INDUCTION OF BACTERIAL LYSIS BY PENICILLIN[1]

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Received for publication January 11, 1957

In the past decade penicillin has become increasingly important clinically, and a great deal has been learned about the action of this antibiotic on susceptible organisms (Florey *et al.*, 1949). Numerous effects have been described in microorganisms exposed to penicillin; however, it is likely that most of these are a consequence of some primary process not yet well understood. Cooper (1956) has reviewed data on the primary action of penicillin and has suggested that a number of effects may follow an initial damage to the osmotic barrier of the cell. Demonstration of interference with transport processes are consistent with this view (Cooper, 1955; Gale and Taylor, 1947). The work to be reported here bears out this hypothesis and gives evidence of very early changes in the osmotic barriers of *Escherichia coli* and *Bacillus megaterium* exposed to penicillin. The conclusion is drawn from experimental data that formation of a substance, very likely an enzyme, is induced by penicillin, that this substance attacks the bacterial membrane and cell lysis ensues.

MATERIALS AND METHODS

Bacteria used were *E. coli* strain B, mutants of other strains of *E. coli* (550-460, uracilless; 15T$^-$, thymineless; and 1K-4, threonineless), and *B. megaterium* strain KM. *E. coli* were grown in a minimal medium, containing per L: K_2HPO_4, 7 g; KH_2PO_4, 2 g; Na_3 citrate$\cdot 5H_2O$, 0.5 g; $MgSO_4\cdot 7H_2O$, 0.1 g; $(NH_4)_2SO_4$, 1 g; and glycerol, 5 g (Lederberg, 1950a) at 37 C, with aeration by shaking. Uracil (10 μg/ml), thymine (20 μg/ml), or threonine (20 μg/ml) were added to the media for growth of the respective mutants. *B. megaterium* were grown at 30 C on 2 per cent peptone, on an asparagine-salts medium (Northrup, 1953), or on the above salts-glycerol medium plus 0.05 per cent peptone, with aeration by shaking.

[1] This work was supported in part by the University of California Cancer Research Funds, and the Rockefeller Foundation.

Chemicals. Crystalline lysozyme was obtained from Mann Research Laboratories, Synthetic chloramphenicol (Chloromycetin) was obtained from Parke, Davis and Co., and crystalline penicillin G was obtained from Eli Lilly and Co. The penicillin solution in water was prepared not over 1 hr before use to avoid loss of activity through decomposition.

Assay. Protein was determined directly on cell suspensions, or after trichloracetic acid (TCA) precipitation, by the Folin method (Lowry *et al.*, 1951); ribonucleic acid (RNA) in bacterial suspensions or in TCA precipitates was assayed by the orcinol method (Schneider, 1945); and deoxyribonucleic acid (DNA) in TCA precipitates of bacteria was measured by the method of Ceriotti (1952).

Enzyme determinations. Assays of β-galactosidase in *E. coli* were carried out following the procedure of Koppel *et al.* (1953). D-Serine deaminase was determined as described by Pardee and Prestidge (1955). Ribonuclease (RNase) was estimated by a modification of the method of Pardee and Kunkee (1952).

Viable cell counts. Suitable dilutions of bacteria suspended in melted agar were plated on tryptone-agar plates.

Cell extracts. Broken cell preparations were obtained by one of the following methods: treatment for 5 min in a 9KC Raytheon Sonic Oscillator; grinding with alumina, 2.5 × weight of wet cells, for 3 min; or treatment for 5–10 min in a Mickle disintegrator with 0.1–0.15 mm glass beads equal to about ½ the volume of cell suspension.

Protoplasts (Weibull, 1956). *B. megaterium* was grown to a concentration of 3×10^7/ml on asparagine medium; or to 1×10^8/ml on 2 per cent peptone, at 30 C. The bacteria were centrifuged, washed once with 0.03 M sodium phosphate (pH 7.0) containing 0.2 M sucrose, and resuspended in 1/10 volume of the latter solution at 25 C. Between 30 and 50 μg/ml of lysozyme was added and the protoplasts were formed at 25 C.

RESULTS

RNA breakdown. The observation which initiated this work was that the RNA of *E. coli* exposed to penicillin became soluble in 5 per cent TCA. This was striking because the RNA of intact *E. coli* had shown little tendency to break down (Pardee, 1955) and an agent capable of causing its solubilization was not known. Therefore, it was decided to determine how this RNA breakdown was brought about and to study its relation to the mechanism by which penicillin kills bacteria, if possible.

How is one to decide whether the solubilization of RNA is related to the lethal action of penicillin? Three elementary criteria may be set up. First, the concentration of penicillin that causes loss of viability (measured by plating) should be similar to that which causes solubilization of RNA. It is seen from figure 1 that exposure of growing cultures of *E. coli* for 80 min to penicillin at different concentrations resulted in similar losses of viability and RNA; also syntheses of macromolecules were inhibited similarly. Second, conditions which prevent the lethal action of penicillin should also prevent solubilization of RNA. Exposure of mutants IK-4 or 550-460 under conditions of growth to 150 μg/ml of penicillin resulted in both loss of viability and breakdown of RNA; however, these mutants were partly protected against both the lethal action (Lederberg, 1950a) and RNA solubilization in the absence of threonine and uracil, respectively. Third, the time of exposure to penicillin required for lethal action should be similar to that required for solubilization of RNA. It is seen from figure 2 that the TCA-insoluble RNA per ml of culture commenced to decrease 20 min after exposure to 150 μg/ml of penicillin, in contrast to a culture not exposed to the antibiotic. For comparison, at 25 min the viability had decreased to 11 per cent; by 35 min there were only 2 per cent survivors (table 1). RNA breakdown and viability loss are correlated in time. The figure also shows that TCA soluble "RNA" (material that gives the orcinol test) was formed, so that the total "RNA" increased for about 25 min and then remained constant. Protein synthesis was also halted about 25 min after penicillin was added. DNA synthesis stopped at about the same time. It is concluded that RNA breakdown is correlated with viability loss with respect to concentration, conditions, and time.

