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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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

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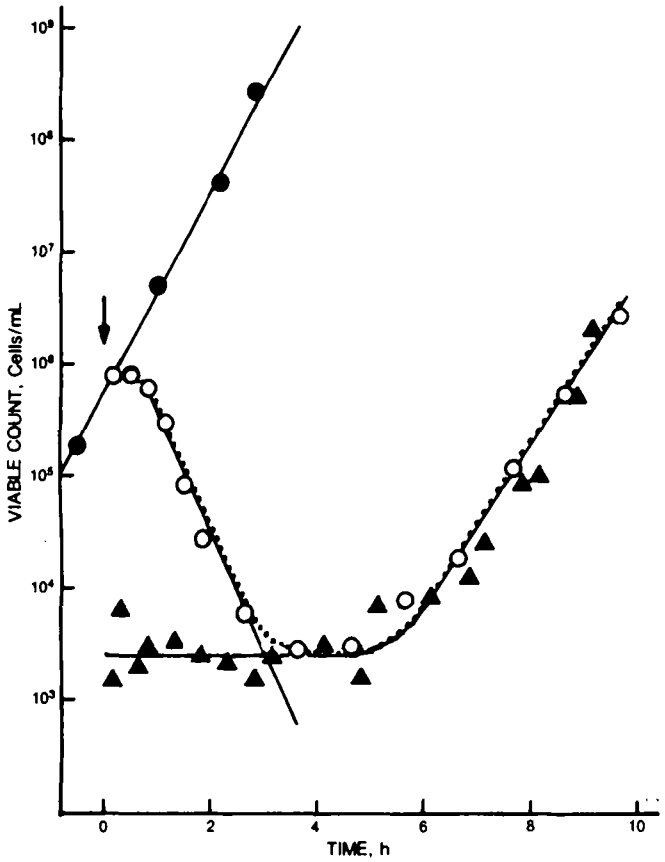

Figure 1—*Generation curves of* E. coli *B/r in antibiotic medium*[3] *at pH 7.0 and 37°C in the absence and presence of 0.5 μg of ampicillin/mL, based on viable counts. Key: (●) control generation curves; (○) drug-affected viable counts in drug-free agar plates; (▲) drug-affected viable counts in drug-containing agar plates. The dotted line was generated from Eqs. 1 and 2 by using the appropriate kinetic parameters (Table I.)*

drug-resistant mechanisms of organisms (7); however, very few reports (2, 6) have focused on the mechanism of regrowth after addition of antibacterial agents to the culture, even though it is very important to prevent the regrowth of organisms in chemotherapy.

The purposes of this study were to establish a mathematical model to describe the entire generation curve of organisms as a function of time and to clarify the nature of regrowth after the bactericidal action of β-lactam antibiotics.

EXPERIMENTAL SECTION

Microorganisms—Replicate slants of *E. coli* NIHJ JC-2[1] and *E. coli* B/r[2] [the same strains as used previously (2)] were used. The slants were prepared from a single isolated colony of the appropriate organism and stored at 4°C.

Culture Medium—Antibiotic medium[3] was rehydrated according to the specifications of the manufacturer and filtered twice through a 0.45-μm filter[4]. The medium was autoclaved at 120°C for 20 min. This pH 7.0 medium was used for all experiments.

Materials—Ampicillin sodium[5] (955 μg/mg), penicillin G potassium[6] (1600 U/mg), and cefazolin sodium[7] (958 μg/mg) were used as received. All other chemicals were of reagent grade and were used without further purification.

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

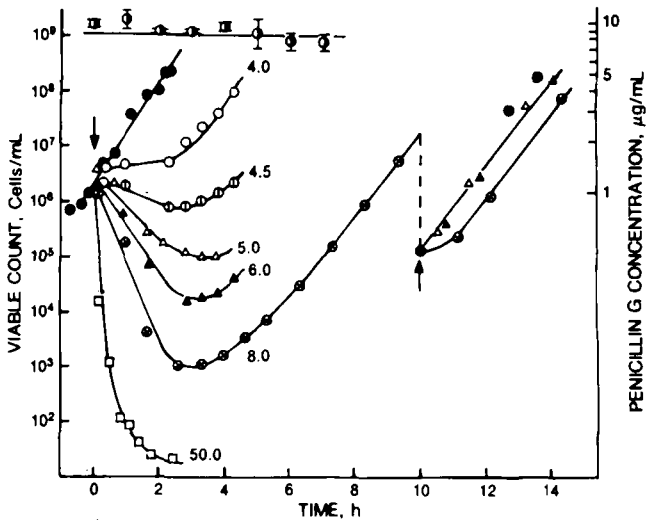

Figure 2—*Generation curves of* E. coli *B/r in antibiotic medium*[3] *at pH 7.0 and 37°C in the absence and presence of various concentrations of penicillin G. The numbers inside the figure are the drug concentrations in micrograms per milliliter. After the first addition (↓) of 8.0 μg of penicillin G/mL, the culture was diluted 100-fold with fresh medium at the second addition (↑). The observed drug concentrations are indicated (○) for 8 μg/mL (nominal) (right-hand axis).*

