STUDIES IN THE PHYSIOLOGY AND BIOCHEMISTRY OF PENICILLIN-INDUCED SPHEROPLASTS OF *ESCHERICHIA COLI*[1]

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In the course of an investigation on biosynthetic mechanisms of *Escherichia coli*, we found it convenient to use preparations according to Lederberg (1956), who reported that these organisms form osmotically fragile spheres when subjected to high concentrations of penicillin. The problems of reproducibility raised by such preparations necessitated a more detailed study of the effects of this antibiotic.

Park and Strominger (1957) reported accumulation of uridine diphosphate derivatives in *Staphylococcus aureus* exposed to penicillin, and suggested that cell wall synthesis is blocked. No similar accumulation has been shown to occur in *E. coli* to our knowledge.

Trucco and Pardee (1958) have reported experiments which seem to show that the *E. coli* cell wall is synthesized during exposure to penicillin. Unfortunately, these experiments, like the interesting experiments of Prestidge and Pardee (1957), were performed in synthetic medium rather than broth, and at a penicillin concentration about 25 per cent of that used by Lederberg (1956) and by us.

Moreover, Lederberg (1956) found penicillin-induced spheroplast formation in a minimal medium to be irregular and incomplete. This has been confirmed by Reiner (*unpublished data*) who found that in a synthetic medium, even at high penicillin levels, spheroplasts were not formed; instead, filamentous forms which reverted to normal rods after 4 to 5 hr were observed.

The effect of exposure of *E. coli* to high levels of penicillin in hypertonic (osmotically stabilizing) medium has two readily apparent results: (1) the rods round up into spheres; and (2) a marked increase in osmotic fragility makes the cells subject to lysis when the stabilizing medium is diluted. Lederberg (1956) has referred to these forms as protoplasts. In view of current uncertainty as to the fate of the cell wall in this case, we shall use "spheroplasts" (McQuillen, 1958, *personal communication*) as a neutral term.

According to Lederberg's technique (1956) the bacteria are exposed to 1000 units of penicillin in penassay broth fortified with 20 per cent sucrose and 0.2 per cent $MgSO_4$. In such a medium, *E. coli* forms spheres which undergo lysis on dilution of the sucrose content below 10 per cent. These lysates, and ghosts separated from these lysates by centrifugation, have been shown to synthesize deoxyribonucleic acid, ribonucleic acid, protein, and β-D-galactosidase under proper conditions.

One difficulty in using these spheroplasts as sources of bacterial enzymes is in following the time course of the process. Most workers apparently have used a visual estimate of the extent of formation of spheres. In our hands, this method proved to be unreliable except at the end point. Even at the end point, precise timing turns out to be critical for obtaining biosynthetically active preparations, as will be seen later. In this report a method for determination of extent of penicillin-induced spheroplast formation from *E. coli* is described. This method is based on the osmotic fragility of the spheroplasts in hypotonic solution and relies on the assumption that a spheroplast suspension diluted by a large amount of distilled water should show a marked decrease in turbidiy when lysis occurs. Furthermore, in a mixture of whole cells and spheroplasts, the degree of change of turbidity should be a function of the proportion of lysable spheroplasts to total cells.

METHODS

E. coli strain B was inoculated from a nutrient agar slant into 6 ml of penassay broth and incubated overnight (18 to 19 hr) at 37 C on a roller

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tube assembly. The bacteria were then centrifuged at 6000 × G for 10 min and resuspended in 6 ml of fresh penassay broth. This suspension was then poured into a 150-ml flask containing 20 ml penassay broth, fortified with 20 per cent sucrose and 0.2 per cent $MgSO_4$, and containing 1000 units (0.67 mg) of penicillin G per ml. This suspension was incubated aerobically by swirling on an Arthur H. Thomas rotating apparatus at 37 C for the indicated time periods. For turbidity measurements, 0.4 ml of the cellular suspension was diluted with 7.5 ml of distilled water and the light scatter was measured in a Coleman model 7 photonephelometer in Nephelos units.

