KINETIC STUDIES OF AMPICILLIN ACTION ON *ESCHERICHIA COLI* AND THEIR SPHEROPLASTS

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Kinetic studies of ampicillin action were made on exponentially growing *Escherichia coli* and on *E. coli*-spheroplasts using a range of inhibitory and subinhibitory concentrations of ampicillin. For each concentration, the value $(k_o - k_a)$ representing the difference in generation rates of ampicillin-free culture (k_o) and the generation rate of the culture with ampicillin (k_a) was calculated and plotted against ampicillin concentration. A straight line relation was obtained with *E. coli* cells, its intersection with the abscissa, where $(k_o - k_a) = 0$, give the concentration of ampicillin which exerts no inhibitory action on the cells (0.25 μg/ml). When k_a was plotted against ampicillin concentration, the relation was also linear. Its intersection with the abscissa gives the minimum lethal concentration of ampicillin on the bacterial cells (1.05 μg/ml). With *E. coli*-spheroplasts such plots were non-linear which means that a different order of reaction was involved. This difference is probably due to a different mechanism of ampicillin action as revealed by the electroscanning microscopy.

Generation rates of growing cultures containing subinhibitory concentrations of antibacterial agents have been used as parameters to study the kinetics of the effects of these agents and to predict the mechanism of their antimicrobial activity[1~5]. Kinetic studies were made on chemotherapeutic agents[3], and some antibiotics[6,7]. With ampicillin, however, no such studies were reported. For this purpose, the action of ampicillin at different concentrations was measured against *Escherichia coli* growing cells and *E. coli*-spheroplasts. Furthermore, the action of ampicillin on *E. coli*-spheroplast was elucidated using the electroscanning microscope.

Theory

In order to study the kinetics of ampicillin action on *E. coli,* the generation rates of the bacterial growth without ampicillin and in the presence of ampicillin were determined from the generation-time curves and the following equations: Since *E. coli* during its course of multiplication followed an exponential order, then

$$S_f = S_0 \, e^{k_0 t} \qquad (1)$$

where S_0 is the initial number of survivors, S_f is the number after time t in the absence of ampicillin and k_0 is the generation rate. In the presence of ampicillin, the generation rate (k_0) decreases and with some concentrations has a negative sign indicating a lethal effect. Thus the relation could be represented as

$$S_a = S_0 \, e^{k_a t} \qquad (2)$$

where S_a is the number of survivors after the same time t, in the presence of ampicillin, and k_a is the apparent generation rate. In order to determine the change in the generation rates of the bacterial

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growth due to the effect of a specific concentration of ampicillin, equation (1) was divided by (2) thus

$$S_f/S_a = e^{(k_0-k_a)t} \tag{3}$$

and

$$\ln S_f - \ln S_a = (k_0 - k_a)t \tag{4}$$

Therefore, the plot of the logarithm of the ratio S_f/S_a as a function of time represent a straight line which has a slope equal to the value $k_0 - k_a$.

Materials and Methods

Materials

Ampicillin was supplied by Bayer, Leverkusen West Germany, 857 μg/mg. Potassium salt of benzyl penicillin was obtained from Serva, Feinbiochemica, Heidelberg, W. Germany, 1,538 units/mg, and penicillinase from Riker Pharma, GmbH Borken Wesfalen, Deutschland. *Escherichia coli,* type 1 (IMViC+ + − −, 44°+).[8]

Growth media

Nutrient broth, 16 g/liter (Merck, Darmstadt, Deutschland) adjusted to pH 6.7 with phosphate buffer. Nutrient agar, nutrient broth (Merck) 16 g/liter supplemented with Ion agar No. 2 (Oxoid) 1%. Spheroplasts media; Spheroplasts growth (SG) broth g/liter: Neopeptone (Difco Lab. Detroit, Michigan, USA) 15.0, yeast extract (Difco) 3.0, sucrose 105, magnesium sulphate 2.0, pH 7.0. Spheroplasts nutrient (SN) broth g/liter: nutrient broth (Merck) 16.0, sucrose 105, magnesium sulphate 2.0 adjusted to pH 6.7 with phosphate buffer. All spheroplasts media when measured with an osmometer read osmotic pressures of 500∼550 Milli-osmosis. (Halbmicro-osmometer type M Knauer, Deutschland). All nutrient media were sterilized by autoclaving at 121°C for 15 minutes.

