

## **Transport of chloramphenicol into sensitive strains of *Escherichia coli* and *Pseudomonas aeruginosa***

**Saad Abdel-Sayed**

*Institut de Microbiologie de l'Université de Lausanne, Centre Hospitalier Universitaire  
Vaudois, Rue du Bugnon 44, 1011 Lausanne, Switzerland*

The uptake of chloramphenicol by susceptible strains of *Escherichia coli* and *Pseudomonas aeruginosa* was measured as the depletion of <sup>14</sup>C-chloramphenicol from the supernatant of centrifuged cultures. Chloramphenicol did not bind to non-growing cells or isolated cell envelopes. Chloramphenicol was recovered from cells in an unchanged form and was intracellularly concentrated several times above external concentrations. The net accumulation of the drug was reduced by an inhibitor of electron transport, by an oxidative phosphorylation uncoupler, by an inhibitor of high energy phosphate synthesis, and by lowering the temperature to +15°C. The initial uptake of drug was saturated at 1.98 mM chloramphenicol in the medium. A 100-fold excess of each of the unlabelled isomers: L-threo, D-threo, and L-erythro chloramphenicol in cultures of either strain effectively reduced the uptake of <sup>14</sup>C-chloramphenicol. These results indicate that chloramphenicol enters Gram-negative bacteria by means of an energy-dependent process.

### **Introduction**

Chloramphenicol is an antibiotic that inhibits protein synthesis in procaryotic cells. It binds primarily to protein L 16 of bacterial ribosomes and blocks peptide bond formation by inactivating peptidyltransferase (Nierhaus & Nierhaus, 1973). Since the target for chloramphenicol is intracellular, the drug must penetrate the cell envelope of bacteria.

The accumulation of chloramphenicol by *Staphylococcus aureus* strain Duncan and *Bacillus megaterium* strain KM (NCIB 9521) was described by Vasquez (1963, 1966). He showed that the rate of uptake was initially high but fell rapidly and that after two minutes a much slower rate was established. The magnitude of uptake was directly related to the external drug concentration and could be greatly reduced by peptidyl transferase inhibitors such as ostreogrycins group A and group B, but was unaffected by tetracyclines, inhibitors of the binding of aminoacyl-tRNA to the 30 S ribosomal subunit or by puromycin, an inhibitor of peptide chain elongation functioning as an analogue of terminal aminoacyl-tRNA.

Irvin & Ingram (1980) isolated two chloramphenicol-resistant mutants of *Pseudomonas aeruginosa* which also exhibited decreased amino-acid accumulation. Neither of these mutants inactivate chloramphenicol by acetylation and both possess ribosomes sensitive to the action of chloramphenicol. Since uptake of amino-acids by bacteria involves active transport (Anraku, 1978), the data of Irvin & Ingram (1980) could be interpreted as showing an energy-dependent chloramphenicol uptake.

However, the chloramphenicol-resistant mutants may accumulate decreased amounts of amino-acids and chloramphenicol owing to loss of outer membrane porins (Foulds & Chai, 1978; Pugsley & Schnaitman, 1978). Thus, the evidence for active accumulation of chloramphenicol remains equivocal.

Bacterial resistance to chloramphenicol can be produced by at least three different mechanisms: enzymatic covalent modification of the molecular structure, alteration of the cells' permeability, and alteration of the ribosomal binding site (Foster, 1983). Studies on the uptake of chloramphenicol by a number of Gram-positive bacteria that do not covalently modify it have shown that there is a correlation between the rate of uptake of the antibiotic by the organisms and their sensitivity to it (Vasquez, 1964). In all the studies reported above the cells were washed to remove external molecules before intracellular concentration of chloramphenicol was determined. Chloramphenicol is so weakly held in the cells that washing itself can remove intracellular antibiotic (Vasquez, 1963, 1964). In the investigation of chloramphenicol transport into bacteria reported here a centrifugation method was used to determine chloramphenicol uptake without washing of the cells.

### Materials and methods

#### *Bacterial strains*

The organisms used for this study were *Escherichia coli* K 12 strain 703 (Hammersmith wild type F<sup>-</sup>) and *P. aeruginosa* ATCC 9027. The MIC of chloramphenicol for the *E. coli* strain was 6.25 mg/l, and for the *P. aeruginosa* strain 25 mg/l, determined by dilution technique in BYGNaCl medium (see below).

#### *Media and growth conditions*

The organisms were maintained on nutrient agar slants at room temperature. When used for experiments, cells were grown aerobically until mid-exponential phase at 37°C on a gyratory Adolf Kühner shaker at 250 r.p.m. Erlenmeyer flasks (100 ml) each containing 30 ml of growth medium, BYGNaCl medium: 13 g nutrient broth (Oxoid), 5 g yeast extract (BBL), 5 g NaCl, 1 g glucose per litre, adjusted to pH 7 (Lennox, 1955), was used for most of these studies. Where noted a BM2 medium composed of 2.5 g glucose, 7 g K<sub>2</sub>HPO<sub>4</sub>, 3.4 g KH<sub>2</sub>PO<sub>4</sub>, 1 g (NH<sub>4</sub>)SO<sub>4</sub>, and 0.024 g MgSO<sub>4</sub> · H<sub>2</sub>O per litre was used (Gilleland, Stinett & Eagon, 1974).

#### *Chloramphenicol uptake*

Cells grown in BYGNaCl medium were harvested by centrifugation at 6000 r.p.m. for 10 min and then resuspended in the same volume of unused prewarmed BYGNaCl medium at 37°C. Of this suspension 1–1.8 ml were transferred to 25-ml Erlenmeyer flasks, and a mixture of <sup>14</sup>C-chloramphenicol (46.9 mCi/mmol) with unlabelled chloramphenicol was added to produce final volumes of 2 ml with various concentrations of chloramphenicol. Final specific activities ranged from 0.63 to 32.3 mCi/mmol. The flasks were incubated at 37°C on a reciprocal shaking water bath. At 5 min intervals, aliquots of 0.2 ml were removed and centrifuged in a Kontron centrifuge (type Hermle z635-K) at 6000 r.p.m. for 1 min at 25°C. The upper 0.1 ml of the supernatant was carefully removed to a scintillation vial containing 10 ml of

scintillation fluid. This was composed of ethanol, Triton X-100 and toluene in the ratio of 1 : 3.3 : 5.7 containing 0.572% (w/v) diphenyloxazole and 0.014% (w/v) 1.4-bis[2-(4-methyl-5-phenyloxazonyl)]-benzene. The radioactivity was determined with a Unilux model scintillation spectrometer (Nuclear Chicago). The amount of cell-associated chloramphenicol was determined by subtracting the amount of the residual  $^{14}\text{C}$ -chloramphenicol in the supernatant from the amount of  $^{14}\text{C}$ -chloramphenicol in the cell suspension.

