Microbial Kinetics of β-Lactam Antibiotics against *Escherichia coli*

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Abstract □ Microbial kinetics of *Escherichia coli* NIHJ JC-2 and *E. coli* B/r were investigated in the presence of β-lactam antibiotics. To maintain a constant drug concentration during the experiment, a novel technique, using a dialysis membrane tube containing the drug solution, was successfully employed. The drug-affected generation curves of *E. coli* exhibited common features. After the addition of drug, an apparent lag period was noted, followed by a first-order decrease of the sensitive organisms and, 6 h later, by a regrowth of resistant organisms, depending on the antibiotic concentration used. The relationship between the apparent generation rate constant, k_{app}, and the antibiotic concentration was found to be nonlinear. This phenomenon is consistent with a saturable receptor site model for the drug action. A good linear free energy relationship was observed between the microbial kinetic parameter, k_{max}, and the alkaline degradation rate constants, k_{OH}, of the cephalosporins studied.

Keyphrases □ Kinetics—microbial, β-lactam antibiotics against *Escherichia coli* □ β-Lactam antibiotics—microbial kinetics against *Escherichia coli*

In dosage regimens for infectious diseases, plasma levels of antibiotics based on the value of minimum inhibitory concentration (MIC) have been used as convenient indices of pharmacological effectiveness. The MIC is defined as the lowest concentration that results in complete inhibition of microbial generation during prolonged contact with an antimicrobial agent (~20 h). However, the quantitative meaning of the MIC is still obscure, and the value does not give any indication of the kinetic behavior of microbial generation in relation to the concentration of the drug remaining in the body at relatively short times after administration of an antibiotic. To evaluate the potency or efficacy of antibiotics more effectively and to establish more reasonable therapeutic guidelines and dosage regimens, it is necessary to investigate the microbial generation kinetics as a function of antibiotic concentration.

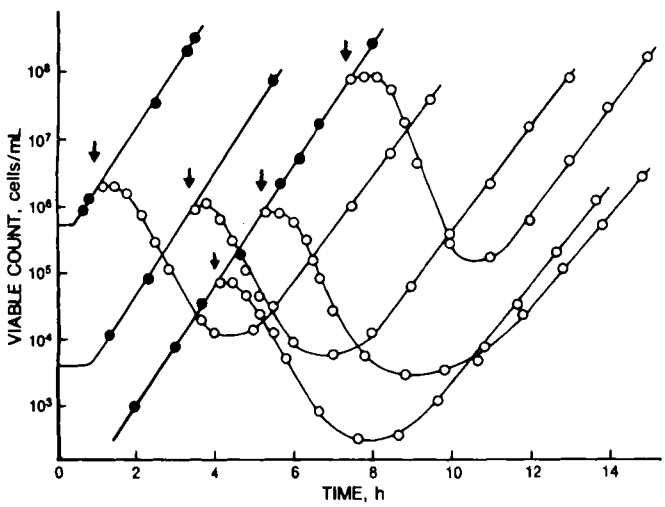

Figure 1—*Effect of organism population at the time of drug addition on the generation curves of* E. coli *B/r in the presence of 0.5 μg of ampicillin/mL and effect of inocula sizes on the generation curves of* E. coli *B/r in antibiotic medium[9] at pH 7.0 and 37°C. The time of drug addition is indicated by the arrow. Key: (●) control generation curves; (○) drug-affected generation curves.*

Several microbial kinetic studies in the presence of antibacterial agents have been reported (1–10); however, there are only a few reports on β-lactam antibiotics (4, 8). Elkhouly and Führer (8) have determined the apparent generation rate constants of *Escherichia coli* in the presence of various concentrations of ampicillin; however, it remains uncertain whether the apparent generation rate constants can be validly expressed as a function of drug concentration under their experimental conditions, because chemical and/or enzymatic degradation of ampicillin may occur under the conditions used in the study. To overcome this possible degradation, we introduced a novel method to keep the drug concentration constant.

The purposes of this study were to establish suitable experimental conditions for the microbial kinetic study of β-lactam antibiotics at constant drug concentrations and to elucidate the kinetic behavior of microbial generation in the presence of β-lactam antibiotics.

EXPERIMENTAL SECTION

Antibiotics—Penicillin G potassium[1] (1600 U/mg), ampicillin sodium[2] (955 μg/mg), methicillin sodium[3] (853 μg/mg), dicloxacillin sodium[2] (900 μg/mg), cefazolin sodium[4] (958 μg/mg), cephalexin monohydrate[5] (925 μg/mg), cefadroxil monohydrate[6] (947 μg/mg), and cephaloridine[5] (971 μg/mg) were used as received. All other chemicals, unless otherwise stated, were of reagent grade and were not further purified.

