

## INHIBITION OF AMINO ACID TRANSPORT IN *ESCHERICHIA COLI* CELLS AND ITS CELL MEMBRANES

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### 1. Introduction

In earlier reports Simon et al. [1, 2] found that in *Escherichia coli* cells levorphanol stimulates the efflux and inhibits the uptake of putrescine and the transport of amino acids. Greene and Magasanik [3] have shown that high concentrations of levallorphan cause permeability changes which lead to a loss of ATP and of thiomethylgalactoside (TMG). Gale [4] was able to show that heroin, levorphanol and levallorphan interfere with amino acid transport into the pool of staphylococci, and that [5] this treatment results in a stimulated turnover of the phospholipids.

These observations made it highly probable that these drugs affect some function of the cell membrane. With low concentrations that hardly affect growth we observed that adsorption of MS-2 phages [6] and the formation of mating pairs during R-factor transfer [7] were inhibited. These findings also point to changes in the cell membranes.

In the present paper we show that with low concentrations of levallorphan the uptake of arginine is inhibited, and that the same type of inhibition of arginine uptake occurs in whole cells as well as in membrane preparations.

### 2. Materials and methods

Levallorphan tartrate was generously provided by Hoffmann-La Roche, Basel. The uniformly labelled  $^{14}\text{C}$ -L-amino acids were a gift of Dr. P. Fromageot, C.E.N., Saclay, France. The specific activities were: phenylalanine 126 mCi/mM, alanine 49.6 mCi/mM, aspartic acid 47 mCi/mM, glutamic acid 77 mCi/mM, and arginine 118 mCi/mM.

#### 2.1. Bacterial strains and media

An *E. coli* strain K12 W 3747 (b)  $\text{F}'\text{lac}^+$ ,  $\text{S}^{\text{H}}$ ,  $\text{T}_6^{\text{R}}$ ,  $\text{met}^-$  was used. The cells were grown in a medium described by E.J. Simon and D. van Praag [8], containing per litre: 2 g  $(\text{NH}_4)_2\text{SO}_4$ , 0.5 mg  $\text{FeSO}_4$ , 75 mg KCl, 7.5 g triethanolamine, 138 mg  $\text{NaH}_2\text{PO}_4$ , 0.2 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ . The pH was adjusted to 8.1. As carbon source Na-succinate 0.5% was used and the medium was supplemented with 0.2% of casamino-acids (Difco).

#### 2.2. Measurement of protein synthesis

The incorporation of  $^{14}\text{C}$ -amino acids into protein was followed by precipitation of portions of the culture in 5% trichloroacetic acid (TCA) and filtration on membrane filters (Sartorius, Göttingen, 0.45  $\mu$ , 25 mm). After washing with 5% TCA the filters were dried at 100° and counted in a Packard 3320 scintillation spectrometer. The liquid scintillator was a 0.4% solution of Omnifluor (NEN Chemicals, Frankfurt) in toluene.

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### 2.3. Estimation of free amino acid pools

The uptake of  $^{14}\text{C}$ -labelled amino acids into the pools was estimated by a technique similar to that used by Gale and Folkes [9]. Instead of buffered saline we used the nutrient medium without carbon source, casamino-acids and  $\text{MgSO}_4$ . For convenience we shall call this solution "mineral medium".

The bacteria were grown to an optical density at 550 nm of 0.400 and 10 ml of the exponential culture were centrifuged at 7000 g and  $27^\circ$  for 5 min. The sediment was washed once with 5 ml of mineral medium and resuspended in the same amount of this medium. The reaction was started by addition of 150  $\mu\text{l}$  of this suspension to 850 ml of an incubation mixture. This incubation mixture contained per ml: 75  $\mu\text{l}$  of 10% glucose, 25  $\mu\text{l}$  chloramphenicol (1 mg/ml), 7  $\mu\text{l}$   $^{14}\text{C}$ -amino acid, and, in the case of addition of levallorphan, 70  $\mu\text{l}$  of a 10 mM or 50  $\mu\text{l}$  of a 100 mM solution of the drug. The additions of  $\text{MgCl}_2$  or  $\text{CaCl}_2$  were made in 5  $\mu\text{l}$  volumes. The mixture was filled up to 1 ml with mineral medium. The total volume for each assay was usually 6 ml. From the assay mixture 2 ml samples were taken after different times of incubation at  $27^\circ$ . The samples were divided: 1 ml was filtered on a membrane filter, washed with mineral medium and counted in the scintillation spectrometer, the rest was made 5% with respect to TCA, filtered on membrane filter, washed with 5% TCA and also counted in the scintillation spectrometer. The counts of this background were subtracted from those of the sample which had not been treated with TCA. Treatment with boiling water or boiling TCA did not give markedly different backgrounds.

