine and spermidine, was explored (4), since reports from the laboratory of S. S. Cohen (5, 6) had provided evidence that these compounds may be involved in the regulation of ribosomal RNA synthesis in *E. coli*. During that investigation it was found that treatment of *E. coli* cultures with levorphanol led to the disappearance of most of the cellular putrescine pool (4). We suggested that this might represent an effect on the cell membrane resulting in an accelerated efflux of putrescine from cells into the medium.

Evidence for membrane effects of levorphanol in *E. coli* was also obtained by Greene and Magasanik (7), who reported a rapid disappearance of cellular ATP and GTP and an inhibition of the accumulation of thiomethyl-β-D-galactoside. Effects of levorphanol and related drugs on amino acid transport and lipid metabolism in *Staphylococcus aureus* were recently reported by Gale (8, 9).

This paper describes a detailed study of

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the effect of levorphanol on putrescine transport. Evidence is presented that the disappearance of cellular putrescine is, indeed, largely the result of a drug-induced efflux of putrescine into the medium.

The drug was also found to inhibit effectively the uptake of ^{14}C-putrescine by *E. coli* cells. Our results indicate that the stimulation of efflux and the inhibition of uptake represent separate effects on putrescine transport. Levorphanol also alters the permeability of *E. coli* cells to other small ions and molecules, including most, if not all, amino acids, spermidine, and K^+. All these effects were found to be readily reversible upon removal of the drug.

MATERIALS AND METHODS

E. coli strains K-13 and B leu$^-$ were grown at 37° with vigorous shaking in the minimal medium of Nakada (10) with 0.5% sodium lactate as the carbon source unless indicated otherwise. Leucine (100 μg/ml) was added to the cultures of the auxotroph. Log phase cultures containing 2–3 × 10^8 bacteria per milliliter were collected by centrifugation, washed twice, and resuspended in a modification of medium B described by Tabor and Tabor (11). This medium contains 0.012 M potassium phosphate, 0.1 mM $MgCl_2$, and 0.1% sodium lactate at pH 7.3. Additions or modifications are given in the legends to tables and figures. The culture was shaken at 37° for 30 min to remove traces of ammonium chloride and to deplete the amino acid pool in order to minimize endogenous polyamine synthesis. The concentration and radioactivity of the ^{14}C-putrescine or ^{14}C-spermidine added is indicated in the legends to tables and figures. For uptake studies, 2-ml samples were removed at various time intervals, rapidly filtered through Millipore filters (pore size 0.45 μ, diameter, 24 mm), and washed three times with cold water.[2] Corrections for physical adsorption were made by subtracting radioactivity adhering to bacteria treated in the same manner at 4°. For studies of efflux, cultures in which putrescine uptake had been allowed to proceed for 30 min were centrifuged and resuspended in the same medium free of labeled putrescine (and of nonradioactive putrescine unless otherwise indicated). Cultures were incubated again, and samples were removed at intervals and treated as described above.

Studies of the labeling of the acid-soluble pool with various amino acids in the presence and absence of levorphanol were carried out in the presence of chloramphenicol (100 μg/ml) to minimize protein turnover. Pool radioactivity was calculated by subtracting acid-precipitable counts, obtained as described previously (3), from total cell counts, obtained as described above for the polyamines. Millipore filters were dried under an infrared lamp and placed in counting vials containing 10 ml of a toluene-based scintillation solution purchased as the concentrate Permafluor from the Packard Instrument Corporation. Samples were counted in a Packard Tri-Carb liquid scintillation counter.

Cellular K^+ content was measured by collecting 4-ml aliquots of culture on HA Millipore filters, washing with 0.3 M sucrose, digesting with concentrated nitric acid, and analyzing by flame photometry, using LiCl as internal standard as described by Epstein and Schultz (13). ^{42}K influx was measured as described by the same authors, and radioactivity was measured in a Packard liquid scintillation counter (14).

ATP determinations were performed for us by Dr. F. Welsch, Department of Biochemistry, Dartmouth Medical School, using the luciferin-luciferase assay (15).

The chemical determination of putrescine in cells and growth medium was performed by the method of Raina and Cohen (5).

The radioactive putrescine, spermidine, amino acids, and uracil were purchased from New England Nuclear Corporation. Unlabeled putrescine was obtained from Mann Research Laboratories. The levorphanol was a generous gift of Hoffmann-La Roche, Inc. ^{42}K was purchased from Isoserve Corporation, Cambridge, Mass.

