INDUCTION OF STAPHYLOCOCCAL PENICILLINASE BY BENZYLPENICILLIN: EFFECT OF pH, CONCENTRATION OF FERROUS ION AND INDUCER, AND DURATION OF EXPOSURE OF CELLS TO INDUCER

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ABSTRACT

LEITNER, FELIX (Michael Reese Hospital and Medical Center, Chicago, Ill.), HELEN M. SWEENEY, T. F. MARTIN, AND SIDNEY COHEN. Induction of staphylococcal penicillinase by benzylpenicillin: effect of pH, concentration of ferrous ion and inducer, and duration of exposure of cells to inducer. J. Bacteriol. **86**:717–727. 1963.—The kinetics of induction of penicillinase by benzylpenicillin in exponentially multiplying *Staphylococcus aureus* strain 55-C-1 were shown to vary with the pH. At pH 7.3 in the absence of free inducer, the rate of increase of penicillinase activity rapidly declined and came to a halt. At pH 5.4 to 5.5 and in the presence of optimal concentrations of Fe^{++}, the penicillinase activity of the induced culture increased linearly with time for 2.5 or more generations, but the rate of increase usually declined eventually. Evidence was advanced to support the concept that the acidic pH and optimal Fe^{++} concentration maintain the induced formation of enzyme. The induced increase in penicillinase activity appeared 3 to 4 min after the addition of benzylpenicillin. The degree of induction of penicillinase varied with the duration of exposure of the staphylococci to the inducer and with the concentration of inducer. Maximal induction under our conditions was attained by exposure for 15 min to benzylpenicillin at an initial concentration between 0.6 and 2 units/ml.

Although the inducibility of staphylococcal penicillinase has been known for some time (Geronimus and Cohen, 1957; Steinman, 1961; Crompton et al., 1962), more information on the characteristics of this system, comparable with that developed for classical systems such as penicillinase in *Bacillus cereus* and β-galactosidase in *Escherichia coli*, has been lacking. In this paper, we present results of studies of the kinetics of induction of staphylococcal penicillinase. We have found that the kinetics of induction of this enzyme in exponentially multiplying cultures of *Staphylococcus aureus* vary markedly with pH and Fe concentration. At an acidic pH in the range from 5.4 to 5.5 and a favorable Fe concentration, penicillinase activity increases linearly with time in the absence of free inducer, in a fashion kinetically similar to but usually not so prolonged as that described by Pollock (1950) for *B. cereus*. Other aspects of induction in this system, including the effects of time of exposure of cells to inducer, are also presented.

MATERIALS AND METHODS

Organisms. S. aureus strain 55-C-1 (Geronimus and Cohen, 1957) was the routine test organism. Drops of overnight broth cultures taken up on porcelain beads and then desiccated over silica gel served as routine inocula. The dried beads, stored at 4 C, were used for periods up to 6 months. A few experiments were repeated with several other penicillinase-producing strains of *S. aureus* to test the generality of the observations. These strains were stored and inoculated from nutrient agar slants.

Media. Cultures were grown in tryptic digest broth (TD), lot no. 003607 (BBL), unless otherwise stated. Several other lots failed to sustain growth at pH 5.5. It was eventually found that acetic acid added to the tryptic meat digest during the preparation of the broth was probably responsible for the inhibition. Batches of tryptic digest of beef heart prepared by us according to Fields (1956) supported good growth at pH 5.5 when HCl was substituted for acetic acid.

TD lot no. 003607 contained 0.001 M inorganic phosphate (Leitner and Cohen, 1962). To avoid

undesired changes in pH during experiments and to improve growth at acidic pH, 0.01 to 0.05 M sodium phosphate was added to the broth prepared from this lot as indicated in the text.

Broth was sterilized routinely at pH 7.4 in an autoclave at a pressure of 12 to 14 psi for 15 min. The sterile broth was adjusted to pH 5.4 to 5.5, when desired, by addition of 1 N HCl.

Soluble penicillinase. Soluble staphylococcal penicillinase was prepared from induced cells of strain 55-C-1, possessing penicillinase activity from 140 to 210 units/mg of protein. Aqueous suspensions of washed organisms (12 to 26 mg of protein/ml) were sonically disrupted, in instruments made by Raytheon Co. (10 kc, model DF 101) or by Branson Instruments, Inc. (model LS-75). With the Raytheon instrument, the cells were treated for 1 hr; with the Branson, for 5 min in 30-sec periods alternating with cooling. Cell debris was removed by centrifugation for 30 min at 105,000 $\times$ *g* (average) in a model L Spinco ultracentrifuge. The supernatants, containing 580 to 3760 units/ml, were divided into 1- to 1.5-ml portions and stored at -20 C.