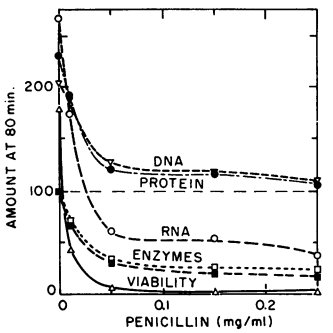

Figure 1. Effect of various concentrations of penicillin on syntheses and viability. Exponentially growing *Escherichia coli* strain B at a concentration of 3×10^8/ml in salts-glycerol medium at 37 C were exposed for 80 min to various concentrations of penicillin. Lactose (0.1 per cent) and DL-serine (0.1 per cent) were present as inducers of β-galactosidase and D-serine deaminase during this period. Aliquots were taken into TCA at 0 and 80 min for protein, RNA, and DNA determinations and other aliquots were taken for viability and enzyme determinations. Results for viability, RNA, DNA, and protein are plotted as per cent of the initial values. Enzyme activities are plotted as per cent of the final values found in the absence of penicillin (initial values were zero).

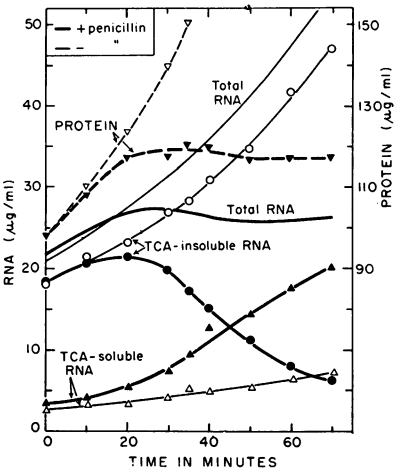

Figure 2. Changes in RNA and protein at various times caused by penicillin. Conditions were the same as those used to obtain figure 1, except that 150 μg/ml penicillin was added to one culture, and no penicillin to the control. Aliquots were taken at intervals for protein, TCA-insoluble RNA, and TCA-soluble "RNA" (material giving color with the orcinol reagent). Total RNA is the sum of the TCA-soluble and insoluble RNA.

TABLE 1

Loss of viability of Escherichia coli exposed to penicillin and Chloromycetin

Time	Survivors	Chloromycetin Added	Survivors at 60 Min	Saved
	%	*min*	%	%
0	100	0	51	51
3	93	5	25	33
6	61	8	25	40
10	64	12	11	22
14	58	16	6	16
18	34	20	4	12
25	11	30	(1)	(5)
35	2	40	(1)	
65	0	50	0	

Exponentially growing *E. coli* were diluted into glycerol-salts medium containing 150 μg/ml penicillin. Aliquots were diluted and plated to determine per cent of survivors at various times. Other aliquots were diluted at various times into media containing 150 μg/ml penicillin and 20 μg/ml Chloromycetin at various times. These cultures were incubated for a total of 60 min after the bacteria were first exposed to penicillin and the survivors were determined by plating. The final column shows the per cent of the bacteria still viable at the time they were exposed to Chloromycetin which survived to 60 min. In the absence of penicillin, 20 μg/ml Chloromycetin in 40 min did not cause a change in the number of viable bacteria, within experimental error. Numbers in parentheses are not considered significant.

Presumably an enzyme breaks down the RNA. Is penicillin an RNase activator? Although the RNA of intact *E. coli* shows little turnover, extracts contain a high RNase activity (Pardee and Kunkee, 1952) ample to account for the observed breakdown of RNA if activation occurred after penicillin addition. The antibiotic did not cause formation of new RNase, because the RNase content of extracts of *E. coli* exposed 30 min to penicillin was the same as that of controls. The question then is how penicillin activates the RNase. Breaking of bacteria by sonic treatment, toluene, etc. was the only means, other than penicillin action, found to activate RNase. Therefore, an altered internal environment would seem adequate to cause RNA breakdown. There is not a direct relation between death of the bacteria and activation of RNase, for when *E. coli* were irradiated with ultraviolet light, sufficiently for 99 per cent kill, and then shaken 1 hr at 37 C in salts-glycerol medium, no loss of TCA precipitable RNA occurred; RNase of these irradiated bacteria was however active in sonically treated extracts.

Cell barrier changes. It seems possible that when *E. coli* is exposed to penicillin some disruption of the osmotic barrier is first caused by the antibiotic, the internal environment is altered, and RNase is activated as a result. In order to test this possibility, some measure of damage to the osmotic membrane is needed. A direct test was performed to determine whether characteristic intracellular components escaped into the medium after addition of penicillin. It was found that protein, material absorbing at 260 mμ, and β-galactosidase all commenced to escape into the medium at 10 min, and continued to escape for at least an hour (figure 3). The damage to the membrane was great enough to permit gradual escape of large molecules, but not great enough to permit immediate escape of all the cell contents. (Gradual leakage cannot be attributed to heterogeneity of damage to cells, because all protein and RNA syntheses, and therefore synthesis by all cells, ceased at about 25 min.)

A less direct test of changes in the permeability of the cells was made using the enzyme β-galactosidase. Induced bacteria hydrolyze the substrate, *o*-NO_2-phenyl-β-D-galactoside (NPG), only slowly unless they are first disrupted with toluene or other agents (Lederberg, 1950b). If penicillin treatment disrupts the osmotic membrane,

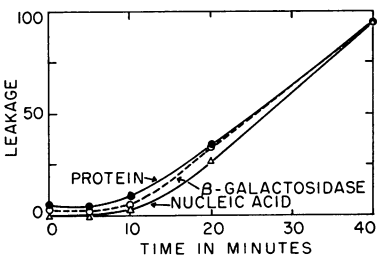

Figure 3. Leakage from *Escherichia coli*. Conditions of growth were like those used to obtain figure 1 except that 0.2 per cent lactose was in the medium, and the bacteria were grown to 5×10^8/ml. Penicillin (150 μg/ml) was added (0 time) and aliquots were removed subsequently, centrifuged to remove the bacteria, and assays were made on the supernatant fluids for protein, β-galactosidase, and nucleic acid (material with absorption at 260 mμ). Results are plotted on an arbitrary vertical scale.

