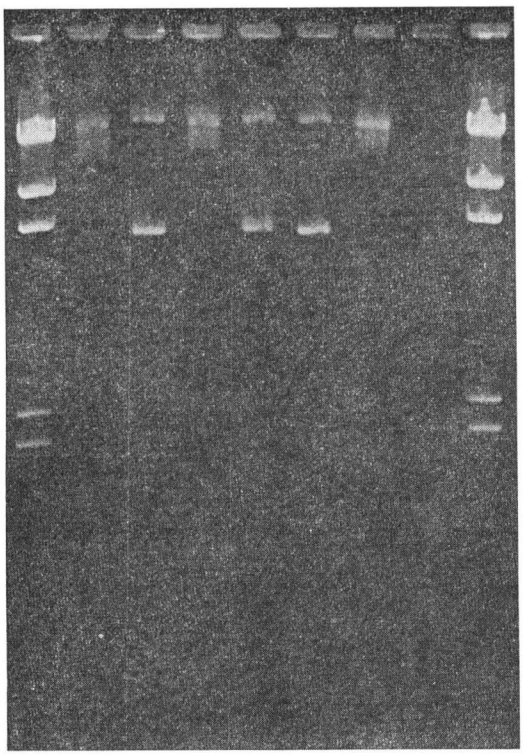
Table 1. Effect of key novobiocin fragments one semiconservative DNA replication in toluenized *E. coli* cells.

Sample		[^{3}H]TMP incorporated (cpm/sample)	Inhibition (%)
Control		17,520	0
Novobiocin sodium	0.05 mM	1,200	94
	0.01 mM	2,940	83
Novenamine	0.05 mM	2,370	87
	0.01 mM	6,040	66
Novobiocic acid	0.05 mM	16,010	9
	0.01 mM	16,750	5
Ethyl novioside	0.05 mM	17,000	3
	0.01 mM	19,000	0

Reaction mixtures contained in a total volume of 0.3 ml: Tris-HCl buffer (pH 8.0) 50 mM, KCl 0.1 M, magnesium acetate 10 mM, ATP 2 mM, dCTP, dGTP, dATP 0.5 mM each, [^{3}H]TTP 0.02 mM containing 2.5 μCi/sample and approximately 2×10^8 *E. coli* cells/sample. Incubation of the reaction mixtures was for 30 minutes at 37°C.

Fig. 2. Sensitivity of DNA gyrase to novobiocin fragments.

Lanes from left to right: λ DNA-*Hin*d III digest, standard; relaxed circular pBR322 DNA, no gyrase; relaxed pBR322 plus gyrase; relaxed pBR 322 plus gyrase plus novobiocin; relaxed pBR322 DNA plus gyrase plus novobiocic acid; relaxed pBR 322 plus gyrase plus benzoic acid derivative; relaxed pBR322 plus gyrase plus novenamine; λ DNA-*Hin*d III digest, standard. Drug concentrations were 0.1 mM. Samples were separated on 1% agarose gel electrophoregrams.



phate buffer containing 1% toluene and stirred at room temperature for 10 minutes[4,5]. Following toluene treatment the cells were removed by centrifugation, the pellet and tube walls were thoroughly rinsed with 0.05 M potassium phosphate buffer, and the pellet was resuspended with the same buffer in 0.1 vol of the original culture medium. This cell suspension was divided into aliquots which were stored above liquid nitrogen until used. Reaction mixtures to assess ATP-dependent DNA replication contained in a total volume of 0.3 ml: Tris-HCl buffer (pH 8.0) 50 mM; KCl 0.1 M, magnesium acetate 10 mM, ATP 2 mM, dCTP, dGTP, dATP 0.5 mM each, [^{3}H]-TTP 0.02 mM containing 2.5 μCi/sample and approximately 2×10^8 cells/sample. Incubations were carried out at 37°C for 30 minutes. The reaction was terminated by the addition of 3-ml portions of cold 10% trichloroacetic acid containing 0.1 M sodium pyrophosphate. The precipitates were collected on 0.45 μm Millipore filters and washed extensively with cold 0.1 N HCl. Radioactivity was determined by conventional liquid scintillation spectrometry techniques.

