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17 December 1956

Penicillin-Induced Lysis of Escherichia coli

This communication reports some results of studies the objective of which was the elucidation of the mechanism of the bactericidal action of penicillin. Those of our findings which are coincidental to the observations of Liebermeister and Kellenberger (*1*) and of Lederberg (*2*) on the penicillin-induced emergence of bacterial protoplasts were established independently.

While several metabolic processes in microorganisms are known to be influenced by penicillin, none of them has been shown conclusively to be the site of the primary action of the antibiotic, and thus, to be originally responsible for the antibacterial action of the drug (*3*). The present report is concerned with the lysis of *Escherichia coli* induced by penicillin, a phenomenon that points to profound disturbances of cell-wall functions.

Escherichia coli strain B was used as the test organism (*4*). The method for spectrophotometric assay of antibiotic action has been described elsewhere (*5*). Figure 1 shows the lysis of *E. coli* B under the influence of 100 units of penicillin per milliliter; the rates of lysis were approximately dependent on penicillin concentrations over a range from 12.5 to 500 units/ml.

Penicillin-induced lysis of *E. coli* occurred only in a nutritional environment that was capable of supporting the growth of the bacteria. Logarithmic cultures of *E. coli* which were washed free of growth medium and then resuspended in fresh media devoid of sources of carbon or nitrogen did not undergo lysis in the presence of penicillin, while bacteria that were resuspended in complete growth medium lysed as usual.

When 100 units of penicillin per milliliter was added to logarithmic mass cultures of *E. coli* B and vigorous aeration was continued, the cultures soon began to foam, and masses of macroscopic, long strands that gave the impression of highly polymerized material appeared. Strands collected by low-speed centrifugation were readily dissolved in 0.5*N* NaOH to form viscous solutions, while some residue remained when the strands were extracted with 5 percent perchloric acid for 30 minutes at 70°C. The ultraviolet absorption spectra of such perchloric acid extracts closely resembled those of nucleic acids. Chemical analysis indicated that about 15 percent of the total nitrogen of the strands went into the perchloric acid extracts. These extracts also contained quantities of pentose and deoxypentose, which suggested the presence of ribonucleic and deoxyribonucleic acids in a ratio of 3.5 to 1.

Another method of lysing bacteria, depolymerization of the cell walls by lysozyme, exposes the bacterial protoplasts (*6*), which, in an unfavorable osmotic environment, are disrupted and yield the cytoplasmic constituents in an alkali-soluble form that contains highly polymerized deoxyribonucleic acid (*7*).

In order to investigate the possibility that protoplasts might become demonstrable also as the result of penicillin action, sucrose was added to logarithmic cultures of *E. coli* B to give molar concentrations of 0.32 or 0.48. Penicillin, to a concentration of 50 units/ml, was added 30 minutes later. Incubation was continued without aeration or mechanical agitation, and samples were taken at 30-minute intervals for spectrophotometric readings and examination under the phase-contrast microscope. Figure 1 shows the time course of penicillin action in the presence of 0.32*M* and 0.48*M* sucrose.

Observation under the phase-contrast microscope (Fig. 2) revealed the following sequence of events: the bacterial rods produced central or terminal globular extrusions that increased in size while the bacterial cell walls became correspondingly empty of cytoplasm. Later, the globes either separated from the cell walls or retained parts of them attached,

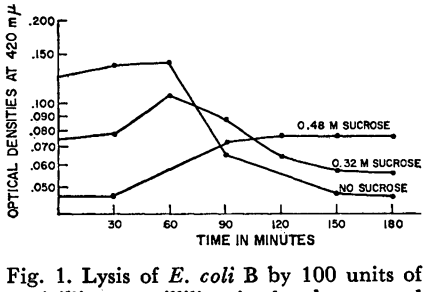

Fig. 1. Lysis of *E. coli* B by 100 units of penicillin per milliliter in the absence and presence of sucrose.

