QUATERNARY HETEROCYCLYLAMINO β-LACTAMS

III. THE MODE OF ACTION OF L-640,876 AND THE EFFECT OF NaCl ON MEMBRANE PERMEABILITY AND BINDING

LAWRENCE R. KOUPAL, BARBARA A. PELAK, PATRICK J. CASSIDY and HANS H. GADEBUSCH

Merck Sharp & Dohme Research Laboratories
P. O. Box 2000, Rahway, New Jersey 07065, U.S.A.

(Received for publication July 12, 1982)

L-640,876, 7-β(1-benzylpyridinium-4-yl)amino-3-{[(1-methyl-1*H*-tetrazol-5-yl)thio]methyl}-ceph-3-em-4-carboxylate, is a potent representative of a new family of β-lactam antibiotics which are similar in some respects to mecillinam. When L-640,876 and mecillinam were compared for effects on growth and morphology of *Escherichia coli*, it was observed that both drugs caused the formation of lemon-shaped cells during the first 30 minutes of exposure and during this period the culture turbidity increased without an appreciable change in culture viability. Unlike mecillinam, after 60 minutes of exposure to L-640,876 the majority of the lemon-shaped cells transformed into spindle-shaped cells and in the continuing presence of the drug formed osmotically fragile spheroplasts. Membrane binding studies indicated that, like mecillinam, L-640,876 was bound to the PBP-2 of *E. coli* and *Proteus morganii*; however, some binding of L-640,876 to the PBP-3 of *E. coli* was detected. In *Staphylococcus aureus* binding differences were more evident as L-640,876 was more rapidly bound to PBP-1 and 2 whereas mecillinam was rapidly bound to PBP-3. The reversal of inhibition of certain strains of Gram-negative bacteria by high ionic strength media could not be directly attributed to a reversal of antibiotic binding to the PBPs. Permeability studies indicated that the superior potency of L-640,876 in *E. coli* was partly due to its higher concentration in the periplasm which was unaffected by the simultaneous addition of drug and NaCl, however, in cells cultured in high ionic strength medium there was a marked reduction in penetration rate of all β-lactams tested.

A newly described cephalosporin, L-640,876, 7-β-(1-benzylpyridinium-4-yl)-amino-3-{[(1-methyl-1*H*-tetrazol-5-yl)-thio]methyl}ceph-3-em-4-carboxylate, synthesized at the Merck Sharp and Dohme Research Laboratories was found to be a potent broad spectrum antibiotic.[1,2] This compound, like mecillinam, was found to be extremely potent against certain members of the *Enterobacteriaceae* and this potency could be partially reversed by increasing the ionic strength of the test medium.[2] Ionic reversal of the antibacterial potency of mecillinam has previously been described and the mechanism of this reversal is not known, although several possible explanations have been advanced.[3~6] There seems to be no ionic induction of β-lactamase activity[7] and the apparent instability of mecillinam in high ionic conditions[4] was not found with L-640,876.[1]

This similarity in bactericidal action of L-640,876 to that of mecillinam suggests that this cephalosporin shares the mode of action exhibited by the penams which lack an acylamino substituent in the 6β-position.[8~11] Studies with mecillinam indicate that at bactericidal concentrations the affected cells first become ellipsoidal and later spherical before undergoing lysis[6,10,12]. This morphological progression is thought to occur because of a specific inhibition of the rod-shape determining function associated with penicillin binding protein 2 (PBP-2). Gram-negative cells exposed to mecillinam exhibit dense cell walls and form trilamellar membranes which are part of the peripheral cytoplasmic membrane.[8]

In this report we describe the lytic cycle of *Escherichia coli* treated with L-640,876 and compare it to the cycle induced by mecillinam. The characteristic binding patterns of L-640,876 to the penicillin binding proteins of *E. coli, Proteus morganii* and *Staphylococcus aureus* are presented to further elucidate the mode of action of this new cephalosporin. The influence of ionic conditions on the strength of binding of this antibiotic to the penicillin binding proteins and on its permeability through the outer membrane of *E. coli* is also examined and discussed.

Materials and Methods

Bacterial Cultures

An R-factor carrying strain of *Escherichia coli* (RP4$^+$) MB-4351 was obtained from J. KNOWLES (Harvard University, Cambridge, MA) and was used for the permeability study. This culture along with a standard screening strain, *E. coli* MB 2884, were stored in the lyophilized state or on brain heart infusion slants at -80°C. Prior to each experiment these cultures were grown in Trypticase soy broth (TSB) at 35°C for 16~20 hours. *Proteus morganii* MB 2833 was a clinical isolate, *Staphylococcus aureus* MB 4447 was obtained from the laboratory of J. STROMINGER (Harvard University) and the *E. coli* KN 126 was obtained from B. G. SPRATT (University of Leicester, Leicester, Great Britain). *Proteus morganii* MB 2833, *S. aureus* (Smith strain) MB 2865 and *S. aureus* MB 4447 cultures were maintained by serial transfer in rich culture medium such as TSB and were stored at 4°C.