Methods

Preparation of bacterial cultures: A 5-ml nutrient broth was inoculated with the organism from a fresh slant and incubated at 37°C for 14∼16 hours. A sample of 0.5 ml was then transferred into 25 ml nutrient broth in 50-ml screw capped bottle and maintained at 37°C in a constant temperature-shaker-water-bath (Köttermann, KG, Deutschland) for 3 hours to obtain a viable count of *ca.* 4×10^8 cells/ml. An aliquot of this culture equivalent to 1 ml/100 ml broth, was transferred to a bulk amount of nutrient broth at 37°C and contained in a flask fitted with a 50-ml glass dispenser. Fifty ml portions of the seeded nutrient broth were then transferred with the aid of the dispenser into sterile screw capped 100-ml bottles and maintained at 37°C in the shaker-water-bath.

Immediately after distributing the seeded broth into the bottles, 1-ml sample from each was withdrawn and a viable count was made.

Effect of ampicillin concentrations on the generation rates of *E. coli*

Fresh solutions of ampicillin were aseptically prepared in phosphate buffer at pH 6.7, 1-ml aliquots of the ampicillin solutions were added to 49 ml seeded broth during the logarithmic phase, usually after 30-minute incubation. At the same time, replicates of the seeded broth but without ampicillin were equally tested. At suitable time intervals, 1-ml samples were withdrawn from each culture, diluted with 0.9% sodium chloride solution and counted using the pour plate technique (from each sample 3 plates were made for viable count). The plates were incubated at 37°C for 48 hours and the developed colonies were counted.

Preparation of spheroplasts culture

A method similar to that described by HIROKAWA[15] was used. Ten ml nutrient broth was inoculated with a loop-full of *E. coli* taken from a nutrient agar slope culture and the broth was incubated at 37°C with shaking for 16 hours. The culture was then diluted five-fold with SG broth containing 1 mg/ml benzyl penicillin, and was left in the shaker at 37°C for 4 hours, where a fresh amount of benzyl penicillin was added (0.5 mg/ml) and the culture was shaken for another 2 hours at 37°C. The culture was then stored without shaking for 16 hours at 4°C. The suspension was centrifuged at 1,500 *g* for 20 minutes at 20°C and the deposited spheroplasts resuspended in SG broth. To inactivate the penicillin, 0.15 ml

of a penicillinase solution (400,000 u/ml) was then added to the spheroplasts suspension and left at 20°C for 60 minutes.   The inactivation of penicillin in the suspension was confirmed by agar diffusion assay using *Staphylococcus aureus* NCTC 4163 as the assay organism.   The spheroplasts were twice centrifuged at 1,500 g for 20 minutes and resuspended in fresh SG broth to remove any penicillinase. The absence of penicillinase activity was confirmed by mixing an aliquot portion of the spheroplasts supernatant solution with an equal volume of 100 μg/ml ampicillin solution and the ampicillin activity was then checked by comparing the inhibition zone produced with that of 100 μg/ml ampicillin solution mixed with equal volume of SG broth containing no penicillinase.   Finally the spheroplasts were suspended in SN broth, standardised to give a count of *ca.* 2×10^7 spheroplasts/ml and were used within 24 hours for generation rate measurements.

Examination of the spheroplasts

The prepared spheroplasts suspension when examined under a phase-contrast microscope has shown rounded bodies of different sizes.   In all samples examined, the normal rod-shaped cells of *E. coli* were not observed.

Effect of ampicillin concentration on the generation rate of *E. coli* spheroplasts

Fresh solutions of ampicillin were aseptically prepared in SN broth and 45-ml quantities were placed in screw capped bottles.   The SN broth was then inoculated with 5 ml of the spheroplasts culture and was left at 37°C in a shaker-water-bath.   Samples were withdrawn at intervals (15~30 minutes), diluted with 0.9% sodium chloride solution and a viable count was made using SN agar.

