New Assay for Penicillinase and Some Results on Penicillinase Induction

JOHN IMSANDE

Department of Biology, Western Reserve University, Cleveland, Ohio

Received for publication 18 January 1965

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 \( \beta \)-galactosidase in Escherichia coli (Pollock, 1959). In spite of this difference, the \( \beta \)-galactosidase system of \( \text{E. coli} \) 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 \( \beta \)-galactosidase system stems, at least in part, from the ease with which the enzyme \( \beta \)-galactosidase can be assayed and from the susceptibility of \( \text{E. coli} \) systems to genetic analysis as compared with the inadequacies of the available penicillinase assays and the dearth of genetic studies on \( \text{B. cereus} \). The studies reported here were undertaken to elucidate further the mechanism of penicillinase induction in \( \text{B. cereus} \) 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. \( \text{B. cereus} \) 569 (ATCC number 10876) was used in these studies. Cultures were grown on a synthetic medium which contained, per liter: trisodium citrate\( \text{2H}_2\text{O} \), 5.9 g; \((\text{NH}_4)\text{SO}_4 \), 2.0 g; \(\text{MgSO}_4\cdot7\text{H}_2\text{O} \), 0.52 g; \(\text{KH}_2\text{PO}_4 \), 2.72 g; \(\text{Casamino Acids} \), 1.0 g; and \(\text{KOH} \) to adjust the \( \text{pH} \) 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 \( 8 \times 10^{-4} \) M hydroxyquinoline, were freed from cells by centrifugation (10,000 \( \times \) g for 5 min) and dialyzed for 2 hr at 2°C against a large volume of \( 10^{-4} \) M ethylenediaminetetraacetate (\( \text{pH} \) 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 X 10^-3 M cacodylate (pH 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 X 10^-3 M cacodylate (pH 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 mu 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:

\[
\begin{align*}
\text{H}_3\text{C} & \text{C} - \text{C} - \text{C} - \text{O}^- + \text{H}_2\text{O} \\
\text{H}_3\text{C} & \text{S} - \text{C} - \text{N} - \text{C} - \text{CH}_3
\end{align*}
\]

The carboxyl group produced by this hydrolysis has a pK_a of approximately 4.7. Hence, at neutral or alkaline pH 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 mu, 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 buffered indicator dye, prepared as described under Materials and Methods, was found to be 1.6 X 10^4. 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
and the addition of deoxyguanosine. If deoxy

guanosine is added immediately after actinomy-
cin, restoration of bacterial growth and penicill-

ase 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 penicillase 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 preferen-
tial effect on penicillase induction, since the

recovery of growth under these conditions appears to be greater than the recovery of penicillase induction.

Effect of penicillin on the synthesis of peni-

cillase. B. cereus 569, once induced by penicillin to produce high levels of penicillase, is known to

continue to synthesize the enzyme at elevated rates for several generations, even when penicillin has been removed from the culture medium (Pol-

lock and Perret, 1951). This observation is sub-

stantiated by the data presented in Fig. 6. More

interestingly, however, these data also suggest

that penicillin influences the synthesis of peni-
cillase by some mechanism in addition to that

of induction of new penicillase-specific messen-
ger. This conclusion is based on the observation

that the enhancement of penicillase synthesis, produced by penicillin, is much greater in pre-

induced cells in which the formation of messenger appears to be strongly inhibited by actinomycin

D (Fig. 6, compare curves C and D) than in non-

induced cells that have received the same actinomycin-penicillase treatment (Fig. 6, curve E).

Furthermore, the stimulation of penicillase formation by penicillin is greater in the actinomy-
cin-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 forma-
tion of a penicillase-specific messenger RNA in B. cereus. Nevertheless, little is known about the mechanism by which penicillin induces penicillase formation.
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 deoxyribonucleoside. 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 case 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 enzyme 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

This investigation was supported by Public Health Service grant A.M. 07310 from the National Institutes of Health. I am indebted to Mary J. Gerber for invaluable technical assistance.

Literature Cited


POLLOCK, M. R. 1963. The differential effect of