Antibiotics

The lithium salt of cefamandole and free acid of cephaloridine were supplied by the Eli Lilly Co. (Indianapolis, Indiana) and the mecillinam was provided by Leo Pharmaceutical Products (Ballerup, Denmark). L-640,876, 7-β-(1-benzylpyridinium-4-yl)amino-3-{[(1-methyl-1*H*-tetrazol-5-yl)thio]methyl}-ceph-3-em-4-carboxylate, and [^{3}H]benzylpenicillin (53 mCi/mg) were prepared at the Merck Sharp and Dohme Research Laboratories.[1,28] Commercial preparations of cephalothin and cefazolin were obtained from a local source and stock solutions of these antibiotics were corrected for potency. Unless otherwise indicated all antibiotic stock solutions were made in distilled water and were used immediately after preparation.

Bactericidal Activity

The bactericidal effect of L-640,876 and mecillinam on *E. coli* MB 2884 was determined by monitoring the turbidity of a 35°C, TSB culture with a Gilford model 250 recording spectrophotometer (Gilford Inst., Oberlin, Ohio) set at 482 nm. Each test broth was inoculated with a 3-hour TSB culture to an initial cell density of approximately 10^6 colony forming units (CFU) per ml and during the incubation period duplicate viable counts were made by a Trypticase soy agar pour plate method. A two-fold broth or agar dilution test[2] was conducted for all antibiotic minimum inhibitory concentration (MIC) determinations made in this study. Unless otherwise specified, each determination was made with approximately 10^6 CFU/ml and was incubated at 35~37°C for 16 hours before recording results. Each MIC was defined as the lowest concentration of antibiotic at which no visible turbidity was seen.

Morphology Study

Concurrent with the bactericidal activity determinations, the morphological response of *E. coli* to L-640,876 and mecillinam was examined. For photographic purposes each 1 ml sample taken from the incubating culture was centrifuged for 1 minute in an Eppendorf Model 5412 micro-centrifuge (Brinkman Inst.). A wet mount slide was made with the cell pellet and the cells examined with a Leitz Dialux phase contrast microscope (E. Leitz Inc., Germany).

Binding of β-Lactams to Penicillin Binding Proteins (PBPs)

The ability of L-640,876 and mecillinam to bind to the PBPs of bacterial membranes was determined by a modification of the competition binding technique of SPRATT[17] using [^{3}H]benzylpenicillin. The bacterial cells not grown in nutrient broth or TSB were grown in Penassay broth (Difco Antibiotic Medium No. 3). One half to 3 liters of culture was grown at 37°C on a rotary shaker to an optical density

of 0.5~1.2 at 600 nm and the cells were chilled, centrifuged and washed with 0.02 M potassium phosphate buffer, pH 7.0 before resuspension in 1/100 volume of buffer. The bacterial suspensions were lysed by sonication in 10 ml portions using a Branson Model LS-75 Sonifier for two 15 second spurts with chilling. The membranes from the lysed cells were isolated by two cycles of low and high speed centrifugation (6,000×*g* for 10 minutes followed by 40,000×*g* for 1 hour) and were resuspended in phosphate buffer to approximately 1/50 of the original culture volume. The membrane preparations were quick-frozen in dry ice-acetone and thawed on the day of use. Typical membrane protein concentrations were 1 to 5 mg/ml.

Prebinding of β-lactam to 0.2 ml membrane preparations was for 10 minutes at 30°C followed by the addition of 30 μg/ml of [^{3}H]benzylpenicillin to *E. coli* KN 126 and *P. morganii* membranes or 3~5 μg/ml to *S. aureus* membranes. Further incubation of the membranes was for 10 minutes at 30°C after which the competition reactions were terminated by the addition of 20 μl of 14% sarkosyl containing 40 mg/ml of unlabeled benzylpenicillin. After a 1-hour 40,000×*g* centrifugation all preparations were subjected to the slab gel electrophoresis system-2 technique of SPRATT *et al.*[18] The gels were fluorographed on Kodak XR-5 X-ray film as described,[18] however, in some cases En^{3}Hance (New England Nuclear Corp.) was used as the fluor which allowed shorter exposure times.

Permeability Determinations

The differential velocity of periplasmic bound β-lactamase *vs.* that of liberated enzyme was utilized to characterize the outer membrane permeability of various β-lactam antibiotics.[13~15] An exponential phase culture of *E. coli* MB 4351 was centrifuged at 5,000×*g* for 15 minutes at 2°C and the cell pellet was resuspended in cold 0.05 M sodium phosphate buffer, pH 7.0. One half of this cell suspension was lysed at 4°C by a 2-minute ultrasonic treatment with a Branson cell disruption (Branson Sonic Power Co., Danbury, Conn.). Complete disruption of the cells was verified microscopically and both the lysed and intact cell solutions were used within 2 hours of preparation. The rate of β-lactamase hydrolysis of each antibiotic was determined at 30°C in a Gilford recording spectrophotometer set at 260 nm for the cephalosporin and at 234 nm for mecillinam. Periplasmic concentration of each antibiotic was estimated using the formula of SAWAI *et al.*[14,15] and the Michaelis-Menten constants were determined by us or were taken from LABIA *et al.*[16]

In this study relative hydrophobicities of the β-lactams were determined by comparing retention time of each antibiotic at room temperature on a 0.4×30 cm μBondapak reverse-phase high pressure liquid chromatography column. The column flow rate was set at 1 ml/minute using aqueous 10% tetrahydrofuran as the solvent. The UV detector was set at 265 nm for all β-lactams tested except for mecillinam which required a detection wavelength of 230 nm. The β-lactam stock solutions were in distilled water, pH 7.0, at 1 mg/ml and 2~8 μl of samples were injected.