#### *Radiochemical purity*

The cells from 20 ml of actively growing culture were suspended in 2 ml of unused BYGNaCl medium maintained at 37°C.  $^{14}\text{C}$ -Chloramphenicol was added to produce a specific activity of 2.5 mCi/l and the cell suspension was incubated at 37°C on a reciprocal shaking water bath. After 5 min of labelling the cells were harvested by filtration on Millipore membrane filters and immediately washed by filtering 2.5 ml of 0.1 M NaCl. The membrane filters bearing the cells were transferred to 25-ml Erlenmeyer flasks containing 3 ml distilled water and the cells were detached from the filter membranes by magnetic stirring. The cell suspension was then submitted to three successive extractions with 3 ml diethyl ether. The pooled extracts were evaporated to dryness in an air stream. The samples, dissolved in 0.1 ml ethyl acetate, were spotted by repeated applications of a total volume of 0.02 ml at the origin of a thin layer silica gel (Kiesel gel 60 F245, 0.25 mm, Merck) alongside a control preparation of  $^{14}\text{C}$ -chloramphenicol. The sheets were submitted to ascending chromatography in a chloroform : methanol 90 : 10 (v/v) solvent system. The chromatograms were air dried and subjected to autoradiography for 66 hours with Kodak X-Omat AR-5 film.

#### *Measurement of protein synthesis*

Exponentially growing cells were harvested and resuspended in BYGNaCl medium at 37°C at  $A_{660} = 0.67$ . From this suspension, 0.97-ml amounts were transferred to 25-ml Erlenmeyer flasks containing 0.03 ml (0.319 mCi) of  $^{35}\text{S}$ -methionine with or without chloramphenicol. The flasks were rapidly mixed and subsequently held on a reciprocal shaking water bath at 37°C. At time intervals, samples of 0.05 ml were removed and spotted on to Whatman no. 3 MM discs pretreated with trichloroacetic acid. After drying at room temperature, all discs were processed simultaneously as described by Levy (1971) to evaluate  $^{35}\text{S}$ -methionine incorporated into trichloroacetic acid-precipitable protein.

#### *Cells preincubated in medium unable to support growth*

Cells grown in BYGNaCl medium were harvested by centrifugation and washed three times with 50 mM phosphate buffer, pH 7. Washed cells were suspended to the original volume of the same buffer and incubated at 37°C with shaking for 2 h. The cells were centrifuged and resuspended in 1 ml phosphate buffer per mg of cell protein for equilibrium dialysis.

### *Transport assays*

The uptake of [ $U\text{-}^{14}\text{C}$ ] protein hydrolysate by both strains was determined at  $37^\circ\text{C}$  to check the active accumulation of amino-acids by cells preincubated in medium unable to support growth. Cells were washed as described above and suspended in 50 mM phosphate buffer, pH 7, to a density of 2 g wet weight per 10 ml. The suspensions were used for uptake assays. The uptake mixtures contained 0.2 ml of cell suspension and 4.4 nanoatom carbon of [ $U\text{-}^{14}\text{C}$ ] protein hydrolysate (5.7 mCi/milliatom carbon) in a final volume of 0.5 ml. The reaction was started by addition of substrate. At time intervals, 0.05 ml samples were removed and delivered over membrane filters (25 mm diameter; 0.45  $\mu\text{m}$  pore size; Millipore corporation, Bedford, MA, U.S.A.) which had been pretreated by filtering 5 ml 0.1 M LiCl. The samples were filtered instantaneously and the filters were immediately washed with 5 ml 0.1 M LiCl. The filters bearing the cells were removed from the suction apparatus and transferred to vials containing 10 ml of the scintillation fluid. The radioactivity was determined as described above.

### *Spheroplast preparation and spheroplast uptake*

Spheroplasts were prepared from the *E. coli* strain according to the method of Weiss (1976) with slight modifications. Cells were grown in BYGNaCl medium. Deoxyribonuclease type I and II, and ribonuclease were added to a final concentration of 0.05 mg/l directly after the addition of lysozyme. Spheroplasts were harvested by centrifugation at 3600 g for 15 min and subsequently washed once with 0.1 M Tris-HCl buffer, pH 8, containing 20% sucrose. Washed spheroplasts were suspended into the original volume of a solution of 20% sucrose in 50 mM phosphate buffer, pH 7, or in sucrose BYGNaCl medium. Samples of 1 ml of these suspensions, when diluted ten times in water, decreased in optical density at 660 nm ten times more than that diluted in 20% sucrose. The spheroplast suspensions were used directly for uptake assays. The uptake mixtures contained 0.9 ml of spheroplast suspension and 10  $\mu\text{g}$  of  $^{14}\text{C}$ -chloramphenicol (6.16 mCi/mmol) in a final volume of 1 ml. The reaction was started by the addition of substrate. After 5 min of incubation at  $37^\circ\text{C}$ , 0.2 ml was removed and centrifuged at 3000 g for 2 min. The upper 0.1 ml of the supernatant was carefully removed and processed as described above to evaluate  $^{14}\text{C}$ -chloramphenicol incorporated into spheroplasts.

### *Miscellaneous assays and techniques*

Protein was determined according to the method of Markwell *et al.* (1978). Cell envelopes were prepared as described by Hancock & Nikaido (1978). Equilibrium dialysis was carried out in a multichamber apparatus by the procedure of Furlong *et al.* (1972).

### *Reagents*

D-threo (dichloro-1,2- $^{14}\text{C}$ ) chloramphenicol (46.9 mCi/mmol) and  $^{35}\text{S}$ -methionine (1058 Ci/mmol) were purchased from New England Nuclear Corp., Boston, Mass., U.S.A.; [ $U\text{-}^{14}\text{C}$ ]-Protein hydrolysate, composed of 16 amino-acids (57 mCi/milliatom carbon), from Amersham, Buckinghamshire, England. Non-radioactive

chloramphenicol was a gift from Le Petit Co., Milan, Italy. Chloramphenicol isomers were a gift from Warner Lambert Co., Ann Arbor, Mich. All other reagents were purchased from normal commercial sources.