Preparation of samples for the electroscanning microscope

Samples were taken, fixed and stained for examination under the electroscanning microscope (Stereoscan 600 Cambridge) according to the method described by GREENWOOD & O'GRADY[9].   A 2.5-ml aliquot from each culture was mixed with equal volume of a fixer consisting of 10% sucrose and 2.0% glutaraldehyde and left overnight at 4°C.   The suspension was then centrifuged at 1,500 g for 20 minutes, washed with distilled water 3 times and finally suspended in 1.0 ml water.   Droplets of each suspension were then placed on a glass cover-slip previously attached to an electron microscope stab.   The droplets were air-dried and stained with 1.0% ferric chloride solution for 10 minutes, rinsed with 2 changes of distilled water and finally dried in a dessicator.   The dried preparations were then coated with a thin-layer of carbon followed by a thin-layer of gold and examined under the electro-scanning microscope.

Results

Effect of Ampicillin Concentration on Growing *E. coli* Cells

The minimum inhibitory concentration of ampicillin was 4~6 μg/ml using inoculumn of 4×10^6 cells/ml.   This concentration was also reported by other workers[10,11].   In this work, therefore, the kinetics studies were made using ampicillin concentrations upto 4.0 μg/ml.   The results obtained are presented in Fig. 1.   Each point is the average of 6 different replicates.   From Fig. 1, the effect of ampicillin was lethal when used at concentrations higher than 1.0 μg/ml.   Although the antibiotic was used at concentrations below the MIC, however, lower viable counts were obtained shortly after the inclusion of the ampicillin.   In this respect, ampicillin behaves differently from other antibiotics like tetracyclines[6], sodium fusidate[5] and lincomycin[7] when similarly tested.

Effect of Ampicillin Concentration on *E. coli* Spheroplasts

The ability of the spheroplasts to revert to normal cells and form visible colonies on nutrient agar was used here to measure the response of the spheroplasts to the ampicillin action.   LEDERBERG[12] has shown that *E. coli* spheroplasts reverted into rods when the penicillin was removed from the medium. Thus the spheroplasts form visible colonies only after their reversion and after being capable to divide like the normal cells[16].   The effect of different concentrations, upto 100 μg/ml, of ampicillin on *E. coli*-

Fig. 1.   Generation-time curves of *E. coli* culture in the absence and in the presence of 0.25, 0.5, 1.0, 1.5, 2.0 and 4.0 μg/ml ampicillin.

The arrow indicates the time at which the ampicillin was added.

