QUANTITATIVE ASPECTS OF PENICILLIN ACTION ON *ESCHERICHIA COLI* IN HYPERTONIC MEDIUM

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Duguid (1946) suggested, as a result of observations on bacteria treated with penicillin, that this antibiotic might inhibit the formation of the outer supporting cell wall. Bonifas (1954) observed the formation of globular bodies when cultures of *Proteus vulgaris* were treated with penicillin, and investigated the effect of hypertonic media, already used to stabilize the lysozyme induced protoplast of *Bacillus megaterium* by Weibull (1953). Lederberg (1956, 1957) demonstrated the conversion of both *Escherichia coli* and *Salmonella typhi* to spherical osmotically fragile bodies by penicillin, provided the medium contained 0.33 M sucrose and preferably 0.2 per cent $MgSO_4 \cdot 7H_2O$. Similar results have been reported for *P. vulgaris* (Liebermaster and Kellenberger, 1956); *E. coli* (Hahn and Ciak, 1957); *Alcaligenes faecalis* (Lark, 1958*a*, *b*); *Cloaca cloacae*, *Citrobacter freundii*, *Klebsiella aerogenes*, *Serratia marcescens*, *Proteus morganii*, *Pseudomonas aeruginosa*, *Pseudomonas hydrophila*, *E. coli* (two strains), *Vibrio cyclosites*, and *Vibrio neocistes* (Hugo, 1958); *E. coli* strain B (Hurwitz, Reiner, and Landau, 1958); *E. coli* strain K12 and *Proteus mirabilis* (Landman, Altenbern, and Ginoza, 1958); and *Xanthomonas phaseoli* (Nozzolillo and Hochster, 1959).

Brenner et al. (1958) suggested the term protoplast should be retained only if complete absence of cell wall is proved and the term round body or spheroplast refers to an observed microscopical appearance without commitment as to presence or absence of certain cell wall components, and the second term will be used hereafter.

Quantitative aspects of the changes occurring during penicillin induced spheroplast formation in cultures of a strain of *E. coli* are reported in this paper.

MATERIALS AND METHODS

Organism and maintenance of strain. The organism used was *E. coli* (formerly NCTC 5934). Tested by the method recommended in the Report of the Coliform Sub-committee (1949) it was designated *E. coli* type I. This strain produces no demonstrable extracellular or intracellular penicillinase, as ascertained by incubating the supernatant fluid from a centrifuged culture and a disintegrated (Mickle, 1948) preparation of the organism with 5 units of penicillin, when no diminution of penicillin activity was found.

The organism was maintained in nutrient broth; at weekly intervals the culture was plated on MacConkey's agar to check upon purity and general colony morphology. A typical smooth, lactose fermenting colony was used to initiate a new weekly culture. All cultures were incubated at 37 C.

Culture media. Nutrient broth contained in each liter: meat extract (Lab Lemco, Oxoid, England), 10 g; peptone (Oxoid), 10 g; sodium chloride, 5 g. When a solid medium was required, agar, 20 g, was included. Fermentation characteristics were determined in peptone water with the appropriate addition. MacConkey's agar was prepared from the dehydrated medium (Difco).

Liquid conversion medium for the study of penicillin action consisted of nutrient broth (defined above) containing magnesium sulfate ($MgSO_4 \cdot 7H_2O$) and sucrose at final concentrations of 0.25 per cent (w/v) and 0.33 M, respectively. This medium has no adverse effect on the growth of the organism, and the mean generation time during the logarithmic phase of growth is of the order of 30 min, which is similar to that in nutrient broth.

Quantitative measurements. Viable counts were made by serial dilution in distilled water and plating in nutrient agar. When viable counts were made from cultures containing penicillin the first dilution tube also contained sufficient penicillinase to destroy any penicillin present. When such counts were performed in cultures containing spheroplasts, it was found that these underwent rapid lysis during the serial dilution and could no longer be detected after this process, but ½

hr was allowed to elapse between dilution and plating to ensure their complete destruction. This process, therefore, gave a count of those cells remaining osmotically stable and capable of growth.

Total spheroplast counts were made using a hemocytometer (0.1 mm depth) or a Helber counting chamber (0.02 mm depth) in conjunction with an interference microscope (C. Baker, London, England). Spheroplast diameter could be determined simultaneously by reference to a calibrated eyepiece micrometer. Culture opacity was determined on a nephelometer (Evans Electroselenium Ltd., Harlow, England).