### 2.4. Preparations of cell membranes

The cell membranes were isolated according to Evans [10] by lysis of lysozyme/EDTA protoplasts.

### 2.5. Assay of amino acid uptake by the membranes

The assay mixture for the uptake of  $^{14}\text{C}$ -amino acids contained per ml: 100  $\mu\text{l}$  of 0.1 M Tris-HCl pH 8.5, 100  $\mu\text{l}$  of 10 mM  $\text{MgCl}_2$ , 1  $\mu\text{l}$  of 0.1 M ATP pH 7.4, 10  $\mu\text{l}$  of membrane suspension and 5  $\mu\text{l}$   $^{14}\text{C}$ -amino acid. In the inhibition assays 70  $\mu\text{l}$  of 10 mM or 50  $\mu\text{l}$  of 100 mM levallorphan were added. The mixture was made up to 1 ml with water for each assay. The total volume was 4 ml and the incubation temp  $37^\circ$ . Samples of 0.5 ml were taken at time

intervals, filtered on membrane filters, washed with 0.01 M Tris-HCl-buffer pH 8.5 and the radioactivity was counted in the scintillation spectrometer.

Control assays to establish uptake, pH-optimum, lack of *in vitro* protein synthesis and dependence on concentration of the membrane suspension were made and will be published elsewhere [11].

## 3. Results

### 3.1. Protein synthesis

When a concentration of 0.7 mM levallorphan was added to exponentially growing cultures of *E. coli* only slight or no inhibition of the incorporation of the amino acids into protein was observed in the initial half hr after addition. Only after about 40 min could a slight inhibition of protein synthesis be observed (fig. 1).

### 3.2. Uptake of arginine into the amino acid pool of the cell

In the presence of chloramphenicol (to prevent protein synthesis) the uptake of  $^{14}\text{C}$ -arginine was strongly inhibited by a concentration of 0.7 mM levallorphan. With 5 mM levallorphan, after a short period of uptake, a decrease in the size of the pool was observed suggesting leakage of the arginine from the cell, (fig. 2a).

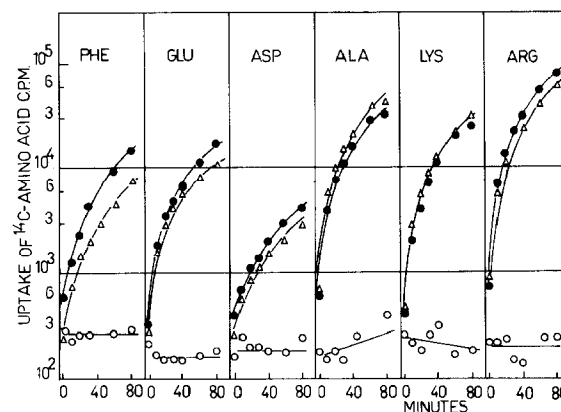


Fig. 1. Protein synthesis in presence of levallorphan. Incorporation of the corresponding amino acid into TCA-precipitable material of *E. coli* cells in the absence  $\bullet$ — $\bullet$ , in the presence of 0.7 mM levallorphan  $\triangle$ — $\triangle$  and in the presence of 5 mM levallorphan  $\circ$ — $\circ$ .