Organisms—Replicate slants of *E. coli* NIHJ JC-2[7] and *E. coli* B/r[8] [the same strains as used previously (4)] were used in the microbial kinetic study. The slants were prepared from single isolated colonies of the respective organisms and were stored at 4°C.

Culture Medium—Antibiotic medium[9] was rehydrated according to the specifications of the manufacturer and filtered twice through a 0.45-μm membrane filter[10]. This pH 7.0 medium was used for all experiments in the microbial generation studies. The medium was autoclaved at 120°C for 20 min.

Bacterial Generation—An aliquot (5 mL) of culture medium was inoculated from a fresh slant, and the culture was allowed to grow for 15 h at 37°C in an incubator. Samples of this culture were appropriately diluted in several steps to achieve an organism concentration of 10^4 cells/mL.

Two 0.5-mL portions of this culture were finally diluted into 49.5 mL of fresh medium for the control growth and 49.0 mL of fresh medium for the drug-affected growth, respectively. The cultures were maintained at 37°C in a constant-temperature water bath equipped with a shaker.

All pipets and media used were kept at 37°C to protect the organisms from temperature shock.

[1] Meiji Seika Kaisha, Tokyo, Japan.
[2] Takeda Chemical Industries, Osaka, Japan.
[3] Banyu Pharmaceutical Co., Osaka, Japan.
[4] Fujisawa Pharmaceutical Co., Osaka, Japan.
[5] Shionogi Co., Osaka, Japan.
[6] Bristol Meyers Co., Tokyo, Japan.
[7] IFO 12734; Institute for Fermentation, Osaka, Japan.
[8] F-57; Institute for Fermentation.
[9] Antibiotic Medium 3; Difco Laboratories, Detroit, Mich.
[10] Sartorius-Membranfilter GmbH, Göttingen, Federal Republic of Germany.

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Table I—Saturable Kinetic Parameters for *E. coli* NIHJ JC-2 and *E. coli* B/r in Antibiotic Medium [a]

Antibiotic	$10^4 k_{max}$, s^{-1}		S_b, μg/mL		C_{mec}, μg/mL	
	NIHJ JC-2	B/r	NIHJ JC-2	B/r	NIHJ JC-2	B/r
Penicillin G	83.8	28.4	56.6	3.80	4.98	3.06
Ampicillin	60.1	30.4	4.83	0.24	0.41	0.35
Methicillin	—	28.1	—	1.74	—	2.71
Dicloxacillin	—	22.4	—	2.44	—	3.32
Cefazolin	47.7	—	1.68	—	1.30	—
Cephalexin	16.9	—	0.60	—	1.76	—
Cefadroxil	27.9	—	2.56	—	3.69	—
Cephaloridine	87.6	—	9.28	—	0.78	—

[a] At pH 7.0 and 37°C.

Viable Count Method—Samples (0.5 mL) were withdrawn from the cultures and serially diluted into sterilized 0.9% NaCl solution in accordance with a preplanned dilution scheme so that 50–150 colonies per plate would be obtained. From these dilutions, aliquots of 0.5 mL were transferred onto each of three replicate agar plates. The plates were incubated for 20 h at 37°C, and the resulting colonies were counted with an electronic colony counter.

Effect of Shaking Speed on Generation Rates—Four replicate, 49.5-mL volumes of the fresh medium were each inoculated with 0.5 mL of appropriately diluted *E. coli* NIHJ JC-2 culture in the logarithmic growth phase. They were cultured at 37°C and pH 7.0 under four different shaking conditions (0, 82, 130, and 190 strokes/min). The viable counts were determined in samples withdrawn at appropriate time intervals.

Effect of Dialysis Membrane Tube—Fresh solutions of ampicillin of two different concentrations were aseptically prepared for each experiment. They were diluted suitably so that aliquots of 0.5 mL added to replicate 49.5-mL volumes of the *E. coli* NIHJ JC-2 seeded culture yielded final concentrations of 4.0 and 1.25 μg/mL. The solutions were added to the cultures growing at 37°C in the logarithmic phase at an organism concentration of about 10^6 cells/mL. A cellulose tube[11] was knotted at both ends to form a bag (length, 10 cm). The bag, which contained 10 mL of ampicllin solution prepared with fresh culture medium to produce the same concentration as the experimental antibiotic concentration, was placed in the culture medium immediately after the drug was added. The dialysis membrane tube was replaced with a fresh tube every 3 h. At appropriate time intervals, aliquots of 0.5 mL were withdrawn, and the ampicillin concentration was assayed by the usual microbial paper disk method employing *Sarcina lutea*[12] as the test organism.