induced bacteria exposed to the antibiotic should hydrolyze NPG at a rate similar to that observed with the toluene treated bacteria. This was found to be the case (figure 4). The rate of NPG hydrolysis by bacteria exposed to 2 mg/ml lactose and not treated with penicillin increased rapidly with time if toluene was used in the assay, and slowly in the intact bacteria. Treatment with penicillin was as effective as toluene in permitting β-galactosidase to act on NPG, by about 25 min. Previous to this time the action of penicillin had not proceeded sufficiently to disrupt the cell barriers completely. (Some of the effects of penicillin may have come about during the enzyme assay, therefore the above times are minimal.) A second conclusion to be drawn from this experiment is that enzyme synthesis in the bacteria treated with penicillin was halted at 20 min. It is significant that the leakage appeared at 10 min, considerably before syntheses of enzymes, protein, and nucleic acid stopped; it is likely therefore that the latter events are due to changes inside the cell brought about by leakage.

E. coli treated with penicillin were examined under oil immersion in a phase-contrast microscope. The exponentially growing bacteria in salts-glycerol medium were exposed to 150 μg/ml penicillin and were observed in a Petroff-Hausser counting chamber at intervals for an hour. No gross changes in shape or size were seen. Owing to their small size, it was not possible to see changes in internal structure of the bacteria; however the outlines of the bacteria became dimmer. Results were similar when the bacteria were observed in the presence of 20 per cent sucrose and 0.1 M $MgSO_4$ in salts-glycerol medium.

Electron microscopy of *E. coli* after addition of penicillin bears out the general impression of a weakening of cell barriers and subsequent loss of high molecular weight material, without a drastic change in the appearance of the bacteria. Preparations of normal bacteria (transferred from the salts-glycerol medium to distilled water prior to spraying for microscopy) showed strong double boundaries surrounding a dense interior (figure 5). The background was free of particles. *E. coli* treated with penicillin for only 20 min had a less electron dense cytoplasm and more diffuse cell wall area, and the effect was enhanced at 60 min. Many small particles were found in the area surrounding the bacteria and these particles, which are known to be inside the cells normally,

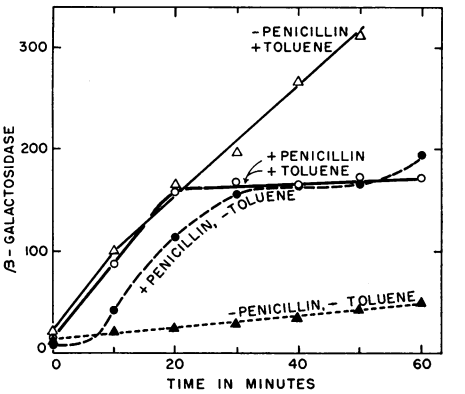

Figure 4. Activation of β-galactosidase by penicillin. Conditions were like those used to obtain figure 1. Lactose (0.2 per cent) was added to the culture, and 20 min later 150 μg/ml penicillin was added to half the culture. Aliquots were removed into 5 μg/ml Chloromycetin in 7 per cent sucrose (final concentrations) at 0 C and assayed for β-galactosidase, both with and without toluene.

must have been released during the process of preparation for microscopy. It is seen that penicillin treated *E. coli* were more fragile to electron microscopic procedures than were untreated cells; but also the damage was not of a sort to show large breaks in the bacterial surface.

Nutritional requirements for penicillin action. In order to approach the question of how penicillin brings about a weakening of the bacterial osmotic barrier, the synthetic processes necessary to support the action of penicillin were next studied in *E. coli*. It has long been known that conditions where "growth" is possible are required for effective penicillin action (Chain *et al.*, 1945), but whether the required "growth" is simply a mass increase, or some specific synthetic process is still unknown. Many processes occur in normally growing bacteria that are not required for the action of penicillin: *E. coli* infected with ultraviolet irradiated T2 bacteriophage showed as much leakage upon exposure to penicillin as did the uninfected bacteria, in spite of the extensive alterations by the phage of nucleic acid and protein synthesis. Also, protection from penicillin was not afforded by the absence of thymine from cultures of the thymineless mutant $15T^-$ (Cohen and Barner, 1955). In this organism the usual early leakage of material with high light absorption at 260 mμ occurred 80 per cent as rapidly in the absence as in the presence of