Induction. Experiments were begun from cultures incubated with slow shaking for approximately 16 hr at 37 C in TD containing 0.05 M phosphate. A portion was diluted sterilely 12- to 50-fold (to a cell density of 11 to 19 μg of protein/ml) with TD of the desired pH, containing sufficient phosphate ion to give a final concentration of 0.01 M. A 50-ml portion of this diluted culture was incubated at 37 C with vigorous shaking (225 rotations/min in a rotatory shaker) in a conical flask of 125-ml capacity with a 19-mm cuvette side arm. After two to three generations of exponential growth, a portion of this culture was diluted again with the same sterile medium to a cell density of about 6 μg of bacterial protein/ml. Clean but nonsterile glassware was used for this dilution and for subsequent manipulations. Repeated cultures and smears confirmed that no significant bacterial contamination took place under these circumstances during the remaining 2 to 3 hr of the experiments. Culture volumes of 50 ml or less were handled in 125-ml flasks, volumes of 50 to 150 ml in 300-ml flasks, and volumes of 150 to 350 ml in 1-liter flasks. In typical experiments, incubation was continued with shaking until a cell density of 10 to 11 μg of protein/ml was attained, at which time inducer was added. In kinetic studies, this point is defined as zero reference time. Additions of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ were usually made 2 min before induction. Growth curves, which were followed through all experiments, were exponential for the periods of the experiments cited, as judged by absorbancy. The generation times of uninduced cultures were 24 to 32 min at pH 7.3 to 7.4 and 26 to 38 min at pH 5.4 to 5.5. In induced cultures, the generation times were 0 to 6 min longer than in the absence of inducer.

The exposure of cells to free inducer was terminated, when desired, by one of two methods. In the first, free penicillin was removed by Millipore filtration followed by washing and resuspension of the organisms with fresh broth at 37 C (Pardee and Prestidge, 1961). Incubation with shaking at 37 C was then resumed. In this manipulation, some of the cell crop was not recovered, as judged by absorbancy of the resuspended cells. The reported penicillinase activity per ml of culture is corrected for the fraction lost experimentally. Alternatively, the inducer was destroyed without interrupting the incubation by addition of soluble penicillinase (Pollock, 1952). The inactivation of the antibiotic was followed by the loss of its inducing power. Table 1 gives the results of a typical experiment. Induction by penicillin was markedly reduced after 30 sec, especially at pH 5.5, and was undetectable, at either pH, after exposure of the inducer to the added enzyme for 90 sec. In the actual induction experiments, the organisms' own penicillinase activity, low at the start, increased rapidly and soon became comparable with the activity of the added enzyme. Under these circumstances, the inducer must have been destroyed appreciably faster than is shown in the table.

Penicillinase assay. Samples of cultures were removed at appropriate times, mixed with chloramphenicol (100 μg/ml), and chilled promptly in a Dry Ice-acetone bath. The penicillinase activity was determined iodometrically according to Perret (1954) by using phosphate buffer at pH 5.8, the optimal pH for the staphylococcal enzyme (Novick, 1962). A manometric assay of cell-bound penicillinase was employed in some experiments, either when the enzymatic activity per ml of culture was too low to be measured without concentration or when soluble penicillinase had been added to the culture to terminate induction more sharply than was possible by filtration. In these cases, samples were taken without chloramphenicol and were chilled promptly in a Dry Ice-acetone bath. The organisms were collected

TABLE 1. *Inactivation of benzylpenicillin by soluble staphylococcal penicillinase**

Time of addition of cells	Cell-bound penicillinase (units/mg of protein)	
	pH 5.5	pH 7.4
sec		
30	19.5	26.6
60	9.5	12.7
90	7.9	4.5
120	8.0	4.3
Controls		
Enzyme + cells	7.7	4.5
Penicillin + cells	167	80.0

* Cells of strain 55-C-1 grown for 2.5 generations with shaking in TD (first shipment of lot no. 003607) with 0.01 M added phosphate at pH 5.5 and 7.4 were harvested by brief centrifugation at room temperature and resuspended in water. Two sets of fresh broth of corresponding pH values, containing penicillin (2 units/ml) and shaken at 37 C, received soluble penicillinase to a final concentration of 0.95 units/ml, followed, at the indicated times later, by the corresponding inoculum. (Initial bacterial concentration of the acid and neutral cultures were 8 and 12 μg of protein/ml.) In the first control (enzyme + cells), penicillin was omitted. In the second (penicillin + cells), the order of addition was reversed: the cells were added to the penicillin-containing broth first, followed by the enzyme 10 min later. Figures represent the specific activities of cell-bound penicillinase in washed organisms collected 55 min (acid cultures) and 35 min (neutral cultures) after inoculation. The corresponding specific activities of the acid and neutral inocula were 4.8 and 2.5 units/mg of protein, respectively. Penicillinase was determined manometrically.

by centrifugation at 4 C. If soluble penicillinase had been used, they were washed once with cold 0.05 M $NaHCO_3$ solution and once with cold 0.017 M $NaHCO_3$; otherwise, they were washed only with the latter. The cell-bound enzyme activity was determined manometrically as previously described (Leitner and Cohen, 1962). It was confirmed that added soluble enzyme was removed by the washing and did not interfere in this assay. The extracellular enzyme fraction was not measured in this assay, but the results reported were corrected for it, when desirable, by data from independent experiments on the distribution of penicillinase activity between cells and supernatant.