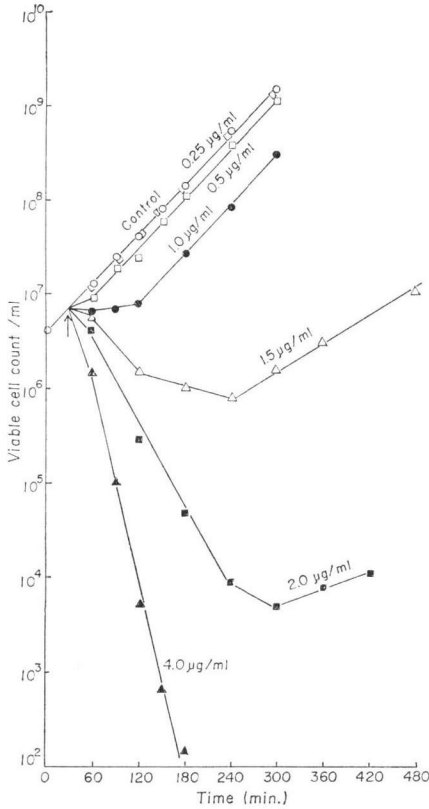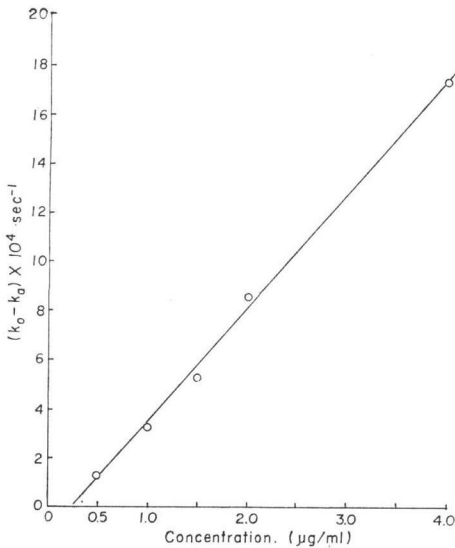Fig. 2.   Survivors number ratios of ampicillin-free culture of *E. coli* (S_f) to the culture with different concentrations (0.5, 1.0, 1.5, 2.0 and 4.0 μg/ml) of ampicillin (S_a) in relation to time.
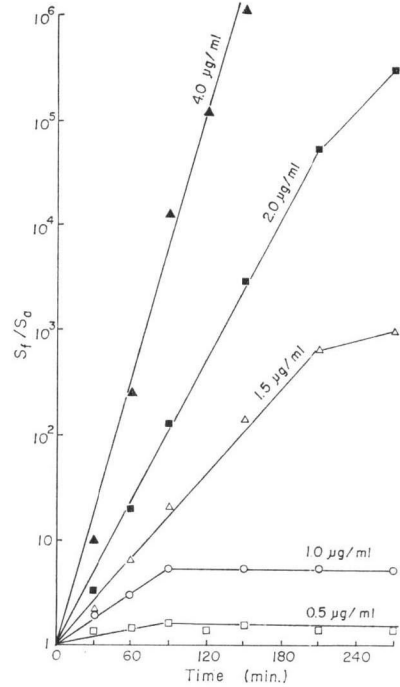
Fig. 3.   Plot of the difference between the generation rate of *E. coli* in the absence of ampicillin (k_o) and the apparent generation rate in the presence of ampicillin (k_a) as a function of ampicillin concentration.
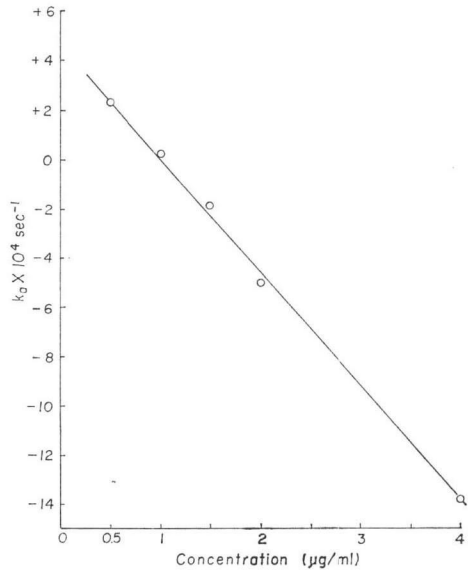
Fig. 4.   Dependence of the apparent generation rate (k_a) of *E. coli* on the ampicillin concentration in the culture medium.

spheroplasts is shown in Fig. 5. In the absence of ampicillin, the viable count followed a logarithmic relation with a generation rate $= 6.24 \times 10^{-4}$ sec^{-1}. This rate was nearly double that of the normal cells, 3.6×10^{-4} sec^{-1}. Providing that the reverted cells multiplied at the same rate as the normal cells,

Fig. 5. Generation-time curves of *E. coli* sphero-
plasts in the absence and in the presence of 2, 4,
10, 20, and 100 μg/ml ampicillin.