Results

Bactericidal Activity and Morphology Studies

The changes in viable count and culture mass of *E. coli* MB 2884 were monitored in medium containing various concentrations of L-640,876 or mecillinam (Figs. 1 and 2). After a lag of about 1 hour the bactericidal activity of each antibiotic was pronounced, with the greatest decline in viable count occurring within 3 hours. At subinhibitory concentrations of either antibiotic the optical density of the culture initially increased at a rate indistinguishable from the control; however, later both the viable count and culture density rapidly decreased. When compared to mecillinam the bactericidal effect of L-640,876 was very similar even though lower concentrations of L-640,876 were required. The MIC of L-640,876 against this *E. coli* strain was found to be 1 μg/ml after an incubation period of 16 hours in broth; however, continued incubation for 36 hours increased the MIC to 4~8 μg/ml. Microscopic observation of each antibiotic dilution revealed that even at a concentration of 0.002 μg/ml any turbidity present was solely due to the presence of pleomorphic forms which persisted throughout a 13-day

incubation period. Subculture of the pleomorphic forms in antibiotic free broth caused reversion to the normal bacillary form.

A complex sequential morphological response to *E. coli* to various concentrations of L-640,876 was seen. Immediately following exposure of the cells to the antibiotic normal cell division occurred. At 30-minute incubation the cells became larger than normal and grew ellipsoidal in shape (Fig. 3a). This cellular morphology closely resembled that induced by mecillinam but differed in that the ellipsoidal shaped cells rapidly converted to a propeller or spindle form (Fig. 3b). The spindle forms persisted for 2 hours of incubation after which the spherical, generally osmotically-fragile forms appeared. This morphological sequence did not seem to be dependent upon the concentration of L-640,876 because it occurred at all concentrations of antibiotic tested above or below the MIC. In our experiments all

Fig. 1. The effect of L-640,876 on the growth of *E. coli* MB 2884.

Antibiotic (μg/ml) was added at 0 hour; ●—●, viable count; ------, optical density of culture treated with 4 μg/ml of antibiotic.

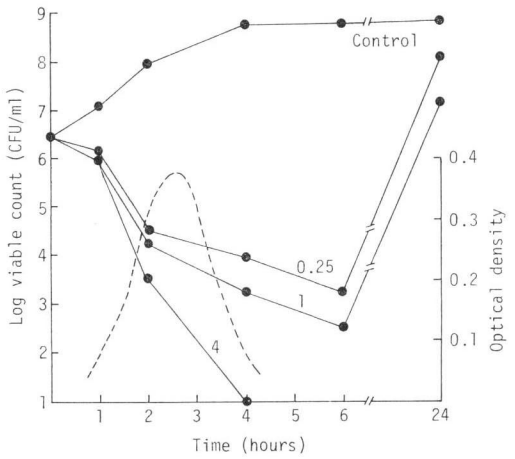


Fig. 2. The effect of mecillinam on the growth of *E. coli* MB 2884.

Antibiotic (μg/ml) was added at 0 hour; ●—●, viable count; ------, optical density of culture treated with 128 μg/ml of antibiotic.

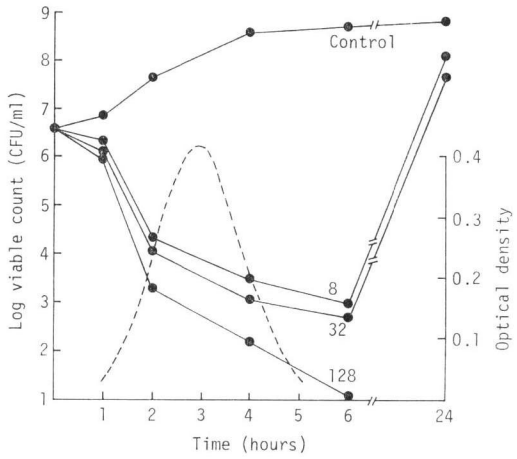
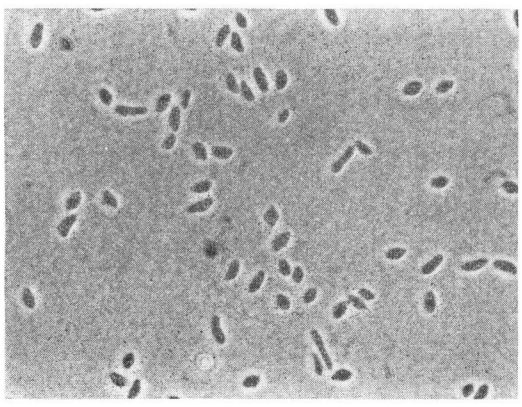
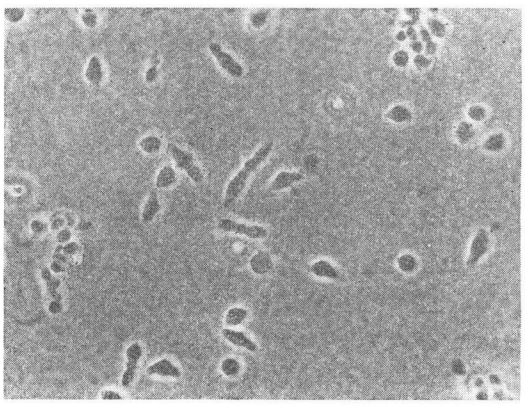