One unit of penicillinase was defined as the amount of enzyme that hydrolyzes 1 μmole of penicillin per hr at 30 C and pH 5.80, the conditions of the iodometric assay. Specific activity was defined as units of penicillinase per mg of bacterial protein. This unit was virtually identical with that based on the manometric assay, performed at 37 C and pH 7.46 (Leitner and Cohen, 1962). The ratio of manometric to iodometric units in eight experiments was 1.0 ($\pm$ 0.07 SD). Accordingly, results obtained by either method were used interchangeably without correction.

Protein determination. The concentration of staphylococci was followed by absorbancy at 540 mμ in a Coleman junior spectrophotometer. These values were related to bacterial protein by experimentally determined curves (Leitner and Cohen, 1962).

Iron determination. The iron content of the broth was determined colorimetrically by the *o*-phenanthroline method (Schales, 1958) after digestion with nitric and perchloric acids.

Chemicals. Sodium benzylpenicillin, a gift of Chas. Pfizer & Co., Inc., is referred to in this paper as penicillin. Concentrations are given in Oxford units.

Chloramphenicol was a gift of Parke, Davis & Co.

Chemicals were reagent grade.

RESULTS

Effect of pH on kinetics of induction. The kinetics of the increase in penicillinase activity of cultures of *S. aureus* 55-C-1 exposed for a limited time to free inducer while growing exponentially at pH 5.4 and 7.3 are compared in Fig. 1. In the unsupplemented acidic medium after a short lag, the rate of increase of penicillinase activity per ml of culture was constant for about one generation (40 min), and then declined progressively (Fig. 1a). In earlier experiments with other samples of TD, including the first shipment of lot no. 003607, a constant rate of increase of penicillinase activity was maintained for three or more generations. In trying to reconcile these conflicting results, we were influenced by our earlier observations of the striking potentiation by Fe^{++} of the constitutive synthesis of penicillinase in strain 55-C-1 at pH 4.7 (Leitner and Cohen, 1962). The TD (lot no. 003607) used in the experiments shown in Fig. 1 contained 1.75×10^{-5} M iron. The addition of 0.5×10^{-5} M $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ permitted linear increase in penicillinase activity for about 2.5 generations