## Results

### *Uptake of chloramphenicol*

Figure 1 illustrates the time course of the uptake of chloramphenicol for *E. coli* and *P. aeruginosa* in BYGNaCl medium at concentrations of 10 and 40 mg/l respectively. Uptake of the drug by either bacterium demonstrated biphasic kinetics: a rapid uptake, which ceased at the time the first sample was taken (1 min) and a slower uptake, which then continued for 5 min; thereafter, the uptake reached a plateau of a period of 5–30 min. With a value of 2.7 ml cell water per g dry weight of bacteria assumed (Winkler & Wilson, 1966), the concentration of cell-associated chloramphenicol after 1 min of incubation for *E. coli* was 689.7 mg/l cell water at a chloramphenicol concentration in the medium of 10 mg/l. For *P. aeruginosa*, the concentration of cell-associated chloramphenicol was 2595.3 mg/l cell water at a chloramphenicol concentration in the medium of 40 mg/l. Thus, after 1 min of incubation the concentration of chloramphenicol accumulated by *E. coli* and *P. aeruginosa* reached about 69 and 65 times the concentration in the medium, respectively. After 5 min of uptake the concentration of chloramphenicol respectively accumulated by *E. coli* and *P. aeruginosa* exceeded about 140 and 97 times the concentration in the medium. Lowering the reaction temperature to +15°C produced a marked change in both rates of uptake by either type of bacterium. Furthermore, the slow uptake appeared to last longer than usually seen at 37°C (Figure 1).

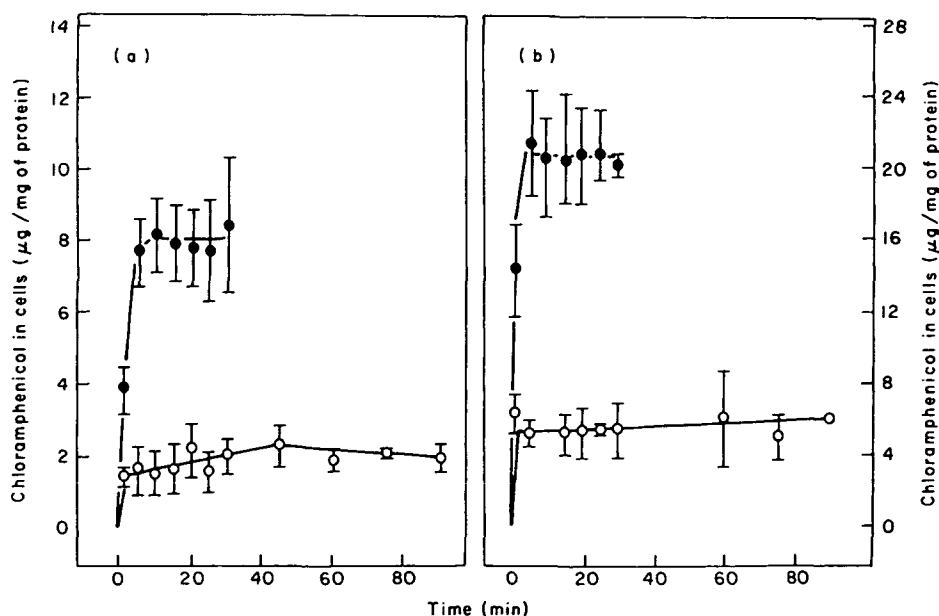


Figure 1. Uptake of chloramphenicol with time in BYGNaCl medium at 37°C (●), and 15°C (○), in the presence of 10 mg chloramphenicol/l for *E. coli* (a) and 40 mg chloramphenicol/l for *P. aeruginosa* (b). Bars represent standard errors of the mean for four experiments.

**Table I.** Effect of unlabelled chloramphenicol and chloramphenicol isomers on  $^{14}\text{C}$ -chloramphenicol uptake by *E. coli* and *P. aeruginosa*

Bacteria	Unlabelled substance	$^{14}\text{C}$ -CM taken up <sup>a</sup> ( $\mu\text{g}/\text{mg}$ protein)	% inhibition of uptake
<i>E. coli</i>	Control	132.00 $\pm$ 54.00	0
	CM	0	100
	L-threo-CM	45.00 $\pm$ 14.00	66
	D-erythro-CM	0	100
	L-erthryo-CM	25.00 $\pm$ 8.00	81
<i>P. aeruginosa</i>	Control	73.00 $\pm$ 10.00	0
	CM	3.15 $\pm$ 2.22	96
	L-threo-CM	1.30 $\pm$ 1.14	98
	D-erythro-CM	0	100
	L-erythro-CM	25.00 $\pm$ 8.00	66

<sup>a</sup>CM, chloramphenicol; in the presence of  $^{14}\text{C}$ -CM and unlabelled substance at concentrations of  $3 \times 10^{-6}$  M and  $3 \times 10^{-4}$  M respectively; accumulation period 1 min in BYGNaCl medium. Values were obtained from two experiments.

Measured after 1 min, the uptake of  $^{14}\text{C}$ -chloramphenicol by either type of bacterium was greatly reduced by the unlabelled chloramphenicol isomers: L-threo and L-erythro when each was added at 100 times the concentration of  $^{14}\text{C}$ -chloramphenicol (Table I). The unlabelled chloramphenicol and the D-erythro isomer completely inhibited the uptake of  $^{14}\text{C}$ -chloramphenicol.  $\text{MgSO}_4$  reduced the rate of uptake of chloramphenicol by both strains (Table II) and also elevated the MIC respectively two and four times for *E. coli* and *P. aeruginosa*, when added to a final concentration of 5 mM. Similar results were obtained with  $\text{MgCl}_2$  (Table II) indicating that the