Fig. 3. Phase contrast micrographs of *E. coli* MB 2884 exposed to 0.25 μg/ml of L-640,876 and incubated at 35°C (magnification 980×).

a. Ellipsoidal cells 30 minutes after addition of L-640,876.



b. Spindle shapes appear within 60 minutes of incubation.



mecillinam concentrations of 4 to 1,000 μg/ml induced only the typical mecillinam-like ellipsoidal and later spherical forms.

Binding Studies of L-640,876 to Penicillin Binding Proteins

In competition binding studies with [^{3}H]benzylpenicillin, L-640,876 was bound almost exclusively to the PBP-2 of *E. coli* KN 127 with a 50% binding concentration (I_{50}) of about 0.01 μg/ml. Some binding to PBP-3 was also observed with a substantially higher I_{50} of 2.5 μg/ml. Under identical conditions mecillinam did not bind to PBP-3 (Fig. 4, Table 1).

Similarly the mecillinam-sensitive PBP-2 of *Proteus morganii* MB 2833 was also the exclusive target of L-640,876. The I_{50} for the PBP-2 was about 0.01 μg/ml, however, no noticeable binding of L-640,876 or mecillinam was observed to any other penicillin binding protein at concentrations as high as 2.5 μg/ml (Fig. 5, Table 1).

The addition of 1% sodium chloride to the membrane binding reactions did not effect the I_{50} of L-640,876 or mecillinam with *E. coli* and *P. morganii* membranes, nor did culture of these bacteria in media of disparate ionic strength (*i.e.* nutrient broth and TSB) noticeably alter the affinity of the PBPs to these antibiotics (Table 2).

Fig. 4. Binding patterns of L-640,876 and mecillinam to *E. coli* PBPs.

Membrane preparations of *E. coli* KN 126 were exposed to various concentrations of L-640,876 (left half) or mecillinam (right half) for 10 minutes at 30°C, followed by the addition of 30 μg/ml of [^{3}H]benzylpenicillin for an additional 10 minutes.

a) Membrane binding patterns with and without the addition of 10 mg/ml exogeneous NaCl (see Methods). b) Membrane binding patterns of cells grown in nutrient broth (NB) and binding patterns of cells grown in Trypticase soy broth (TSB). Protein bands labeled X are thought to be artifacts caused by [^{3}H]benzylpenicillin degradation products not competed for by unlabeled benzylpenicillin. The concentrations of L-640,876 and mecillinam used were (μg/ml): O, none; A, 0.004; B, 0.01; C, 0.02; D, 0.05; E, 0.10 and F, 0.20.

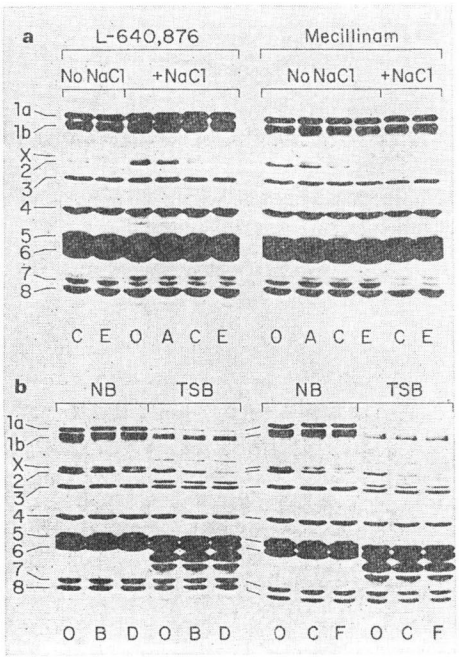


Fig. 5. Binding patterns of L-640,876 and mecillinam to *P. morganii* PBPs.

Experimental conditions were the same as those in Fig. 4.

a) The effect of the addition of 10 mg/ml exogeneous NaCl upon binding. b) Binding patterns of membranes isolated from cells grown in nutrient broth (NB) or in Trypticase soy broth (TSB). Paired gel patterns at lower right depict PBP patterns of the benzylpenicillin control of cells grown in TSB (T) and NB (N). Concentrations of L-640,876 and mecillinam used were (μg/ml): O, none; A, 0.002; B, 0.004; C, 0.01; D, 0.02; E, 0.05; F, 0.10; G, 0.20 and H, 0.25.