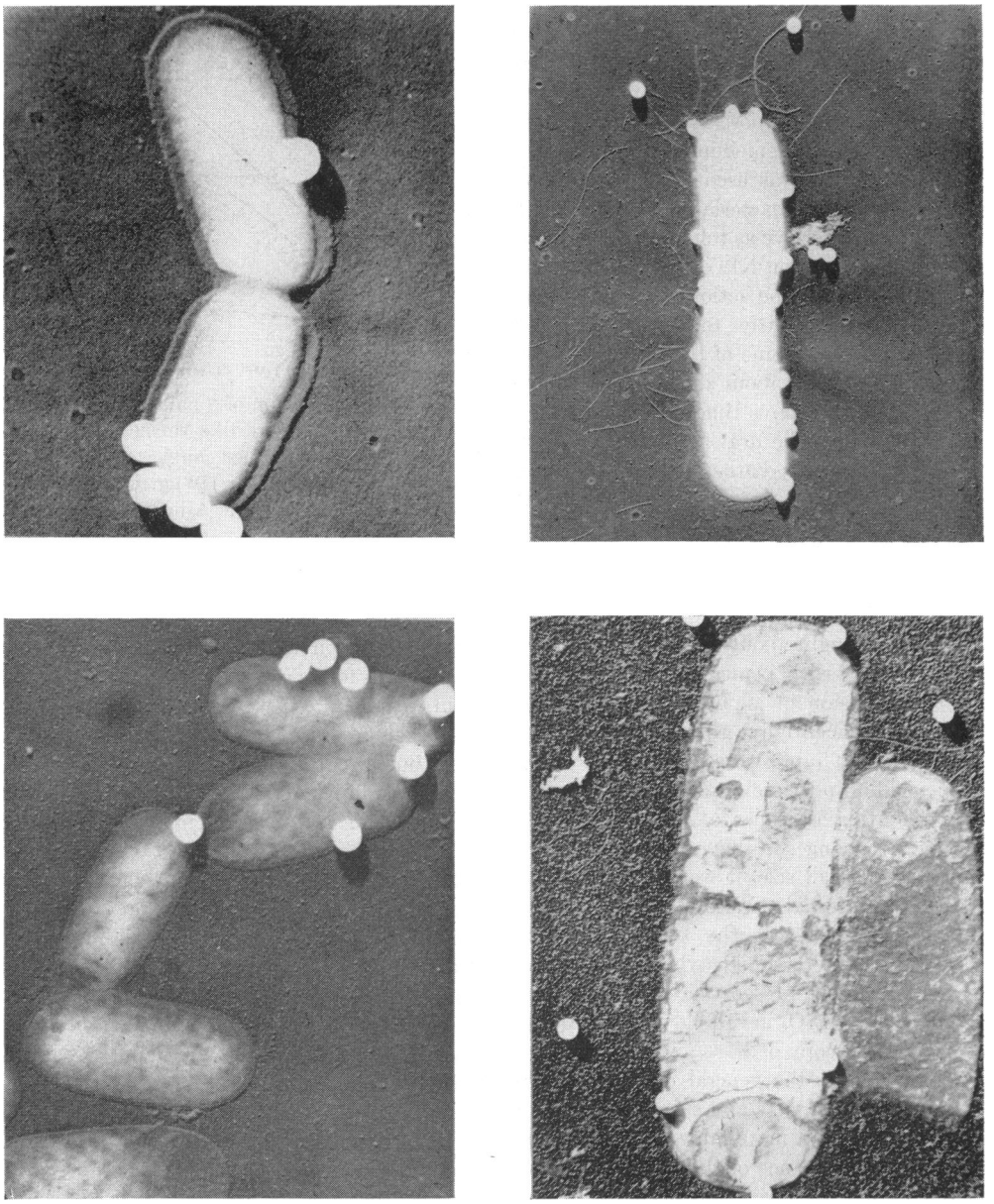

Figure 5. Electron micrographs of *Escherichia coli* and *Bacillus megaterium*. *E. coli* were grown as described in the legend for figure 1. Penicillin (150 μg/ml) was added, aliquots were removed from the culture at 0, 30 and 60 min, centrifuged, resuspended in ice water, and sprayed for electron microscopic observation. *B. megaterium* were grown on 2 per cent peptone to 2×10^7/ml, exposed to 15 μg/ml penicillin and subsequently treated like the *E. coli*. The small spheres are polystyrene latex particles, added to give a measure of size. Upper left: *E. coli*, 0 min exposure. Lower left: *E. coli* 60 min exposure. Upper right: *B. megaterium*, 0 min exposure. Lower right: *B. megaterium*, 60 min exposure to penicillin.

thymine, after the addition of penicillin. The antibiotic was equally effective under both conditions in halting protein and RNA synthesis after about 20 min. This leads to the conclusion that DNA synthesis has no major role in the action of penicillin. Interest was shifted to studies of effects of alteration of protein and RNA synthesis.

It has already been mentioned that if interference of protein and RNA synthesis is brought about by withholding amino acids or uracil from mutants requiring these nutrients, the bacteria become resistant to lethal action of penicillin and the RNA of these cells does not break down. This implies that protein or RNA synthesis, or some related process, is required for penicillin action, but it does not distinguish between these processes since uracil is required for protein synthesis (Pardee, 1954) and amino acids are required for RNA synthesis (Pardee and Prestidge, 1956).

Chloromycetin is known to inhibit protein synthesis specifically (Gale and Folkes, 1953) and its antagonism to penicillin (Jawetz *et al.*, 1951) suggests that protein synthesis is an important requirement in the action of penicillin. Experiments were designed to test the protective effects of chloromycetin at various times in the course of penicillin action (table 1). When the two antibiotics were added together (or if chloromycetin was added first) 50 per cent of the bacteria were viable after 60 min, showing that Chloromycetin antagonized the bactericidal effect of penicillin. However, if Chloromycetin was added after exposure to penicillin, a smaller fraction of those bacteria still viable was saved until 60 min; for example, 25 per cent of the initial bacteria survived 20 min exposure to penicillin, but only 10 per cent of these survivors (or 2.5 per cent of the original number) were able to form colonies after a subsequent 40 min exposure to the combined antibiotics. The same antagonistic effect had been shown much earlier with the bacteriostatic antibiotic helvolic acid (Chain *et al.*, 1945).

A similar experiment was performed in which leakage from the cells instead of colony counts was used as a measure of penicillin action. The results (figure 6) showed first of all, the marked increase of OD_{260} of the medium from the penicillin treated bacteria as compared to the slight release of material from the controls. When 20 μg/ml of Chloromycetin was added at the same

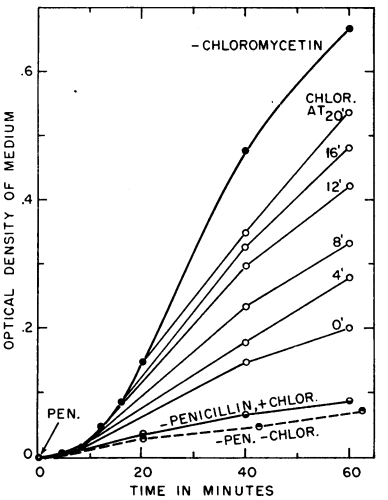

Figure 6. Protection against penicillin by Chloromycetin. The experiment was performed in a manner similar to that used to obtain table 1, except that optical densities of the supernatant fluids of aliquots were determined, instead of viability. Optical densities, corrected for densities due to the antibiotics, are plotted vs. times after addition of penicillin.

time as penicillin, the rate of leakage was only 20 per cent of that in the absence of Chloromycetin. Material was released into the medium more rapidly as the time of Chloromycetin action was delayed, and reached 80 per cent of the maximum rate when Chloromycetin was added at 20 min. By this time apparently almost all the protein synthesis necessary to insure death and lysis of the bacteria has occurred.