[2] After the appearance of the paper by C. W. Tabor (12) reporting that acetylation occurs mainly in the cold, we repeated a number of experiments using water at room temperature for the washing procedure and making certain that samples were never chilled. No differences were found in our results.

RESULTS

The putrescine transport system. The transport of putrescine and other polyamines by *E. coli* has been studied by Tabor and Tabor (11). A number of features of the system were reinvestigated, and the previous findings were confirmed and extended.

Both the rate and extent of putrescine uptake were found to be maximal in medium B of Tabor and Tabor and were decreased by changes in carbon source, pH, or ionic strength. The uptake was particularly sensitive to ionic strength. Thus, the addition of 0.12 M NaCl decreased the rate of uptake by about 80%. Increasing the potassium phosphate concentration 10-fold to 0.12 M virtually abolished putrescine uptake. The substitution of lactate for glucose decreased uptake by only 10%. This latter change was adopted in our experiments to permit better control of pH because of the previously observed dependence of levorphanol activity on pH.

The uptake of putrescine by *E. coli* is dependent on temperature, with little uptake occurring at 0°. Nevertheless, deprivation of a carbon source or treatment with metabolic inhibitors, such as 2,4-dinitrophenol or sodium arsenite, caused only a slight decrease in putrescine uptake. Tabor and Tabor (11) reported a 40% decrease of putrescine uptake upon removal of glucose. We found a variable decrease ranging from 10 to 30%.

The pH dependence of putrescine uptake is shown in Fig. 1. The uptake exhibits a broad optimum between pH 6.6 and 7.3 and drops at both lower and higher pH values. This finding appears to apply to both rate and extent of uptake.

We have found that the efflux of putrescine from *E. coli* cells exhibits a strict requirement for metabolic energy. When a control culture of *E. coli* K-13 is placed in a medium devoid of a carbon source and starved for 30 min, it exhibits a considerable reduction of the putrescine efflux normally observed when this strain is incubated in a complete growth medium (Fig. 2). The exchange with ^{12}C-putrescine in the medium is also greatly diminished.

Figure 3 shows that similar effects on putrescine exchange were produced by the addition of the metabolic inhibitors 2,4-dinitrophenol (2.5 mM) and, though it was less effective, sodium arsenite (1 mM). Most of the experiments of Tabor and Tabor (11) were carried out in the presence of this con-

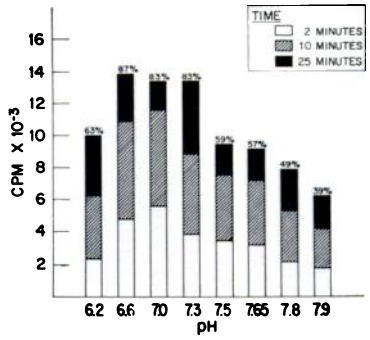

FIG. 1. *Variation of putrescine uptake by E. coli with pH*

Cultures of *E. coli* B leu⁻ were incubated in the modified medium B described under MATERIALS AND METHODS, adjusted to the pH values shown. ^{14}C-Putrescine (specific activity, 5 mCi/mmole) was added at a final concentration of 1 μM. During the period of observation pH values remained constant. Aliquots were removed at the indicated intervals and treated as described in the text.

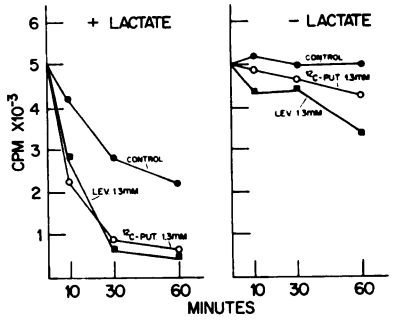

FIG. 2. *Putrescine efflux in the presence and absence of a carbon source*

This experiment was performed with a culture of *E. coli* K-13 in the triethanolamine-buffered medium described previously (2), containing ammonium chloride as a source of nitrogen and supplemented with leucine (100 μg/ml) and, where indicated, with sodium lactate (0.1%). The concentration and specific activity of ^{14}C-putrescine used to label cells were the same as described in Fig. 1. After 30 min the labeled cells were washed and treated as described under MATERIALS AND METHODS.