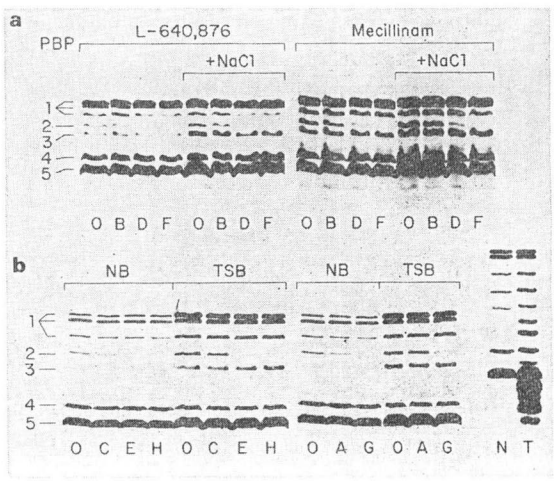


Table 1. The effect of growth medium and *in vitro* addition of NaCl to membrane preparations on the binding of L-640,876 and mecillinam to *E. coli* and *P. morganii* penicillin binding proteins.

Strain	Antibiotic	PBP[a]	Binding affinity (I_{50}, μg/ml)			
			In vitro NaCl[b] addition		Growth medium[c]	
			+	−	NB	TSB
E. coli KN 126	L-640,876	2	0.01	0.01	0.02	0.01
		3	2.5	2.5		
	Mecillinam	2	0.006	0.006	0.01	0.004
		3	>12.5	>12.5		
P. morganii MB 2833	L-640,876	2	0.01	0.01	0.01	0.015[d]
		3	>2.5	>2.5		
	Mecillinam	2	0.01	0.01	0.01	0.01[d]
		3	>2.5	>2.5		

[a] Penicillin binding protein.
[b] Growth medium was Penassay broth (Difco), addition of 10 mg/ml NaCl was to membrane preparation immediately prior to binding study.
[c] NB=nutrient broth, TSB=Trypticase soy broth.
[d] In one experiment a much higher value was obtained. See text.

Table 2. Comparison of binding affinities of L-640,876 and mecillinam to *S. aureus* penicillin binding proteins.

Strain	Antibiotic	Binding affinity to PBP (I_{50} μg/ml)[a]				MIC (μg/ml)[b]
		1	2	3	4	
H (MB 4447)	L-640,876	0.016	0.5	16	>16	1
	Mecillinam	8	16	1	>64	8
Smith (MB 2865)	L-640,876	0.031	2	>8	>8	1
	Mecillinam	8	16	4	>32	8

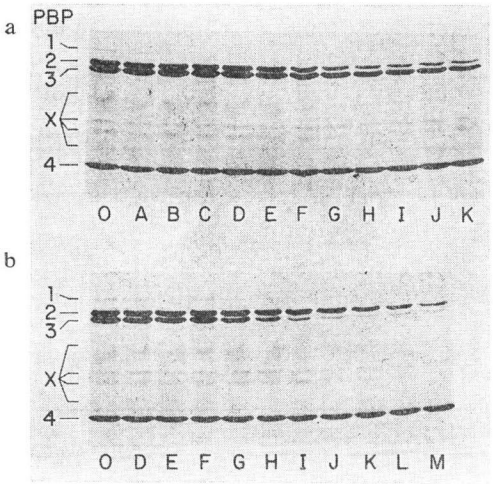
[a] Estimates of the concentration of antibiotic required to competitively reduce [^{3}H]benzylpenicillin binding by 50%. Estimated from densitometer traces and visual examination of fluorograms.
[b] A broth dilution assay with Penassay medium and an inoculum of 10^4 CFU/ml was used. The MIC was defined as the lowest concentration at which no growth or barely visible growth was seen after 18-hour incubation at 37°C.

However, in one of the three *P. morganii* membrane preparations from a TSB culture a distinctly different pattern of PBP binding was observed (Fig. 5, lines N and T). In this preparation no single PBP had the exact electrophoretic mobility of PBP-2, nor did any PBP bind L-640,876 or mecillinam at concentrations to 2.5 μg/ml. This pattern was suggestive of proteolytic degradation of the PBPs and is believed to be an artifact of the membrane preparation procedure.

The MIC of L-640,876 against the *S. aureus* strains was observed to be significantly lower than those of mecillinam.[2] The PBP pattern in the two *S. aureus* strains show that L-640,876 binds more rapidly to PBP-2 whereas mecillinam binds more rapidly to PBP-3 (Fig. 6, Table 2). This is in contrast to the binding results in *E. coli* and *P. morganii* where no significant differences exist other than the low binding affinity of L-640,876 to PBP-3. A substantial difference in the degree of binding to PBP-1 was also seen in *S. aureus*, however, this PBP is believed not to be essential to growth.[19]

Fig. 6. Binding patterns of L-640,876 and mecillinam to *S. aureus* H membranes.