Experiments of the same sort were performed with the tryptophan analogue 5-methyltryptophan (20 μg/ml) and the results were virtually identical with those obtained with Chloromycetin. Sodium azide (0.02 M), an inhibitor of energy supply, completely prevented leakage when added to *E. coli* at the same time as penicillin, or 10 min later. It seems likely this compound inhibits lytic activity in progress, unlike Chloromycetin.

These results strongly support the idea that protein synthesis is necessary before penicillin can cause the lysis of *E. coli*. But is this protein synthesis a specific one or is it required for a mass increase? Experiments with 7-azatryptophan were performed to attempt an answer to this question. This tryptophan analogue does not inhibit pro-

TABLE 2

Antagonism of penicillin by 7-azatryptophan

	AT (μg/ml)					
	0			100		
	Penicillin (μg/ml)					
	0	150	Diff.	0	150	Diff.
	Optical density increase at 260 mμ					
min						
15	0.025	0.045	0.020	0.00	0.035	0.035
30	0.025	0.185	0.165	0.075	0.135	0.060
45	0.035	0.370	0.335	0.110	0.220	0.110
60	0.055	0.510	0.455	0.135	0.365	0.230

Exponentially growing *Escherichia coli* (3×10^8/ml) were exposed to penicillin in the presence and absence of DL-7-azatryptophan (AT). Aliquots were taken at 15 min intervals, centrifuged, and the optical densities at 260 mμ of the supernatant fluids were determined. The table shows optical density increases above the values at the start of the experiment and the differences (diff.) caused by penicillin.

tein synthesis but inhibits appearance of a number of enzyme activities, presumably because the analogue is incorporated into the enzyme protein (Pardee *et al.*, 1956). Penicillin action should not be inhibited by 7-azatryptophan if a simple mass increase is required; however, if some specific protein synthesis is involved, 7-azatryptophan might antagonize penicillin. It was found that 100 μg/ml DL-7-azatryptophan added at the same time as 150 μg/ml penicillin decreased the rate of leakage by about 50 per cent (table 2). Also, the analogue preserved the viability of 20 per cent of the bacteria for 60 min in the presence of penicillin. This inhibition by 7-azatryptophan favors the hypothesis that formation of a specific protein is required for penicillin action in *E. coli*.

Experiments with B. megaterium. The gram-positive organism *B. megaterium* was next investigated in order to determine whether a sequence of events could be observed on addition of penicillin similar to those demonstrated for the gram negative *E. coli*. Such experiments were of interest with respect to the generality of the mechanism of action of penicillin.

Effects of penicillin on RNA and DNA synthesis are shown in table 3. By 30 min RNA and DNA synthesis was halted. Similar results were obtained in other media, at times approximately proportional to the growth rates of the cultures. Consistent results were not obtained for protein determinations by the Folin method and are not presented. In order to determine whether functional protein synthesis continued, assays were made for β-galactosidase under the conditions employed by Landman and Spiegelman (1955). In asparagine medium, β-galactosidase formation ceased 35 min after addition of 4 μg/ml penicillin. Leakage of β-galactosidase, protein, and material absorbing light at 260 mμ commenced at 60 min in this medium. In peptone medium, both protein and RNA escaped from penicillin treated *B. megaterium* within about 30 min (table 3). The RNA in the medium was still TCA precipitable, unlike that from *E. coli*; probably because the RNase of *B. megaterium* extracts was essentially inactive in the growth medium.

Phase contrast microscopy of *B. megaterium* in growth media plus penicillin showed gradual appearance over several hours of cells that seemed empty except for a few large, dense granules. There was no obvious distortion or swelling of the bacteria, and the empty cells remained in

TABLE 3

Changes brought about by penicillin in a culture of Bacillus megaterium

Time	μg/ml of Culture							
	RNA, total		DNA, total		Protein, sup.		RNA sup.	
	−*	+	−	+	−	+	−	+
min								
0	14	13	1.1	1.1	0.0	0.0	0.0	0.0
10	18	17	1.3	1.3	1	−1	0.6	1.0
20	21	19	1.5	1.5	−1	−1	1.1	0.9
40	24	20	2.0	1.7	0	6	1.1	2.5
60	26	19	2.5	1.7	1	15	1.8	4.4

A culture of exponentially growing *Bacillus megaterium* in 0.5 per cent peptone was divided into two portions and 15 μg/ml penicillin were added to one portion. Aliquots were taken for TCA precipitation and determination of RNA and DNA in the precipitates (total). Other aliquots were centrifuged to remove the bacteria, and TCA insoluble RNA and protein were determined in the supernatant fluids (sup.) Amounts of materials in the media at the start of the experiment were subtracted from amounts found later to give values in the table.

* − = without penicillin; + = penicillin added.

chains with ones in which penicillin action had not yet become apparent. Electron microscopy (figure 5) showed that penicillin treated *B. megaterium* were more fragile than the untreated cells. Particularly striking was the high density of small particles surrounding the flattened bacteria. A considerable number of cells were broken, perhaps on spraying since breakage before washing would have resulted in loss of the granules.

The effect of penicillin on *B. megaterium* protoplasts (Weibull, 1956) was also tested. Addition of penicillin to protoplasts in 0.03 M phosphate, pH 7, containing 0.2 M sucrose had no effect on turbidity. Protoplasts in nutrient media plus sucrose were not stable long enough for meaningful measurements to be made. *B. megaterium* exposed 2 hr to penicillin formed protoplasts of definitely lower stability than unexposed bacteria. Such a result is difficult to interpret, owing to the variety of conditions that can affect protoplast stability.