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

Two 0.5-mL portions of this cultture were finally diluted into 49.5 mL of fresh medium for control growth and 49.0 mL of fresh medium for drug-affected growth, respectively. The cultures were maintained at 37°C in a constant-temperature water bath equipped with a shaker set at 130 strokes/min. The drug solutions were added to the cultures in the logarithmic growth phase at 37°C. As soon as a drug was added to the cultures, a dialysis membrane tube[8] containing 10 mL of fresh culture medium with the same drug concentration as the organism culture medium was placed in the culture medium and replaced every 3 h with a new one (1). To keep the antibiotic concentration constant during the experiment, a membrane tube was used in all generation studies.

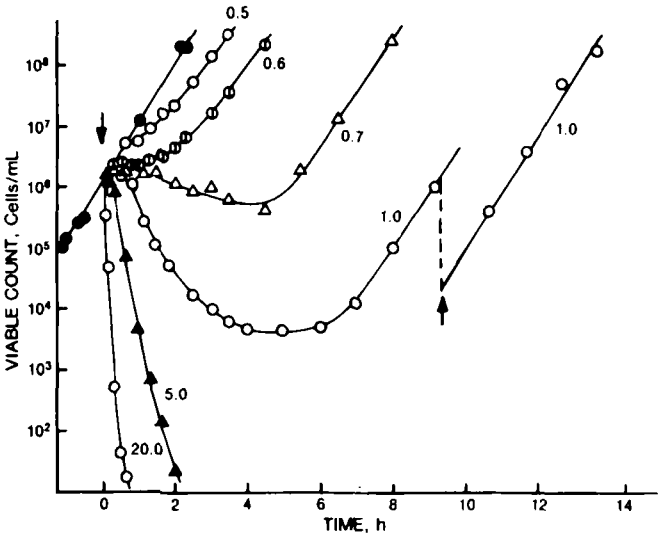

Figure 3—*Generation curves of* E. coli *NIHJ JC-2 in antibiotic medium*[3] *at pH 7.0 and 37°C in the absence and presence of various concentrations of cefazolin. The numbers inside the figure are drug concentrations in micrograms per milliliter. After the first addition (↓) of 1.0 μg of cefazolin/mL, the culture was diluted 100-fold with fresh medium at the second addition (↑).*

[8] Seamless cellulose tubing 20/32; Visking Co.

Table I—Microbial Kinetic Parameters of *E. coli* B/r and *E. coli* NIHJ JC-2 in Antibiotic Medium[3] [a]

Organism	Ns_0, cells/mL	Nr_0, cells/mL	k_{app}, s^{-1}	k_g, s^{-1}	L_1, min	L_2, min
E. coli B/r	8.0×10^5	3.2×10^3	-7.01×10^{-4}	4.67×10^{-4}	53	322
E. coli NIHJ JC-2	1.0×10^6	1.2×10^1	-1.04×10^{-3}	4.95×10^{-4}	55	326

[a] At pH 7.0 and 37°C in the presence of 0.5 and 1.5 μg of ampicillin/mL for *E. coli* B/r and *E. coli* NIHJ JC-2, respectively.

At appropriate time intervals, aliquots (0.5 mL) were withdrawn, and the penicillin G concentration was determined by the usual microbiological paper disk method, employing *Staphylococcus aureus* 209P[9] as the test organism.

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 result. From these dilutions, aliquots (0.2 mL) were transferred onto each of three replicate peptone agar plates. The plates were incubated for 20 h at 37°C, and the resulting colonies were counted with an electronic colony counter.

To obtain the viable count of drug-resistant organisms shown in Figs. 1–5, the procedure was the same as that described above, except for the use of peptone agar plates which contained the drug at the desired concentration.

Effect of Second Addition of Antibiotics—Aliquots (0.5 mL) of penicillin G and cefazolin solutions were added to *E. coli* B/r and *E. coli* NIHJ JC-2 cultures, respectively, in the logarithmic growth phase of the culture to give final concentrations of 8.0 μg/mL for penicillin G (culture A) and 1.0 μg/mL for cefazolin (culture B). The drug-affected cultures A and B were allowed to generate for ~10 h at 37°C in an incubator. Portions of these solutions were then diluted 100-fold with fresh medium. Aliquots of the *E. coli* B/r culture were then treated with penicillin G concentrations of 5.0, 6.0, and 8.0 μg/mL, and an aliquot of the *E. coli* NIHJ JC-2 culture was treated with 1.0 μg of cefazolin/mL. One colony of *E. coli* NIHJ JC-2 harvested from culture B in the logarithmic growth phase of regrowth was subcultured, and then the subcultured organism was used as a test organism. The effect of the second addition of cefazolin on this subcultured resistant organism was studied in the same manner as described above.