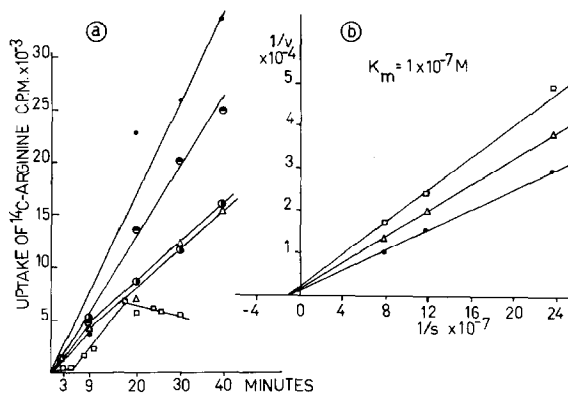


Fig. 2. Uptake of  $^{14}\text{C}$ -arginine into the amino acid pool of *E. coli* cells. a) Uptake of  $^{14}\text{C}$ -arginine in the absence  $\bullet$ — $\bullet$ , in presence of 0.7 mM levallorphan  $\triangle$ — $\triangle$  and 5 mM levallorphan  $\square$ — $\square$ . Uptake in presence of 0.7 mM levallorphan and 2 mM  $\text{Mg}^{2+}$   $\circ$ — $\circ$ , and uptake in presence of 0.7 mM levallorphan and 2 mM  $\text{Ca}^{2+}$   $\circ$ — $\circ$ . All samples were treated with 50  $\mu\text{g}/\text{ml}$  of chloramphenicol. b) Lineweaver-Burk plot of the uptake kinetics of  $^{14}\text{C}$ -arginine. To an exponentially growing culture 50  $\mu\text{g}/\text{ml}$  chloramphenicol were added. After 5 min the culture was divided into portions of 1.5 ml. One series received  $4.22 \times 10^{-9}$  M,  $8.45 \times 10^{-9}$  M, and  $1.26 \times 10^{-8}$  M  $^{14}\text{C}$ -arginine, another series received additionally 0.7 mM of levallorphan and to a third series levallorphan was added in a concentration of 5 mM. After 10 min at  $27^\circ$  the uptake was measured. No levallorphan  $\bullet$ — $\bullet$ , in presence of 0.7 mM levallorphan  $\triangle$ — $\triangle$  and in presence of 5 mM levallorphan  $\square$ — $\square$ . All symbols represent an average of 2 assays.

As Boquet et al. [12] have pointed out that  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  have an antagonistic effect, the ability of these ions to reverse the inhibition was tested. In fig. 2a it is shown that  $\text{Mg}^{2+}$  had a strong counter-effect on the levallorphan action while  $\text{Ca}^{2+}$  at the same concentration had only a slight effect.

The  $K_m$  of the arginine uptake was  $1 \times 10^{-7}$  M over the concentration range utilized, (fig. 2b). This implies a somewhat lower affinity for arginine than the value of  $2.6 \times 10^{-8}$  described by Rosen [13]. Probably this is due to differences in the bacterial strain. The inhibition exerted by levallorphan is of the non-competitive type, similar to that described by Simon [2] for the uptake of putrescine.

### 3.3. Uptake of arginine by cell membranes (ghosts)

When bacterial ghosts, obtained by lysis of EDTA/lysozyme protoplasts, are assayed for uptake of  $^{14}\text{C}$ -

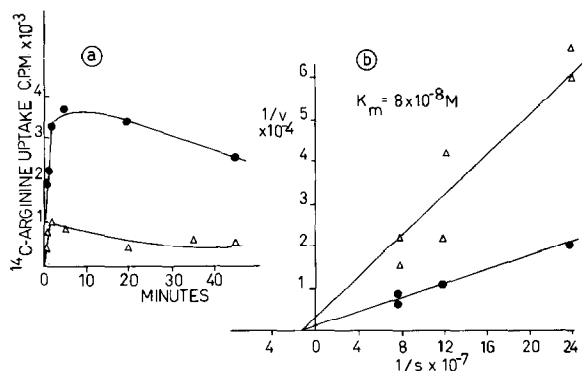


Fig. 3. Uptake of  $^{14}\text{C}$ -arginine by cell membranes of *E. coli*. a) Uptake of  $^{14}\text{C}$ -arginine by 30  $\mu\text{l}$  of membrane preparation per ml of incubation mixture. No levallorphan addition  $\bullet$ — $\bullet$ , in presence of 0.7 mM levallorphan  $\triangle$ — $\triangle$ . b) Lineweaver-Burk plot of the uptake kinetics of  $^{14}\text{C}$ -arginine into membranes. The arginine concentrations were as in fig. 2b. After incubation in the incubation mixture for 3 min at  $37^\circ$  the uptake was measured. Without levallorphan  $\bullet$ — $\bullet$ , in presence of 0.7 mM levallorphan  $\triangle$ — $\triangle$ . All symbols represent an average of 2 assays.