Action of bacterial extracts on protoplasts. Combination of the observations that penicillin somehow damages the bacterial membrane, and that formation of a specific protein is required suggests the involvement of a newly formed lytic enzyme. Exposure of membranes to an extract of penicillin treated bacteria and determination of a change in their properties provides a direct test of this hypothsis. This is conveniently done, and with high sensitivity, by use of protoplasts as the substrate, because an attack on the protoplast membrane results in decreased turbidity. The results of such an experiment are shown in figure 7. The protoplasts alone were quite stable for an hour but the addition of a fraction of penicillin treated *E. coli* caused a rapid loss of turbidity (measured in the Beckman Spectrophotometer). A fraction prepared similarly from untreated *E. coli* had no effect on the protoplasts. Evidently treatment with penicillin produced some new active factor.

Extracts of untreated bacteria usually were less active than extracts of penicillin treated bacteria, but fractionation of the *E. coli* extracts was necessary to show a clear difference between the two. The pellet formed by centrifugation for 10 min at 8,000 × G contained much of the lytic activity of the penicillin treated bacteria, and little of that from the control. Another means of differentiation was by heating: an extract of the control cells kept for 10 min at 100 C largely

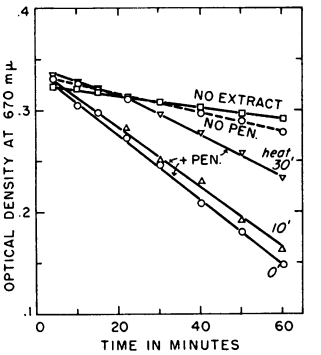

Figure 7. Action of *Escherichia coli* extracts on protoplasts. *E. coli* were grown as described for figure 1. 200 ml of the culture was exposed to 150 μg/ml penicillin for 35 min and an equal volume was kept as a control. The cultures were centrifuged and the bacteria were resuspended in 10 ml 0.03 M phosphate, pH 7.5, treated 5 min in a Raytheon sonic oscillator, and the extract was centrifuged for 10 min at 20,000 × G. The pellets were resuspended in 12 ml phosphate buffer plus 7.5 per cent sucrose. Portions were heated 10 and 30 min at 100 C, 1 ml aliquots at 25 C were added to 2 ml *Bacillus megaterium* protoplasts in sucrose-phosphate and turbidities were determined at 670 mμ with the Beckman Spectrophotometer.

lost its activity, but the activity of the penicillin treated bacteria was destroyed only on longer heating. This result favors the concept of formation of a new substance rather than transfer of a pre-existing active material to the membrane (unless it is assumed that binding to the membrane promotes heat stability).

Exact shapes of the curves obtained in experiments of this sort varied considerably; however, the results were always qualitatively the same. This variability is attributed to differences in the protoplasts, stability of which was highly dependent on the age of the bacteria from which they were prepared. Also, the degree of destruction of *E. coli* by sonic oscillation was probably quite variable. Finally, the activity of a given extract on a suspension of protoplasts was highly dependent upon many conditions of the experiment. For example, monovalent salts such as NaCl were inhibitory at concentrations greater than 0.1 M, and $HgCl_2$, $MgSO_4$ or $CoCl_2$ were inhibitory at 0.0005 M concentration. The rate of lysis of protoplasts by *E. coli* extracts depended on pH: the maximum rate appeared to be at pH 6.5. Activity was not lost by dialysis of the ex-

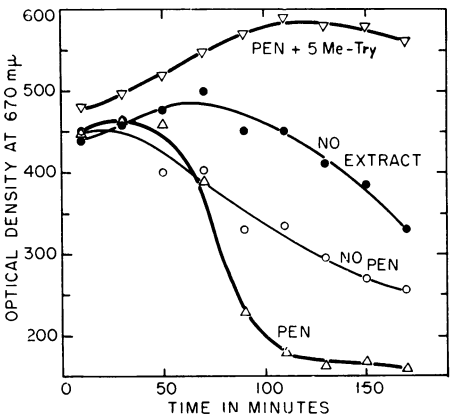

Figure 8. Inhibition of formation of the lytic factor by 5-methyl-tryptophan. Extracts made from exponentially growing *Escherichia* coli (○), *E. coli* exposed to 150 μg/ml penicillin for 30 min (△) and exposed to 5-methyl-tryptophan (5-me-try) for 15 min and then to 5-me-try plus penicillin for 30 min (▽) were added without fractionation to suspensions of *Bacillus megaterium* protoplasts, as described in figure 7, and lysis of the protoplasts was followed by measurement of turbidity at 670 mμ in the Beckman Spectrophotometer. A control of protoplasts alone is included (●).

tracts or storage for several days at 4 C, but the activity varied from day to day. Lysis was not homogeneous throughout the suspension, but a pattern of clear areas appeared as if caused by convective disturbances.

If the lytic factor in extracts is connected with the lethal action of penicillin, it should be found only in bacteria which have been exposed to the antibiotic under conditions where the *E. coli* are killed. Thus, if penicillin is added to a growing culture to which 5-methyl-tryptophan is also added, the lethal action is greatly reduced, and the lytic activity of extracts of these bacteria should be small. This result was found (figure 8): protoplasts were completely lysed between 50 and 110 min by the unfractionated extract of penicillin treated *E. coli* but the extract of bacteria exposed in the presence of 5-methyl-tryptophan permitted an increase in turbidity of 12 per cent in the same period (experiment by Dr. R. E. Trucco), and the protoplasts were more stable than the control. Partial inhibition of the lethal action of penicillin by uracil deficiency (with a uracilless mutant) or by Chloromycetin also reduced the activities of *E. coli* extracts, definitely but not completely.

Great difficulty has been experienced in attempts to fractionate the cells to obtain the active material, owing to variability of the assay. At present, it is questionable whether these experiments can be carried much further with these techniques.