Effect of Organism Population on Drug-Affected Generation Rates—Three sets of the culture medium, with initial inoculum sizes of 6×10^4, 6×10^5, and 6×10^6 cells/mL, were prepared and used to determine generation curves of the *E. coli* B/r culture in the presence of 0.5 μg of ampicillin/mL at 37°C. The viable counts were obtained at appropriate time intervals.

Effect of Antibiotic Concentrations on Generation Rates—Aliquots (0.5

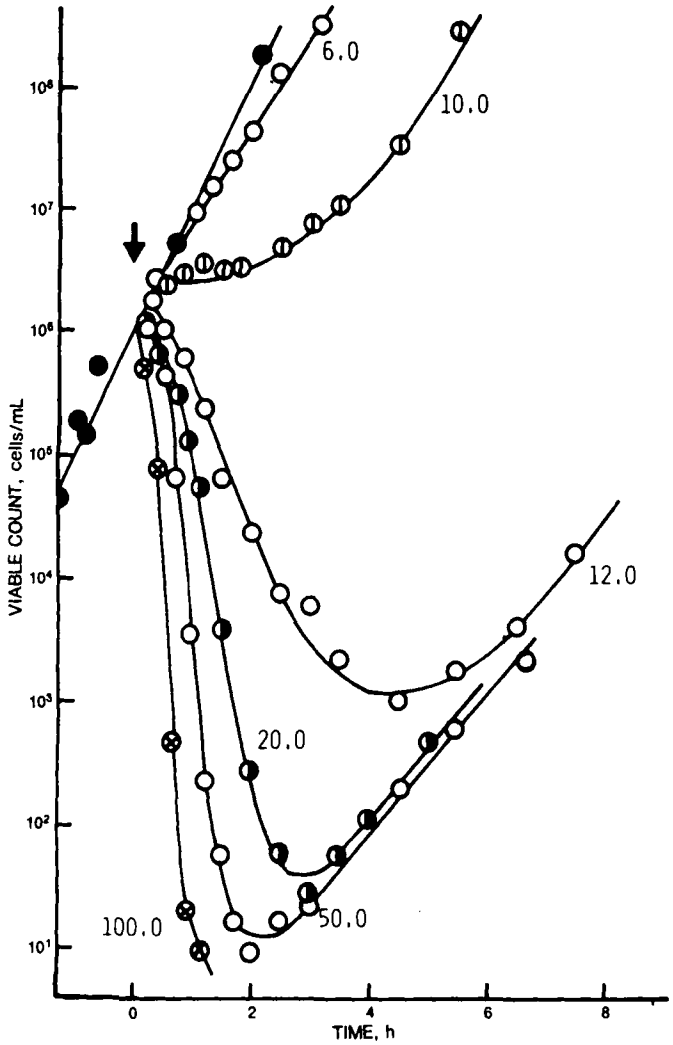

Figure 2—*Generation curves of* E. coli *NIHJ JC-2 in antibiotic medium[9] at pH 7.0 and 37°C in the absence (●) and presence (other symbols) of various concentrations of penicillin G. The numbers inside the figure are drug concentrations in micrograms per milliliter. The time of drug addition is indicated by the arrow.*