Population Distribution of Drug-Resistant Organisms—Replicate agar plates containing ampicillin concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 0.55, 0.6, 0.65, and 0.7 μg/mL were prepared. Samples (0.5 mL) were withdrawn from *E. coli* NIHJ JC-2 culture at an organism concentration of 2×10^6 cells/mL in the logarithmic growth phase and serially diluted. From these dilutions, samples (0.2 mL) were transferred onto the replicate agar plates containing drug. The plates were incubated for 20 h at 37°C, and the number of colonies was counted.

Single Colonization—*E. coli* NIHJ JC-2 obtained from a fresh slant was suspended in 2 mL of 0.9% NaCl. A portion of this solution was transferred onto peptone agar plates with a standard platinum loop and cultured for 20 h at 37°C. One colony from this agar plate was again treated in the same way; the colony that appeared the next day was used as the test organism.

Drug-Affected Growth and Effect of Second Addition—The experimental procedure for drug-affected growth and the effect of the second addition of antibiotic was the same as that described previously (1).

Minimal Inhibitory Concentration of Subcultured Drug-Resistant Organisms—The organism obtained from the regrowth of *E. coli* NIHJ JC-2 after ampicillin treatment (1.5 μg/mL) was purified by single colonization and subcultured up to 29 times on peptone agar plates. The minimal inhibitory concentration (MIC) was determined periodically.

The MIC values were determined by the agar dilution method. Overnight cultures of *E. coli* NIHJ JC-2 were diluted to final concentrations of ~10^6 and 10^8 cells/mL, and 5-μL aliquots of each culture were inoculated and incubated on antibiotic medium[3] agar plates containing serial twofold dilutions of ampicillin. The MIC values were recorded after 18 h of incubation at 37°C.

Effect of Ampicillin Concentrations on Regrowth Rates—Aliquots (0.5 mL) of aqueous solutions of ampicillin were aseptically added to replicate 49.5-mL volumes of *E. coli* NIHJ JC-2 cultures to yield concentrations of 0.55, 0.6, 0.7, and 1.1 μg/mL.

Preparation of Synchronous Cell Cultures—Aliquots (0.5 mL) of phenethyl alcohol solutions were aseptically added to replicate 49.5-mL volumes of *E. coli* NIHJ JC-2 culture in the logarithmic growth phase to yield desired concentrations. The cell concentration at the time of drug addition was ~10^5 cells/mL. A portion of the culture was diluted 1000-fold with fresh medium at 90 min so that the effect of phenethyl alcohol became negligible. A synchronized cell culture was generated from the medium containing 10^5 cells/mL. Aliquots (0.5 mL) of ampicillin solution were added to the synchronized cell culture at an organism concentration of ~2×10^5 cells/mL to achieve final concentrations of 1.25 and 1.5 μg/mL. The viable counts were monitored with an electronic colony counter.

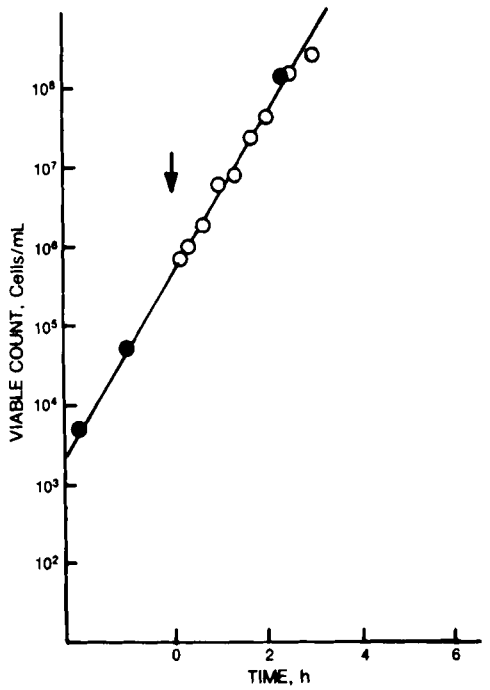

Figure 4—*Semilogarithmic plots of* E. coli *NIHJ JC-2 obtained from the logarithmic regrowth phase after the first addition of cefazolin (1.0 μg/mL) at pH 7.0 and 37°C. The experiment was effected 24 h after development and isolation of resistant organisms.*