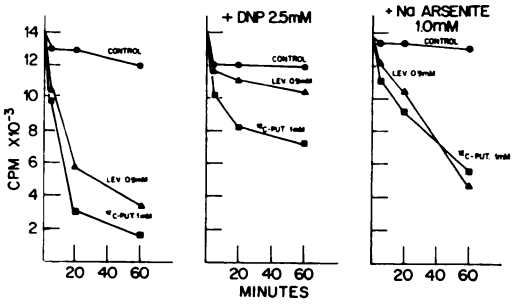

FIG. 3. *Effect of metabolic inhibitors on ^{14}C-putrescine efflux induced by levorphanol and by exchange with medium putrescine*

A culture of *E. coli* B leu⁻ was incubated in medium B and labeled with ^{14}C-putrescine. Cells were washed with the same medium and resuspended in this medium with the additions shown in the figure. DNP, 2,4-dinitrophenol.

centration of sodium arsenite to prevent acetylation of polyamines. The rather slow rate of putrescine exchange observed by these investigators may be explained by our finding.

Effect of levorphanol on putrescine efflux. Analysis of *E. coli* cells for total and free putrescine indicated that upon treatment with levorphanol, 90% of the putrescine disappeared within 60 min (4). At that time it was suggested, but not proven, that this represented putrescine efflux into the medium. A similar experiment, in which putrescine was determined in both cells and medium, is shown in Table 1. In this experiment the cellular putrescine content in levorphanol-treated cells fell to 29% of its initial value in 60 min, while the amount of putrescine in the control cells increased more than 2-fold. When the cellular putrescine levels were corrected for differences in cell density (determined from absorbance at 550 mμ) of the cultures, the control pool was found to be relatively constant within experimental error, while the drug-treated cells lost 80% of their putrescine pool. Considerable amounts of putrescine were found in the medium of both control and treated cultures. The ratio of medium putrescine to cell putrescine was also relatively constant in the control culture, while it increased from 1.2 to 7.3 in the levorphanol-treated cultures. The major portion of the putrescine lost from the cells was thus recovered in the medium.

Experiments with ^{14}C-labeled putrescine provided confirmation that the cell putrescine decrease is largely the result of efflux. The lefthand portions of Figs. 2 and 3 show that cells previously labeled with ^{14}C-putres-

TABLE 1

Effect of levorphanol on total putrescine content of E. coli cells and medium

E. coli K-13 was incubated in minimal medium with 0.5% glucose as carbon source. Analysis was carried out on 200-ml aliquots of the cultures by the method of Raina and Cohen (5). The values represent nanomoles of total putrescine (free and acetylated) per milliliter of culture. The concentration of levorphanol was 1.35 mM.

Sample	Time	Cell putrescine	Cell putrescine / Absorbance of culture	Medium putrescine	Cell plus medium putrescine / Absorbance of culture	Medium putrescine / Cell putrescine
	min					
	0	6.2	6.2	7.2	13.1	1.2
Control	10	6.2	5.8	7.2	12.4	1.2
Levorphanol	10	4.5	4.2	—[a]	—[a]	—[a]
Control	25	6.7	5.1	9.4	12.1	1.4
Levorphanol	25	2.5	2.0	9.8	9.8	3.9
Control	60	14.3	6.3	16.4	13.4	1.2
Levorphanol	60	1.8	1.2	12.2	9.5	6.8
Control	120	16.5	4.0	37.0	12.5	2.2
Levorphanol	120	2.1	1.2	15.3	9.7	7.3

[a] Sample was lost.

cine manifest a rapid loss of radioactivity upon treatment with levorphanol. In the experiment depicted in Fig. 2, levorphanol effects a marked increase in the already considerable efflux of putrescine shown by *E. coli* K-13 in a complete growth medium. The effect of levorphanol was more striking in the experiment shown in Fig. 3, in which *E. coli* B leu⁻ incubated in a nitrogen-free medium showed little spontaneous leakage of putrescine.

Figures 2 and 3 also show that the levorphanol-induced efflux is virtually abolished when cells are deprived of a carbon source or treated with 2,4-dinitrophenol. Treatment with sodium arsenite slows down the efflux. The stimulation of efflux by levorphanol was also effectively prevented by keeping the cultures at 4°.

Analysis of medium radioactivity by paper electrophoresis revealed the presence of free and acetylated putrescine. No other radioactive molecules were detected.