Deoxyribonucleic acid was measured by the Burton (1956) modification of the Dische diphenylamine procedure. Deoxyribonucleic acid in the residue was extracted and washed with cold 0.2 N $HClO_4$ (in some experiments, with cold 5 per cent trichloracetic acid). The washed residue was then hydrolyzed in 0.5 N $HClO_4$ at 80 C for 30 min (in some experiments with 5 per cent trichloracetic acid at 95 C for 15 min). One volume of this supernatant was then incubated with 2 volumes of Burton's deoxyribonucleic acid reagent overnight in the dark and then read at 600 mμ with the Beckman DU spectrophotometer using microcuvettes.

Deoxyribonucleic acid in the supernatant was treated somewhat differently. A small amount (0.1 ml of 1 per cent solution) of fraction V bovine albumin was added to the chilled solution to aid in precipitation. The solution was then brought to 5 per cent trichloracetic acid concentration by addition of 80 per cent trichloracetic acid to precipitate the deoxyribonucleic acid. The washed residue was then hydrolyzed with 5 per cent trichloracetic acid at 95 C for 15 min and the supernatant was then incubated with deoxyribonucleic acid reagent and determined as above.

The ability of cellular suspensions (both whole cells and spheroplasts were treated alike) to synthesize β-galactosidase was measured by the procedure described by Reiner (*unpublished data*). The suspension (26 ml) was centrifuged and washed once with 20 ml of cold 20 per cent sucrose and then resuspended by homogenization in 4 ml of cold 1 per cent NaCl. Standard amounts (0.05 ml) of this suspension were then incubated at 37 C for 2 hr with a mixture of amino acids, purines, and pyrimidines. Lactose was present as an inducer. The incubation mixture also contained 0.2 per cent $MgSO_4$ and was buffered at pH 7.2 with potassium phosphate.

At the end of the 2-hr incubation, chloramphenicol was added to stop synthesis, and toluene was added to make cryptic enzyme accessible to substrate. *Ortho*-nitrophenyl-β-D-galactoside was then added to measure enzyme activity. This reaction was stopped after a given time interval (depending on the activity of the preparation) by addition of M Na_2CO_3. The tubes were centrifuged 3 to 5 min to clarify the solution and then read on a Klett colorimeter using a no. 42 filter. Activity was recorded as adsorption in Klett units resulting from liberation of *o*-nitrophenol by cleavage of the galactoside in 30 min.

Viable counts were made by surface plating in quadruplicate on nutrient agar after dilution in distilled water at room temperature.

RESULTS

Since spheroplasts are osmotically fragile in a hypotonic environment, it was felt that on dilution of a cellular suspension in distilled water, the spheroplasts would lyse, causing a decrease in turbidity. A comparably treated suspension of whole cells would not lyse under these conditions and would therefore show no similar decrease in turbidity.

Preliminary work at room temperature showed that lysis occurred when a spheroplast suspension was diluted with distilled water. However, the rate of clearing after dilution was very slow, in contrast to lysis of lysozyme preparations (Repaske, 1956). It took 40 to 45 min before maximum clearing occurred. It was observed, however, that the rate of clearing after dilution increased rapidly with temperature. At 56 C maximum clearing resulted in less than 3 min. Therefore, as a routine procedure, lysis was measured as a function of decrease in turbidity 4 min after 0.4 ml of the cellular suspension was diluted with 7.5 ml of distilled water at 56 C. The turbidity of a whole cell suspension similarly diluted was taken as the value showing no lysis, that is, the zero time value of penicillin exposure.

Figure 1 shows a typical fragility curve in which the lysis, tested in this manner, is plotted against time of exposure of *E. coli* to penicillin under Lederberg's (1956) condition. When 20 per cent sucrose was used as a diluent instead of distilled water, no lysis was observed.

Because spheroplasts are more osmotically fragile than whole cells, it was reasoned that