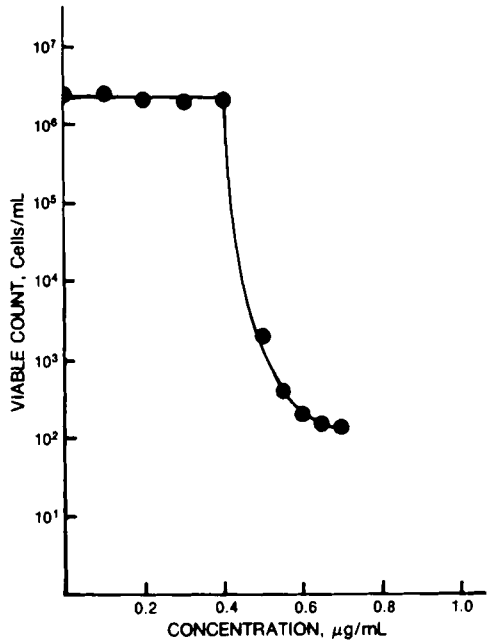

Figure 5—*Population diagram of ampicillin-resistant* E. coli *NIHJ JC-2. The viable counts were performed in agar plates with drug with various ampicillin concentrations after isolation of organisms from drug-free culture.*

[9] IFO 12732; Institute for Fermentation.

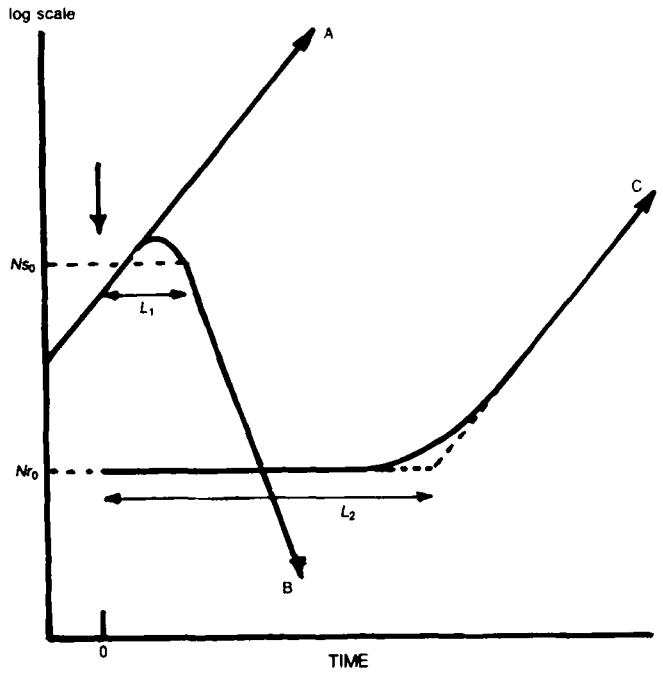

Figure 6—*Generation model for drug-affected organism. Key: (A) control generation curve; (B) generation curve of sensitive organisms; (C) generation curve of resistant organisms; (L_1 and L_2) lag times for sensitive and resistant organisms, respectively; (Ns_0 and Nr_0) initial organism concentrations for sensitive and resistant organisms, respectively; (time 0) time of drug addition.*

Assay of β-Lactamase—Antibiotic medium[3] in a 1.0-L flask was inoculated with 25 mL of an overnight culture of *E. coli* NIHJ JC-2 in the presence of 8 μg of penicillin G/mL at 37°C. After 4 h, the bacteria at a concentration of ~5 × 10^8 cells/mL in the logarithmic growth phase were harvested by centrifugation and washed once with 0.05 M phosphate buffer (pH 7.0). The packed cells were resuspended in 50 mL of 0.05 M phosphate buffer (pH 7.0), and the suspensions were intermittently treated in an ultrasonic disintegrator[10] for 3 min. The disrupted suspensions were centrifuged at 12,000 rpm for 30 min at 5°C to remove broken cells and cell debris; the cell-free extract was retained as the crude enzyme preparation. Activity of β-lactamase was then assayed spectrophotometrically (8) by measuring the decrease in the absorbance at the substrate-specific wavelengths of 233 and 260 nm for penicillin G and cephaloridine, respectively.

RESULTS

Development of Drug-Resistant Organisms—A typical semilogarithmic plot of the viable counts against time is shown (Fig. 1) for the action of 0.5 μg of ampicillin/mL on *E. coli* B/r. After a certain lag period following drug addition, the logarithm of the viable count against time decreased exponentially with time. After a definite period of organism killing, there is a subsequent generation of viable organisms. The slope of the regrowth phase in the presence of drug was found to be very similar to that for the plot in the absence of ampicillin. This result indicates a negligible drug effect in the terminal segments, even though the drug concentration was kept constant by the use of the dialysis membrane tube. A similar result was obtained for the *E. coli* NIHJ JC-2 culture treated with 1.5 μg of ampicillin/mL.

