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New Assay for Penicillinase and Some Results on Penicillinase Induction

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ABSTRACT

IMSANDE, JOHN (Western Reserve University, Cleveland, Ohio). New assay for penicillinase and some results on penicillinase induction. J. Bacteriol. **89**:1322-1327. 1965.—A rapid, sensitive, and reliable assay of penicillinase activity is described. Studies conducted on the induction of penicillinase, with use of the new assay, show that actinomycin D inhibits growth and the induced synthesis of penicillinase in *Bacillus cereus* 569. These inhibitory effects can be reversed to various degrees by deoxyguanosine, depending upon the time lapse between the addition of the antibiotic and the addition of the deoxynucleoside. Inhibition of growth is reversed more readily than inhibition of penicillinase induction, and it is suggested that actinomycin D may preferentially inhibit the induced synthesis of penicillinase. Studies conducted in an attempt to ascertain the role of penicillin in inducing penicillinase formation in *B. cereus* 569 suggest that the inducer, penicillin, enhances penicillinase synthesis in some manner in addition to its probable role of inducing the formation of a penicillinase-specific ribonucleic acid messenger.

Bacillus cereus 569 produces a highly active penicillinase when induced by penicillin. However, the induction of penicillinase in this organism differs greatly from induction of β-galactosidase in *Escherichia coli* (Pollock, 1959). In spite of this difference, the β-galactosidase system of *E. coli* has received much more attention as a model system for studying enzyme induction than has the penicillinase system (Jacob and Monod, 1963). The greater popularity of the β-galactosidase system stems, at least in part, from the ease with which the enzyme β-galactosidase can be assayed and from the susceptibility of *E. coli* systems to genetic analysis as compared with the inadequacies of the available penicillinase assays and the dearth of genetic studies on *B. cereus*. The studies reported here were undertaken to elucidate further the mechanism of penicillinase induction in *B. cereus* in order that this system might be examined as a possible model system for investigating the universality of certain features of enzyme induction.

Described in this communication is a rapid, sensitive, and reliable assay for penicillinase activity. With use of this assay, data have been obtained which indicate that penicillin plays some role in the induction of penicillinase in addition to that of inducing a penicillinase-specific messenger.

MATERIALS AND METHODS

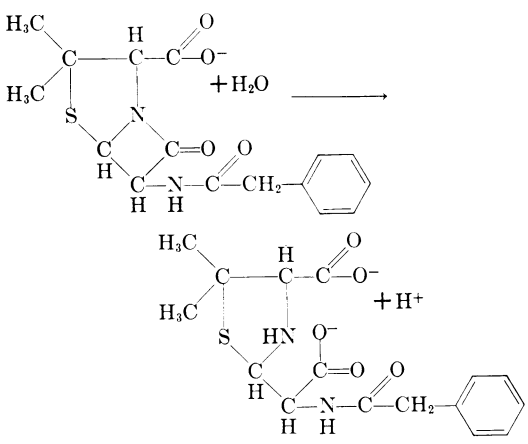
Organism and medium. B. cereus 569 (ATCC number 10876) was used in these studies. Cultures were grown on a synthetic medium which contained, per liter: trisodium citrate·$2H_2O$, 5.9 g; $(NH_4)_2SO_4$, 2.0 g; $MgSO_4 \cdot 7H_2O$, 0.52 g; KH_2PO_4, 2.72 g; Casamino Acids, 1.0 g; and KOH to adjust the *p*H to 7.2. At the time of inoculation, 2 g per liter of glucose were added to the medium. Bacterial cultures were grown overnight, aerobically with swirling, at 36 C and were maintained in exponential phase prior to each experiment. For experiments, cells were removed from such cultures, washed on a Millipore filter, and suspended in fresh medium supplemented with 0.1% gelatin. Cell growth was followed with a Klett-Summerson colorimeter (filter no. 54) and is expressed as $\log_{10}$ of the observed Klett reading. One unit of penicillin per milliliter of culture medium was used to induce penicillinase formation. Penicillin and deoxyguanosine were obtained from Calbiochem, and actinomycin D was a gift of Merck & Co., Inc., Rahway, N.J.