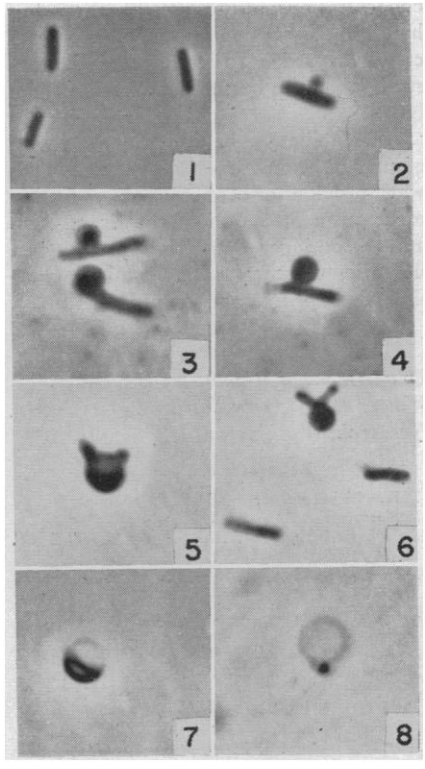

Fig. 2. Sequential phases of penicillin-induced lysis of *E. coli* B: 1, Bacteria immediately after addition of penicillin; 2, 3, and 4, emergence of globular extrusions; 5 and 6, "rabbit ear" forms; 7, partially vacuolized globular structure; 8, "ghost" form of a cytoplasmic membrane.

giving a typical "rabbit-ear" appearance. Finally, the globular structures underwent partial vacuolization, showing many crescent-shaped forms; eventually they released their entire content, leaving as formed elements only circular "ghosts" that probably represented empty cytoplasmic membranes.

The emergence from bacteria of spherical structures, called "large bodies," has been the subject of a literature more extensive than conclusive. The present morphological observations resemble those of Liebermeister and Kellenberger (*1*) concerning the action of penicillin on *Proteus vulgaris* in liquid cultures; these two authors also have emphasized the similarity between "large bodies" and bacterial protoplasts. For *E. coli,* this idea has been further expanded by Lederberg (*2*).

The present findings on the lysis of *E. coli* by penicillin are consistent with the following interpretation: the drug induces a metabolic change that affects the integrity of the bacterial cell walls in such a way that the content of the bacteria, enclosed in a cytoplasmic membrane, extrudes as a globular structure. Without protection against osmotic and mechanical disruption, the globes disintegrate and release the cytoplasmic material. In the presence of appropriate

concentrations of sucrose, on the other hand, the disruption of the globes is sufficiently retarded to permit the demonstration of the sequential phases of the lysis.

An elucidation of the primary action of penicillin on bacterial cell walls may well provide one of the clues to the mechanism of action of the drug. Park and Strominger (*8*) have provided a definitive chemical basis for an explanation of the primary action of penicillin.

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31 October 1956

Effect of Digoxin on Myokinase Activity

In a study of "energetic-dynamic cardiac insufficiency," Munchinger (*1*) reported that strophanthin and digilanid enhanced the adenosine triphosphatase (ATPase) activity of rat-heart homogenate. Attempts to repeat this observation using actomyosin prepared from heart gave results that varied from 40 percent to zero activation, depending on the relative purity (by reprecipitation) of the actomyosin preparation. The loss of digoxin enhancement on reprecipitation indicated that a component other than actomyosin was sensitive to digoxin. The present report suggests that this component may be myokinase (*2*).

Actomyosin (myosin B) was prepared from dog heart by the method of Szent-Gyorgi (*3*). Adenosine triphosphatase activity was measured by incubating myosin B and adenosine triphosphate (ATP) in KCl (0.15*M*)- veronal buffer (0.02*M*) at *p*H 7.3 At the end of the incubation time, 20-percent trichloroacetic acid was added, the mixture was centrifuged, and phosphorus was determined on an aliquot of the supernatant by the method of Fiske and Subbarow (*4*).

Myokinase was prepared according to Kalckar's method (*5*). The final trichloroacetic acid precipitation was omitted. The myokinase solution was dialyzed against distilled water and finally centrifuged at 18,000 *g* for 30 minutes. The final product contained 0.28 mg of N per milliliter and showed only one major peak when subjected to electrophoresis for 90 minutes in 0.1*M* veronal buffer at *p*H 8.5.

Myokinase activity was determined following the procedure of Bendall (*6*). A myosin B preparation was used as a specific ATPase hydrolyzing only the terminal phosphate group of ATP. Because the myosin B was in sufficient excess to hydrolyze 45 to 46 percent of the 10-minute acid-labile phosphate of ATP in 2 minutes, the extra phosphate liberated in 10 minutes was a function of the myokinase concentration and was taken as myokinase activity. The myokinase preparations, when present in optimum concentration, liberated 100 percent of the 10-minute labile phosphate.