Chemicals. The penicillin used was the sodium salt of benzylpenicillin of the British Pharmacopoeia 1958, and contained no added citrate, or surface active agent. All other chemicals were of analytical reagent quality.

Penicillin determination. The penicillin content of conversion medium was determined by the cylinder plate method upon a sample of the medium sterilized by passage through a cellulose acetate membrane.

RESULTS

In a typical experiment, 10 ml of a 17-hr culture of *E. coli* grown at 37 C were added to 190

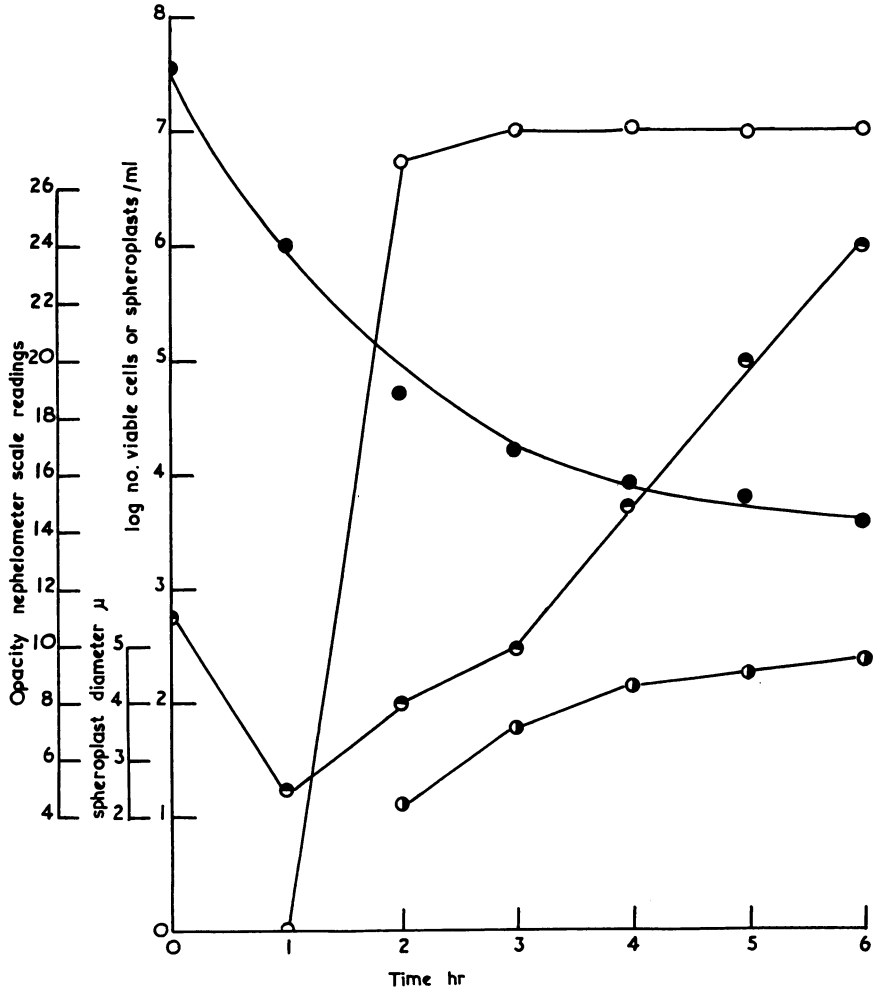

Figure 1. Changes in viable population ●——● opacity ◓——◓ and formation, ○——○, and diameter ◑——◑ of spheroplasts when a culture of *Escherichia coli* is treated with 5,000 u/ml penicillin at 37 C in hypertonic medium.