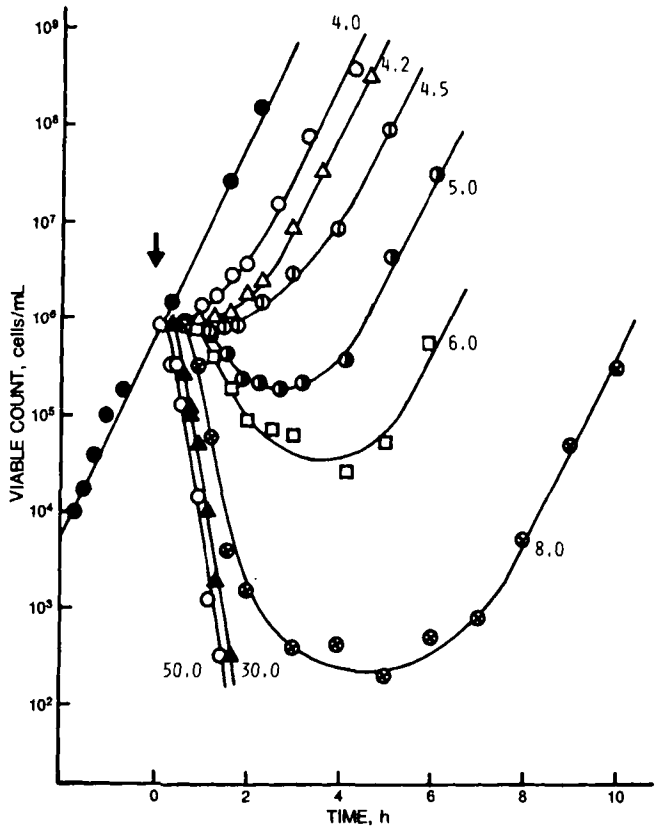

Figure 3—*Generation curves of* E. coli *NIHJ JC-2 in antibiotic medium[9] at pH 7.0 and 37°C in the absence (●) and presence (other symbols) of various concentrations of cefadroxil. The numbers inside the figure are drug concentrations in micrograms per milliliter. The time of the drug addition is indicated by the arrow.*

[11] Visking dialysis membrane, type 20/32; Union Carbide Corp., Chicago, Ill.
[12] IFO 12708; Institute for Fermentation.

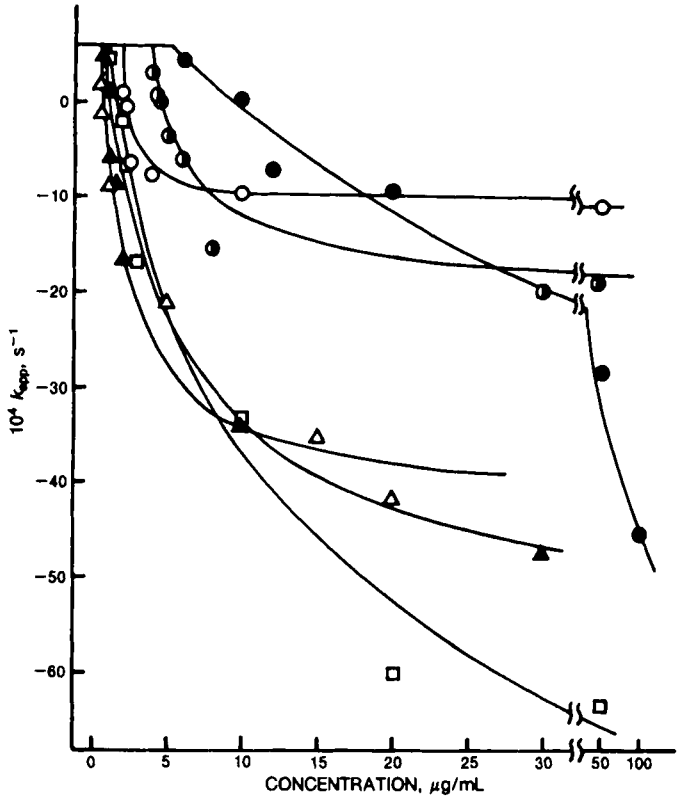

Figure 4—*Dependence of the apparent generation rate constant,* k_{app}, *for* E. coli *NIHJ JC-2 in antibiotic medium*[9] *at pH 7.0 and 37°C on drug concentration. Key: (●) penicillin G; (▲) ampicillin; (○) cephalexin; (△) cefazolin; (◑) cefadroxil; (□) cephaloridine.*

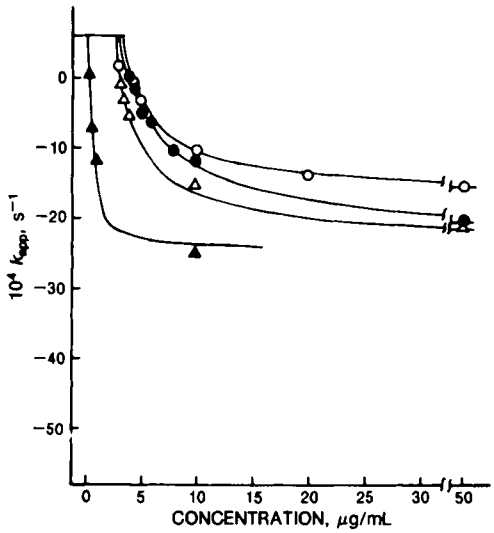

Figure 5—*Dependence of the apparent generation rate constant,* k_{app}, *for* E. coli *B/r in antibiotic medium*[9] *at pH 7.0 and 37°C on drug concentration. Key: (●) penicillin G; (▲) ampicillin; (○) dicloxacillin; (△) methicillin.*