**Table II.** Inhibition of chloramphenicol uptake by magnesium sulphate

Bacteria	$\text{Mg}^{++}$ added (mM)	Cell associated CM ( $\mu\text{g}/\text{mg}$ protein)
<i>E. coli</i> <sup>a</sup>	0.00	7.85 $\pm$ 0.15
	0.50	4.90 $\pm$ 0.20
	1.00	4.10 $\pm$ 0.60
	1.50	2.35 $\pm$ 0.35
	2.00	1.85 $\pm$ 0.05
	2.00	(1.10 $\pm$ 0.14) <sup>c</sup>
<i>P. aeruginosa</i> <sup>b</sup>	0.00	21.40 $\pm$ 3.00
	0.50	15.45 $\pm$ 1.25
	1.00	14.70 $\pm$ 3.10
	1.50	13.65 $\pm$ 0.25
	2.00	11.15 $\pm$ 1.55
	2.00	(6.15 $\pm$ 0.21)

CM, chloramphenicol; <sup>a</sup>chloramphenicol concentration, 10 mg/l, accumulation period, 5 min in BYGNaCl medium; <sup>b</sup>chloramphenicol concentration, 40 mg/l; accumulation period, 5 min in BYGNaCl medium; <sup>c</sup>the numbers in parentheses are the uptake of CM in the presence of  $\text{MgCl}_2$ ; values were obtained from two experiments.

inhibition of chloramphenicol uptake by  $\text{MgSO}_4$  was due to  $\text{Mg}^{++}$  effect and not to sulphate. Cells grown in minimal BM2 medium exhibited a reduced uptake of chloramphenicol compared with those grown in BYGNaCl medium, with values of 27% and 14% for *E. coli* and *P. aeruginosa*, respectively. Cells grown in BM2 medium were thus phenotypically more resistant. For example, the MIC for the *P. aeruginosa* strain harvested from BM2 medium was 100 mg/l, whether the test medium was BM2 or BYGNaCl. In contrast, cells grown in BYGNaCl and transferred into BM2 were not able to incorporate  $^{14}\text{C}$ -chloramphenicol.

#### *Chloramphenicol extracted from cells*

More than 90% of accumulated chloramphenicol was recovered from cells by ether extraction. Autoradiography of thin layer chromatograms demonstrated that the  $R_f$  value (0.68) of extracted chloramphenicol was identical to that of a control preparation of  $^{14}\text{C}$ -chloramphenicol (Figure 2). These results clearly indicated that the majority of accumulated chloramphenicol was recovered from cells in an unchanged form.

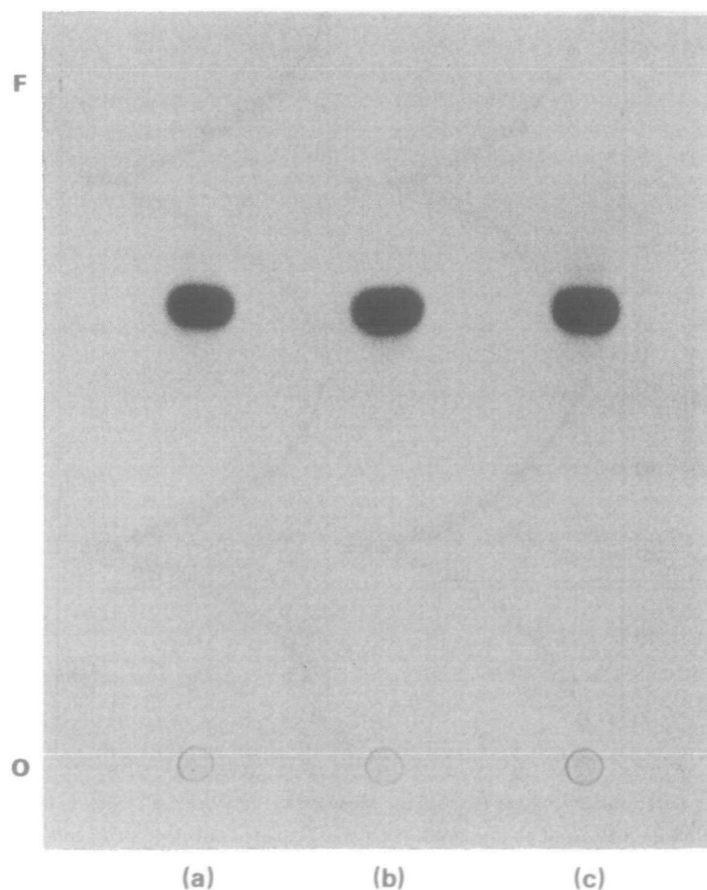


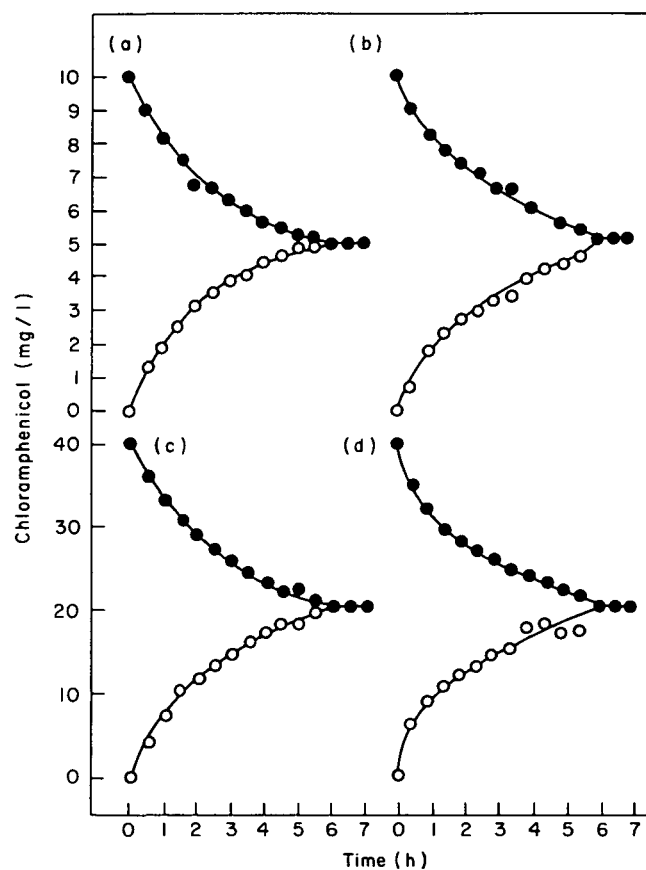
Figure 2. Autoradiographic demonstration of a thin layer chromatogram of chloramphenicol extracted from prelabelled cells of *E. coli* (a), *P. aeruginosa* (c), and of a control preparation of  $^{14}\text{C}$ -chloramphenicol (b). The designations O and F refer to origin and solvent front respectively.