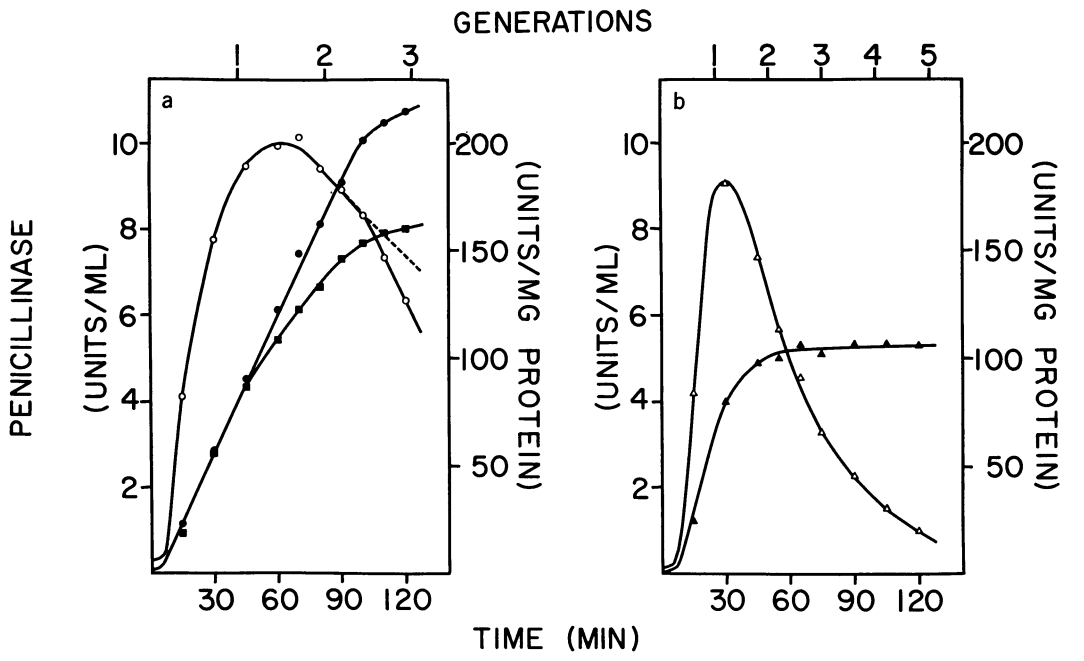

FIG. 1. *Kinetics of penicillinase induction in cultures of Staphylococcus aureus growing exponentially at pH 5.4 (a) and 7.3 (b). (a) Strain 55-C-1 was grown with shaking at 37 C in TD with 0.01 M added phosphate at pH 5.4 for 3.5 generations. The culture was divided into two portions, one receiving a supplement of 0.5 × 10^{-5} M $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 2 min before induction. At zero time, both cultures were induced with penicillin (2 units/ml) at a cell concentration of 11 μg of protein/ml. After 10 min, induction was terminated by Millipore filtration. During the subsequent growth period, samples were taken at 10- to 15-min intervals for iodometric penicillinase assay. Symbols: ■ = penicillinase, units/ml in the culture without added iron; ● = penicillinase, units/ml and ○ = penicillinase, units/mg of staphylococcal protein in the culture supplemented with Fe^{++}. The specific penicillinase activity before induction was 6.6 units/mg of staphylococcal protein. (b) Strain 55-C-1 was grown with shaking at 37 C in TD with 0.01 M added phosphate at pH 7.3 for 3.5 generations. At 2 min before induction, 0.5 × 10^{-5} M $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ was added. At zero time, the culture was induced with penicillin (2 units/ml) at a cell concentration of 10 μg of protein/ml. After 10 min, the inducer was removed by Millipore filtration. Samples were taken at 10- to 15-min intervals for iodometric penicillinase assay. Symbols: ▲ = penicillinase, units/ml; △ = penicillinase, units/mg of staphylococcal protein. The specific penicillinase activity before induction was 3.1 units/mg of staphylococcal protein.*

(Fig. 1a), and, at other times, for the duration of the experiments (as long as 3.5 generations). This concentration of iron was without effect on the growth rate at pH 5.4, but higher concentrations reduced the duration of exponential growth. In the medium at pH 7.3 (Fig. 1b), after a similar lag, the rate of increase in penicillinase activity was roughly constant for less than one generation (18 min), and progressively declined thereafter, becoming nil after two generations. At this pH, addition of 5 × 10^{-5} M Fe^{++} did not affect growth nor alter the kinetics of penicillinase induction.

Specific penicillinase activities reached peak values at 1.5 generations of increase in enzymatic activity at pH 5.4 (Fig. 1a) and at 1 generation at pH 7.4 (Fig. 1b). The sudden rapid decline, 100 min after induction, of the specific activity of the acid-grown culture is the reflection of the concomitant drop in the rate of increase of penicillinase activity of the whole culture. The dotted line illustrates the curve that the specific penicillinase activity would have followed if enzymatic activity per ml of culture had continued to increase at a constant rate.

The two kinetic patterns could be converted one into the other during the early phases of induced enzyme formation by the appropriate

change in pH after elimination of free penicillin. Thus, the results cannot be ascribed to an effect of pH upon the uptake of inducer by the cells. Figure 2 illustrates the results of neutralization, 12 min after induction, of a culture growing at pH 5.4. At 15 min after neutralization, the rate of increase of penicillinase activity started to decline progressively, reaching zero about 70 min after neutralization. The generation time, 36 min during the acidic phase of growth, fell to 33 min after neutralization. The reciprocal procedure at 12, 32, and 42 min is shown in Fig. 3. After acidification at 12 min, linear increase in enzymatic activity persisted, although at a rate slightly slower than that prevailing at the time of acidification. Acidification at 32 min, when the rate of increase of penicillinase activity in the neutral culture was about to decline, caused only a transitory prolongation of the increase in penicillinase activity. Acidification at 42 min, when penicillinase activity in the neutral culture was stationary, had virtually no effect. In these experiments, generation times after acidification increased only from 0 to 2 min.

The different kinetics of induction at acid and neutral pH could not be shown to result from a higher rate of inactivation of enzyme in the neutral medium. This point was tested by stopping protein synthesis at varying times after induction at pH 7.3 by the addition of chloramphenicol and assaying the constancy of the penicillinase activity of the culture during a further period of incubation. The results (Fig. 4) indicate a slow loss of activity, amounting to 10 to 13% per hr, a rate of inactivation that obviously cannot account for the difference in induction in the acid and neutral cultures.

The cells induced at neutral pH did not contain unrecognized, cryptic enzyme, for their penicillinase activity did not increase appreciably when they were broken in a sonic oscillator. Similarly, crypticity could be excluded as an explanation for the lower penicillinase activities of cultures induced at pH 5.4 without added Fe^{++}.

The mechanism of the effect of Fe^{++} on induction of penicillinase remains obscure. We were unable to obtain any evidence suggesting that it functioned as a cofactor with penicillinase. Thus, addition of Fe^{++}, up to 10^{-4} M, to broken-cell preparations of organisms induced either at pH 5.4 and 0.8×10^{-5} M Fe or at pH 7.3 and 1.3×10^{-5} M Fe did not alter their penicillinase activities significantly (Table 2). In the same broken-cell preparations, addition of *o*-phenanthroline, α,α'-dipyridyl, or sodium ethylenediaminetetraacetate did not alter the penicillinase activities.