mL) of aqueous solutions of antibiotics were aseptically added to replicate 49.5-mL samples of *E. coli* cultures to yield the desired concentrations. The solutions were added to the cultures growing at 37°C in the logarithmic phase at an organism concentration of 10^6 cells/mL. As soon as the drug solution was added to the culture medium, a dialysis membrane bag containing the drug solution was put into the culture medium, as described above, to keep the drug concentration constant during the experimental period. Samples were withdrawn at appropriate time intervals, and the organism concentrations were determined by the viable count method.

The *E. coli* NIHJ JC-2 culture was carried out in the presence of selected concentrations of penicillin G, ampicillin, cephalexin, cefazolin, cefadroxil, and cephaloridine. The *E. coli* B/r culture was also carried out in the presence of various concentrations of penicillin G, ampicillin, dicloxacillin, and methicillin. As a control, one culture without any drug added was studied in each set of experiments.

RESULTS AND DISCUSSION

Effect of Shaking Speed on Generation Rates—To establish suitable experimental conditions for the kinetic study of antibiotic action on *E. coli*, the bacterial generation rates without antibiotics were determined from the generation time curves obtained at various shaking speeds. The culture of *E. coli* showed an exponential phase which could be expressed as:

$$\ln N = \ln N_0 + k_0 t \qquad \text{(Eq. 1)}$$

where N is the number of organisms per unit volume of broth medium at time t; N_0 is the number of organisms per unit volume of broth medium at the initial time, 0, in the logarithmic generation phase; and k_0 is the apparent generation rate constant for the drug-free culture. The mean generation time, tg, was calculated by:

$$tg = 0.693/k_0 \qquad \text{(Eq. 2)}$$

The generation rate constant, k_0, and the generation time, tg, were calculated by Eqs. 1 and 2. In the absence of agitation, the mean generation time was 22 min (slightly larger than the values obtained for cultures under agitation); however, very similar values of 17–18 min were obtained within the range of 82–190 strokes/min. Therefore, the shaking speed was fixed at 130 strokes/min in this microbial kinetic study.

Table II—Microbial Kinetic Parameter k_{max} of *E. coli* NIHJ JC-2 and Alkaline Degradation Rate, k_{OH}, of Cephalosporins

Cephalosporin	$10^4\ k_{max}, s^{-1}$	$10^2\ k_{OH}, M^{-1}\ s^{-1}$
Cephaloridine	87.6	108[a]
Cefazolin	47.7	31.7[a]
Cephalexin	16.9	7.33[a]
Cefadroxil	27.9	7.06[b]

[a] Reference 17. [b] Reference 18.

Effect of Dialysis Membrane Tube—Ampicillin concentrations in the *E. coli* NIHJ JC-2 culture in the absence or presence of a dialysis membrane tube containing drug solution of the same concentration as that of the culture medium was measured at suitable time intervals.

When the ampicillin concentration was 4.0 μg/mL, the drug concentrations remained nearly constant, both with and without a dialysis membrane tube. On the other hand, at the initial concentration of 1.25 μg/mL in the absence of a dialysis membrane tube, the concentration decreased significantly, with a half-life of ~7 h. However, with replacement of the dialysis membrane tube every 3 h, a constant ampicillin concentration was successfully maintained during the experimental period.

These results indicate the importance of keeping the drug concentration constant during the experiment and demonstrate that the method in which the dialysis membrane tube is utilized is suitable for this purpose. A membrane tube containing antibiotic solution of the appropriate concentration was, therefore, used in all microbial kinetic studies.

Effect of Organism Population on Drug-Affected Generation Rates—Semilogarithmic plots of the viable count *versus* time for *E. coli* B/r cultures treated with 0.5 μg of ampicillin/mL are illustrated in Fig. 1, in which the effects of different initial inoculum sizes and organism populations at the time of drug addition can be seen. No significant effects of the initial inoculum size and organism population at the time of drug addition are apparent.

Effect of Antibiotic Concentrations on Generation Rates—*Shape of Generation Curves*—Typical semilogarithmic plots of viable counts *versus* time for *E. coli* NIHJ JC-2 and *E. coli* B/r cultures in the presence of various concentrations of several β-lactam antibiotics are shown in Figs. 2 and 3 for penicillin G and cefadroxil, respectively.