These observations indicate that the apparent generation curves in the presence of drugs (Fig. 1) consist of a first-order decrease of susceptible organisms and a first-order increase of resistant organisms, both of which occur after certain lag times. The lag time is the elapsed time from the time of drug addition to the time at which a decrease or an increase of the viable count begins. Therefore, the apparent generation curves may be expressed by the generation model depicted in Fig. 6, in which t is the time after addition of drug: Ns_0 is the number of susceptible organisms present at time L_1; Nr_0 is the number of resistant organisms at time L_2; L_1 and L_2 are the lag times for the appearances of the first-order decrease of susceptible organisms and the first-order increase of resistant organisms, respectively. This generation

[10] Ultrasonic Disruptor model UR-200P; Tomy Seiko Co. Ltd., Tokyo, Japan.

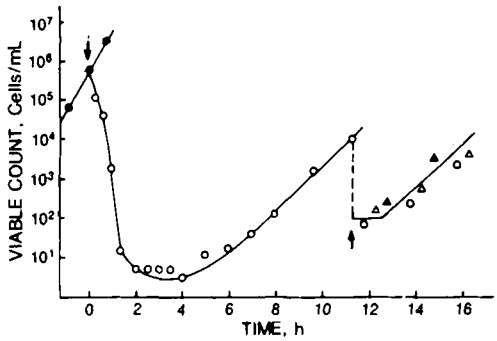

Figure 7—*Generation curves of the purified* E. coli NIHJ JC-2 *in antibiotic medium*[3] *at pH 7.0 and 37°C in the absence and presence of 1.5 μg of ampicillin/mL. After the first addition (↓) of ampicillin, the generating culture was diluted 100-fold with fresh medium at the second additions (▲) of various ampicillin concentrations. Key: (○) 1.5 μg/mL; (△) 1.2 μg/mL; (▲) 1.0 μg/mL.*

model leads to the following equations (see below for the derivation) after a lag period $t > L_1$ (when $L_1 \leqq t \leqq L_2$):

$$N_{app} = Ns_0 \cdot e^{k_{app}(t-L_1)} + Nr_0 \qquad \text{(Eq. 1)}$$

when $t > L_2$:

$$N_{app} = Ns_0 \cdot e^{k_{app}(t-L_1)} + Nr_0 \cdot e^{k_g(t-L_2)} \qquad \text{(Eq. 2)}$$

where N_{app} is the apparent number of organisms present at time t and k_{app} and k_g are the apparent generation rate constants for the susceptible and resistant organisms, respectively. The relationship between the observed number of viable counts and the simulation based on Eqs. 1 and 2 with the appropriate parameters listed in Table I seems to support the model depicted in Fig. 6.

Effect of Second Addition of Antibiotics—The effects of the second addition of penicillin G and cefazolin in the case of resistant organisms are shown in Figs. 2 and 3, respectively. In these experiments, the membrane tube containing the drug solution was used.

In a typical case shown in Fig. 2, a penicillin G concentration of 8 μg/mL

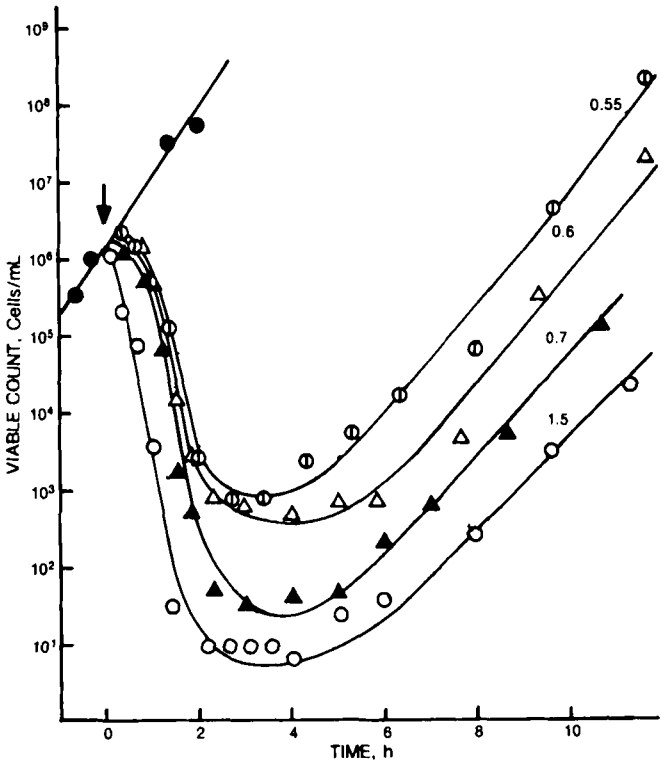

Figure 8—*Generation curves of the purified* E. coli *NIHJ JC-2 in antibiotic medium*[3] *at pH 7.0 and 37°C in the absence and presence of various concentrations of ampicillin. The curves are labeled according to drug concentrations in micrograms per milliliter.*