*Equilibrium dialysis*

As shown by Figure 3, chloramphenicol passed through the dialysis membrane without being bound by either non-growing cells or isolated cell envelopes.

*Uptake of amino-acids by cells preincubated in medium not supporting growth*

Figure 4 illustrates the time-course of the uptake of labelled amino-acids for both strains in a mineral solution at a concentration of 8.8 nanoatom carbon/ml. After 60 s of incubation, the preincubated cells of *E. coli* and *P. aeruginosa* accumulated labelled amino-acids, respectively 49.5 and 191.8 times over the external concentration, as determined on the basis of cell water (Winkler & Wilson, 1966). Thereafter, the uptake reached a plateau over a period of measurements (60–120 s). The freshly washed cells of both strains accumulated amino-acids 1.3–1.5 times less than the preincubated cells. These results indicated that 2 h preincubation of cells in mineral solution did not significantly alter the active accumulation of amino-acids.



**Figure 3.** Equilibrium dialysis of chloramphenicol against isolated envelope of *E. coli* (a), cell envelope of *P. aeruginosa* (c), cells in non-growth conditions of *E. coli* (b), and *P. aeruginosa* (d). The dialysis chambers contained as appropriate: 0.5 mg isolated envelope protein; 1 mg cell protein; 0.031 mM chloramphenicol against isolated envelope or cells of *E. coli*; and 0.124 mM chloramphenicol against isolated envelope or cells of *P. aeruginosa*. ○, Radioactivity of samples taken from the compartments containing isolated envelope or cells; ●, radioactivity of samples taken from the compartments containing <sup>14</sup>C-chloramphenicol.



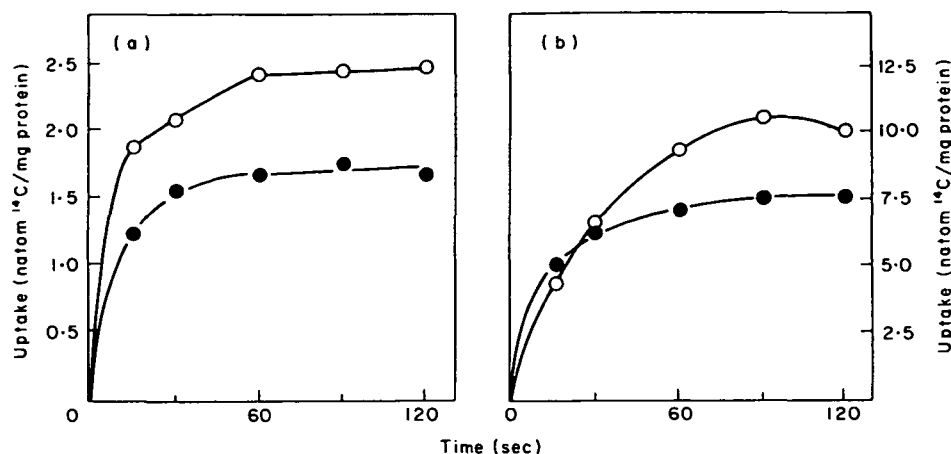


Figure 4. Uptake of [U-<sup>14</sup>C] protein hydrolysate by cells preincubated in medium not supporting growth (○), and freshly washed cells (●), of *E. coli* (a), and *P. aeruginosa* (b).

#### Effect of energy inhibitors on chloramphenicol uptake

Uptake of chloramphenicol was decreased or inhibited by a variety of energy inhibitors when added to the cells 5 min before the addition of chloramphenicol. As shown by Figure 5(a), cyanide and azide, inhibitors of electron transport (Lehninger, 1976) and arsenate, an inhibitor of high energy phosphate synthesis (Lehninger, 1976), all reduced to various degrees the rapid uptake by *E. coli*; thereafter, the cells progressively released the accumulated chloramphenicol into the supernatant. Rapid uptake by *E. coli* was insensitive to 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation (Lehninger, 1976), but the cells were unable to maintain the chloramphenicol gradient; about 81% of the rapid uptake was released into the

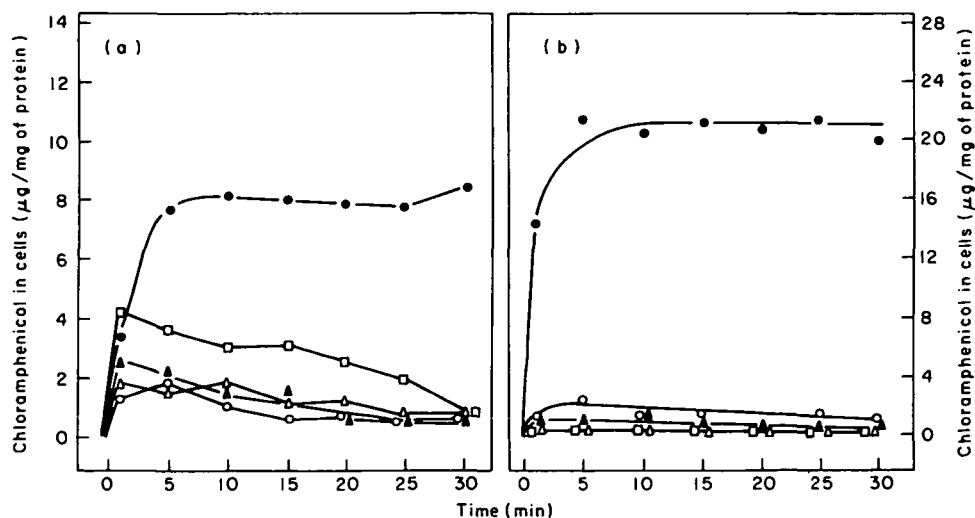


Figure 5. Effect of energy inhibitors on chloramphenicol uptake by *E. coli* (a), and *P. aeruginosa* (b), in the presence of 10 mg and 40 mg chloramphenicol/l, respectively. Cells preincubated for 5 min without any inhibitor (●), or individually with 1 mM DNP (□), 2 mM KCN (▲), 5 mM azide (△), and 5 mM arsenate (○).