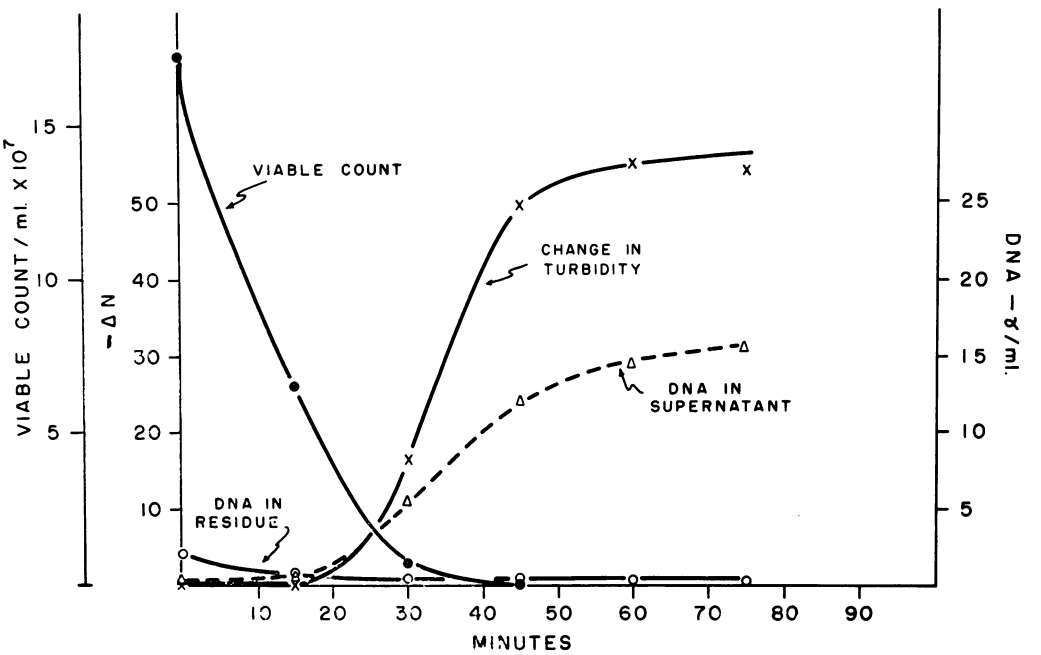

Figure 1. Osmotic fragility is plotted as the decrease in turbidity ($-\Delta N$) 4 min after the suspension is diluted in distilled water at 56 C. The turbidity of the whole cell suspension diluted under the same conditions was taken as the zero time value. Turbidity was measured in Nephelos units (N) with a Coleman photonephelometer. DNA = deoxyribonucleic acid.

exposure to hypotonic solution should release deoxyribonucleic acid from the spheroplast residue into the supernatant. If the fragility curve is a valid measure of spheroplast formation, the deoxyribonucleic acid content of the residue should decrease as turbidity decreased, while its content in the supernatant should increase. One-ml samples were diluted with 18.75 ml of water at 56 C at the designated time intervals. After the turbidity was determined, the solution was centrifuged and the deoxyribonucleic acid contents of the residue and of the supernatant were determined. Figure 1 shows the relationship of the deoxyribonucleic acid distribution to the fragility curve. The deoxyribonucleic acid in the residue decreased to almost zero at about the time the cells showed evidence of becoming osmotically fragile while its content in the supernatant increased sharply. It is interesting to note that a large amount of net deoxyribonucleic acid synthesis occurred during spheroplast formation.

Viability counts were also made of the bacterial suspension at the same time intervals. Here it was reasoned that, if penicillin kills by making the bacteria susceptible to lysis in a hypotonic solution, the viable count should decrease as fragility increased, providing the bacteria were diluted in distilled water and plated on unsupplemented nutrient agar to promote lysis of the spheroplasts. Lederberg and St. Clair (1958) noted that about 50 per cent of spheroplasts survived after being washed and diluted in sucrose and then plated on sucrose-containing nutrient agar. Under our conditions, the viable count decreased sharply, well before fragility became appreciable (figure 1). From this, it would seem that penicillin must have an effect on viability which precedes the effect on susceptibility to lysis.

Finally, the synthesizing ability of the bacteria exposed to penicillin was correlated with the fragility curve. In this experiment, a sample of the bacterial suspension was separated by centrifugation at indicated time intervals, washed with 20 per cent sucrose, and then exposed to the lytic effect of resuspension in cold 1 per cent NaCl by means of a tight-fitting glass homogenizer. The resulting suspensions were then incubated in a medium required for synthesis of β-galactosidase as described under Methods. In this experiment,