Fig. 6. Survivors number ratios of ampicillin-free
culture of *E. coli* spheroplasts (S_f) to the culture
with different concentrations (2, 4, 10, 20 and
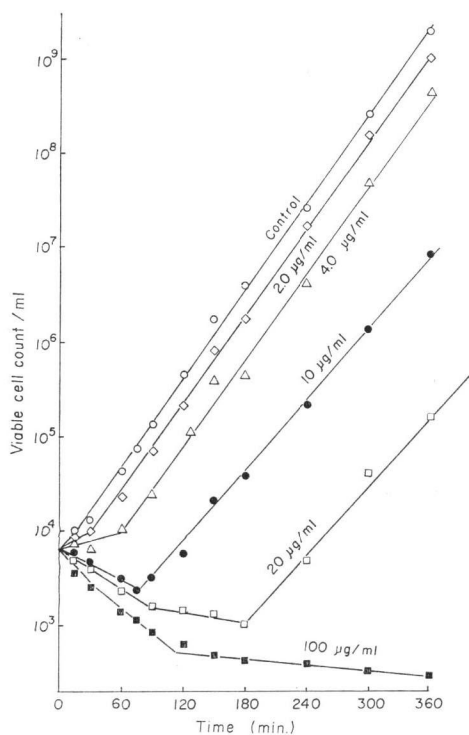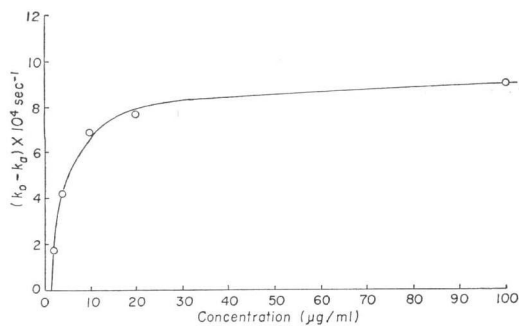100 μg/ml) of ampicillin (S_a) in relation to time.

Fig. 7. Plot of the difference between the generation
rate of *E. coli* spheroplasts in the absence of ampicil-
lin (k_o) and the apparent generation rates in the
presence of ampicillin (k_a) as a function of ampicil-
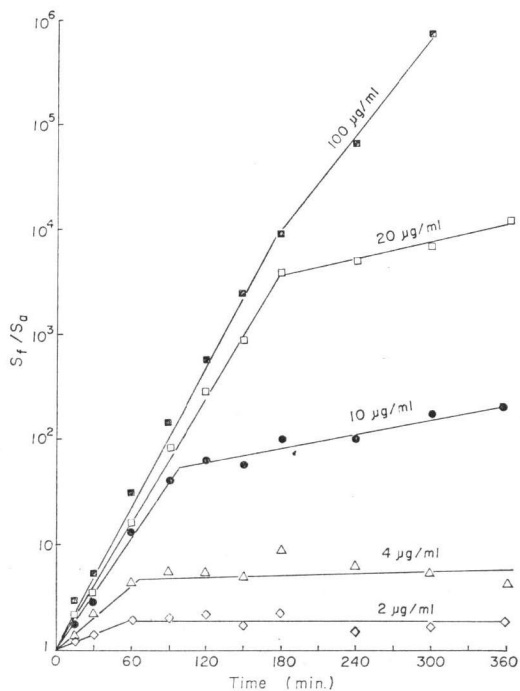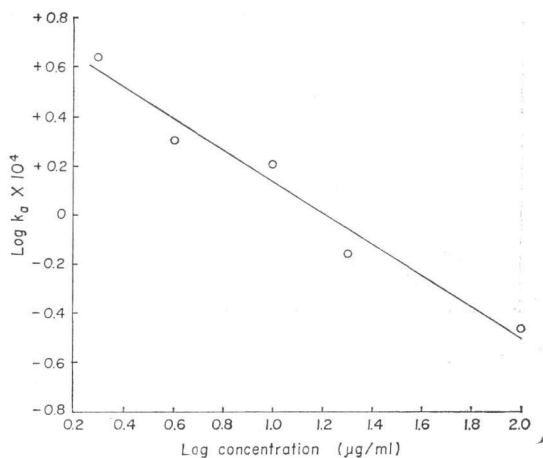lin concentration.

Fig. 8. Dependence of the apparent generation rate
(k_a) of *E. coli* spheroplasts on the ampicillin con-
centration in the culture medium.

then the difference between the two rates is the apparent reversion rate of the spheroplasts and $=2.84 \times 10^{-4} \sec^{-1}$. In the presence of ampicillin, the generation rate did not change upto 10 μg/ml concentrations but the growth was preceeded by a lag period which varied according to the ampicillin concentration. At ampicillin concentrations above 10 μg/ml, the growth was preceeded by a period during which the viable count was decreasing with a rate depending upon the ampicillin concentration.