Effect of pH and magnesium concentration on levorphanol-induced efflux. Figure 4 reveals that pH has a marked influence on the effectiveness of levorphanol in accelerating the efflux of putrescine, quite analogous to our previous finding that pH influences the ability of levorphanol to inhibit bacterial growth and RNA synthesis (1, 2). While levorphanol at 1.3 mM caused a rapid efflux of radioactivity at pH 7.8, it was virtually ineffective at pH 5.8 even at a concentration of 3.6 mM.

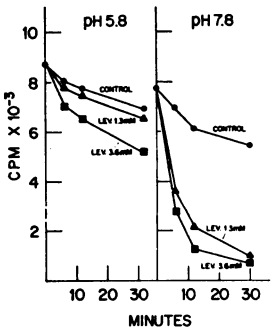

FIG. 4. *Effect of pH on stimulation of putrescine efflux by levorphanol*

E. coli B leu⁻ cells labeled with ^{14}C-putrescine were incubated in medium B adjusted to the pH values shown. The pH remained constant throughout the experimental period. The specific activity of ^{14}C-putrescine was the same as in Fig. 1.

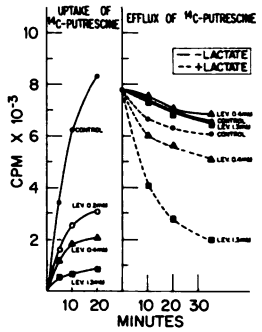

FIG. 5. *Effect of levorphanol on ^{14}C-putrescine uptake and efflux in the absence of a carbon source*

Experiments were carried out as usual with *E. coli* B leu⁻ in medium B, but the uptake of putrescine was studied in the absence of a carbon source. Efflux was measured in the absence as well as the presence of a carbon source. During efflux, ^{12}C-putrescine was added at the concentration at which ^{14}C-putrescine was used for uptake (1 μM).

Again in agreement with our finding concerning the inhibition of RNA synthesis, the addition of high concentrations of magnesium to the medium reduced or prevented the levorphanol-induced loss of cellular putrescine. A concentration of levorphanol (1.3 mM) which stimulated putrescine efflux markedly at 0.1 mM Mg^{2+} was without effect at 10 mM Mg^{2+}.

Inhibition of putrescine uptake by levorphanol. The accumulation of ^{14}C-putrescine by *E. coli* was found to be markedly reduced by levorphanol. The results depicted in Fig. 5 provide evidence that reduced accumulation is not merely a reflection of the increased rate of putrescine efflux but represents a separate inhibition by levorphanol of putrescine uptake. It is apparent that putrescine uptake was inhibited even in the absence of a carbon source. Under identical conditions (no carbon source, addition of 1 μM putrescine), levorphanol caused no acceleration of efflux even at 1.3 mM. The inhibition of accumulation was therefore clearly not the consequence of increased efflux. Nor can increased efflux be readily explained by prevention of putrescine re-entry, since entry is blocked to the same extent in the presence and absence of a carbon source, while rates of efflux differ greatly. Moreover, putrescine uptake was found to be the most levorphanol-sensitive reaction so far observed by

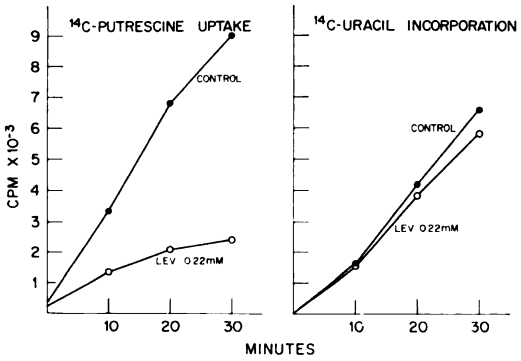

FIG. 6. *Effect of 0.2 mM levorphanol on putrescine uptake and RNA synthesis*

A log phase culture of *E. coli* B leu⁻ was incubated in medium B to which were added 2% NH_4Cl, leucine (100 μg/ml), and 0.1% lactate. ^{14}C-Putrescine was added to a portion of the culture, and aliquots were treated as described under MATERIALS AND METHODS. To another portion of the culture, ^{14}C-uracil (0.02 μCi/μg/ml) was added. Aliquots (2 ml) were collected in an equal volume of 10% trichloracetic acid and washed three times with 5% trichloracetic acid on a Millipore filter. Dried Millipore membranes were counted as described under MATERIALS AND METHODS.