Various concentrations of L-640,876 a) or mecillinam b) were incubated with *S. aureus* membranes for 10 minutes at 30°C after which 3 μg/ml [^{3}H]-benzylpenicillin was added for an additional 10 minutes. Protein bands labeled X are thought to be degradation artifacts. Antibiotic concentrations (μg/ml): O, none; A, 0.0156; B, 0.031; C, 0.062; D, 0.125; E, 0.25; F, 0.5; G, 1.0; H, 2.0; I, 4.0; J, 8.0; K, 16; L, 32 and M, 64.



Effect of NaCl on Periplasmic Concentration

SAWAI *et al.*[14,15] and ZIMMERMAN and ROSSELET[13] demonstrated that β-lactam antibiotics penetrate into the periplasm of Gram-negative bacteria at a constant diffusion rate and after a short lag period the concentration of antibiotic achieved can be easily estimated. If the starting concentrations of all β-lactams under test are equivalent the periplasmic concentration reached by each can be used to estimate the effect of NaCl on the steady state maintenance of these antibiotic concentrations in the *E. coli* periplasm.

The periplasmic concentrations of various β-lactam antibiotics including L-640,876 were estimated and compared to the corresponding MICs against *E. coli* cells which were grown in media with and without exogenous sodium chloride. The data indicated that the addition of 0.5% sodium chloride to the growth medium served to increase the MIC of L-640,876 and mecillinam by at least 10 fold, however, the MIC of cephaloridine, cefamandole, cephalothin and

Table 3. The effect of growth medium composition on periplasmic concentrations and minimum inhibitory concentration of certain β-lactam antibiotics with *Escherichia coli* MB 4351.

Antibiotic	*Km*[a] (μM)	Retention[b] time (minutes)	Culture medium			
			Basal[c]		Basal+0.5% NaCl	
			Periplasmic[d] concentration (μM)	MIC[e] (μg/ml)	Periplasmic concentration (μM)	MIC (μg/ml)
L-640,876	560	15.8	54.9	0.03	36.3	0.25
Cephaloridine	2,100	7.0	21.9	16	10.5	16
Cefamandole	670	4.7	7.3	8	2.3	8
Cephalothin	350	4.6	7.6	8	3.7	16
Mecillinam	530	4.2	7.2	4	2.5	64
Cefazolin	680	2.6	29.0	4	10.1	8

[a] The Michaelis constants for the *E. coli* TEM2 cephalosporinase were from our unpublished data and from that of LABIA *et al.*[16]

[b] Retention times were determined on a μBondapak reverse-phase high pressure liquid chromatography column. Greater retention times indicate greater hydrophobicity.

[c] The basal medium contained: 1.7% Tryptose peptone, 0.3% Phytone peptone, 0.25% potassium phosphate and 0.25% glucose.

[d] The antibiotic concentrations in the periplasm were determined with 100 μM of antibiotic outside the bacterial cell and were calculated from β-lactamase hydrolysis rates of intact and lysed cells using the formula of SAWAI *et al.*[14]

[e] The minimum inhibitory concentration (MIC) of each antibiotic was determined with an inoculum of 10^4 colony forming units in an agar dilution assay.[2]

cefazolin were not significantly affected (Table 3). The dramatic increase in MIC of mecillinam and L-640,876 by salt could not be directly attributed to an equivalent decrease in periplasmic concentration. Indeed the subculturing of this strain of *E. coli* in medium containing exogenous 0.5% sodium chloride seemed to alter the periplasmic levels of all β-lactams tested. The cephalosporin, L-640,876, suffered the lowest decrease in periplasmic concentration (approximately 33%) whereas shifts of 51% for cephalothin and 68% for cefamandole were seen. The relatively high periplasmic concentrations of cefazolin and cephaloridine were decreased by 65% and 52%, respectively, when the bacteria were subcultured under higher ionic conditions. The addition of 0.5% NaCl to cells immediately prior to the measurements had no effect on periplasmic concentration of antibiotic regardless of the cultural source of the cells.

Of the β-lactam antibiotics used in this study, L-640,876 was the most hydrophobic in character and by the criterion of retention time on a μBondapak reverse phase column, L-640,876 was twice as hydrophobic as cephaloridine (Table 3). When comparing periplasmic levels of antibiotic, L-640,876 consistently had levels of approximately double those seen with cephaloridine or cefazolin and 8 to 15 fold higher than mecillinam. The relatively high periplasmic concentration of L-640,876, may account for its potency advantage over the other β-lactams tested in this microorganism.