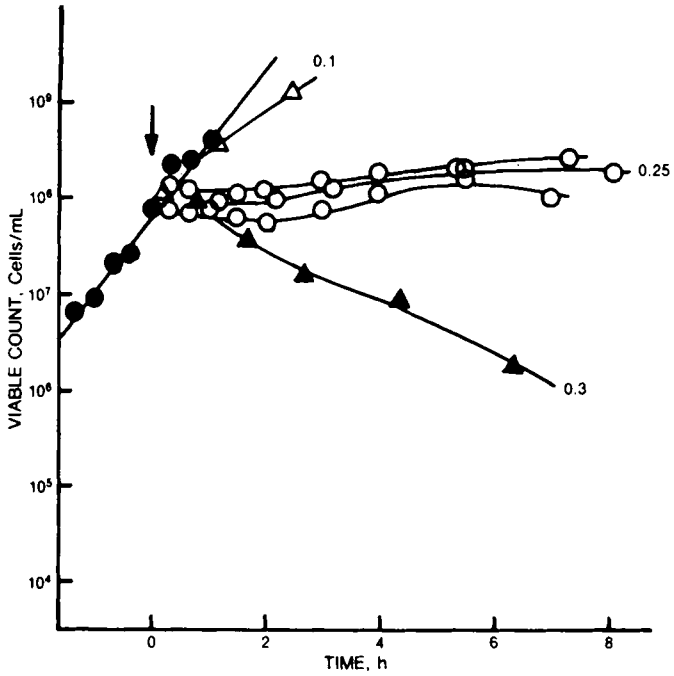

Figure 9—*Generation curves of* E. coli *NIHJ JC-2 in antibiotic medium*[3] *at pH 7.0 and 37°C in the absence and presence of phenethyl alcohol. The curves are labeled according to percent concentrations of phenethyl alcohol.*

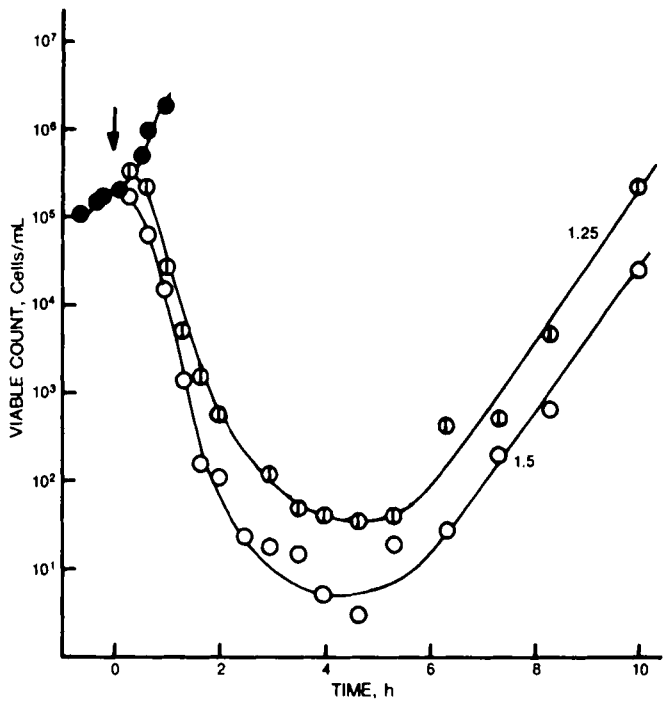

Figure 10—*Generation curves of the synchronized* E. coli *NIHJ JC-2 culture in antibiotic medium*[3] *at pH 7.0 and 37°C in the absence and presence of ampicillin. The curves are labeled according to ampicillin concentrations in micrograms per milliliter.*

was confirmed to be maintained essentially constant. At about 10 h after the first drug addition, when the drug-resistant organisms were in a logarithmic growth phase, the membrane tube was removed, and a 100-fold dilution was made with fresh medium to make the original drug action negligible. The second additions of penicillin G did not greatly affect the generation rate at the concentrations of 5.0 and 6.0 μg/mL (Fig. 2). Even though the first addition of penicillin G at these concentrations markedly reduced the viable counts, the generation rate constants for these concentrations were found to be similar to those of the drug-resistant organism at the first addition, although a slight lag time was observed at 8.0 μg/mL. The result indicates that once the organism has acquired resistance (after the first addition), it is resistant to all drug concentrations lower than that of the first addition. The same result was obtained for cefazolin with *E. coli* NIHJ JC-2 (Fig. 3).