Enzyme assay. Under the growth conditions employed, penicillinase is excreted into the medium (Pollock and Perret, 1951). Enzyme samples, obtained by transferring 3-ml fractions of culture medium to tubes containing 0.6 ml of 8×10^{-4} M hydroxyquinoline, were freed from cells by centrifugation (10,000 × *g* for 5 min) and then dialyzed for 2 hr at 2 C against a large volume of 10^{-4} M ethylenediaminetetraacetate (*p*H 7.2). The dia-

lyzed enzyme solution (0.1 ml, unless otherwise stated) was incubated with 2.4 ml of a weakly buffered indicator solution [40 mg of bromothymol blue per liter of 3×10^{-3} M cacodylate (*p*H 7.3)] at 30 C for 10 min in a total volume of 2.94 ml. At the termination of the incubation period, 0.06 ml of penicillin [100 mg of penicillin per ml of 3×10^{-3} M cacodylate (*p*H 7.3)] was added to a reaction cuvette and the incubation mixture was immediately transferred to the reaction cuvette. The decrease in optical density at 620 mμ was measured with a Zeiss spectrophotometer at zero-time and at 3-min intervals thereafter for 12 min. The rate of the reaction was calculated from the slope of a best-fit straight line drawn through the five experimental points. Enzyme activity described below is expressed as the change in optical density per 10 min. Reaction mixtures minus penicillin served as a blank.

RESULTS

Spectrophotometric assay of penicillinase activity. Penicillinase catalyzes the hydrolysis of the cyclic amide bond of benzylpenicillin as follows:



The carboxyl group produced by this hydrolysis has a pK_a of approximately 4.7. Hence, at neutral or alkaline *p*H the products of this hydrolysis are the salt of penicilloic acid and a proton. Experimental results presented in Fig. 1 show that, with the aid of the color indicator bromothymol blue, low hydrogen ion concentrations can be measured accurately with a spectrophotometer. Measurements are made at 620 mμ, since at this wavelength the difference in spectra of the reduced and oxidized forms of bromothymol blue is greatest, and the assay has maximal sensitivity. Under these conditions, hydrogen ions are produced stoichiometrically during the enzymatic hydrolysis of penicillin (Fig. 1). From the linear portion of the curve the molar extinction coefficient of the

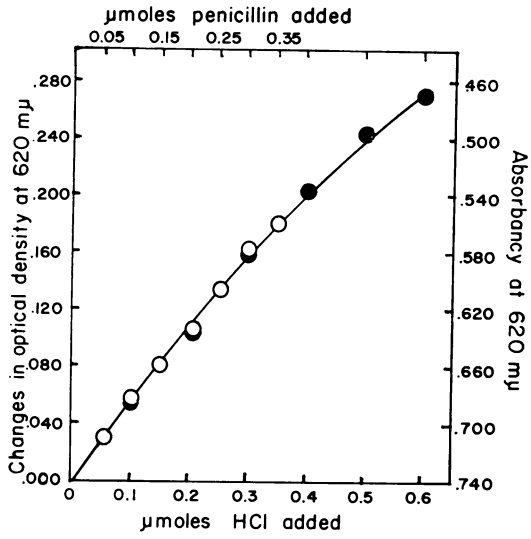


FIG. 1. *Standard curve for the quantitative determination of penicillinase-catalyzed hydrolysis of penicillin. Closed circles (●) represent values obtained when 50 μliters of a standardized solution of HCl are substituted for penicillin and penicillinase in the reaction cuvette. Open circles (○) represent values obtained when an excess of crystalline penicillinase and a known amount of penicillin are added to the reaction cuvette.*

buffered indicator dye, prepared as described under Materials and Methods, was found to be 1.6×10^3. Hence, the hydrolysis of penicillin can be readily determined quantitatively by this procedure. As shown in Fig. 2, the spectrophotometric assay for penicillinase activity is sensitive enough to measure the low level of penicillinase produced by noninduced cells and also reliable for the determination of high levels of induced penicillinase activity.

Inhibition by actinomycin D and its reversal by deoxyguanosine. The antibiotic actinomycin D is known to inhibit deoxyribonucleic acid (DNA)-dependent ribonucleic acid (RNA) synthesis in gram-positive bacteria. This inhibition probably results from the binding of the actinomycin D to the deoxyguanosine regions of the bacterial DNA (Reich, 1964). Data presented in Fig. 3 show that actinomycin D inhibits bacterial growth and the induction of penicillinase, and that this inhibition appears to be completely prevented by deoxyguanosine when the deoxynucleoside is added to the culture medium immediately prior to the antibiotic. However, as shown in Fig. 4, the degree of restoration of both bacterial growth and penicillinase induction is dependent upon the time interval between the addition of actinomycin