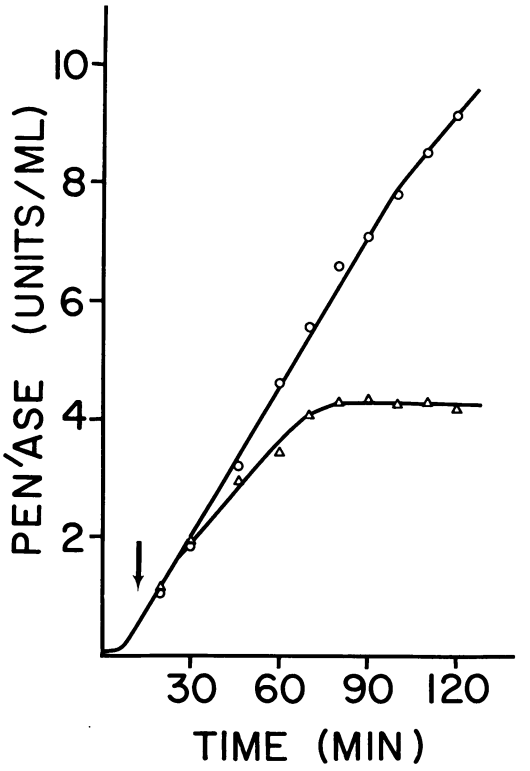

FIG. 2. *Kinetics of penicillinase synthesis in an exponential culture of strain 55-C-1 induced at pH 5.4 and a portion subsequently adjusted to and grown at pH 7.3. The organisms were grown with shaking at 37 C in TD with 0.01 M added phosphate at pH 5.4 for four generations. At 2 min after the addition of 0.5×10^{-5} M $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, the culture was induced with penicillin (2 units/ml) at a cell concentration of 11 μg of protein/ml. At 10 min after induction, the inducer was removed by Millipore filtration. At 12 min after induction, the culture was divided into two portions, one being adjusted to pH 7.3. Samples were removed at indicated times for iodometric penicillinase assay. Symbols: ○ = culture at pH 5.4; △ = culture at pH 7.3; arrow indicates time of neutralization.*

The distribution of penicillinase activity between cells and broth is shown in Table 3. After induction at pH 5.4, 9 to 13% of the penicillinase activity was found in the supernatant 60 to 120 min after induction. Washing the cells with $NaHCO_3$ solution removed little or no enzyme,

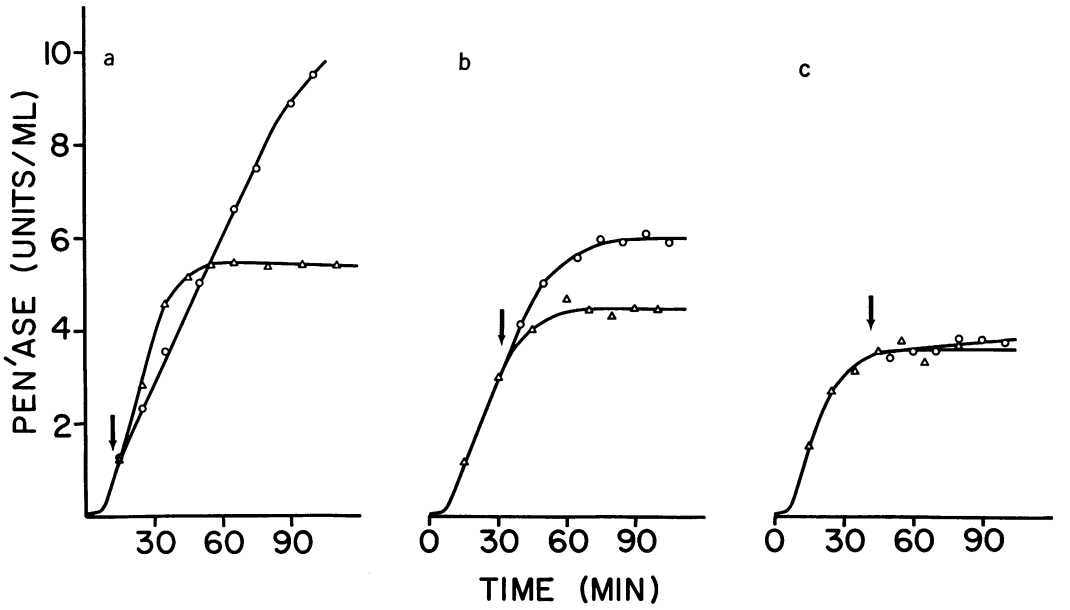

FIG. 3. *Kinetics of penicillinase formation in exponential cultures of Staphylococcus aureus induced at pH 7.3 and portions subsequently acidified and grown at pH 5.4. Three cultures of strain 55-C-1 were grown with shaking at 37 C in TD with 0.01 M added phosphate at pH 7.3 for 3.5 generations. At 2 min after the addition of 0.5 × 10^{-5} M $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, they were induced with penicillin (2 units/ml) at cell concentrations of 10 to 11 μg of protein/ml. At 10 min after induction, the inducer was removed by Millipore filtration. At 12 min (a), 32 min (b), and 42 min (c) after induction, each culture was divided into two portions, one of each pair being adjusted to pH 5.4. Samples were taken at 10- to 15-min intervals for iodometric penicillinase assay. Symbols: △ = cultures at pH 7.3; ○ = cultures at pH 5.4; arrows indicate time of acidification.*

although this procedure had been shown to remove added soluble penicillinase. Thus, the cell-bound enzyme did not include significant amounts of adsorbed extracellular enzyme. After induction at pH 7.3, 13 to 19% of the enzyme was extracellular. Here, too, no additional enzyme was removed from the cells 80 to 120 min after induction by washing with $NaHCO_3$ solution, but detectable amounts were removed from cells collected at 20 to 60 min.