The organism which was made resistant by the addition of 1.0 μg of cefazolin/ml was subcultured and again treated with cefazolin (1.0 μg/mL) on the next day. This drug-affected culture exhibited a generation rate similar to that of the drug-free culture (Fig. 4). This result shows that, once acquired, drug resistance is retained 48 h later.

Population Distribution of Drug-Resistant Organisms—To obtain a population diagram of ampicillin-resistant *E. coli* NIHJ JC-2, viable counts were performed in the agar plates with drug after isolation of organisms from drug-free culture. In Fig. 5 are plotted the resultant viable counts against ampicillin concentrations in the agar plates. A sharp decrease of viable counts was observed when the ampicillin concentration was increased from 0.4 to 0.5 μg/mL. This result suggests that the number of drug-resistant organisms depends on the drug concentration used.

Single Colony Subcultures—*Drug-Affected Generation Curve*—When the organism was purified by repeated subcultures of a single colony and then employed in the drug-affected generation experiment, the drug-resistant organisms increased again after about a 5-h lag period (Fig. 7). This result raises the possibility that some population among the presumably genetically identical organisms may have acquired drug resistance by contact with the drug. If some organisms such as these were originally insensitive to the ampicillin concentration of 1.5 μg/mL, they would not have shown a decrease of viable count after the first addition.

A second addition of antibiotic was also tested. Treatment with concentrations below that of the first addition also did not affect the generation rate of the resistant organisms.

Minimal Inhibitory Concentration for Subcultured Organisms—The MIC of ampicillin for the original strain of *E. coli* NIHJ JC-2 was 3.13 μg/mL at organism concentrations of both 10^6 and 10^8 cells/mL. The resistant organisms obtained after the first addition of ampicillin at 1.5 μg/mL were subcultured serially. The MIC values were in the ranges of 12.5–25 μg/mL at an organism concentration of 10^6 cells/mL and 25–50 μg/mL at 10^8 cells/mL. The MIC values remained essentially unchanged during 29 serial subcultures. This means that the resistance, once acquired, was persistent.

Effect of Drug Concentration on Generation Rate of the Purified Organism—Semilogarithmic plots of viable counts *versus* time for the *E. coli* NIHJ JC-2 culture treated with various concentrations of ampicillin are shown in Fig. 8. The generation curves are clearly concentration dependent, suggesting that the degree of resistance caused by contact with the drug might depend on the drug concentration.

Effect of Cell Cycle at the Time of Drug Addition—*Effect of Phenethyl Alcohol on Bacterial Generation*—The effect of phenethyl alcohol on the generation of *E. coli* NIHJ JC-2 was measured to ascertain the concentration which would effectively retard bacterial generation by the inhibition of DNA synthesis (9). The results (Fig. 9) indicate that 0.25% phenethyl alcohol treatment is suitable for the inhibition of DNA synthesis. Treatment with 0.1% phenethyl alcohol failed to inhibit DNA replication, whereas 0.3% phenethyl alcohol treatment killed the organisms.

Effect of Ampicillin Concentration on Generation Rate—The stationary-phase cells of *E. coli* NIHJ JC-2 were exposed to 0.25% phenethyl alcohol for 90 min. The inhibition was then released by 1000-fold dilution of the inhibitor with fresh medium, so that the effect of phenethyl alcohol became negligible. After this dilution, the synchronous culture started to grow again. Aliquots of ampicillin solution were added to yield concentrations of 1.25 and 1.5 μg/mL when the viable count had doubled. Generation curves similar to that of the nonsynchronous culture were observed (Fig. 10).

DISCUSSION

The apparent drug-affected generation curves for *E. coli* treated with β-lactam antibiotics seem to be the summation of the viable counts for drug-susceptible and drug-resistant organisms (Fig. 1). Based on this hypothesis, a mathematical model for the generation curve is presented in Fig. 6. The apparent number of viable counts can be expressed by:

$$N_{app} = Ns + Nr \qquad \text{(Eq. 3)}$$

where Ns and Nr are the numbers of susceptible and resistant organisms, respectively. When t is in the range of $L_1 \leqq t \leqq L_2$:

$$Ns = Ns_0 \cdot e^{k_{app}(t-L_1)} \qquad \text{(Eq. 4)}$$

$$Nr = Nr_0 \qquad \text{(Eq. 5)}$$

Here, $t = 0$ when the drug is added. Substitution of Eqs. 4 and 5 into Eq. 3 yields Eq. 1 (when t is $t > L_2$):

$$Ns = Ns_0 \cdot e^{k_{app}(t-L_1)} \quad \text{(Eq. 6)}$$

$$Nr = Nr_0 \cdot e^{k_g(t-L_2)} \quad \text{(Eq. 7)}$$

Replacement of Ns and Nr in Eq. 3 by Eqs. 6 and 7 gives Eq. 2.