TABLE 1

Effect of penicillin concentration on the formation of spheroplasts and the number of survivors when Escherichia coli is treated with the antibiotic in a hypertonic medium

Penicillin Concn	Viable Count in Medium, 0 hr	Viable Count in Medium, 5 hr	Spheroplast Count in Medium, 5 hr
units/ml	*per ml*	*per ml*	*per ml*
100	4×10^7	7.7×10^4	3.0×10^7
1,000	3.9×10^7	4.4×10^3	2.0×10^7
5,000	3.9×10^7	6.7×10^3	1.4×10^7

ml of conversion medium of nutrient broth containing, at a final concentration, sucrose, 0.33 M; $MgSO_4 \cdot 7H_2O$, 0.25 per cent (w/v; 0.01 M); and penicillin, 5,000 u/ml, in a conical Roux flask of 18-cm diameter base. A sample was immediately withdrawn for a viable count and the flask incubated at 37 C. At hourly intervals samples were removed and viable count, spheroplast count and diameter, and culture opacity were determined. The results of such an experiment are shown in figure 1.

During the first hour of the experiment, few spheroplasts can be found in the medium. Those seen are small (<2 μ in diameter), and not numerous enough for reliable counts to be made. They are thus represented as zero in figure 1. After 2 hr incubation, almost the maximal number are formed, and a constant value is attained after 3 hr incubation. From 2 to 6 hr there is an increase in average diameter from 2.2 to 4.8 μ, and the opacity of the culture increases. The viable count falls to a residual 10^3–10^4 organisms/ml. After 24 hr incubation, the viable count falls to 10^3/ml, after 48 hr to 10^2/ml, and at 72 hr is zero. The spheroplasts gradually disintegrate over this period. If the experiment is repeated omitting sucrose and magnesium sulfate, no spheroplasts form and the fall in viable cell numbers follows the same pattern as shown in the corresponding viable count curve of figure 1. In 0.16 M sucrose, very few spheroplasts are formed, but in 0.66 M sucrose, a conversion picture similar to that shown in figure 1 is obtained but the equilibrium spheroplast diameter is lower.

Effect of penicillin concentration. This was studied at concentrations of 100, 1,000, and 5,000 u/ml. The final concentrations of $MgSO_4 \cdot 7H2O$ and sucrose were constant at 0.25 per cent (w/v), and 0.33 M, respectively. Results are shown in table 1.

TABLE 2

Effect of magnesium sulfate concentrations on penicillin-induced spheroplast formation in Escherichia coli at a penicillin concentration of 5000 u/ml

Concn Added $MgSO_4 \cdot 7H_2O$	Viable Count in Medium, 0 hr	Viable Count in Medium, 5 hr	Spheroplast Count in Medium, 5 hr
% *w/v*	*per ml*	*per ml*	*per ml*
Absent	3.6×10^7	7×10^2	1.6×10^6
Absent*	3×10^7	3.9×10^2	None observed
Absent†	3×10^7	1.2×10^3	None observed
0.05	3.5×10^7	5.7×10^3	4.5×10^6
0.2	3.4×10^7	2.3×10^3	1.1×10^7
0.25	3.9×10^7	6.7×10^3	1.4×10^7
1.0	4.2×10^7	6.8×10^3	1.1×10^7

* Medium contained 0.1 per cent (w/v) trisodium citrate.

† Medium contained 0.1 per cent (w/v) EDTA (tetrasodium salt).

Effect of magnesium concentration. This was also studied at penicillin concentration of 5000 u/ml, and sucrose constant at 0.33 M. The production of spheroplasts could be prevented by the addition of the tetrasodium salt of ethylenediaminetetraacetic acid (EDTA) or trisodium citrate (0.1 per cent, w/v, in each case) to nutrient broth containing 0.33 M sucrose and to which no $MgSO_4 \cdot 7H_2O$ had been added. Results are shown in table 2.

Problem of persisting viable rods. The striking feature of these quantitative investigations was the constant surviving population of viable rods. At first appraisal, the organisms resisting conversion to round bodies or suffering rapid killing in hypertonic medium or resisting lysis or rapid killing in normal medium would be classified as resistant mutants or resistant members of a nonhomogenous population and this contention was subjected to experiment. First, these survivors were subjected to a recycling process through a normal conversion process. A sample of osmotically stable survivors for each recycle was conveniently obtained from the first (1 in 10) dilution prepared when performing the viable count after 5-hr penicillin treatment, and a portion of these cells was used to prepare a 17-hr

TABLE 3

Effect of penicillin (5000 u/ml) on an inoculum derived from the survivors from a previous day's experiment

Day	Ratio of No. of Viable Cells at 0 hr to No. of Viable Cells at 5 hr	Ratio of No. of Viable Cells at 0 hr to No. of Spheroplasts at 5 hr
1	5800:1	2.8:1
2	6200:1	3.5:1
3	100,000:1	3.1:1

inoculum for conversion treatment on the following day. The results are shown in table 3.