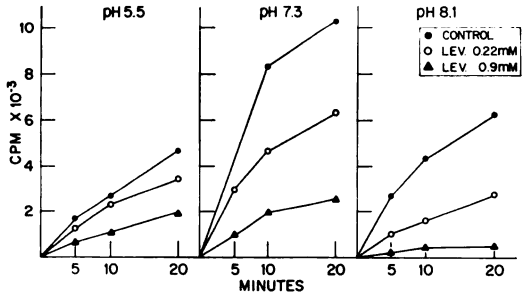

FIG. 7. *Effect of pH on inhibition of putrescine uptake by levorphanol*

E. coli B leu⁻ was incubated in medium B adjusted to the appropriate pH values. ^{14}C-Putrescine was added at zero time, and uptake of radioactivity was measured at indicated intervals as described under MATERIALS AND METHODS.

us. Figure 6 indicates that putrescine uptake was decreased 70% by 0.22 mM levorphanol, a concentration virtually without effect on RNA synthesis under identical conditions. The stimulation of efflux, however, is minimal at 0.4 mM and requires 1 mM or more levorphanol for a clear-cut effect. Finally, inhibition of uptake was less sensitive to changes in pH than the acceleration of efflux. Figure 7 indicates that, while levorphanol becomes a more effective inhibitor as the pH is raised, this increased effectiveness is less striking than that seen in Fig. 4 for stimulation of efflux.

ATP levels in levorphanol-treated E. coli cultures. Table 2 shows the level of ATP in *E. coli* in the presence of several concentrations of levorphanol. These determinations were carried out in a culture suspended in the nitrogen-free medium (see MATERIALS AND METHODS) used for most of the transport studies. A similar determination in a logarithmically growing culture of *E. coli* B in a complete minimal medium gave an ATP level of 10 nmoles/mg dry weight. The results with levorphanol were similar to those shown here. A slight decrease in ATP level was observable at, or slightly above, 1 mM levorphanol. At the levels at which putrescine uptake is strongly inhibited (0.2–0.4 mM), no decrease of ATP content was seen.

Kinetics of putrescine uptake. The possibility that the inhibition of putrescine uptake might be due to competition between putrescine and levorphanol for a binding site

TABLE 2

Effect of levorphanol on ATP level of E. coli

A log phase culture of *E. coli* B leu⁻ was resuspended in the nitrogen-free medium used for the transport studies (see MATERIALS AND METHODS). At 15 and 45 min after levorphanol addition, 1-ml aliquots of the culture were treated with 0.1 ml of cold 5.9 M perchloric acid. Precipitates were removed by Millipore filtration, and filtrates were neutralized with 5.0 M KOH. $KClO_4$ precipitates were removed by centrifugation. All steps were carried out at 0–4°. The supernatant solutions were frozen and kept at −20° until analyzed. The ATP determinations were carried out by means of the luciferin-luciferase assay (15).

Levorphanol	ATP	
	15 min	45 min
mM	*nmoles/mg dry wt*	
0	4.3	4.7
0.22	5.4	6.3
0.45	5.1	5.4
0.90	5.4	4.7
1.8	2.2	1.5

or a carrier was explored by studying the kinetics of putrescine uptake. To obtain linear rates of uptake, which permitted the calculation of initial rates, the experiments were carried out with bacterial cultures diluted 5–10-fold. The K_m of the putrescine uptake system for its substrate was found to be about 1 μM (0.8–1.25 μM in three experiments), which is somewhat higher than the value (0.2 μM) reported by Tabor and Tabor (11).

A representative kinetic experiment, in which putrescine concentrations were varied over a 5-fold range in the presence and absence of levorphanol, is plotted according to the method of Lineweaver and Burk in Fig. 8. It can be seen that the inhibition is not competitive over the range of concentrations studied.

Reversibility of levorphanol effects. The effects of levorphanol on putrescine transport, just like those on bacterial growth and RNA synthesis, are readily reversible upon removal or dilution of the drug. The experiments in Fig. 9 show this reversibility for the levorphanol-depleted putrescine pool and the inhibition of RNA synthesis in the same culture of *E. coli*, and at the same time inquire into the relationship between cellular putrescine concentration and rate of RNA synthesis. The results show that replenishment of the putrescine pool begins at a linear rate (when expressed per cell) immediately upon removal of the drug. The rate of RNA synthesis becomes essentially normal after a lag of no more than 3 min. RNA is thus made at or near the normal rate when the cellular putrescine pool is less than 20% of its normal size.