**Table III.** Uptake of chloramphenicol by *E. coli* spheroplasts in sucrose phosphate buffer and sucrose BYGNaCl medium

Uptake medium	<sup>14</sup> C-CM taken up <sup>a</sup> (μg/mg protein)
Sucrose phosphate buffer	1.20 ± 0.44
Sucrose BYGNaCl medium	0.71 ± 0.26

<sup>a</sup>After 5 min of incubation in the presence of 10 mg CM/l. Values were obtained from three experiments.

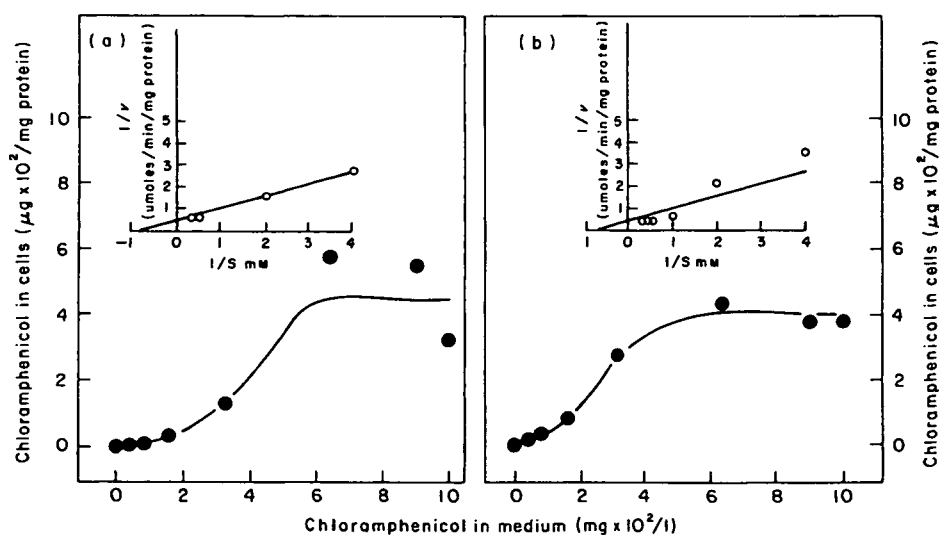
supernatant within 29 min. The inhibitors were more effective with *P. aeruginosa* than with *E. coli*. DNP and sodium azide completely inhibited the uptake of chloramphenicol by *P. aeruginosa* in both the rapid and the slow phases (Figure 5).

#### *Uptake of chloramphenicol by spheroplasts in phosphate buffer*

Table III shows the uptake of <sup>14</sup>C-chloramphenicol at 37°C by spheroplasts prepared from *E. coli* cells grown in BYGNaCl medium. After 5 min of incubation, the spheroplasts assayed in sucrose phosphate buffer incorporated chloramphenicol 1.6 times more than those assayed in sucrose BYGNaCl medium. These results clearly indicated, therefore, that the outer membrane and peptidoglycan deprived cells were not sensitive to the shift down of the environmental conditions and thus incorporated chloramphenicol even in phosphate buffer.

#### *Uptake as a function of chloramphenicol concentration in medium*

Measured after 1 min, the uptake of chloramphenicol by *E. coli* and *P. aeruginosa* increased as the concentration in medium was increased from 40 to 640 mg/l. Beyond



**Figure 6.** Uptake of chloramphenicol by *E. coli* (a), and *P. aeruginosa* (b), in BYGNaCl medium at various chloramphenicol concentrations. The inset is a Lineweaver-Burk plot of the kinetic data.

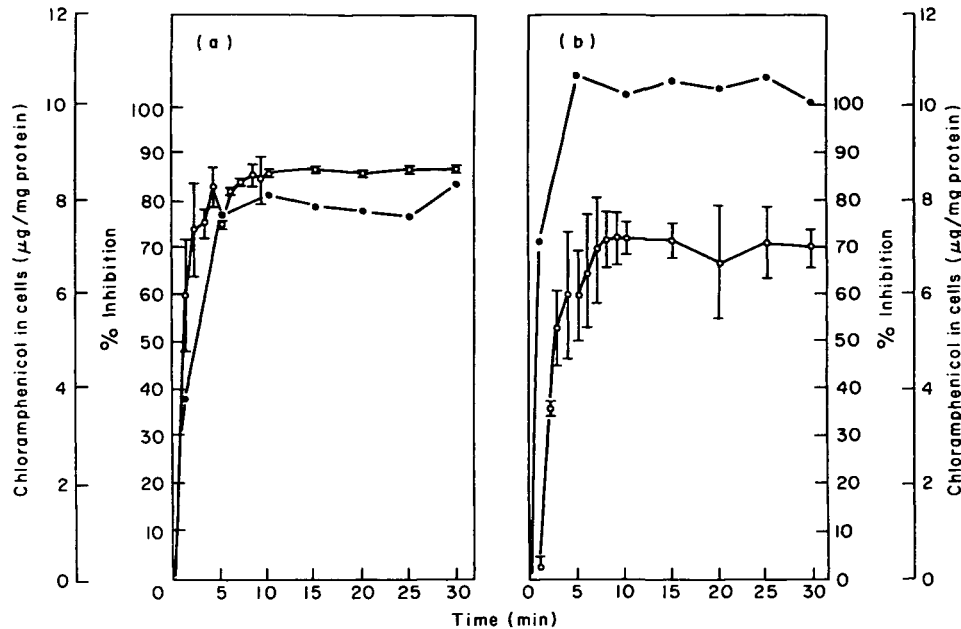


Figure 7. Relationship of chloramphenicol uptake (●), to inhibition of protein synthesis (○), in cells of *E. coli* at 10 mg chloramphenicol/l (a), and *P. aeruginosa* at 40 mg chloramphenicol/l (b). Bars represent standard errors of the mean for two experiments.

that range of concentration, the uptake by either strain of bacterium reached a plateau. A plot of the data in Lineweaver-Burk form revealed an apparent  $K_m$  ( $K_i$ ) of 1.8 mM and a  $V_{max}$  of 2.5 mmoles/min/g protein, for both strains (Figure 6).