In contrast to the current observations, extracellular penicillinase was not detected in earlier experiments of shorter duration (Geronimus and Cohen, 1957). Novick (1962) and Swallow and Sneath (1962) found 15 to 45% of induced staphylococcal penicillinase to be extracellular, larger proportions being found several hours after induction. The presence of a lower proportion of induced extracellular staphylococcal penicillinase at pH 5.4 than at pH 7.3 resembles an earlier observation with *B. subtilis* penicillinase (Pollock, 1961*b*).

Comparable differences in induction at neutral and acidic pH were obtained with strain 55-C-1 growing in Difco Heart Infusion Broth and with three randomly selected strains of penicillinase-producing staphylococci in TD.

Lag period. The pH of the culture medium had no detectable influence on the lag period. An increase in penicillinase activity was noted 3 to 4 min after addition of the inducer (Fig. 5). These results resemble those of Pardee and Prestidge (1961), who found a lag period of 3 to 4 min for several inducible enzymes in *E. coli*. Extrapolation of the straight-line portion of the plot in Fig. 5 intercepts the abscissa between 7 and 8 min. This may be compared with the value of 14 (±1.7) min found upon induction of the *B. cereus* penicillinase system at 35 C (Pollock, 1952). It should be noted that in Pollock's experiments on the lag period, inducer was eliminated after 1 min by the addition of soluble penicillinase, but in ours it was not. The possibility has not been excluded that the length of the lag period may be

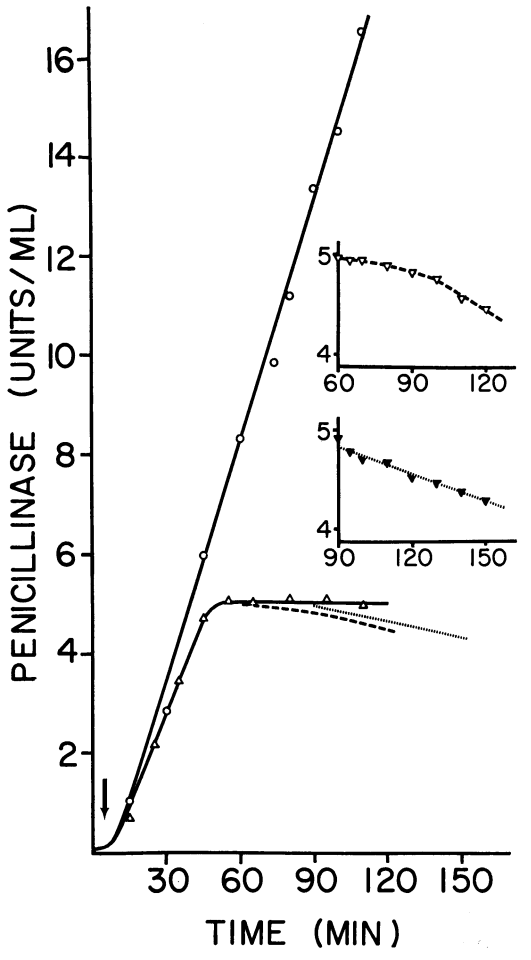

FIG. 4. *Rate of inactivation of penicillinase at pH 7.3 in an induced culture of strain 55-C-1. The organisms were grown exponentially with shaking at 37 C in TD with 0.01 M added phosphate at pH 5.4 for 3.5 generations. At 2 min after the addition of 0.5 × 10⁻⁵ M $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, the culture was induced with penicillin (2 units/ml) at a cell concentration of 11 μg of protein/ml. At 5 min after induction (indicated by arrow), the culture was divided into two portions, one being adjusted to pH 7.3. Both cultures were sampled for iodometric penicillinase determination at the times indicated in the figure (○ = culture at pH 5.4; △ = culture at pH 7.3). At 60 and 90 min after induction, portions of the neutral culture received chloramphenicol (100 μg/ml). They were incubated with shaking at 37 C for 60 min, samples for iodometric penicillinase determination being taken at 5- to 10-min intervals. Inserts show in detail the penicillinase activities of the chloramphenicol-treated cultures.*

TABLE 2. *Effect of Fe^{++} and of chelating agents on the penicillinase activity of sonically disrupted cells of strain 55-C-1**

Addition	Penicillinase (units/ml)	
	pH 5.4	pH 7.3
None (unincubated control)	8.90	8.88
None (incubated control)	8.56	8.38
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (10^{-5} M)	8.64	—
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (3 × 10^{-5} M)	8.68	8.23
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ (10^{-4} M)	8.33	8.19
o-Phenanthroline (5 × 10^{-4} M)	8.51	8.76
o-Phenanthroline (10^{-3} M)	8.37	8.74
α,α′-Dipyridyl (5 × 10^{-4} M)	8.55	8.56
α,α′-Dipyridyl (10^{-3} M)	8.58	8.78
EDTA† (5 × 10^{-4} M)	8.26	—
EDTA (10^{-3} M)	8.29	8.25
EDTA (3 × 10^{-3} M)	8.29	8.19

* Sonically disrupted cells from cultures induced in TD (lot no. 203662) with 0.01 M added phosphate at pH 5.4 and 7.3 were appropriately diluted and incubated for 1 hr at 37 C in 0.01 M tris(hydroxymethyl)aminomethane buffer (pH 7.2) containing the indicated additions. Penicillinase was determined iodometrically. The acid culture medium contained 0.8 × 10^{-5} M iron; the neutral culture medium contained 1.3 × 10^{-5} M iron. Specific activities of the acid and neutral cultures at the time of harvesting were 41.8 and 22.7 units/mg of protein, respectively.