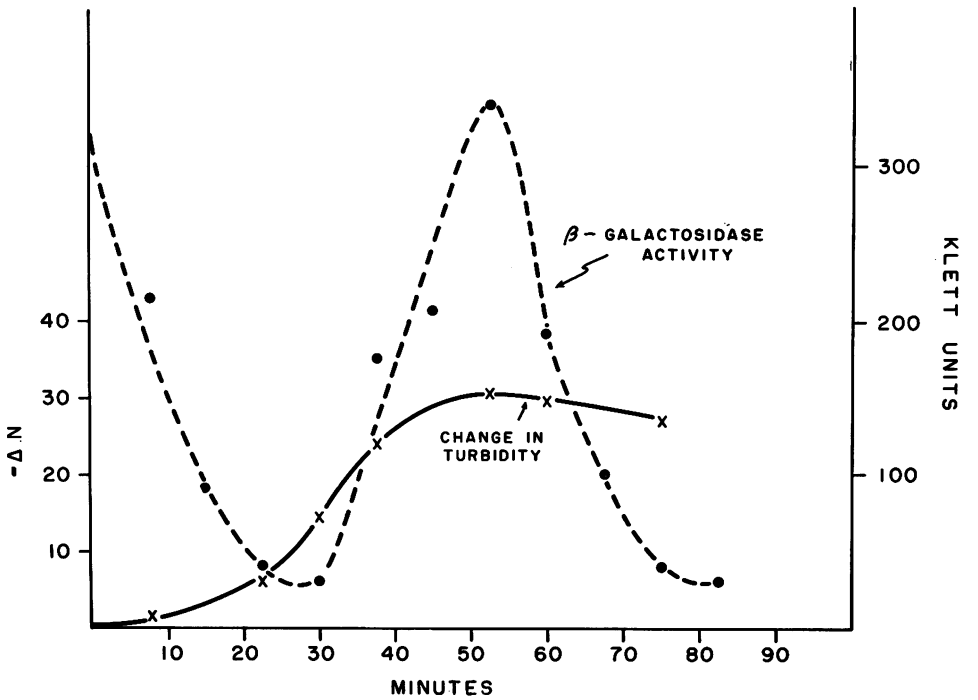

Figure 2. Correlation of osmotic fragility with biosynthetic capacity. Biosynthetic activity refers to the ability of the lysate, prepared after the indicated time of exposure to penicillin, to synthesize β-galactosidase under conditions described in the text.

large volumes were used for spheroplast formation so that all samples exposed to penicillin were taken from the same flask. The ratio of bacteria to spheroplast inducing medium was kept constant, however, and at each time interval, a 26-ml sample was taken for further treatment.

The results are found in figure 2. The synthesizing ability at zero time is that of whole cells before exposure to penicillin. The ability to synthesize β-galactosidase dropped rapidly and reached a minimum at about 10 min after osmotic fragility began to increase, then rapidly rose and reached a sharp maximum at about 50 min following exposure to penicillin. The maximum synthesis has always occurred at the same time of penicillin exposure, even though different preparations have shown variations in the time that fragility became manifest. The 50 min maximum appears to be characteristic, at least for this substrain under these conditions.

DISCUSSION

The decrease in turbidity after dilution at 26 C appears to be a valid, practical method for determining the extent of spheroplast formation induced by penicillin treatment of *E. coli*. This direct relationship between decrease in turbidity and osmotic fragility is corroborated by the observed decrease in deoxyribonucleic acid content of the residue coincident with the sharp increase of its content released into the supernatant.

Therefore, accepting the fragility curve as a valid measure of spheroplast formation, one may deduce several interesting relationships from the data presented.

The data indicate that a lag period precedes the onset of susceptibility to lysis. The length of the lag period, 15 to 30 min, indicates that time for about one generation is required to produce osmotic instability. Lederberg (1956) has proposed that this osmotic fragility results from a block in cell wall synthesis, as in the case of *S. aureus*. If this were so, the lag period would be accompanied by both an increase in protoplasmic synthesis and a block in the synthesis of cell wall material. Our experiments showed no evidence of increase in viable cells, total number of cells, or

even in size of cells during this lag period. In fact, viable count decreased rapidly, turbidity remained stable as did deoxyribonucleic acid content of the residue, and direct microscopic cell counts showed no increase in numbers. The increase in total deoxyribonucleic acid occurred after spheroplast formation was well under way.