#### Relationship of chloramphenicol uptake to inhibition of protein synthesis

As shown by Figure 7, when chloramphenicol was added simultaneously with  $^{35}\text{S}$ -methionine to growing cells of *E. coli*, 60% inhibition of protein synthesis occurred within 1 min; thereafter, an increasing inhibition occurred reaching 87% within 10 min. For the *P. aeruginosa* strain, 53% inhibition occurred within 3 min and thereafter, an increasing inhibition reached 72% within 10 min. Thus, it seems that the rapid phase of chloramphenicol uptake was more effective with *E. coli* than with *P. aeruginosa* in inhibiting cellular protein synthesis.

#### Discussion

Our experimental results showed that chloramphenicol did not bind to either non-growing cells or to isolated cell envelopes of *E. coli* and *P. aeruginosa*. Therefore, we measured the uptake of chloramphenicol by exponentially growing cells as the depletion of  $^{14}\text{C}$ -chloramphenicol from the supernatant of centrifuged cultures, assuming the missing  $^{14}\text{C}$ -chloramphenicol to be found within the cells. Filtration could not be used to collect cells because of the retention of label by membrane filters.

Hurwitz & Braun (1967) proposed that chloramphenicol crosses the cytoplasmic membrane of *E. coli* by passive diffusion. This proposal was supported by data

indicating that concentration of chloramphenicol by bacteria probably results from binding of the antibiotic to ribosomes (Harvey & Koch, 1980). The results of the present study show that accumulation of chloramphenicol after 5 min by sensitive strains of *E. coli* and *P. aeruginosa* reaches respectively about 140 and 97 times the concentration in the medium. When the amount of ribosome-bound chloramphenicol, calculated on the basis of 26,000 ribosomes per cell (Goldstein & Lowney, 1964) and one specific binding site per ribosome (Wolfe & Hahn, 1965; Das, Goldstein & Kenner, 1966; Hurwitz & Braun, 1967), was subtracted from the total amount of cell-associated chloramphenicol, it appeared that chloramphenicol concentrations in the cell "pool" of *E. coli* and *P. aeruginosa* were respectively 136.3 and 96.5 times the concentration in the medium. Thus, these results clearly indicate that concentration of chloramphenicol by bacteria does not result simply from binding of the antibiotic to ribosomes, but that it is accumulated against a concentration gradient. Results reported here showed that chloramphenicol accumulation by either strain required energy, presumably to transport chloramphenicol across the cytoplasmic membrane, since it is well recognized that ribosomal binding of chloramphenicol is not energy dependent (Vasquez, 1963, 1964).

Irvin & Ingram (1982) pointed out that acquisition of chloramphenicol resistance in *P. aeruginosa* was a function of the concentration of free  $Mg^{++}$  in the growth medium. The antagonism of chloramphenicol activity by  $Mg^{++}$  was essentially immediate and was not a result of prolonged growth in  $Mg^{++}$  limiting medium. More recently, however, Harvey & Koch (1980) demonstrated that  $Mg^{++}$  enhances in-vitro binding of chloramphenicol to ribosomes up to a concentration of 10 mM. Our experimental results show that  $Mg^{++}$  reduces the uptake of chloramphenicol and raises the MICs of the antibiotic for both strains of *E. coli* and *P. aeruginosa*. Thus, the effect of  $Mg^{++}$  is the reverse of that noted on ribosomal binding. It is plausible, therefore, that the interaction of  $Mg^{++}$  with the cell envelope of Gram-negative bacteria retards the movement of chloramphenicol across the cell envelope (diffusion through the outer membrane and subsequent transport across the cytoplasmic membrane). This in turn would permit bacterial growth in the presence of otherwise inhibitory concentrations of chloramphenicol.

The results reported here demonstrate that the accumulation kinetics of chloramphenicol are biphasic: an initial rapid phase and a second slower phase. In addition, uptake of the drug measured in minimal BM2 medium is much less than that measured in enriched medium. These findings agree with those previously reported by Vasquez (1966) with Gram-positive bacteria. However, our results indicate that unlabelled chloramphenicol isomers effectively prevent the uptake of  $^{14}C$ -chloramphenicol (Table I); in other words, the isomers compete with chloramphenicol in cellular penetration. These findings do not agree with those previously reported by Vasquez (1966) with Gram-positive bacteria. He demonstrated that unlabelled chloramphenicol isomers: L-threo, D-erythro, and L-erythro did not reduce the uptake of  $^{14}C$ -chloramphenicol by *B. megaterium* after 15 min of incubation with the drug; but the uptake of  $^{14}C$ -chloramphenicol was reduced by 77.9% when unlabelled chloramphenicol was added at ten times the concentration of  $^{14}C$ -chloramphenicol. However, it is doubtful that chloramphenicol uptake could be measured accurately by washing the cells twice with 15 ml of 10 mM Tris-HCl buffer. Most probably washing the cells removed most of the unbound chloramphenicol from the bacterial "pool" and the apparent reduction of uptake of  $^{14}C$ -chloramphenicol

could be explained by competition between the labelled and unlabelled molecules for binding to ribosomes. The lack of effect of chloramphenicol isomers was most probably due to inability of isomers to prevent stereospecific binding of chloramphenicol. Vasquez (1966) showed in his studies that all the compounds which inhibit uptake of chloramphenicol by intact bacteria, with the exception of ostreogrycins B group, also prevent binding of chloramphenicol to bacterial ribosomes in a cell-free system.

Cells grown in BYGNaCl and assayed in BM2 medium do not transport chloramphenicol. Such cells, when transferred to BM2 medium, exhibited a lag period over 6 h before the growth initiation. During the lag period the cells do not incorporate chloramphenicol. During growth in BM2 medium, they incorporate chloramphenicol, but however at a lower rate than in BYGNaCl medium. Cells transferred from BYGNaCl or from BM2 medium to phosphate buffer do not incorporate chloramphenicol. These results indicate that non-growing cells do not transport chloramphenicol. However, such cells actively accumulate amino-acids even after 2 h of preincubation in medium unable to support growth (Figure 4). Thus, the failure of non-growing cells to transport chloramphenicol cannot simply be attributed to loss of energy coupling for uptake. The data reported here demonstrate that spheroplasts prepared from cells grown in BYGNaCl medium and assayed in phosphate buffer transport chloramphenicol slightly more than those assayed in BYGNaCl medium. These findings clearly indicate that the permeability barrier to chloramphenicol in non-growing cells is located outside the cytoplasmic membrane.