As can be seen, the ratios of spheroplast to inocula are of the same order, whereas the ratio of inoculum to survivors is increasing. If, in fact, the persistors represented resistant mutants, it would be expected that the ratio of spheroplast to inoculum would decrease with each recycle, and that the ratio of survivors would increase. It can be seen that in the third cycle the survivors (persistors) in fact decrease in number.

Additional experiments were performed in which the concentrations of penicillin were determined which would just permit, and just inhibit, growth from small inocula (8 to 76 cells) of cells derived from (a) a "normal" (penicillin untreated) 17-hr culture, (b) persistors, and (c) a 17-hr culture derived from these persistors.

In each case, it was found that whereas all these types of cells grew in the presence of 40 u/ml, they were inhibited by 50 u/ml, suggesting that there was no difference in their sensitivity to penicillin.

DISCUSSION

The action of penicillin in hypertonic media on our strain of *E. coli* presents a stable general pattern, characterised by the appearance after 2 hr of spheroplasts and a residual population of persistors. The spheroplasts undergo a 7-fold increase in volume from the 2nd to the 4th hr and this may be due to further growth unhampered by rigid cell wall restriction or to the fact that the spheroplasts are acting as osmometers and are slowly reaching osmotic equilibrium during this period. From a consideration of the composition of the normal liquid environment and the lack of stability of the spheroplasts in 0.16 M sucrose the internal osmotic pressure is likely to be of the order of from 4 to 8 atmospheres. The persisting cells are indistinguishable from "normal" cells in diagnostic biochemical properties and colony appearance on MacConkey's agar. Furthermore, they have the same sensitivity to lower penicillin concentrations. The organism does not produce detectable penicillinase, and there is no significant fall in penicillin titer during the conversion period, so that it is not possible to invoke penicillin destruction as an explanation for persistence. If the property of persistence is due to the presence of resistant mutants, an increase in the number of persistors would be expected during the recycling experiment, but table 3 shows that this is not the case.

A source of magnesium ions appears necessary for stable spheroplast production. In nutrient broth containing 0.33 M sucrose a count of 10^6 is achieved, this is increased 10-fold at $MgSO_4 \cdot 7H_2O$ concentrations above 0.05 per cent. If compounds capable of chelating magnesium are added to conversion media, no spheroplast formation occurs. Presumably stabilized nutrient broth with sucrose contains enough Mg^{++} for some spheroplast formation to occur.

Inspection of the counts for inoculum, spheroplasts and persistors in the experiment with 5000 u/ml shows that some two-thirds of the inoculum are unaccounted for, appearing neither as countable spheroplasts nor as colony-forming persistors. Two explanations for this are possible. First, that the cells are killed by a toxic effect other than cell wall inhibition, or second, that some of the spheroplasts are osmotically unstable, and burst as soon as they are formed, so that they may never be counted. We do not favor the second explanation, as preliminary experiments in conversion media containing 0.66 M sucrose have shown that there is no increase in the number of spheroplasts. In fact, except that the equilibrium spheroplast diameter is smaller, the conversion picture is the same.

Our results lead us to believe that, with the 17-hr culture employed throughout, only those cells which can reach the point of division are able to form spheroplasts, which might well explain why a greater yield of spheroplasts is obtained with 100 u/ml than with 5000 u/ml.

In similar studies, Lederberg (1956) and Lederberg and St. Clair (1958) showed that in the case of *E. coli* strains K12 and Y10, there was a "1-for-1" conversion of cells to protoplasts.

Landman et al. (1958), however, reported only a 50 per cent conversion of *E. coli* strain K12 when treated with 1,000 u/ml penicillin in a semisynthetic conversion medium. Hurwitz et al. (1958) have suggested that penicillin has an effect on viability which precedes the effect on susceptibility to lysis, and our results give support to this contention.

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SUMMARY

When *Escherichia coli* is treated with penicillin in a hypertonic medium the inoculum appears to suffer two lethal events, first, a killing which is initially rapid and then slows considerably, resembling a typical disinfection curve and second, an interference with cell wall synthesis which, unless it takes place in hypertonic media, is also lethal.

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