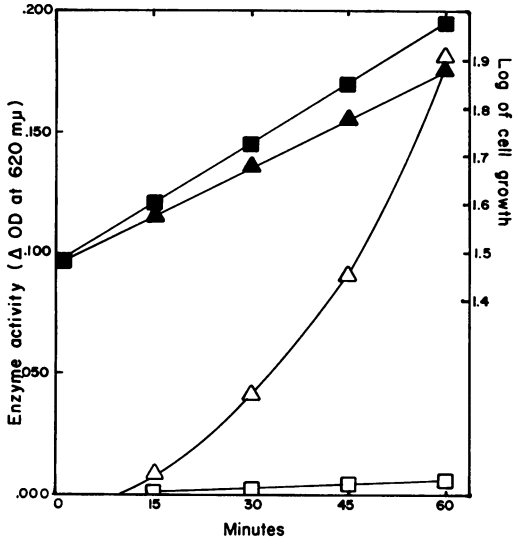


FIG. 2. *Reproducibility and sensitivity of the penicillinase assay. The upper lines represent growth curves of a control culture of Bacillus cereus 569 without supplement (■) and a culture to which 1 unit/ml of penicillin was added (▲). The lower curves (open symbols) show the amount of penicillinase activity found in a 0.1-ml sample of the spent medium of the induced culture (△) and a 0.5-ml sample of the spent medium of the noninduced culture (□). Each enzyme assay was conducted in triplicate and each set of values were in excellent agreement.*

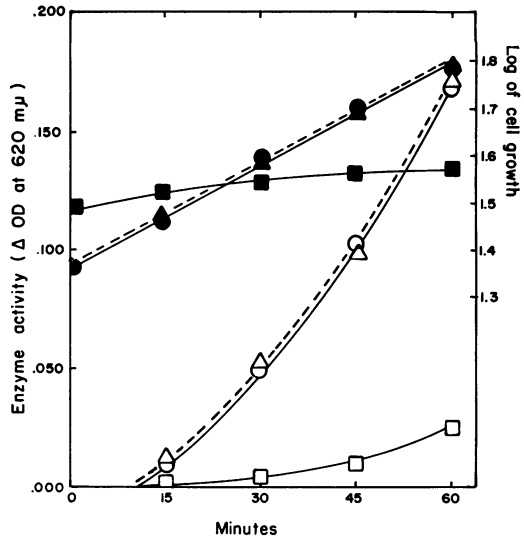


FIG. 3. *Inhibition of growth and penicillinase induction by actinomycin D and reversal of these inhibitions by deoxyguanosine. Growth curves shown are for a culture of Bacillus cereus 569 induced with 1 unit/ml of penicillin (▲), a penicillin-induced culture to which 0.12 μg/ml of actinomycin D was added (■), and a culture to which deoxyguanosine (1 mg/ml), actinomycin, and penicillin (in that order) were added (●). Penicillinase activity for each culture is represented by the corresponding open symbols.*

and the addition of deoxyguanosine. If deoxyguanosine is added immediately after actinomycin, restoration of bacterial growth and penicillinase induction appears complete. On the other hand, if the deoxyguanosine is added 10, 20, 30, or 40 min after the addition of actinomycin, the restoration of growth and penicillinase induction are delayed and progressively less pronounced. Data shown in Fig. 5, which is a summary of the data presented in Fig. 4, suggest that a prolonged treatment with actinomycin produces a preferential effect on penicillinase induction, since the recovery of growth under these conditions appears to be greater than the recovery of penicillinase induction.

Effect of penicillin on the synthesis of penicillinase. B. cereus 569, once induced by penicillin to produce high levels of penicillinase, is known to continue to synthesize the enzyme at elevated rates for several generations, even when penicillin has been removed from the culture medium (Pollock and Perret, 1951). This observation is substantiated by the data presented in Fig. 6. More interestingly, however, these data also suggest that penicillin influences the synthesis of penicillinase by some mechanism in addition to that of induction of new penicillinase-specific messenger. This conclusion is based on the observation that the enhancement of penicillinase synthesis, produced by penicillin, is much greater in preinduced cells in which the formation of messenger appears to be strongly inhibited by actinomycin D (Fig. 6, compare curves C and D) than in noninduced cells that have received the same actinomycin-penicillin treatment (Fig. 6, curve E). Furthermore, the stimulation of penicillinase formation by penicillin is greater in the actinomycin-treated preinduced cells (Fig. 6, compare curves C and D) than in preinduced cells in the absence of actinomycin (Fig. 6, compare curves A and B).