Nakae & Nikaido (1975) demonstrated that the outer membrane does act as a limiting barrier for the penetration of hydrophilic substances, but not the peptidoglycan. Although chloramphenicol is relatively hydrophobic, it penetrates the outer membrane of *Salmonella typhimurium* and *E. coli* via aqueous (porin) pathways (Nikaido, 1976; Chopra & Eccles, 1978; Pugsley & Schnaitman, 1978). The data presented here suggest that diffusion of chloramphenicol across the outer bacterial membrane depends on active growth. It seems possibly a conformational change occurs in non-growing cells that prevents chloramphenicol recognizing the porin channels.

#### Acknowledgements

I am grateful to Professor Valentin Bonifas for discussions and for critical reading of this manuscript. The able technical assistance of Hong Fritschy and France Schmid is acknowledged.

#### References

- Anraku, Y. (1978). Active transport of amino acids. In *Bacterial transport* (Rosen, B. P., Ed.), p. 171. Marcel Dekker, New York.
- Chopra, I. & Eccles, S. J. (1978). Diffusion of tetracycline across the outer membrane of *Escherichia coli* K-12: Involvement of protein I<sub>a</sub>. *Biochemical and Biophysical Research Communications* **83**, 550-7.
- Das, H. K., Goldstein, A. & Kenner, L. C. (1966). Inhibition by chloramphenicol of the growth of nascent protein chains in *Escherichia coli*. *Molecular Pharmacology* **2**, 158-70.
- Foster, T. J. (1983). Plasmid-determined resistance to antimicrobial drugs and toxic metal in bacteria. *Bacteriological Reviews* **47**, 361-409.

- Foulds, J. & Chai, T. J. (1978). New major outer membrane protein found in an *Escherichia coli* to 1F mutant resistant to bacteriophage T4. *Journal of Bacteriology* **133**, 1478–83.
- Furlong, C. E., Morris, R. G., Kandrach, M. & Rosen, B. P. (1972). A multichamber equilibrium dialysis apparatus. *Analytical Biochemistry* **47**, 514–26.
- Gilleland, H. E., Stinett, J. D. & Eagon, R. G. (1974). Ultrastructural and chemical alterations of the cell envelope of *Pseudomonas aeruginosa* associated with resistance to ethylenediamine-tetraacetate resulting from growth in Mg<sup>++</sup>-deficient medium. *Journal of Bacteriology* **117**, 302–11.
- Goldstein, A. D. B. & Lowney, L. I. (1964). Protein synthesis at 0°C in *Escherichia coli*. *Journal of Molecular Biology* **9**, 213–35.
- Hancock, R. E. W. & Nikaido, H. (1978). Outer membranes of Gram-negative bacteria. XIX. Isolation from *Pseudomonas aeruginosa* PA01 and use in reconstitution and definition of the permeability barrier. *Journal of Bacteriology* **136**, 381–90.
- Harvey, R. J. & Koch, A. L. (1980). How partially inhibitory concentrations of chloramphenicol affect the growth of *E. coli*. *Antimicrobial Agents and Chemotherapy* **18**, 323–37.
- Hurwitz, C. & Braun, C. B. (1967). Measurement of binding of chloramphenicol by intact cells. *Journal of Bacteriology* **93**, 1671–6.
- Irvin, J. E. & Ingram, J. M. (1980). Chloramphenicol-resistant variants of *Pseudomonas aeruginosa* defective in amino acid transport. *Canadian Journal of Biochemistry* **58**, 1165–71.
- Irvin, J. E. & Ingram, J. M. (1982). Divalent cation regulation of chloramphenicol resistance in *Pseudomonas aeruginosa*. *FEMS Microbiology Letters* **13**, 63–7.
- Lehninger, A. I. (1976). *Biochemistry*, p. 495. Worth Publishers, New York.
- Lennox, E. S. (1955). Transduction of linked genetic characters of host by bacteriophage P1. *Virology* **1**, 190–206.
- Levy, S. B. (1971). Physical and functional characteristics of R-factor deoxyribonucleic acid segregated into *Escherichia coli* minicells. *Journal of Bacteriology* **108**, 300–8.
- Markwell, M. A. K., Haas, S. M., Birber, L. L. & Tolbert, H. E. (1978). A modification of the Lowry procedure to simplify protein determination in membrane lipoprotein samples. *Analytical Biochemistry* **87**, 206–310.
- Nakae, T. & Nikaido, H. (1975). Outer membrane as a diffusion barrier in *Salmonella typhimurium*. *Journal of Biological Chemistry* **250**, 7359–65.
- Nierhaus, D. & Nierhaus, K. H. (1973). Identification of the chloramphenicol-binding protein in *Escherichia coli* ribosomes by partial reconstitution. *Proceedings of the National Academy of Sciences U.S.A.* **70**, 2224–8.
- Nikaido, H. (1976). Outer membranes of *Salmonella typhimurium*: Transmembrane diffusion of some hydrophobic substances. *Biochimica et Biophysica Acta* **433**, 118–32.
- Pugsley, A. P. & Schnaitman, C. A. (1978). Outer membrane proteins of *Escherichia coli*: VII. Evidence that bacteriophage-directed protein 2 functions as a pore. *Journal of Bacteriology* **133**, 1181–9.
- Vasquez, D. (1963). Antibiotics which affect protein synthesis: The uptake of <sup>14</sup>C-chloramphenicol by bacteria. *Biochemical and Biophysical Research Communications* **12**, 409–13.
- Vasquez, D. (1964). Uptake and binding of chloramphenicol by sensitive and resistant organisms. *Nature* **203**, 257–8.
- Vasquez, D. (1966). Binding of chloramphenicol to ribosomes. The effect of a number of antibiotics. *Biochimica et Biophysica Acta* **114**, 277–88.
- Weiss, R. L. (1976). Protoplast formation in *Escherichia coli*. *Journal of Bacteriology* **128**, 668–70.
- Winkler, H. H. & Wilson, T. H. (1966). The role of energy coupling in the transport of β-galactosides by *Escherichia coli*. *Journal of Biological Chemistry* **241**, 2200–11.
- Wolfe, A. D. & Hahn, F. E. (1965). Mode of action of chloramphenicol IX. Effects of chloramphenicol upon a ribosomal amino acid polymerization system and its binding to bacterial ribosome. *Biochimica Biophysica Acta* **95**, 146–5.