Scanning Electron-microscopy of Ampicillin-treated *E. coli* and their Treated Spheroplasts

Samples of *E. coli* cells taken during their growth in the absence of ampicillin as well as after 1-hour contact with different concentrations of ampicillin were examined under the Stereoscan 600 (Cambridge). Electron-micrographs of untreated and ampicillin-treated *E. coli* are presented in Plates 1 and 2. With *E. coli* spheroplasts, electron micrographs showing the shape of the *E. coli* spheroplasts (Plate 3) and the effect of ampicillin at 100 μg/ml concentrations on the integrity of the spheroplasts (Plate 4) are presented.

Plate 1. Electron micrograph of untreated *E. coli* cells during their growth. ×2,000.

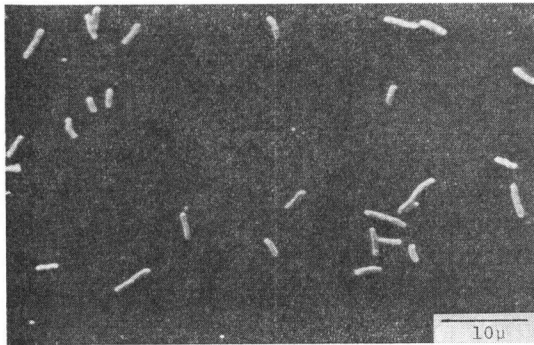Plate 2. Electron micrograph of *E. coli* growing cells treated with 1.5 μg/ml ampicillin, showing emergent spheroplast. ×10,000.
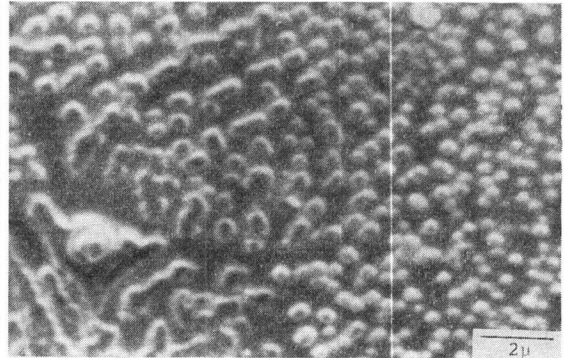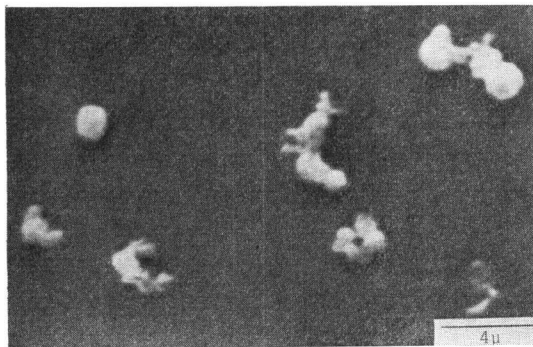

Plate 3. Electron micrograph of *E. coli* spheroplasts. ×5,000.
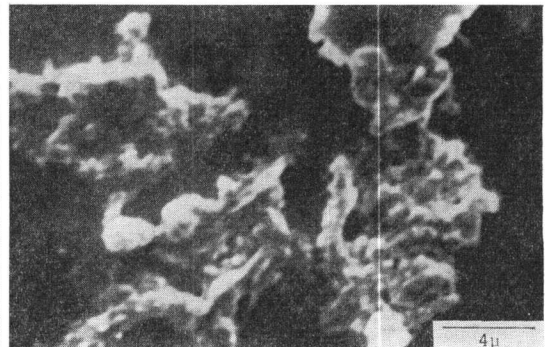
Plate 4. Electron micrograph showing the effect of ampicillin at 100 μg/ml on the integrity of the *E. coli* spheroplasts. ×5,000.