Discussion

The antibacterial activity of L-640,876 is similar to that of mecillinam[1,2,20,21] in that both antibiotics induce lemon-shaped forms, competitively bind mostly to PBP-2 of *E. coli* and are strongly affected by ionic growth medium components.[2,3] Studies by others with mutants of *E. coli* indicate that the major morphological effects may be attributable to inhibition of PBP-2 function.[6] However, certain differences can be distinguished in the antibacterial action of L-640,876. In *E. coli* a distinctly separate phase of induced morphogenesis occurs which manifests itself in the formation of spindle shapes. Although there is one instance in which these forms were described with extremely high concentrations of mecillinam,[11] this could not be reproduced by us using concentrations of mecillinam as high as 1 mg/ml. The induced morphogenic progression of *E. coli* from rods, to lemon-shapes, to spindle forms and finally to spheres has also been seen when synergistic combinations of mecillinam and cephalexin were used.[22] The formation of spindle-shapes with a mixture of these two antibiotics is possibly due to the simultaneous inhibition of both PBP-2 and PBP-3. With L-640,876, the apparent weak inhibition of PBP-3 would not at first seem sufficient to mimic the effect of cephalexin in the synergistic combination. However, it is possible that L-640,876 inhibits PBP-3 in a reversible fashion not observable by our binding experiments. Such a reversable inhibition could result in the close similarity of the morphological effects of L-640,876 to those of the mecillinam-cephalexin combination.

However, similarity of binding of L-640,876 and mecillinam is less evident in *S. aureus*. The MIC values for L-640,876 against two strains of *S. aureus* are 8 to 64 times lower than those of mecillinam, likewise the pattern of competitive binding to membrane protein is also different. Of the PBP's of *S. aureus*, only PBP-2 and PBP-3 appear to be required for cell-wall synthesis.[19,25] For these two, the concentration of L-640,876 required to half-saturate PBP-2 is lower than that for PBP-3. With mecillinam, the reverse relation holds. The apparently nonessential PBP-1 is half-saturated at L-640,876 concentrations lower than for any other *S. aureus* PBP. The two *S. aureus* strains used in this study appear to differ in their tolerance for PBP-3 inactivation and only a partial correlation can be seen between MIC and extent of PBP-binding. It nevertheless is clear that the similarities between the effects of L-640,876 and mecillinam on *E. coli* do not carry over to *S. aureus*. These observations demonstrate that the binding sites of critical cell wall enzymes in unrelated bacteria may differ sufficiently so that structure-activity correlations established in one species are not applicable to others.

We could not determine the reasons for the effect of ionic conditions on the antibacterial action of

L-640,876 and mecillinam although some possibilities were ruled out. No direct effect of sodium chloride or growth medium on PBP affinity of either antibiotic can be seen. The influence of salt on antibiotic permeability of the outer-membrane of Gram-negative bacteria has been suggested.[4,7,21,27] If we assume that in this case the reduction in periplasmic levels are due to altered permeability of the outer-membrane, our data indicate that growth in higher ionic conditions alters the permeability of all the β-lactams by approximately twofold. However, no direct relationship between the extent of the permeability reduction and the MIC increase could be seen, nor was there any immediate effect on permeability due to direct addition of NaCl to the cells undergoing test. The apparent reduction of the permeability of *E. coli* seems to require an adaptation period of growth and is likely to be due to repression of porin synthesis[24] rather than a direct effect of salt on the properties of the porin channels.[26,27] However, the high relative hydrophobicity and periplasmic concentration of L-640,876 compared to cephaloridine suggest that this antibiotic penetrates the outer envelope of Gram-negative bacteria in an efficient manner and may in fact penetrate *via* a route other than the hydrophilic porin channels.

Despite ionic reversals in lethal effects of L-640,876 and mecillinam the full morphological cycle of each antibiotic is usually manifested at concentrations far below the expected MIC. Since no detectable increase in β-lactamase activity[2,7] and no reversal of morphogenesis is seen, a simple ionic stabilization of the normally fragile spheroplast or some effect on the ability of the distorted cells to divide may cause retention of viability.[6]

Acknowledgment

We thank Dr. J. HANNAH of Merck Sharp & Dohme Research Laboratories for his generous assistance in the chromatographic analysis of these β-lactams.

References

1) HANNAH, J.; C. R. JOHNSON, A. F. WAGNER & E. WALTON: Quaternary heterocyclylamino β-lactams: A generic alternative to the classical aminoacyl side chain. J. Med. Chem. 25: 457~469, 1982
2) KOUPAL, L. R.; B. WEISSBERGER, D. L. SHUNGU, E. WEINBERG & H. H. GADEBUSCH: Quaternary heterocyclylamino β-lactams. II. The *in vitro* antibacterial properties of L-640,876, a new type of β-lactam antibiotic. J. Antibiotics 36: 47~53, 1983
3) TYBRING, L. & N. H. MELCHIOR: Mecillinam (FL 1060), a 6 β-amidinopenicillanic acid derivative: Bactericidal action and synergy *in vitro*. Antimicrob. Agents Chemother. 8: 271~276, 1975
4) NEU, H.: Mecillinam, a novel penicillanic acid derivative with unusual activity against Gram-negative bacteria. Antimicrob. Agents Chemother. 9: 793~799, 1976
5) GREENWOOD, D. & F. O'GRADY: FL 1060: A new beta-lactam antibiotic with novel properties. J. Clin. Path. 26: 1~6, 1973
6) SPRATT, B. B.: The mechanism of action of mecillinam. J. Antimicrob. Chemother. 3: 13~19, 1977
7) BONGAERTS, G. P. A. & K. M. BRUGGEMAN-OGLE: Effect of beta-lactamase and salt on mecillinam susceptibility of enterobacterial strains. Antimicrob. Agents Chemother. 18: 680~686, 1980
8) LORIAN, U. & B. ATKINSON: Comparison of the effects of mecillinam and 6-aminopenicillanic acid on *Proteus mirabilis, Escherichia coli* and *Staphylococcus aureus*. Antimicrob. Agents Chemother. 11: 541~552, 1977
9) LUND, F. J.: 6β-Amidinopenicillanic acids — synthesis and antibacterial properties. *In* Recent Advances in the Chemistry of β-Lactam Antibiotics. pp. 25~45, Special Publication No. 28, The Chemical Society, London, 1977
10) GREENWOOD, D. & F. O'GRADY: Trimodal response of *Escherichia coli* and *Proteus mirabilis* to penicillins. Nature 228: 457~458, 1970
11) MATSUHASHI, S.; T. KAMIRYO, P. M. BLUMBERG, P. LINNETT, E. WILLOUGHBY & J. L. STROMINGER: Mechanism of action and development of resistance to a new amidino penicillin. J. Bacteriol. 117: 578~587, 1974
12) MELCHIOR, N. H.; J. BHOM, L. TYBRING & A. BIRCH-ANDERSON: Light and electron microscopy of the early response of *Escherichia coli* to a 6 β-amidinopenicillanic acid (FL 1060). Acta Path. Microbiol. Scand. 81: 393~407, 1973