DISCUSSION

Recent reports (Pollock, 1963; Harris and Sabath, 1964) indicate that penicillin induces the formation of a penicillinase-specific messenger RNA in *B. cereus*. Nevertheless, little is known about the mechanism by which penicillin induces penicillinase formation.

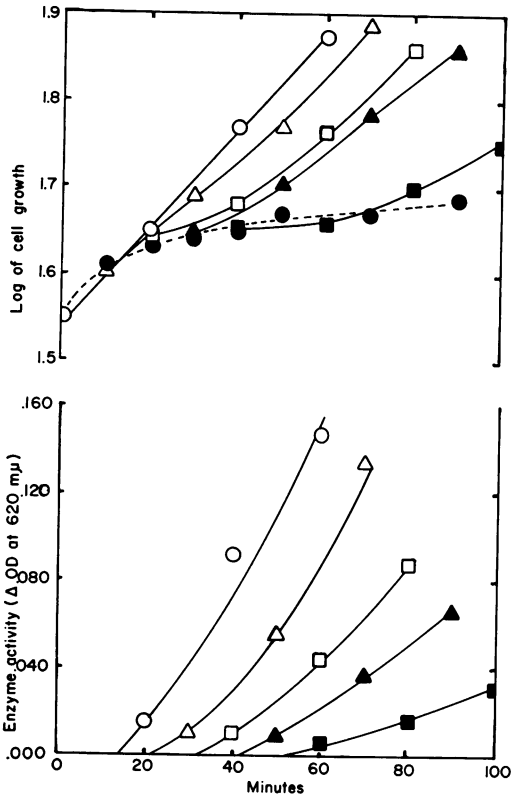


FIG. 4. *Deoxyguanosine-mediated reversal of inhibition produced by actinomycin D. The upper family of curves represents the growth curve of actinomycin-treated (0.12 μg/ml) Bacillus cereus (●) and subcultures to which deoxyguanosine and penicillin were added at zero time (○); 10 min (△); 20 min (□); 30 min (▲); and 40 min (■). The lower family of curves shows the accumulation of penicillinase activity in the culture designated by the corresponding symbol.*

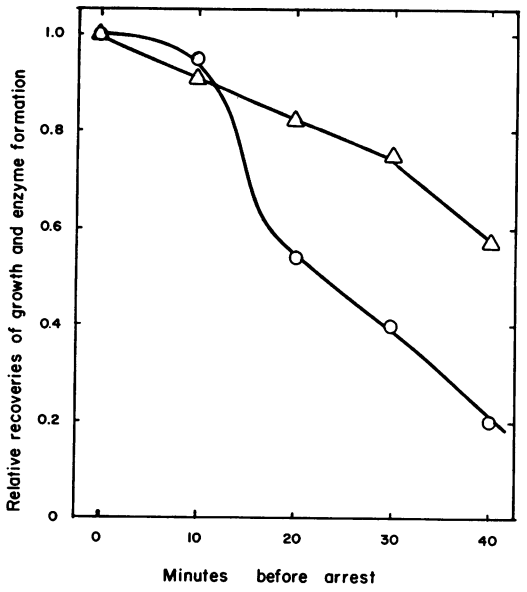


FIG. 5. *Relative inhibition of growth rate and penicillinase inducibility. The decline in growth rate is represented by the open triangles (△) and the reduction in penicillinase induction is represented by the open circles (○). The curves depicted in this figure are a summary of the data shown in Fig. 4.*

Evidence provided in Fig. 3 supports the notion that RNA synthesis must occur after the addition of the inducer for the rate of formation of active penicillinase to increase, since penicillinase induction is inhibited by actinomycin D. This notion receives further support from the data presented in Fig. 4, which show that deoxyguanosine prevents or reverses the inhibition produced by actinomycin. Reversal of actinomycin inhibition by deoxyguanosine is thought to result from the complexing of the antibiotic with the deoxynucleoside. Formation of such a complex lowers the concentration of the antibiotic available to react with the deoxyguanosine residues of the DNA and thereby prevents or reverses inhibition. More interestingly, however, the data presented in Fig. 4 and 5 suggest that actinomycin inhibits penicillinase induction to a greater extent than it does cell growth. It will be noted that removal of the actinomycin by deoxyguanosine, after a 20- to 40-min exposure, is accompanied by a relatively greater recovery of growth rate than of penicillinase inducibility. One possible explanation of these results is that actinomycin binds more strongly to the DNA region which constitutes the penicillinase gene than to the average gene. Another possibility is that actinomycin inhibits penicillinase induction, and perhaps other biological processes, by some unknown mechanism (excluding messenger breakdown). If actinomycin is bound preferentially to the penicillinase gene, this might suggest that this particular segment of DNA is unusually rich in deoxyguanosine residues. Furthermore, if this were the case, then the messenger produced would be high in cytidine (C) or guanosine (G), depending upon which strand of DNA is transcribed. Since the ease with which two strands of annealed nucleic acids can be separated is inversely related to the number of G-C pairs (Marmur and Doty, 1962), one would expect messenger rich in G-C to possess more secondary structure or to complex more strongly with other nucleic acids and perhaps with ribosomes than G-C-poor RNA. Indeed, a relatively stable association of the penicillinase messenger