Transport of other small molecules by E. coli in the presence of levorphanol. The effects of levorphanol on putrescine transport led us to examine its effect on the transport of other small molecules and ions. Experiments on the transport of the amino acid leucine are shown in Fig. 10. In the presence of levorphanol there is some accumulation of ^{14}C-leucine in the acid-soluble pool of chloramphenicol-treated *E. coli* during the first 5 min, followed by a rapid loss of most of the radioactivity. When levorphanol is added after labeling of the leucine pool, over 90% of the acid-soluble radioactivity is lost within 15 min. Figure 11 shows that incorporation of several other amino acids into the acid-soluble pool was also decreased upon levorphanol treatment. A mixture of 16

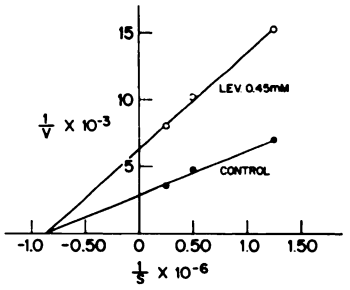

FIG. 8. *Lineweaver-Burk plot of kinetic experiment of putrescine uptake in the presence and absence of levorphanol*

The concentrations of ^{14}C-putrescine used were 1, 2, and 4 μM at the specific activity of 5 mCi/mmole. Initial rates were calculated in terms of uptake (counts per minute) per minute from the linear portions of the uptake curves. Cultures of *E. coli* B leu$^-$ in medium B were diluted 10-fold to 3–5 × 10^7 cells/ml to facilitate the determination of initial rates. The size of the aliquots taken for filtration and counting was increased to 5 ml to provide adequate radioactivity at short intervals.

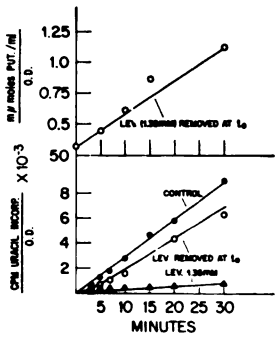

FIG. 9. *Reversibility of levorphanol effect on putrescine pool and RNA synthesis*

E. coli B leu$^-$ cells were grown in complete minimal medium (see MATERIALS AND METHODS). The culture was treated with 1.35 mM levorphanol for 30 min, centrifuged to remove the drug, and placed in drug-free medium. Levorphanol was added again to the portion of the culture used to measure RNA synthesis in the presence of drug. ^{14}C-Uracil incorporation into the acid-precipitable portion was measured in 15-ml portions of the culture as described in Fig. 6. Total cell putrescine was determined in 200-ml aliquots by the method of Raina and Cohen (5).

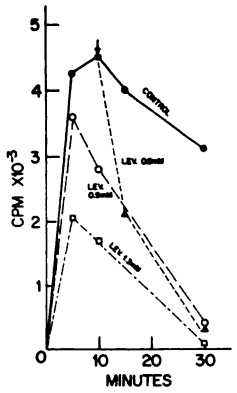

FIG. 10. *Effect of levorphanol on leucine transport*

E. coli B leu⁻ was incubated in medium B and treated with chloramphenicol (100 μg/ml). ^{14}C-Leucine (0.2 μCi/1.3 μg/ml) was added at zero time. Levorphanol was added in the concentrations indicated at zero time and at 10 min (indicated by the arrow). Samples were taken at indicated intervals, and acid-soluble radioactivity was measured as described under MATERIALS AND METHODS.

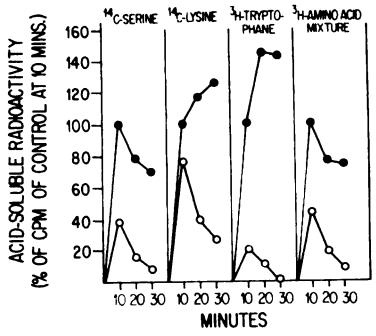

FIG. 11. *Transport of amino acids in the presence and absence of levorphanol*

The experiment was performed as indicated in the legend to Fig. 10. To permit the presentation of different experiments in the same figure, the acid-soluble radioactivity is plotted as a percentage of the control value at 10 min. The concentration of levorphanol was 1.35 mM. Specific activities were: ^{14}C-serine, 0.01 μCi/0.33 μg/ml; ^{14}C-lysine, 0.1 μCi/2 μg/ml; ^{3}H-tryptophan, 0.06 μCi/1.6 μg/ml; amino acid mixture (^{3}H), 0.1 μCi/0.7 μg/ml. ●, control; ○, levorphanol.

labeled amino acids from an algal hydrolysate also showed this decline, indicating that most amino acids are probably affected.