13) ZIMMERMAN, W. & A. ROSSELET: Function of the outer membrane of *Escherichia coli* as a permeability barrier to beta-lactam antibiotics. Antimicrob. Agents Chemother. 12: 368~372, 1977
14) SAWAI, T.; K. MATSUBA & S. YAMAGISHI: A method for measuring the outer membrane-permeability of β-lactam antibiotics in Gram-negative bacteria. J. Antibiotics 30: 1134~1136, 1977
15) SAWAI, T.; K. MATSUBA, A. TAMURA & S. YAMAGISHI: The bacterial outer-membrane permeability of β-lactam antibiotics. J. Antibiotics 32: 59~65, 1979
16) LABIA, R.; M. BARTHELEMY, C. FABRE, M. GUIONIE & J. PEDUZZI: Kinetic studies of three R-factor mediated β-lactamases. *In* Beta-Lactamases. J. M. T. HAMILTON-MILLER & J. T. SMITH, *Eds.*, pp. 429~442 Academic Press, London, 1979
17) SPRATT, B. G. & A. B. PARDEE: Penicillin-binding proteins and cell shape in *E. coli*. Nature 254: 516~517, 1975
18) SPRATT, B. G.; V. JOBANPUTRA & V. SCHWARZ: Mutants of *Escherichia coli* which lack a component of penicillin-binding protein 1 are viable. FEBS Lett. 79: 374~378, 1977
19) CURTIS, N. & M. V. HAYES: A mutant of *Staphylococcus aureus* deficient in penicillin-binding protein 1 is viable. FEMS Microbiol. Lett. 10: 227~229, 1981
20) LUND, F. & L. TYBRING: 6β-Amidinopenicillanic acids — a new group of antibiotics. Nature (London), New Biol. 236: 135~137, 1972
21) RICHMOND, M. H.: *In vitro* studies with mecillinam on *Escherichia coli* and *Pseudomonas aeruginosa*. J. Antimicrob. Chemother. 3: 29~39, 1977
22) OTSUKI, M.: Synergistic effect of cephalexin with mecillinam. J. Antibiotics 34: 739~752, 1981
23) CURTIS, N.; D. ORR, G. ROSS & M. BOULTON: Competition of β-lactam antibiotics for the penicillin binding proteins of *Pseudomonas aeruginosa, Enterobacter cloacae, Klebsiella aerogenes, Proteus rettgeri* and *Escherichia coli*: Comparisons with antibacterial activity and effects upon bacterial morphology. Antimicrob. Agents Chemother. 16: 325~328, 1979
24) HARDER, K. J.; H. NIKAIDO & M. MATSUHASHI: Mutants of *Escherichia coli* that are resistant to certain beta-lactam compounds lack the OMP F porin. Antimicrob. Agents Chemother. 20: 549~552, 1981
25) HAYES, M.; N. CURTIS, A. W. WYKE & J. B. WARD: Decreased affinity of a penicillin-binding protein for β-lactam antibiotics in a clinical isolate of *Staphylococcus aureus* resistant to methicillin. FEMS Microbiol. Lett. 10: 119~122, 1981
26) NIKAIDO, H.: Permeability of the outer membrane of bacteria. Angew. Chem. 18: 337~420, 1979
27) VAN ALPHEN, W.; N. VAN SELM & B. LUYTENBERG: Pores in the outer membrane of *Escherichia coli* K12: Involvement of proteins b and e in the functioning of pores for nucleotides. Molec. Gen. Genet. 159: 75~83, 1978
28) ROSEGAY, A.: High specific activity [phenyl-^{3}H]benzylpenicillin *N*-ethylpiperidine salt. J. Labelled Cmpds. Radio Pharm. 18: 1337~1340, 1981