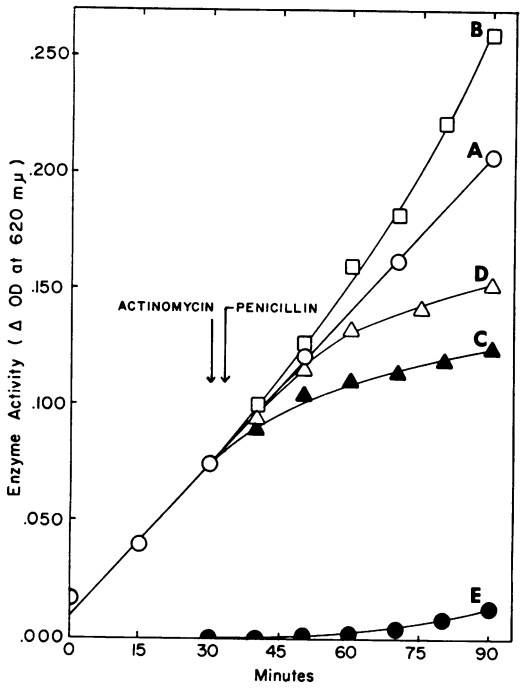


FIG. 6. *Stimulation of penicillinase formation in preinduced, actinomycin-treated cells by penicillin (preinduced cells refers to cells that were induced with 1 unit of penicillin per milliliter for 30 min, then Millipore-filtered, washed, and suspended in fresh medium lacking penicillin). Accumulation of penicillinase activity in preinduced (control) cells is indicated by curve A (○). At 30 min, three samples were withdrawn from the control culture and placed in culture flasks. At the times indicated, actinomycin (0.12 μg/ml) was added to cultures C (▲) and D (△), and penicillin (1 unit/ml) to cultures B (□) and D. Curve E represents penicillinase accumulation in a noninduced culture to which actinomycin D and penicillin were added at the indicated times. Samples were taken from each culture for enzyme analysis at times indicated. Each point represents an average of 8 to 10 determinations.*

with some cellular component might also be inferred from the relatively long half-life (40 min) of the penicillinase messenger (Pollock, 1963), since partially annealed RNA appears somewhat more resistant to digestion by ribonuclease than RNA with little or no secondary structure.

It should be noted that *B. cereus*, once induced by penicillin to produce an elevated level of penicillinase, continues to produce the enzymes at an increased rate for several (approximately eight) generations after the inducer, penicillin, has been removed from the culture medium. It has been suggested (Pollock and Perret, 1951) that the continued production of penicillinase in the absence of exogenous inducer might be promoted by the approximately 200 molecules of penicillin contained in the average induced *B. cereus* cell. The data presented in Fig. 6 substantiate Pollock's finding that preinduced *B. cereus* produces penicillinase at an elevated rate in the absence of exogenous inducer. However, these data also suggest that penicillin plays a role in penicillinase formation other than that of inducing a penicillinase-specific messenger, since penicillin stimulates penicillinase formation in preinduced actinomycin-treated cells. No clue as to the mechanism of this stimulation has been found. It is not due to protection or stabilization of penicillinase, because enzyme activity in spent medium does not decrease when shaken at 37 C in the absence of penicillin, nor does enzyme activity increase when spent medium is shaken at 37 C in the presence of 1 unit per ml of penicillin. Furthermore, when actinomycin-treated cells are added to spent medium obtained from induced cells, the small decrease in enyzme activity observed is not influenced by 1 unit/ml of penicillin. In addition, the amount of penicillinase activity associated with the cells collected from cultures C and D, Fig. 6, did not differ. It is therefore concluded that penicillin, in addition to its probable role of inducing the formation of a penicillinase-specific messenger, enhances penicillinase synthesis in some other manner.

ACKNOWLEDGMENTS

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