Figure 12 shows the inhibition by levorphanol of the uptake of the polyamine spermidine. While the effect is not as striking as that described for putrescine, the inhibition is real and reproducible. When cells previously labeled with ^{14}C-spermidine are treated with levorphanol, there is no detectable disappearance of radioactivity from the cells. This may be due to the binding of most of the cellular spermidine to macromolecules.

The reduced accumulation of thiomethyl-β-D-galactoside reported by Greene and Magasanik (7) at 10 mM levorphanol (or 5 mM levallorphan) was confirmed by us and found to occur even at 1.3 mM levorphanol.[3]

The cellular K^+ concentration was decreased in the presence of levorphanol, but the relatively small effect (30–50%) could have resulted from leakage from a small number of damaged or dead cells. We therefore measured K^+ influx by the two methods depicted in Figs. 13 and 14. The reaccumulation of K^+ by K^+-depleted cells as well as the influx of ^{42}K into normal *E. coli* was decreased by levorphanol.

As already seen with putrescine transport, all of the above effects of levorphanol were readily reversible upon dilution or removal of the drug.

DISCUSSION

In the course of this investigation a number of interesting features of putrescine transport in *E. coli* were studied. One of these is the nature of the pH dependence of putrescine uptake first reported by Tabor and Tabor (11). The uptake of basic compounds generally increases with rising pH, while that of putrescine declines above pH 7.3. This result is difficult to interpret. The increase in uptake of basic compounds with pH is usually thought to be the consequence of increased concentration of the more permeable unprotonated base molecules at higher pH. In the case of putrescine, we suggest that this may be more than offset by the pH dependence of the state of ionization of a functional group of a carrier or binding component for putrescine.

Another feature of putrescine transport we have observed is the stringent requirement

[3] N. Wurster and E. J. Simon, unpublished observations.

for metabolic energy exhibited by the efflux of putrescine from *E. coli* cells. We found that ^{14}C-putrescine efflux, whether spontaneous, induced by levorphanol, or the result of exchange with medium putrescine, is greatly reduced by carbon source starvation or by treatment with inhibitors of energy metabolism, such as 2,4-dinitrophenol or sodium arsenite. Since the effect is seen in cultures incubated in media lacking a source of nitrogen, it is not likely to be the result of interference with putrescine biosynthesis. In agreement with Tabor and Tabor (11), we observed little effect on putrescine uptake by energy source starvation or metabolic inhibitors. Our results suggest, therefore, that putrescine efflux is an energy-requiring process analogous to that recently reported for magnesium efflux from *E. coli* by Lusk and Kennedy (16).

Our conclusion that the inhibition of uptake and the stimulation of efflux of putrescine represent two separate effects of levorphanol rests on the following findings: (a) inhibition of uptake is quite marked at intervals at which loss of putrescine by efflux is negligible, especially in the absence of a carbon source; (b) uptake is more sensitive to levorphanol than efflux; and (c) the inhibition of putrescine uptake is less sensitive than the acceleration of efflux to changes in pH. The possibility that the apparent stimulation of efflux results entirely from prevention of re-entry of putrescine into the cells is made unlikely by the finding that rates of efflux induced by levorphanol in the presence and absence of a carbon source differ markedly, while entry is inhibited to about the same degree under both conditions.

The question was raised whether the in-

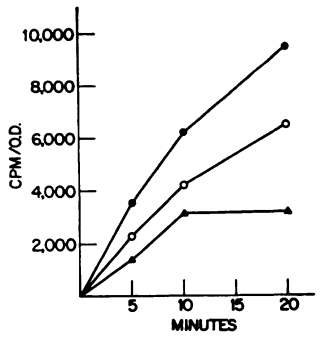

FIG. 12. *Effect of levorphanol on spermidine uptake*

^{14}C-Spermidine (0.04 μCi/0.01 μmole/ml) was added to a culture of *E. coli* B leu$^-$ in medium B. Aliquots were taken at indicated intervals and treated as described under MATERIALS AND METHODS. ●, control; ○, 0.67 mM levorphanol; ▲, 1.35 mM levorphanol.