DNA Gyrase

DNA gyrase isolated from *Micrococcus luteus* was obtained from Bethesda Research Labs., Gaithersburg, Maryland. The enzyme was assayed in reaction mixtures containing in a total volume of 25 μl: Tris-HCl (pH 7.5) 35 mM, $MgCl_2$ 20 mM, KCl 20 mM, EDTA·Na_2 0.1 mM, 2-mercaptoethanol 10 mM, spermidine 2 mM, ATP 1 mM, 10% glycerol, 0.5 μg of relaxed covalently closed pBR322 DNA and 1

unit of gyrase. Incubation was at 37°C for 30 minutes.

Relaxed circular covalently closed DNA was prepared by digesting supercoiled pBR322 DNA with a ten-fold excess of *Eco*R I restriction enzyme followed by ligation with T_4 DNA ligase according to standard procedures. The religated product obtained consisted mostly of dimers of pBR322.

Results and Discussion

DNA Replication in Toluenized *E. coli* Cells

The parent compound novobiocin caused almost complete cessation of replicative DNA synthesis when present at a concentration of 0.05 mM (Table 1). Ethyl novioside (the sugar portion of novobiocin) and novobiocic acid were both inactive. Novenamine on the other hand, was nearly as inhibitory as novobiocin itself at a concentration of 0.05 mM.

DNA Gyrase

Novobiocin, as expected, did inhibit the formation of supercoiled DNA from the circular relaxed form of pBR322 (Fig. 2). Novobiocic acid and the benzoic acid moieties each did not inhibit DNA gyrase. Novenamine proved as inhibitory as novobiocin in this system. All compounds were studied at 0.1 mM concentrations.

Only novobiocin exerts significant antibacterial activity against whole *Staphylococcus aureus* cells whereas all the novobiocin fragments studied are virtually inactive against this organism. None of the fragments tested inhibits whole *E. coli* cells and novobiocin itself is a very weak inhibitor of this latter organism but it is very potent in toluenized *E. coli* cells. The reason that novobiocin has only weak activity against whole *E. coli* cells must thus be due to insufficient penetration of the *E. coli* cell envelope by the drug.

Novenamine is essentially as active as novobiocin in inhibiting DNA replication and DNA gyrase. Since none of the other fragments tested exerted any inhibition of DNA replication or DNA gyrase, novenamine represents the minimal structural entity present within the novobiocin molecule essential for interaction with DNA gyrase. The fact that novenamine is inactive against whole cells suggests that this molecule can not penetrate the cell envelope efficiently and in turn this indicates that the benzoic acid moiety which does not interact with DNA gyrase fulfills a transport function only in intact novobiocin.

References

1) STAUDENBAUER, W. L.: Novobiocin — A specific inhibitor of semiconservative DNA replication in permeabilized *Escherichia coli* cells. J. Mol. Biol. 96: 201~205, 1975
2) REUSSER, F.: Ficellomycin and feldamycin; inhibitors of bacterial semiconservative DNA replication. Biochemistry 16: 3406~3412, 1977
3) GELLERT, M.; M. H. O'DEA, T. ITOH & J. T. TOMIZAWA: Novobiocin and coumermycin inhibit DNA supercoiling catalyzed by DNA gyrase. Proc. Natl. Acad. Sci. U.S.A. 73: 4474~4478, 1976
4) MOSES, R. E. & C. C. RICHARDSON: Replication and repair of DNA in cells of *Escherichia coli* treated with toluene. Proc. Natl. Acad. Sci. U.S.A. 67: 674~681, 1970
5) MOSES, R. E.: Replicative DNA synthesis in a system diffusible for macromolecules. J. Biol. Chem. 247: 6031~6038, 1972

EXAMINATION OF MODEL ENZYME AND PENETRATION SYSTEMS IN RELATION TO ANTIBACTERIAL ACTIVITY

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Recent work has shown that the activity of cephalosporins in inhibiting exocellular DD-peptidases from *Streptomyces* R61 and *Actinomadura* R39 are, at best, only poorly related to minimum inhibitory concentrations against pathogenic isolates. Taking into account the rate at which cephalosporins diffuse through porin channels, such as exist in certain Gram-negative organisms, does not help in establishing a relationship between MIC data and the kinetic data on the model enzymes. Most published cell wall permeability studies, the porin ones being a principal exception, have not examined long enough series of structurally related compounds to establish property-activity relationships.