DISCUSSION

The observations of this paper are summarized as follows: specific protein synthesis is required in the first few minutes after penicillin is added to *E. coli*, and this is considered to be the period during which a lytic enzyme is produced. Leakage of the cell contents commences at 10 min and continues for at least an hour. Between 10 and 25 min, permeability changes are also shown by the activation of β-galactosidase. RNase is activated at 20 min as a result of the changed intracellular environment, and RNA breakdown commences at this time. Syntheses of macromolecules cease abruptly at about 25 min. This general pattern was observed in *B. megaterium* as well as in *E. coli*. The lethal action would appear to be due to changes in the intracellular environment brought about as a consequence of damage to the cell barriers. This conclusion is in accord with the earlier results of Gale and Taylor (1947) and Cooper (1955), as well as with the observations of Lederberg (1956) which appeared while this manuscript was in preparation. It is worth noting that the action of polymyxin is attributed to permeability changes (Newton, 1956), and the sequence of events is remarkably similar to those described here.

Damage to the cell membrane rather than to the wall must be directly responsible for leakage and permeability changes; this is concluded from reports of the effects of removal of the wall alone (McQuillen, 1956) or damage to the membrane (Gale and Folkes, 1955) on synthetic processes. Several observations favor the idea that damage to the membrane is like that expected if fine holes were created, rather than if there were a gross rupture of the entire membrane: electron and phase microscope observations showed no gross damage to the barriers of penicillin treated *E. coli* and many *B. megaterium* cells also were found intact; the cessation of syntheses by penicillin treated *E. coli* at about 25 min showed that all the cells were damaged at this time, yet leakage continued for at least an hour; finally, the decreased stability of *B. megaterium* proto-

plasts favored the idea of gradual damage to the membrane.

How does penicillin cause damage to the membrane? One mechanism would be "direct" by attack on the membrane itself; a second would be "indirect" by a local weakening of the wall and subsequent breakage of the membrane by osmotic pressure. The demonstration of a factor produced by penicillin which attacks the membrane favors the first, direct mechanism. However, the recent report by Lederberg (1956) that *E. coli* exposed to penicillin in 20 per cent sucrose and 0.2 M $MgSO_4$ in a rich medium form large round bodies that are unstable when diluted into water, and which can reform normal *E. coli* in sucrose medium upon removal of penicillin shows that under these conditions penicillin affects cell wall stability or formation in some way and that the membrane remains intact. It is possible that in hypertonic medium penicillin inhibits wall synthesis with subsequent escape of the cytoplasm, or lyses the wall, or permits uncontrolled proliferation of the wall. Similar results have been obtained more recently by Hahn and Ciak (1957). The observations favor an indirect action on the membrane in hypotonic media as the simplest explanation. However, it may be objected that the formation of protoplasts by penicillin is very dependent on the experimental conditions; these effects may not be the same as those leading to loss of viability in hypotonic media. Also, protoplasts are formed slowly relative to the leakage effects described in this communication. It is not easy to understand how a process dependent on specific wall protein synthesis, even at a specific site on the wall, could in 10 min alter the cell wall sufficiently to affect the membrane.

In an attempt to reconcile these observations and present a unified mechanism for penicillin action, it is suggested that the primary action of penicillin is to cause the formation of an active protein which attacks the membrane. The consequences of this attack would depend on the concentration of penicillin, the medium, and the condition of the bacteria (and probably the strain of bacteria). Under conditions of exponential growth and with a high concentration of penicillin, as in the present experiments, damage to the membrane would be irreparable and after a short time the membrane would break. Under less drastic conditions, as at low concentrations of antibiotic, the function of the membrane would be impaired, but repair processes would keep the membrane physically intact. One could reasonably expect that damage to the membrane would have a strong effect on cell wall synthesis since the membrane must be involved in transport of building blocks from the cytoplasm to the wall, if not in actual synthesis of the wall. Sublethal action could result in production of aberrant forms (Dienes and Weinberger, 1951) or of Lederberg's "protoplasts." Damage to the mechanism for wall synthesis might also result in the appearance of precursors of the cell wall, such as the uridine compounds, as described by Park and Strominger (1957).

What is the nature of the lytic factor produced in *E. coli* by penicillin? The requirement of protein synthesis suggests an enzyme induction. This idea is perhaps made more reasonable when it is recalled that penicillin causes the induced formation of at least one enzyme, penicillinase (Pollock, 1953). Also, appearance of new lytic enzymes has been demonstrated after bacteriophage infection (Panijel and Huppert, 1954; Ralston *et al.*, 1955; Brown, 1956). The ability of the active factor to be centrifuged indicates that it is adsorbed to the wall or membrane of *E. coli*, and this is not inconsistent with the idea of an enzyme-substrate complex. Its relatively great heat stability argues against its being an enzyme although a number of enzymes (lysozyme, myokinase, and peroxidase) are stable to brief boiling. Efforts are being made to find a better assay system which may be used to characterize the lytic material.

ACKNOWLEDGMENTS

We are indebted to Mr. Joseph Toby for electron micrographs, Drs. E. A. Adelberg and S. S. Cohen for mutants, to Parke, Davis and Co. for a gift of Chloromycetin, and to Dr. M. M. Robison for a gift of 7-azatryptophan.

SUMMARY

Exposure of exponentially growing *Escherichia coli* in salts-glycerol medium for 20–30 min to 150 μg/ml penicillin G caused loss of colony forming ability. A search was made for alterations in the bacteria which under these conditions could result in death. It was found that 20 min after addition of penicillin the RNA content of the bacteria decreased, and at 25 min syntheses of protein, RNA, DNA, and enzymes stopped.

Leakage of large molecules was seen at 10 min and other indications of permeability changes occurred at this time. Similar observations were made with *Bacillus megaterium*. Inhibitors of protein synthesis largely prevented this leakage in *E. coli*; inhibitors were more effective the earlier they were added. It is supposed that penicillin causes formation of an enzyme which attacks the cell membrane and as a result the cell contents escape. In the search for such an enzyme, it was found that fractions of extracts of penicillin treated *E. coli* possess the ability to cause lysis of protoplasts made from *B. megaterium*; similar fractions of untreated *E. coli* or from bacteria exposed to penicillin in the presence of 5-methyl-tryptophan were ineffective. The results are discussed in terms of the mechanism of the action of penicillin.