arginine accumulation can be observed which is resistant to washing with Tris-HCl buffer (0.01 M, pH 8.8). This accumulation is not due only to physical adsorption because (i) there is a concentration optimum, higher concentrations of the ghost preparation in the assay mixture showing less accumulation, (ii) a pH optimum is observed at about 8.8, at lower pH values less accumulation being observed, and (iii) a time dependence is found which reaches the optimal value after 5 min (fig. 3a), a time period too long to account for physical adsorption to protein. Boiling of the ghost preparation always resulted in a much lower accumulation of arginine despite the expectation that denatured protein might adsorb more  $^{14}\text{C}$ -arginine.

ATP-concentrations of more than 1 mM decrease the uptake of arginine. The washing of the ghosts with buffer solution containing casamino acids gave a decrease of accumulated arginine after a short period of stimulation. Chloramphenicol had no effect on the accumulation indicating that no *in vitro* protein synthesis occurred. A test for the  $\text{Mg}^{2+}(\text{Ca}^{2+})\text{ATPase}$  [14] showed the presence of this enzyme in the membranes and its dependence on the correct concentration of  $\text{Mg}^{2+}$  and also  $\text{Ca}^{2+}$ .

The  $K_m$  of the uptake was  $8 \times 10^{-8}$  M (fig. 3b), a

value which implies an affinity for arginine only 3 times less than that described by Rosen [13] for whole cells, and it is only slightly different from the  $K_m$  obtained *in vivo* in our strain ( $1 \times 10^{-7}$  M). The inhibition of the uptake was also of the non-competitive type and was found with the same concentration of levallorphan to be even stronger than with the whole cells. From these data we suggest that the same action of levallorphan as is found with whole cells is responsible for the inhibition of uptake in the ghost preparation.

#### 4. Discussion

Levallorphan has been shown to have an effect on permeability [3] and on transport of putrescine and amino acids [12]. Other effects concerning the adsorption of MS-2 phage [6] and formation of mating pairs [7] can also be attributed to changes of the membrane of the cell. We have shown that the uptake of arginine into the pool of free amino acids is strongly inhibited at a concentration of 0.7 mM levallorphan. At the same concentration the uptake of some other amino acids (lysine, aspartic acid, phenylalanine and glutamic acid, not shown here) was usually less inhibited. The concentration is not sufficient to inhibit the growth of the bacteria or their protein synthesis significantly. The inhibition of uptake is of the non-competitive type. The  $K_m$  of the uptake by whole cells lies in the same order of magnitude as that of the high affinity system described by Rosen [13]. However, with a ghost preparation the uptake of  $^{14}\text{C}$ -arginine was also found to have about the same affinity, the inhibition of the uptake by levallorphan being of the same type. This could mean that the shock proteins are not detached from the membranes during formation or lysis of the protoplasts.

With high concentrations of levallorphan (5 mM) the transport system seems to become completely blocked and the membrane becomes permeable to the amino acid.

$\text{Mg}^{2+}$  is able to reverse the inhibition of levallorphan

*in vivo* to a great extent. As no complex is formed *in vitro* between levallorphan and  $\text{Mg}^{2+}$  [15] this cannot be due only to competition of levallorphan for  $\text{Mg}^{2+}$ .  $\text{Ca}^{2+}$  seems to have only little effect in this respect. From the data obtained we suggest that the inhibition of the arginine uptake by levallorphan observed in whole cells is of the same type as that found in the membrane fraction. This means that an effect of levallorphan can be studied with low concentrations in a cell-free system.

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