All antibiotics studied gave very similar generation curves of *E. coli*. After the addition of a drug to the medium, a definite lag period was observed (Figs. 1–3) before the semilogarithmic plots of viable counts decreased linearly with time. As is apparent from the results shown in Figs. 2 and 3, these lag periods tend to become shorter at higher drug concentrations. These periods may depend on the time required for the antibiotic concentration on both sides of the bacterial membrane to reach steady state after drug addition to the broth medium. Garrett and Won (4) have reported similar lag periods in their study on the bactericidal effects of penicillin G, kanamycin, and rifampin against *E. coli* and have suggested that these intervals reflect the drug partitioning process into the bacterial membrane. In this study, however, we were not able to express the lag period as a function of drug concentration.

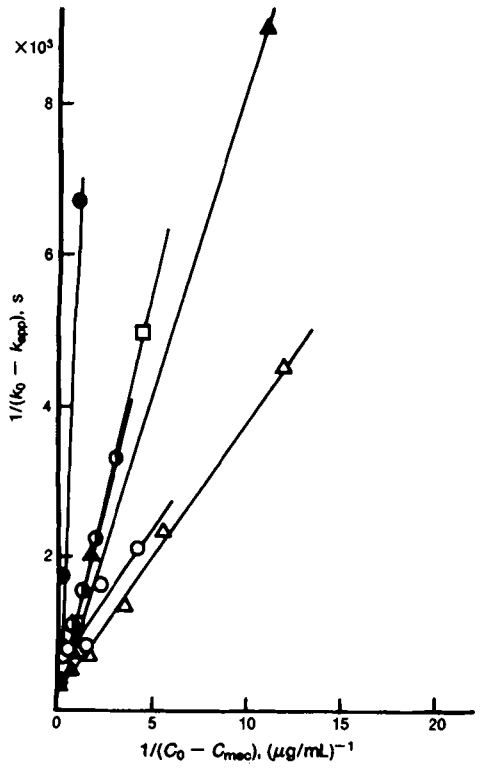

Figure 6—*Applicability of saturation kinetics to the dependency of apparent generation rate constants, k_{app}, of drug-affected* E. coli *NIHJ JC-2 on drug concentration at pH 7.0 and 37°C. Key: (●) penicillin G; (▲) ampicillin; (○) cephalexin; (△) cefazolin; (◑) cefadroxil; (□) cephaloridine.*

At higher drug concentrations, the number of viable cells decreased linearly in the semilogarithmic plots obtained immediately after drug addition. At lower drug concentrations, subsequent to the deviation from the curve of the drug-free culture, a new steady-state generation phase was established.

Apparent generation rate constants, k_{app} (in s^{-1}), of drug-affected cultures were obtained from the slopes of the linear portions of ln N *versus* t plots in accordance with the general rate expression:

$$\ln N = \ln N_0 + k_{app}t \qquad \text{(Eq. 3)}$$

Concentration Dependence of Generation Rate—The apparent generation rate constants, k_{app}, for the drug-affected *E. coli* NIHJ JC-2 and *E. coli* B/r cultures are plotted against antibiotic concentrations in Figs. 4 and 5, respectively. The plots for all antibiotics studied show a marked decrease of k_{app} with an increase in the drug concentration, C, and a tendency to reach maximum rate constants. These nonlinear plots indicate that the apparent generation rates of the drug-affected cultures obey the well-established kinetics of the saturable receptor site model proposed for other antibacterial agents (1, 2, 5–10) and can be expressed by:

$$k_{app} = k_0 - [k_{max} \times (C - C_{mec})]/[S_b + (C - C_{mec})] \qquad \text{(Eq. 4)}$$

where k_{max} and S_b are the proportionality constants relating to drug availability in the cell and drug affinity to receptor or binding sites, and C_{mec} is the minimum effective concentration manifesting antibacterial activity (defined as $C_{mec} = C$ when $k_{app} = k_0$). As suggested previously by Garrett and Won (4), C_{mec} may reflect the binding or removal of effective antibiotic by the components of the medium. The constants of k_{max}, S_b, and C_{mec} were determined by Eq. 4 using the nonlinear regression program NONLIN (11)[13] and are listed in Table I.