REFERENCES

BROWN, A. 1956 A study of lysis in bacteriophage infected *Escherichia coli*. J. Bacteriol., **71,** 482–490.

CERIOTTI, G. 1952 A microchemical determination of deoxyribonucleic acid. J. Biol. Chem., **198,** 297–303.

CHAIN, E., DUTHIE, E. S., AND CALLOW, D. 1945 Bactericidal and bacteriolytic action of penicillin on the staphylococcus. Lancet, **248,** 652–657.

COHEN, S. S. AND BARNER, H. D. 1955 Enzymatic adaptation in a thymine requiring strain of *Escherichia coli*. J. Bacteriol., **69,** 59–86.

COOPER, P. D. 1955 The site of action of penicillin: some changes in *Staphylococcus aureus* during the first two hours growth in penicillin media. J. Gen. Microbiol., **13,** 22–38.

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

DIENES, L. AND WEINBERGER, I. H. 1951 The L forms of bacteria. Bacteriol. Revs., **15,** 245–288.

FLOREY, H. W., CHAIN, E., HEATLEY, N. G., JENNINGS, M. A., SANDERS, A. G., ABRAHAM, E. P., AND FLOREY, M. E. 1949 Penicillin. In *Antibiotics*, **2,** 631–1293. Oxford University Press, London.

GALE, E. F. AND FOLKES, J. P. 1953 The assimilation of amino acids by bacteria. 15. Action of antibiotics on nucleic acid and protein synthesis in *Staphylococcus aureus*. Biochem. J. (London), **53,** 493–498.

GALE, E. F. AND FOLKES, J. P. 1955 The assimilation of amino acids by bacteria. 20. The incorporation of labelled amino acids by disrupted staphylococcal cells. Biochem. J. (London), **59,** 661–675.

GALE, E. F. AND TAYLOR, E. S. 1947 The action of penicillin in preventing the assimilation of glutamic acid by *Staphylococcus aureus*. J. Gen. Microbiol., **1,** 314–326.

HAHN, F. E. AND CIAK, J. 1957 Penicillin-induced lysis of *Escherichia coli*. Science, **125,** 119–120.

JAWETZ, E., GUNNISON, J. B., SPECK, R. S., AND COLEMAN, V. R. 1951 Studies on antibiotic synergism and antagonism. The inference of chloroamphenicol with the action of penicillin. Arch. Internal Med., **87,** 349–359.

KOPPEL, J. L., PORTER, C. J., AND CROCKER, B. F. 1953 The mechanism of the synthesis of enzymes. I. Development of a system suitable for studying this phenomenon. J. Gen. Physiol., **36,** 703–722.

LANDMAN, O. E. AND SPIEGELMAN, S. 1955 Enzyme formation of protoplasts of *Bacillus megaterium*. Proc. Natl. Acad. Sci. U. S., **41,** 698–704.

LEDERBERG, J. 1950*a* Isolation and characterization of biochemical mutants of bacteria. Methods in Med. Research, **3,** 5–22.

LEDERBERG, J. 1950*b* The β-D-galactosidase of *Escherichia coli* strain K-12. J. Bacteriol., **60,** 381–392.

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

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J. 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem., **193,** 265–275.

MCQUILLEN, K. 1956 Capabilities of bacterial protoplasts. In *Bacterial anatomy*, pp. 127–149. Cambridge University Press, Cambridge, England.

NEWTON, B. A. 1956 The properties and mode of action of the polymyxins. Bacteriol. Revs., **20,** 14–27.

NORTHRUP, J. H. 1953 Growth and phage production of *Bacillus megaterium*. J. Gen. Physiol., **36,** 581–599.

PANIJEL, J. AND HUPPERT, J. 1954 Mise en évidence et titration d'une substance lytique non-reproductible en série (lysine) accompagnant la production d'un bactériophage. Compt. Rend. Acad. Sci., **238,** 745–747.

PARDEE, A. B. 1954 Nucleic acid precursors and protein synthesis. Proc. Natl. Acad. Sci. U. S., **40,** 263–270.

PARDEE, A. B. 1955 Effect of energy supply on enzyme induction by pyrimidine requiring mutants of *Escherichia coli*. J. Bacteriol., **69,** 233–239.

PARDEE, A. B. AND KUNKEE, R. E. 1952 Enzyme activity and bacteriophage infection. J. Biol. Chem., **199,** 9–24.

PARDEE, A. B. AND PRESTIDGE, L. S. 1955 Induced formation of serine and threonine deaminases by *Escherichia coli*. J. Bacteriol., **70,** 667–674.

PARDEE, A. B. AND PRESTIDGE, L. S. 1956 The dependence of nucleic acid syntheses on the presence of amino acids in *Escherichia coli*. J. Bacteriol., **71,** 677–683.

PARDEE, A. B., SHORE, V. G., AND PRESTIDGE, L. S. 1956 Incorporation of azatryptophan into proteins of bacteria and bacteriophage. Biochim. et Biophys. Acta, **21,** 406–407.

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

POLLOCK, M. R. 1953 Stages in enzyme adaptation. In *Adaptation in Microorganisms*, pp. 150–177. Cambridge University Press, Cambridge, England.

RALSTON, D. J., BAER, B. S., LIEBERMAN, M., AND KRUEGER, A. P. 1955 Virolysin: A virus induced lysin from staphylococcal phage lysates. Proc. Soc. Exptl. Biol. Med., **89,** 502–507.

SCHNEIDER, W. C. 1945 Phosphorus compounds in animal tissues. Extraction and estimation of deoxypentose nucleic acid and of pentose nucleic acid. J. Biol. Chem., **161,** 293–303.

WEIBULL, C. 1956 Bacterial protoplasts; their formation and characteristics. In *Bacterial anatomy*, pp. 111–126. Cambridge University Press, Cambridge, England.