† Sodium ethylenediaminetetraacetate.

affected by the length of time that free inducer is present.

Concentration of penicillin and time of exposure. Figure 6 illustrates the effects of variation of the concentration of added inducer, and of the time of exposure of strain 55-C-1 to it, upon induction of penicillinase. The experiments were conducted at pH 5.5, since the linear relation of enzyme formation to time at this pH made it possible to take the specific activity of the cells at a convenient time (45 to 55 min after induction) as a measure of the degree of induction, provided cell concentration at the time of induction and growth rates were constant, as was the case. Figure 6 indicates that, under the conditions of our ex-

periments, the degree of induction was approximately linearly related to the length of exposure to inducer over a 15-min period. This relation cannot be accounted for by the saturation of new receptor sites for penicillin, formed at an unchanging rate, since the cells increased by about 40% and the degree of induction by about fivefold in the interval from 2 to 15 min after addition of penicillin. Similar experiments at pH 7.3 with 2 and 4 units of penicillin/ml gave comparable results.

The degree of induction also varied with the initial concentration of penicillin. Saturation appeared to be reached at an initial penicillin concentration between 0.6 and 2 units/ml. In other experiments with slightly larger concentrations of organisms, 20 units of penicillin/ml gave no greater induction than did 2 units. The data of Fig. 6 indicate that 0.15 unit of penicillin/ml induces penicillinase to approximately half the maximal level attainable by this method. A very slight degree of induction was detected with as little as 0.01 to 0.02 unit/ml.

TABLE 3. *Distribution of penicillinase between cells and medium in cultures of strain 55-C-1**

pH	Time after induction	Penicillinase (units/ml)			
		Total	Cells		Supernatant
			Unwashed	Washed	
	min				
5.4	20	1.87	1.81	1.72	
	40	4.90	4.78	4.62	<0.3
	60	7.75	6.93	6.93	0.70
	80	10.4	9.08	8.80	1.08
	100	13.7	12.4	11.6	1.85
	120	17.1	15.3	14.8	1.94
7.3	20	2.36	2.19	1.86	
	40	4.56	3.85	3.43	0.58
	60	4.81	3.81	3.44	0.87
	80	4.68	3.76	3.67	0.85
	100	4.70	3.72	3.80	0.88
	120	4.50	3.66	3.79	0.67

* The organisms were grown with shaking for four generations in TD with 0.01 M added phosphate at pH 5.4 and 7.3. At 2 min after the addition of 0.5×10^{-5} M $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ each culture was induced with penicillin (2 units/ml) at a cell concentration of 11 μg/ml. Samples were removed at 20-min intervals and divided into three sets. In two sets, the cells were separated from the supernatant by centrifugation at 10,000 × *g* for 15 min (acid culture) and 20 min (neutral culture) at 4 C. One set was washed once with cold 0.05 M $NaHCO_3$, and once with cold 0.017 M $NaHCO_3$. The organisms of both sets were then resuspended to the original volume in fresh broth containing 100 μg/ml of chloramphenicol. Penicillinase was determined iodometrically in the supernatants, the washed and unwashed cells, and the whole cultures.

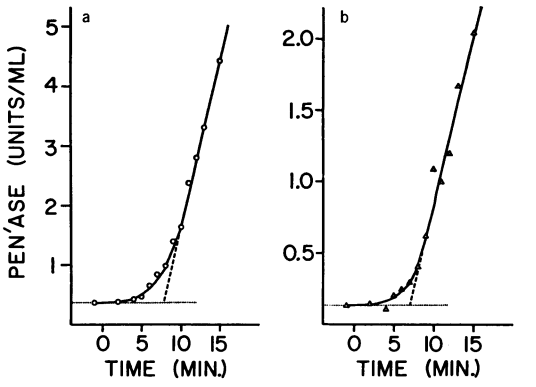

FIG. 5. *The lag phase at pH 5.4 (a) and 7.4 (b). (a) Strain 55-C-1, grown exponentially in TD (lot no. 11956 containing 0.01 M phosphate) at pH 5.4 for four generations, was induced at zero time with penicillin (2 units/ml) at a cell concentration of 57 μg of protein/ml. Samples for manometric penicillinase assay of cell-bound enzyme were removed at 1- to 2-min intervals. (b) Strain 55-C-1 was grown exponentially in TD with 0.01 M added phosphate at pH 7.4 for 3.5 generations. Induction and assay were performed as in a.*

DISCUSSION

The persistence of induced formation of penicillinase, after removal of free inducer, in strain 55-C-1 growing at pH 5.4 resembles that found in *B. cereus* by Pollock (1950) but is less prolonged. In the latter system, penicillinase continues to be formed indefinitely in the absence of free inducer but, in the staphylococcal system, its rate of formation usually declines within three to four generations. The induced production of penicillinase in *B. subtilis* is also of limited duration (Pollock, 1961*a*). The interesting feature of the staphylococcal system is the effect of an increased hydrogen ion concentration which, in conjunction with an appropriate concentration of iron, appears to prolong markedly the formation of enzyme in the absence of free inducer.