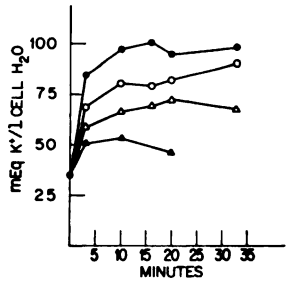

FIG. 13. *Effect of levorphanol on reaccumulation of K$^+$ by K$^+$-depleted E. coli*

A log phase culture of *E. coli* B leu$^-$ in triethanolamine-buffered minimal medium was washed with cold distilled water and resuspended in K$^+$-free medium. After removal of an aliquot to determine the zero time K$^+$ concentration, KCl was added to a final concentration of 20 μEq/ml. The culture was divided and levorphanol added as follows: ●, control; ○, 0.7 mM; △, 1.0 mM; ▲, 1.6 mM. Aliquots of 4 ml were removed at the indicated intervals and analyzed for K$^+$ as described under MATERIALS AND METHODS.

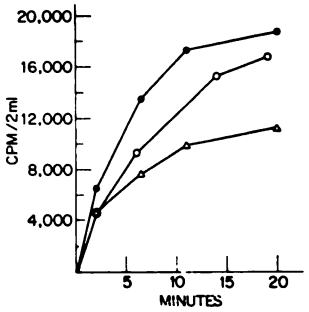

FIG. 14. *Effect of levorphanol on uptake of ^{42}K by E. coli*

To a culture of *E. coli* B leu$^-$ growing logarithmically in triethanolamine-buffered minimal medium was added ^{42}K (0.3 μCi/3.7 μEq/ml). Aliquots of 2 ml were removed at the indicated intervals and analyzed for ^{42}K content as described under MATERIALS AND METHODS. ●, control; ○, 0.45 mM levorphanol; △, 0.81 mM levorphanol.

hibition of putrescine uptake could be explained by competition between two basic compounds, levorphanol and putrescine, for a binding site or a carrier. This possibility was ruled out by experiments on the kinetics of putrescine uptake in the presence and absence of levorphanol, which showed that the inhibition was not competitive.

The findings reported here, together with those of Greene and Magasanik, also in *E. coli* (7), and the results of Gale in *S. aureus* (8, 9), provide evidence that levorphanol alters the properties of bacterial cell membranes. Recent studies on the effect of levorphanol on the electrical activity of the giant axon of the squid (17) and on phagocytosis and the concomitant metabolic changes in rabbit leukocytes[4] show that this effect extends to other biological systems.

The manner in which levorphanol affects the properties of membranes is not understood. Greene and Magasanik have suggested that the primary effect of levorphanol is on the cellular level of ATP and therefore on available metabolic energy. Our findings indicate that a loss of cellular ATP can be observed at or slightly above 1 mM levorphanol. The hypothesis that the primary action of levorphanol is on the supply of metabolic energy is placed in doubt, however, by the observation that not all of the effects of levorphanol are consistent with it. Thus the inhibition of putrescine uptake is quite marked at concentrations of levorphanol which have no effect on the level of ATP. Moreover, the stimulation of putrescine efflux by levorphanol is directly opposite to the effects of treatments presumed to result in energy deprivation, and, indeed, the stimulation of efflux by levorphanol is prevented by carbon source deprivation and treatment with metabolic inhibitors. The increase in phospholipid turnover observed by Gale in *S. aureus* is also difficult to reconcile with a mechanism involving loss of metabolic energy.

Another hypothesis we tend to favor at this time is a direct effect of levorphanol on the cell membrane. Support for this idea derives from the alterations in phospholipid metabolism observed in *S. aureus* (9) and in *E. coli*.[5] The breakdown of ATP might be a secondary effect resulting from the stimulation of a membrane ATPase, frequently observed in mammalian cells after membrane damage but not yet reported in bacteria.

The effects on the membrane produced by levorphanol must be relatively subtle ones, since they are readily reversible and since certain other membrane changes are not brought about by the action of levorphanol. Thus, the rate of uptake of *o*-nitrophenyl-β-D-galactoside by *E. coli* is unaffected (7). The sensitization of *E. coli* RNA synthesis to inhibition by actinomycin D, readily accomplished by treatment with EDTA (18), is not seen after treatment with levorphanol.

It will be of considerable interest to investigate the mechanism by which levorphanol alters the properties of cell membranes and to determine whether or not this effect is related to the inhibition of RNA synthesis by the drug.

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