Although the observed points were scattered around the simulation curve generated from Eq. 1 by incorporating the appropriate parameters (Table I), it appears that the apparent generation curves can be reasonably well described by the present mathematical model.

Garrett and Won (2) have reported the development of penicllin-resistant organisms and proposed the following possible explanation for the regrowth phenomenon: (*a*) the consumption or degradation of drugs, (*b*) production of an inhibitor or inactivator of antibiotic action, (*c*) preexistence of drug-resistant bacteria, and (*d*) acquisition of drug resistance through mutation and/or adaptation by exposure to the drug. Besides these factors, the cell cycle (*e*) may be an important factor in regrowth after the first addition of drug.

In the present study, the drug concentration was kept constant during the experiment by using a dialysis membrane tube containing drug solution. Therefore, possibility *a* is ruled out.

The regrowth pattern appears to depend on the drug concentration (Figs. 2 and 3). The organism, once it acquired resistance after the first drug addition, retained resistance to concentrations below that of the first addition (Figs. 2 and 3), and this persisted for at least 48 h during subculture (Fig. 4). These observations and the population diagram of the resistant organisms (Fig. 5) seem to support the preexistence of insensitive bacteria with different degrees of resistance, although possibilities *b*, *d*, and *e* cannot be excluded completely on the basis of the results shown in Figs. 2–4 and 7.

The subcultured organism from a single colony showed similar apparent regrowth (Fig. 8). The possibility of picking up an organism originally resistant to the ampicillin concentration of 1.5 μg/mL is very small, because the number of organisms which may manifest resistance to 1.5 μg of ampicillin/mL is expected to be <10, judging from the population diagram (Fig. 7).

The possibility of mutation and/or adaptation may be ruled out by the MIC determinations on subcultured organisms. If the resistance was acquired by mutation and/or adaptation, the MIC should increase gradually during repeated subcultures, but in fact it did not change. Furthermore, when the drug was added just at the doubling time (DNA synthesis stage) of the synchronous culture, a similar generation curve was obtained (Fig. 10); so, possibility *e* was ruled out.

The possibility of β-lactamase production still remains; therefore, we assayed β-lactamase activity by using resistant organisms generated with 8 μg of penicillin G/mL, but β-lactamase activity was not detected in the crude preparation. This result excludes the possibility that β-lactamase may have been induced by contact with the drug, leading to the acquisition of resistance.

The remaining and most probable explanation for the bacterial regrowth after the addition of the drug is a selection process and/or change of membrane permeability caused by contact with the drug at various concentrations.

Zimmermann (10) and Sawai *et al.* (11, 12) have reported an indirect method for measuring the outer membrane permeability of β-lactam antibiotics, but it is necessary to use β-lactamase-producing organisms. To confirm the difference in degree of permeability between sensitive and resistant organisms, a direct measurement of membrane permeability is desirable; however, a direct method for such measurement is not available at present.

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Spectrofluorometric Determination of Captopril Plus Captopril Disulfide Metabolites in Plasma

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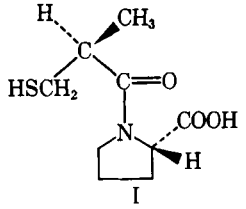
Abstract □ Captopril disulfides and the drug covalently bound to proteins were reduced with tri-*n*-butylphosphine. After sample purification on an XAD-2 column, captopril was treated with 1-(7-dimethylamino)-4-methyl-2-oxo-2*H*-1-benzopyran-3-yl)-1*H*-pyrrole-2,5-dione to form a fluorescent derivative. After acidification, the fluorescent derivative was extracted into toluene and purified on a C_{18} cartridge. The fluorescence of the dimethylformamide eluate was measured at an excitation wavelength of 380 nm and a fluorescence wavelength of 440 nm.

Keyphrases □ Captopril—spectrofluorometric analysis, plasma □ Angiotensin-converting enzyme inhibitors—captopril, spectrofluorometric analysis, plasma

Captopril (1-[(2*S*)-3-mercapto-2-methylpropionyl]-L-proline; I) a potent, specific, and orally active inhibitor of angiotensin-converting enzyme, has been shown (1) by *in vitro* and *in vivo* metabolism studies to exist in blood as unchanged drug, symmetrical disulfide, captopril–cysteine and captopril–glutathione mixed disulfides, and as captopril covalently bound to plasma albumin by S—S linkages. It has been reported that the plasma proteins and mixed disulfides with endogenous thiol compounds may act as a reservoir from which captopril is liberated over time to exert pharmacological effects (2, 3).

Several methods for determining captopril have been re-



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