The fact that the viable count decreased markedly before osmotic fragility became apparent would indicate that penicillin does not kill solely by making the cells susceptible to the possible lytic effects of their environment. The blocking of certain uridine diphosphate-mediated reactions, as reported by Park and Strominger (1957) may be less specific for cell wall metabolism than they suggest. Lederberg and St. Clair (1958) have shown that *E. coli* spheroplasts washed free of penicillin by 20 per cent sucrose and plated on sucrose-containing agar will conserve up to 50 per cent of their viability. These findings indicate that the marked decrease in viability reported in the present study may be a reversible phenomenon dependent upon environmental regulation.

Consideration was given to the possibility that the early drop in viability was an artifact caused by a partial increase in osmotic fragility mechanically aggravated by the necessity of diluting the cells. This was rejected as unlikely, however, because the cells were diluted at room temperature for plate counts, a procedure shown to be far less lytic than dilution at 56 C, the condition for measuring change in osmotic fragility.

The increase in net synthesis of deoxyribonucleic acid during spheroplast formation was a rather striking finding. The formation of β-galactosidase in lysates, as reported here, and the concomitant formation of nucleic acids and protein as reported by Reiner (*unpublished data*), indicate clearly that penicillin spheroplasts retain their biosynthetic capacity when removed from penicillin. However, the deoxyribonucleic acid increase reported here indicates that at least one major biosynthetic ability is retained in the presence of massive amounts of penicillin. It is interesting that deoxyribonucleic acid synthesis should be less susceptible than the reactions leading to increase of total cell mass. In view of the importance of the continuity of genetic material, it is perhaps not really surprising.

Finally, the marked changes in synthesizing ability of the cells, depending upon the length of exposure to penicillin, are of practical importance to those interested in obtaining actively synthesizing preparations. The explanation of these changes is difficult. Two facts seem to stand out. The initial loss of synthesizing ability by the cells seems roughly to parallel the loss of viability, whereas the subsequent sharp increase in activity parallels both spheroplast formation and deoxyribonucleic acid synthesis.

In view of Cooper's (1956) suggestion that penicillin damages the cell wall, the changes in β-galactosidase synthesizing ability may be explained as resulting from alterations in transport mechanisms caused by exposure to penicillin. Prestidge and Pardee (1957) have shown that considerable leakage of nucleic acid and protein occurs upon exposure to the antibiotic. The initial loss of activity, which corresponds to the decrease in viability, may result from leakage of ingredients essential for biosynthesis. The subsequent increase in synthesizing ability, which corresponds to the formation of spheroplasts and synthesis of deoxyribonucleic acid, may reflect an ability of spheroplasts to reverse this process.

SUMMARY

A method for determining the extent of formation of penicillin-induced spheroplasts of *Escherichia coli* is described. The method is based on the decrease in turbidity of the spheroplasts when the sucrose content is diluted with water at 56 C.

The fragility curve obtained was correlated with (1) deoxyribonucleic acid in the residue and the deoxyribonucleic acid released into the supernatant after lysis; (2) the viability count after exposure to hypotonicity; and (3) the ability of the preparations subsequently to synthesize β-galactosidase.

The significance of the results relating to the mechanism of spheroplast and protoplast formation is discussed.

REFERENCES

BURTON, K. 1956 A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. (London), **62**, 315–323.

COOPER, P. D. 1956 Site of action of radiopenicillin. Bacteriol Revs., **20**, 28–48.

LEDERBERG, J. 1956 Bacterial protoplasts induced by penicillin. Proc. Natl. Acad. Sci. U. S., **42,** 574–577.

LEDERBERG, J. AND ST. CLAIR, J. 1958 Protoplasts and L-type growth of *Escherichia coli.* J. Bacteriol., **75,** 143–160.

PARK, J. T. AND STROMINGER, J. L. 1957 Mode of action of penicillin. Science, **125,** 99–101.

PRESTIDGE, L. S. AND PARDEE, A. B. 1957 Induction of bacterial lysis by penicillin. J. Bacteriol., **74,** 48–59.

REPASKE, R. 1956 Lysis of gram-negative bacteria by lysozyme. Biochim. et Biophys. Acta, **22,** 189–191.

TRUCCO, R. E. AND PARDEE, A. B. 1958 Synthesis of *Escherichia coli* cell walls in the presence of penicillin. J. Biol. Chem., **230,** 435–446.