The end point of an MIC assay is the summation of many physical and biochemical events. In order for a β-lactam antibiotic to prevent bacterial growth, it must be able to reach and inhibit target enzymes. The enzymes thought to be associated with killing of bacteria are the high molecular weight transpeptidases in the periplasm of the bacterial envelope[1]. In order to reach these sites the antibiotic molecules must diffuse through the thick peptidoglycan layer of Gram-positive bacteria or through the outer lipid bilayer and peptidoglycan meshwork of Gram-negatives[2]. The rate of entry must allow both specific and nonspecific sites to be occupied and must exceed the rate at which β-lactamases[3] can detoxify the inhibitors. The antibiotic molecules that do reach the active site of target enzymes must have appropriate electronic and steric properties to be recognized as a substrate or transition state analog of the X-D-alanyl-D-alanine substrate[1,4]. The β-lactam ring has to be reactive enough[4] that when the antibiotic molecule collides with the active site serine[5], a covalent ester linkage will form. The half-life of the acylated transpeptidase needs to be long enough that turnover of the enzyme is adversely affected, so that normal bacterial autolysins can operate on the peptidoglycan without a balancing biosynthesis of new crosslinked peptidoglycan[6,7]. The net result is an aberrant cell wall which is not sustainable.

Numerous investigations have separately addressed the behavior of β-lactam antibiotics in β-lactamase resistance, permeability, and enzymic inhibitory assays. Experiments on the last two factors usually rest on some assumptions and have not always been able explain relative microbiological potency of well studied antibiotics. If structure-property relationships were to be established, they could be very valuable in prospectively evaluating the potential activity of new antibiotic structures.

ZIMMERMANN and ROSSELET assumed a steady state model and used β-lactamase hydrolysis rates of intact and sonically disrupted cells to compute the concentration of six cephalosporins and two penicillins inside the outer membrane of *Escherichia coli*[8,9]. More hydrophili compounds were found to have higher concentrations inside the cells, but this does not translate into better *E. coli* MICs. For instance, the periplasmic concentration reached by cephacetrile is ten-fold higher than that of cephalothin, and the diffusion parameter C of cephacetrile is seven times higher than that of cephalothin. Yet the MIC against *E. coli* of the latter compound is two times better than that of cephacetrile[10].

SAWAI, *et al.*, evaluated the ability of five cephalosporins and two penicillins to pass through the outer membrane of *Proteus morganii*, *Citrobacter freundii*, and *E. coli*[11]. This work showed that barriers vary from species to species and that there is a very rough correlation indicating more hydrophilic compounds penetrate better. For this limited number of compounds, a statistically acceptable correlation between the periplasmic concentration of antibiotic and MICs only appears to exist for *E. coli*.

In another study, permeability coefficients were measured for six cephalosporins in regard to penetration of the outer membrane of *Enterobacter cloacae*[12], but the coefficients do not correlate with the MICs. Similarly, no general correlation exists between antibacterial concentration and periplasmic concentration for a set of eight cephalosporins and 1-oxacephalosporins[13]. Still another study determined the transmembrane permeability coefficient for seven cephalosporins and three penicillins[14]. Although the ability to diffuse across the outer membrane of *Haemophilus influenzae* type b is related to the lipophilicity of the compounds, analysis of the data fails to reveal any correlation between permeability coefficients and MICs for either a β-lactamase-positive or a β-lactamase-negative strain of this organism.

A very recent permeability study[15,16] is especially significant because a large enough number of compounds were examined to allow some conclusions to be reached about structure-property relationships. Penetration through the porin channels of *E. coli* K-12 in reconstituted proteoliposomes was determined for a wide range of cephalosporins and other β-lactam antibiotics of current interest[15,16]. In contrast to the murein layer, the outer membrane of Gram-negative organisms presents a formidable barrier to the diffusion of β-lactam antibiotics. Porins are thought to be trimeric[16] or dimeric[17] protein structures which form a channel through the outer membrane of enteric bacteria. The rate of diffusion of cephalosporins through porins depends not just on lipophilicity, but also on ionic charge and steric properties[15]. Some compounds with excellent antibacterial activity, such as cefotaxime and other oxime-containing compounds, pass through porins more poorly than expected. The steric rigidity of the acylamino side chain may retard passage. YOSHIMURA and NIKAIDO concluded that resistance to β-lactamases compensates for the low penetration rate of these compounds, so that an effective periplasmic concentration can, nevertheless, be achieved[15].