When the reciprocal values of the differences between the drug-free generation rate constant, k_0, and the drug-affected generation rate constant, k_{app}, are plotted against the reciprocal of the difference between the drug concentration, C, and the minimum effective concentration, C_{mec}, a good linearity is obtained by:

$$1/(k_0 - k_{max}) = S_b/k_{max}(C - C_{mec}) + 1/k_{max} \qquad \text{(Eq. 5)}$$

The plots are illustrated in Figs. 6 and 7 for *E. coli* NIHJ JC-2 and *E. coli* B/r,

[13] The computer analysis was performed with a FACOM-170F digital computer at the Data Processing Center, Kanazawa University.

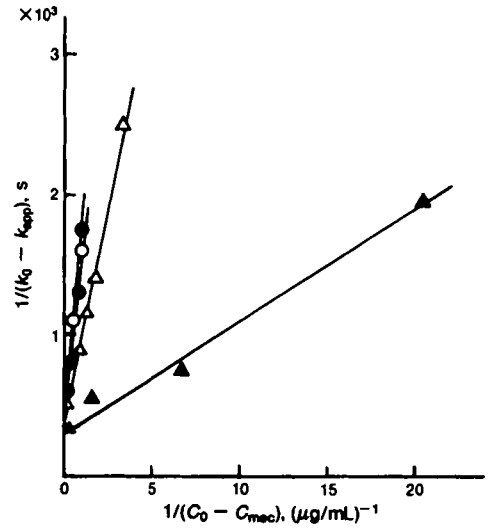

Figure 7—*Applicability of saturation kinetics to the dependency of apparent generation rate constants, k_{app}, of drug-affected* E. coli *B/r on drug concentration at pH 7.0 and 37°C. Key: (●) penicillin G; (▲) ampicillin; (○) dicloxacillin; (△) methicillin.*

respectively. The good agreement of data with Eq. 5 also substantiates the validity of the saturable receptor site model (1, 2, 5–10) for interpretation of the action of β-lactam antibiotics on microbial growth. It is likely that the receptor sites correspond to transpeptidase, which is known to interact with β-lactam antibiotics (12).

The quantitative meaning of the MIC is still unclear, and the magnitude does not always reflect the kinetic behavior of microbial generation in the presence of antibiotics. However, the parameter k_{max} corresponds to the V_{max} in enzyme kinetics and may reflect the intrinsic potency of the antibiotic better than does the MIC.

Structure–Activity Correlation of β-Lactam Antibiotics and Microbial Kinetics—Hermann (13) has suggested that the bactericidal activity of β-lactam antibiotics is parallel to the reactivity of the β-lactam ring, and Indelicato *et al.* (14, 15) and Yamana *et al.* (16, 17) have stated that the alkaline degradation rate, k_{OH}, of cephalosporin parallels antibacterial activity. The mechanism of hydroxide ion-catalyzed degradation of the β-lactam ring in alkaline solution is similar to the mechanism of the acylation of transpeptidase because both reactivities depend on the cleavage energy of the C—O bond of the β-lactam ring. Based on these considerations, a linear free energy relationship can be applied for k_{max} and k_{OH}:

$$G^{\neq}_{k_{max}} = \text{const} \cdot G^{\neq}_{k_{OH}} + \text{const} \qquad \text{(Eq. 6)}$$

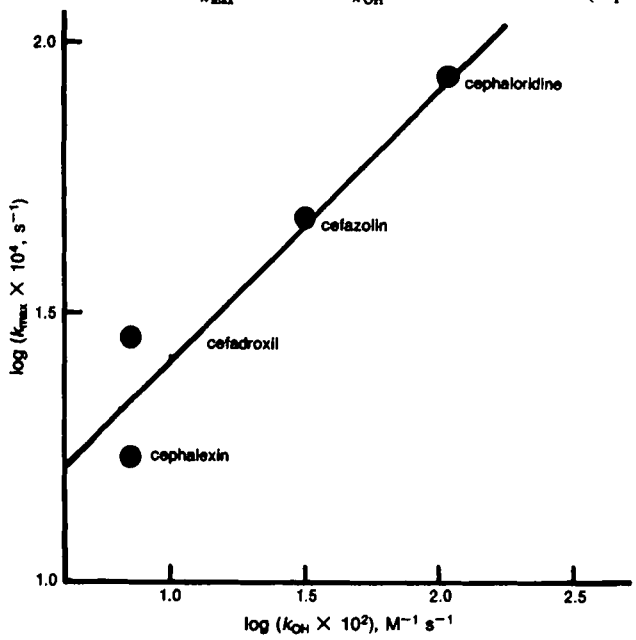

Figure 8—*Relationship between microbial kinetic parameter, k_{max}, of* E. coli *NIHJ JC-2 and alkaline degradation rates, k_{OH}, of cephalosporins.*