We found that myosin B from rat heart was difficult to free from myokinase activity, whereas that from dog heart was relatively easy to free. Myosin B from dog heart was therefore chosen for the test system. Figure 1 shows the effect of digoxin on myokinase activity. The myosin B and ATP concentrations are constant throughout. Bars 1 through 6 denote increasing quantities of myokinase added to the test system. The activating effect of digoxin appears to be confined to the systems in which myokinase is a limiting factor. At high myokinase concentrations (bar 6), the total activity was inhibited, and digoxin had no influence on the reaction. This decrease of the over-all reaction was apparently the result of inhibition of ATPase activity of

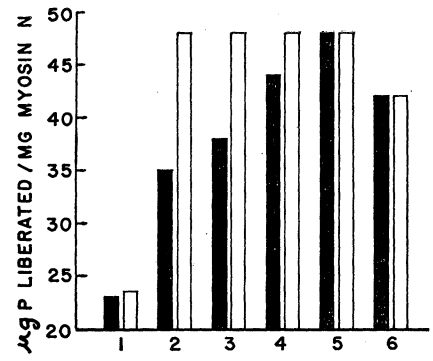

Fig. 1. Effect of digoxin on the activity of myokinase. The incubation tubes contained the following: KCl, 0.15*M*; veronal buffer, 0.02*M* at *p*H 7.3; $CaCl_2$, 0.01*M*; ATP, $3.75 \times 10^{-4}M$, myosin B (0.46 mg N/ml, 0.2 ml; and digoxin, 10 μg/ml. The total volume was 2.0 ml. Black bars, controls; open bars, with digoxin. Myokinase (0.28 mg N/ml) was added as follows: bar 1, none; bar 2, 0.025 ml; bar 3, 0.05 ml; bar 4, 0.1 ml; bar 5, 0.2 ml; bar 6, 0.5 ml.

myosin B rather than to an effect on myokinase activity. At a lower myokinase concentration (bar 5), which appears to be the optimum myokinase concentration in our test system, digoxin was without effect. When the myokinase concentration was decreased below the optimum level (bars 2, 3, and 4), digoxin activated the myokinase reaction, bringing the reaction to the level obtained with optimum concentrations. With no added myokinase, digoxin had no effect on the ATPase activity of myosin B.

Hasselbach and Weber (*7*) have shown that the Marsh (*8*) and Bendall (*9*) relaxing factor of muscle inhibits the ATPase activity of myosin by extending substrate inhibition to physiological concentrations. The demonstration of Bendall (*6*) that the Marsh-Bendall factor exhibited all the characteristics of myokinase prompted us to include in the present study the effect of digoxin on the ATPase-inhibiting characteristics of myokinase. Table 1 shows the effect of digoxin on the myokinase inhibition of ATPase activity of myosin B at two concentrations of ATP. At lower ATP concentrations (0.005*M*), the myokinase inhibition is slight (32 percent) but the ability of digoxin to reverse myokinase inhibition is evident. At higher ATP concentrations (0.01*M*), inhibition approaches 73 to 74 percent, and the ability of digoxin to reverse the myokinase effect is obvious.

According to Weber (*10*), contraction of muscle is associated with ATP breakdown and lasts as long as ATP continues to be hydrolyzed. Relaxation sets in as soon as ATP breakdown is prevented. Physiologically induced relaxation is based on an inhibition of ATP hydrolysis by the Marsh-Bendall factor (myokin-

Table 1. Effect of digoxin on the myokinase inhibition of ATPase activity of myosin B at two concentrations of ATP. The figures are based on 6 individual runs. Three runs were made on one myosin and myokinase preparation and three runs were made on a different myosin and myokinase preparation. The tubes contained the following: KCl, 0.15*M*; veronal buffer, 0.02*M* at *p*H 7.3; $MgCl_2$, 0.005*M*; myosin B (0.46 mg N/ml), 0.15 ml; myokinase (0.28 mg N/ml), 0.5 ml; and digoxin, 10 μg/ml. The total volume was 2.0 ml.

ATP Concn. (*M*)	Phosphorus liberated (μg/mg of myosin N hr)			
	Control	Digoxin	Myokinase	Myokinase and digoxin
0.005	1842 ± 24	1868 ± 28	1270 ± 36	1884 ± 32
0.01	1520 ± 58	1610 ± 32	402 ± 27	1458 ± 60