It has been pointed out that whereas cephalosporins appear to pass through the outer membrane *via* porins, penicillins do not[18]. The mode of penetration varies not only with organism, but also with compound.

Regarding the other factor of enzyme inhibition, MIRELMAN attempted to use *Pseudomonas aeruginosa* cells permeabilized by ether treatment to biochemically assess intrinsic activity, *i.e.*, ability of a compound to inhibit transpeptidase action isolated from the influences of penetration and β-lactamases[19]. However, only a modest correspondence between the enzymic inhibitory activities and MICs could be established for cephalosporins (D. B. BOYD, unpublished work).

Other working models of intrinsic activity are the DD-peptidases from *Streptomyces* R61 and *Actinomadura* R39[20,21]. These β-lactam-sensitive transpeptidases have been subjected to exhaustive biochemical studies because they can be isolated and purified in stable, active form, unlike the target penicillin-binding proteins. Recently enough data for these two model enzymes have emerged to show that they generally have a profile of sensitivity to caphalosporins quite unlike those of 20 clinically important Gram-positive and Gram-negative pathogens[22]. In other words, the model enzymes respond differently to cephalosporins than do membrane-bound PBPs.

Table 1. Organisms studied to determine if MICs can be regressed on data from the model systems.

Staphylococcus aureus X1.1	*Klebsiella pneumoniae* X26
〃 〃 V41	〃 〃 X68
〃 〃 X400	*Enterobacter aerogenes* C32
S. epidermidis Epil	*E. cloacae* EB5
Streptococcus pyogenes C203	*Salmonella heidelberg* X514
Group D *Streptococcus* X66	*Pseudomonas aeruginosa* X528
Haemophilus influenzae C.L.	*Serratia marcescens* X99
Escherichia coli N10	*Shigella sonnei* N9
〃 〃 EC14	*Norganella morganii* PR15
〃 〃 TEM	*Providencia rettgeri* C24

This article addresses the question of whether kinetic constants for the DD-peptidases can be related to microbiological activity if available permeability data are taken into account. Eight of the cephalosporins studied by the Liege group[20,21] were also studied by YOSHIMURA and NIKAIDO[15]. Having a larger number of compounds in a closely related series would be highly preferable. However, it is worth-while to proceed with the available data while being mindful that statistical abnormalities may arise.

MICs of the eight compounds against 20 pathogens (Table 1) are reported elsewhere[22]. Rates of diffusion through the OmpF porin of *E. coli* K-12[15] are expressed relative to that of cephacetrile. Kinetic constants[20,21] for the R61 and R39 DD-peptidases include k_2/K, k_3, and $k_2/(k_3K)$. K is the dissociation constant of the reversible enzyme-inhibitor complex; k_2 is the rate of acylation, and k_3 is the rate of release of the degradation product(s) from the enzyme.

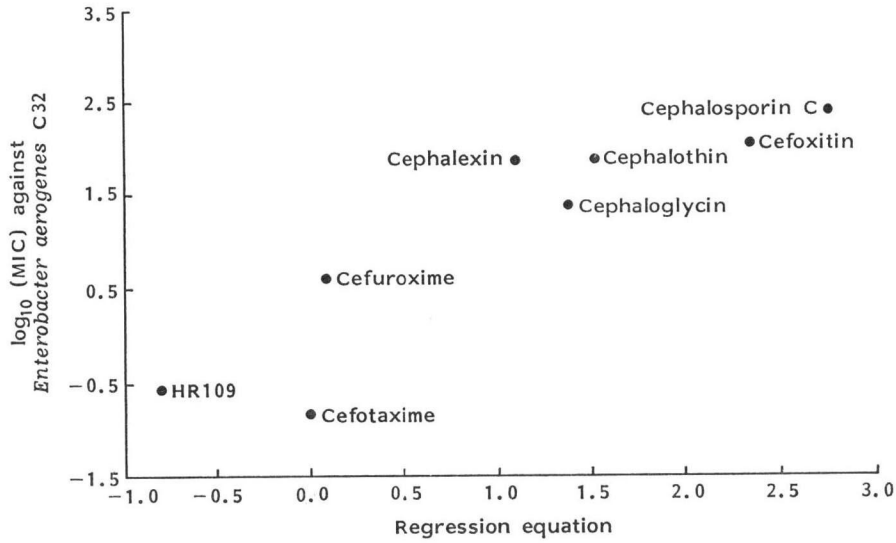
$$E+I \overset{K}{\rightleftharpoons} EI \xrightarrow{k_2} EI^* \xrightarrow{k_3} E+P$$