Discussion

Although ampicillin with *E. coli* cells was present at concentrations below the MIC, it was however taken by the cells and exerts its effect after a short time of contact (15 minutes), as indicated by the re-

duction in the survivors number or by the lag phase in Fig. 1. Ampicillin at 0.25 μg/ml has no harmful effect while at concentrations 1.5, 2.0 and 4.0 μg/ml exhibited a bactericidal effect as appeared from the reduction of the viable counts. This could be explained from the electroscanning microscope studies. Those cultures exhibiting reduction in the viable counts have shown some spheroplasts under the electron microscope, while culture containing 1.0 μg/ml and lower concentrations have not shown any spheroplasts. Such results, therefore, indicate that the loss of viability is directly associated with the formation of spheroplasts.

The kinetics of the effect of ampicillin at the tested concentrations on growing bacteria could determine the order of the reaction possibly involved with the formation of the spheroplasts. When the logarithm of the ratio S_f/S_a was plotted against time, straight line relations were obtained. The slope of each represents the value $(k_0 - k_a)$, which when plotted against the ampicillin concentration produced a straight line (Fig. 3). Also, the plot of k_a versus concentration (Fig. 4) is linear and means that the order of reaction involved is a first order.

In Fig. 3, the intersection of the straight line with the abscissa gives the concentration of ampicillin at which $(k_0 - k_a) = 0$, this was 0.25 μg/ml. This concentration is in agreement with the experimental results using ampicillin at this concentration (Fig. 1). While the intersection of the straight line in Fig. 4 with the abscissa gives the concentration at which $k_a = 0$. This concentration was 1.05 μg/ml and represents the minimum lethal concentration. At higher ampicillin concentrations, k_a has a negative sign, which indicates the reduction in the viable count from the original count due to the effect of ampicillin. It is clear from the results obtained that when the ampicillin concentration exceeds 4 μg/ml the culture maintained a bactericidal effect which persists until all the cells were converted into spheroplasts and lysed.

The effect of ampicillin concentration on *E. coli* spheroplasts was different from that on normal cells. With *E. coli* spheroplasts, higher ampicillin concentrations were required in order to detect reduction in the viable counts. The results obtained from the kinetics studies were also of different behaviour. The sensitivity of the spheroplasts to ampicillin action was much lower than the bacterial cells, as indicated by the apparent generation and degeneration rates for both (Table 1). It is interesting to notice also that the plot of $(k_a - k_0)$ in case of spheroplasts fit a curve (Fig. 7) and not a straight line as it was with normal cells (Fig. 3). This difference in response could indicate that ampicillin acts on the spheroplasts through different receptor sites. Further proof is the lower order of response obtained when log k_a was plotted against log concentration (Fig. 8), this was 0.634. The most likely explanation for the effect exerted by ampicillin on the spheroplasts, which is a concentration dependence, is the suggestion given by PARK[13] who considered the culture of penicillin-spheroplasts to be consisted of a mixture of different stages defected cell-wall cells; some cells were with completely deficient murein sacculus[14], and the others were with partially defected cross-linked murein sacculus. It was the latter type of cells which was affected by the ampicillin and require therefore high concentrations of the antibiotic.

Another effect of ampicillin on the spheroplasts which was observed by the electroscanning microscope, was the damage occurred to them by high ampicillin concentrations (Plate 4). At 100 μg/ml ampicillin concentration, the spheroplasts were completely ruptured and cell fragments were observed. The actual mechanism involved to produce this effect is not quite clear, however, it may be due to a direct action of ampicillin on the protoplast membrane resulting in their lyses, an effect which was not exhibited by benzyl penicillin.

Table 1. Effect of ampicillin concentration on the apparent generation rate (k_a) of *E. coli* cells and *E. coli* spheroplasts.

Ampicillin μg/ml	(k_a) sec^{-1}	
	Bacterial cells	Spheroplasts
0.0	3.6×10^{-4}	6.2×10^{-4}
0.5	2.3×10^{-4}	—
1.0	0.3×10^{-4}	—
1.5	-1.7×10^{-4}	—
2.0	-5.0×10^{-4}	4.4×10^{-4}
4.0	-13.8×10^{-4}	2.0×10^{-4}
10.0	—	-0.6×10^{-4}
20.0	—	-1.4×10^{-4}
100.0	—	-2.9×10^{-4}

Acknowledgement

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