and:

$$k_{max} = \text{const} \cdot \exp(-G^{\neq}_{k_{max}}/RT) \quad \text{(Eq. 7)}$$

$$k_{OH} = \text{const} \cdot \exp(-G^{\neq}_{k_{OH}}/RT) \quad \text{(Eq. 8)}$$

where $G^{\neq}_{k_{max}}$ and $G^{\neq}_{k_{OH}}$ are free energies of activation for k_{max} and k_{OH}, respectively, const is a constant, R is the gas constant, and T is the absolute temperature. Taking the common logarithm of both sides of Eqs. 7 and 8 gives:

$$\log k_{max} = \text{const} - G^{\neq}_{k_{max}}/2.303RT \quad \text{(Eq. 9)}$$

$$\log k_{OH} = \text{const} - G^{\neq}_{k_{OH}}/2.303RT \quad \text{(Eq. 10)}$$

Substitution of Eqs. 9 and 10 for $G^{\neq}_{k_{max}}$ and $G^{\neq}_{k_{OH}}$ into Eq. 6, followed by rearrangement, yields:

$$\log k_{max} = \text{const} \cdot \log k_{OH} + \text{const} \quad \text{(Eq. 11)}$$

Equation 11 indicates a linear relationship between log k_{max} and log k_{OH}.

The k_{max} values for *E. coli* NIHJ JC-2 and k_{OH} values for cephalosporins are listed in Table II, and log k_{max} is plotted against log k_{OH} in Fig. 8 (good linearity was obtained). The regression equation obtained is:

$$\log k_{max} = 0.513 \cdot \log k_{OH} + 0.902 \quad \text{(Eq. 12)}$$

with a regression coefficient of 0.951. This relationship suggests that β-lactam antibiotics possessing high reactivity of the C—O bond in alkaline solution will show high bactericidal activity.

Organisms Resistant to Drug Action—Garrett and Won (4) have suggested the following possible explanations in connection with the regrowth of organisms after the first-order decrease of viable cells in drug-affected cultures (Figs. 1–3): (*a*) the consumption or degradation of the drug, (*b*) the production of an inhibitor or inactivator of antibiotic action, and (*c*) the presence of bacteria able to resist the drug action. This study on the microbial kinetics of various β-lactam antibiotics was conducted at constant drug concentrations (achieved by the dialysis membrane tube method); therefore, possibility *a* can be ruled out, but possibilities *b* and *c* still remain.

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ACKNOWLEDGMENTS

The authors wish to acknowledge the gifts of β-lactam antibiotics from Meiji Seika Kaisha, Takeda Chemical Industries, Banyu Pharmaceutical Co., Fujisawa Pharmaceutical Co., Shionogi Co., and Bristol Meyers Co. They also acknowledge the skillful technical assistance of Mr. Y. Deguchi, Miss M. Takeda, and Miss K. Wakiya.

Kinetic Analysis and Characterization of the Bacterial Regrowth after Treatment of *Escherichia coli* with β-Lactam Antibiotics

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Abstract □ The generation curves of *Escherichia coli* B/r and *E. coli* NIHJ JC-2 in the presence of several β-lactam antibiotics were studied from the kinetic point of view. Apparent first-order regrowth of resistant organisms was observed ~6 h after addition of these antibiotics. The time courses of apparent viable counts could be interpreted in terms of the sum of the viable counts of sensitive and resistant organisms. To clarify the nature of the regrowth, experiments involving a second addition of antibiotic, single colonization by subculture, and synchronous cell culture were carried out. Several possible explanations for the results are discussed, including β-lactamase production, selection in terms of membrane permeability, and mutation to acquire drug resistance. A selection process or a modification of membrane permeability caused by contact with the drug seems to be the most probable reason for the regrowth of the organisms.

Keyphrases □ Kinetics—microbial, analysis and characterization of bacterial regrowth after treatment of *Escherichia coli* with β-lactam antibiotics □ β-Lactam antibiotics—kinetic analysis and characterization of bacterial regrowth after treatment of *Escherichia coli*

In the previous paper (1), we described the microbial kinetics of *Escherichia coli* treated with several β-lactam antibiotics. We also mentioned that, once treated with β-lactam antibiotics, the organisms showed an apparent regrowth in spite of the maintenance of a constant drug concentration by the use of a dialysis membrane tube containing the antibiotic solution (1). This regrowth is a well-recognized phenomenon in drug-affected cultures (2–6). Much work has been done on

0022-3549/84/1000-1422$01.00/0