The statistics programs MINITAB (Minitab Project, The Pennsylvania State University, University Park, Pennsylvania, U.S.A., 1982) and STATPACK (R. HOUCHARD, Western Michigan University, Kalamazoo, Michigan, U.S.A., 1974) were used to determine which, if any, of the MIC's for the 20 organisms could be linearly regressed on two independent variables: (1) one of the six kinetic constants mentioned above and (2) relative diffusion rate R. All quantities were treated in $\log_{10}$ units. Altogether over 120 regression equations were evaluated in terms of (a) the amount of variance in the MICs explained (r^2 in percent), (b) the ratios of the coefficients to the standard deviation of the coefficient, and (c) the probability of the null hypothesis being satisfied.

The strongest correlation found (Fig. 1) involves *Enterobacter aerogenes* C32 MICs and k_2/K for the R61 enzyme. Other strong regressions relate log R and R61 k_2/K to MICs for additional enteric Gram-negatives, *Shigella sonnei* N9, *Klebsiella pneumoniae* X26, and *K. pneumoniae* X68. All these two variable regressions are able to explain 65% or more of the variance in the MIC data and had T-ratios of 2 or more for each term in the regression equation. For the eight compounds in Fig. 1, no strong correlations are found between MICs and the porin R values alone.

When the data in Fig. 1 are examined carefully, one realizes that the *E. aerogenes* correlation is, in fact, "counter-intuitive". The oxime-containing compounds, cefotaxime, HR109, and cefuroxime, are at the low MIC end of Fig. 1, yet are relatively poor at inhibiting DD-peptidases[20,21] and diffusing through porins[15]. At the high MIC end is cephalosporin C, which passes through porins over three times more rapidly than cefotaxime[15] and is exquisitely potent at inhibiting the R39 and especially R61 DD-peptidases[20,21]. Fig. 1 is thus a case of statistics happening to explain the observations when

Fig. 1. Experimental MICs against *Enterobacter aerogenes* C32 plotted against MICs predicted by MIC= $-(3.85\pm1.12)+(0.85\pm0.20)\log(k_2/K)+(2.11\pm0.59)\log R$, n=8, r=0.91, r^2=83%, s=0.63, p=1.24%.



the number of observations is small and the number of variables is sufficient (two in this case).

One must still deal with the fact that for none of the 20 organisms are sensible correlations to be found. One reason is that there are fundamental chemical and physical differences between PBPs and DD-peptidases[22]. Another equally important reason is that there are multiple β-lactam-sensitive proteins, each with selective functions, catalytic activities, and affinities for structurally different antibiotics[1]. Even if a target membrane-bound β-lactam-binding protein can be isolated and studied, it will be challenging to relate the kinetic properties of that one enzyme to microbiological activity. In order to ferret out whatever relationships may exist between whole organism data and model system data, future experiments on biochemistry and penetration should be designed such that structurally related sets of antibiotics are examined and that the number of compounds is large (at least 15~20, preferably 30~40). The number of compounds must be large enough that β-lactamase susceptibilities can be included in the regression analysis as an additional independent variable. In the meantime, data from the model systems can be suggestive of the roles of intrinsic activity and permeability and will have to form the basis of judicious inferences about these factors in property-activity relationships.

References

1) WAXMAN, D. J. & J. L. STROMINGER: β-Lactam antibiotics: Biochemical modes of action. *In* Chemistry and Biology of β-Lactam Antibiotics. Vol. 3. *Eds.*, R. B. MORIN & M. GORMAN, pp. 209~285, Academic Press, New York, 1982
2) COSTERTON, J. W.: Cell envelope as a barrier to antibiotics. *In* Microbiology-1977. *Ed.*, D. SCHLESSINGER, pp. 151~157, American Society of Microbiology, Washington, D.C., 1977
3) KNOWLES, J. R.: Penicillin resistance: The chemistry of β-lactamase inhibition. Acc. Chem. Res. 18: 97~104, 1985
4) BOYD, D. B.: Theoretical and physicochemical studies on β-lactam antibiotics. *In* Chemistry and Biology of β-Lactam Antibiotics. Vol. 1. *Eds.*, R. B. MORIN & M. GORMAN, pp. 437~545, Academic Press, New York, 1982
5) KECK, W.; B. GLAUNER, U. SCHWARZ, J. K. BROOME-SMITH & B. G. SPRATT: Sequences of the active-site

peptides of three of the high-M_r penicillin-binding proteins of *Escherichia coli* K-12. Proc. Natl. Acad. Sci. U.S.A. Biol. Sci. 82: 1999~2003, 1985

6) TIPPER, D. J.: Mode of action of β-lactam antibiotics. Pharmacol. Ther. 27: 1~35, 1985
7) TOMASZ, A.: From penicillin-binding proteins to the lysis and death of bacteria: A 1979 view. Rev. Infect. Dis. 1: 434~467, 1979
8) ZIMMERMANN, 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
9) ZIMMERMANN, W.: Penetration through the Gram-negative cell wall: A co-determinant of the efficacy of beta-lactam antibiotics. Int. J. Clin. Pharmacol. Biopharm. 17: 131~134, 1979
10) GORMAN, M. & C. W. RYAN: Structure-activity relationships of β-lactam antibiotics. *In* Cephalosporins and Penicillins: Chemistry and Biology. *Eds.*, E. H. FLYNN, pp. 532~582, Academic Press, New York, 1972
11) SAWAI, T.; K. MATSUBA, A. TAMURA & S. YAMAGISHI: The bacterial outer-membrane permeability of β-lactam antibiotics. J. Antibiotics 32: 59~65, 1979
12) KOJO, H.; Y. SHIGI & M. NISHIDA: *Enterobacter cloacae* outer membrane permeability to ceftizoxime (FK 749) and five other new cephalosporin derivatives. J. Antibiotics 33: 317~321, 1980
13) MURAKAMI, K. & T. YOSHIDA: Penetration of cephalosporins and corresponding 1-oxacephalosporins through the outer layer of Gram-negative bacteria and its contribution to antibacterial activity. Antimicrob. Agents Chemother. 21: 254~258, 1982
14) COULTON, J. W.; P. MASON & D. DORRANCE: The permeability barrier of *Haemophilus influenzae* type b against β-lactam antibiotics. J. Antimicrob. Chemother. 12: 435~449, 1983
15) YOSHIMURA, F. & H. NIKAIDO: Diffusion of beta-lactam antibiotics through the porin channels of *Escherichia coli* K-12. Antimicrob. Agents Chemother. 27: 84~92, 1985
16) NIKAIDO, H.: Role of permeability barriers in resistance to β-lactam antibiotics. Pharmacol. Ther. 27: 197~231, 1985
17) ZALMAN, L. S. & H. NIKAIDO: Dimeric porin from *Paracoccus denitrificans*. J. Bacteriol. 162: 430~433, 1985
18) SAWAI, T.; A. YAMAGUCHI, R. HIRUMA & M. KANEKO: Mechanism of outer membrane permeation of antibiotics in Gram-negative bacteria. J. Pharmacobiodyn. 8: s45, 1985
19) MIRELMAN, D. & Y. NUCHAMOWITZ: Biosynthesis of peptidoglycan in *Pseudomonas aeruginosa*. 2. Mode of action of β-lactam antibiotics. Eur. J. Biochem. 94: 549~556, 1979
20) FRÈRE, J.-M. & B. JORIS: Penicillin-sensitive enzymes in peptidoglycan synthesis. CRC Crit. Rev. Microbiol. 11: 299~396, 1985
21) GHUYSEN, J.-M.; J.-M. FRÈRE, M. LEYH-BOUILLE, M. NGUYEN-DISTECHE, J. COYETTE, J. DUSART, B. JORIS, C. DUEZ, O. DIDEBERG, P. CHARLIER, G. DIVE & J. LAMOTTE-BRASSEUR: Bacterial wall peptidoglycan, DD-peptidases, and β-lactam antibiotics. Scand. J. Infect. Dis., Suppl. 42: 17~37, 1984
22) BOYD, D. B. & J. L. OTT: Lack of relevance of kinetic parameters for exocellular DD-peptidases to minimum inhibitory concentrations of cephalosporins. Antimicrob. Agents Chemother., submitted