We have mentioned above the possibility that the change in the pattern of penicillinase formation with pH might be due primarily to an increased rate of inactivation of enzyme at neutral pH in the presence of an unchanged rate of synthesis. Possible mechanisms include enzyme inhibition, destruction by proteinases, or surface inactivation (Novick, 1962). Surface inactivation seems unlikely since the experiments were performed in complex broth media, which minimize surface inactivation. Also, the course of induction at neutral pH was unaltered in the presence of 0.5% gelatin. Inactivation by proteinases seems unlikely in view of the relatively slight degree of inactivation of penicillinase in induced cultures at pH 7.3 after treatment with chloramphenicol. It is probable, however, that penicillinase is inactivated principally in its extracellular fraction. If this is true and if its excretion is inhibited by chloramphenicol, as was the case with the *B. subtilis* enzyme (Pollock, 1961*b*), more enzyme might be available for inactivation extracellularly after induction at pH 7.3 in the absence of chloramphenicol than is suggested by Fig. 4. But even if excretion of penicillinase were doubled without chloramphenicol, as was the case with *B. subtilis* (Pollock, 1961*b*), and its inactivation were increased proportionately, the amount of enzyme inactivated would still be less than 2 units per ml per hr, an amount far too small to account for the difference between the acidic and neutral cultures.

An argument against inhibitors, in the sense of compounds combining stoichiometrically with penicillinase, may be derived from the results given in Fig. 3. If the effect of pH on induction were due solely to differences in rates of inactivation of penicillinase, one would expect that acidification of an induced neutral culture, after apparent enzyme formation had declined or ceased, should restore enzyme formation at a rate independent of the time of acidification. The data of Fig. 3 indicate that this is not the case. Thus, acidification at 42 min of the neutral induced culture produced no significant increase in penicillinase activity, although, at this time, organisms from the sample acidified at 12 min still exhibited a linear increase in penicillinase activity. The results would not be changed by reckoning time in bacterial generations, since the growth rates of the freshly acidified cultures were

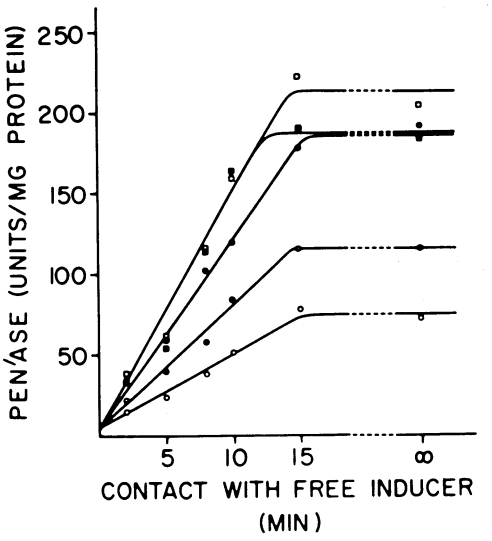

FIG. 6. *Effect of the time of contact with various concentrations of free inducer on penicillinase synthesis in exponentially growing Staphylococcus aureus. Five cultures of strain 55-C-1 were grown with shaking at 37 C in TD (first shipment of lot no. 003607) with 0.01 M added phosphate at pH 5.5 for three to four generations. When the bacterial cultures reached a density of 11 μg of cell protein/ml, inducer was added at the following concentrations: 0.05 (○), 0.15 (◒), 0.6 (●), 2.0 (□), and 4.0 (■) units of penicillin/ml. Immediately after induction, each culture was divided into six portions and growth was continued. In five of the daughter cultures, induction was terminated with soluble penicillinase (0.94 units/ml), the times of addition being 2, 5, 8, 10, and 15 min. No soluble penicillinase was added to the sixth culture (marked ∞). At 45 and 55 min after induction, samples were removed for the manometric assay of cell-bound penicillinase. The mean of the obtained values (corrected for extracellular enzyme) is plotted against the time of contact of the cells with free inducer. The penicillinase content of the cells before induction ranged from 3.7 to 4.4 units/mg of protein. For the cultures receiving 0.05, 0.15, and 0.6 units of penicillin/ml, recrystallized inducer was used.*

virtually unchanged from those of the neutral cultures. A comparable argument applies to the results of acidification at 32 min. Although, in this case, the change in pH was followed by a slight prolongation of the increase in penicillinase activity, this declined at 45 min and soon stopped altogether, again much sooner than was the case with a culture exposed to neutral pH for a shorter

time, i.e., the sample acidified at 12 min. We consider that the results of these experiments are best understood on the interpretation that the penicillinase-synthesizing capabilities of induced organisms deteriorate more rapidly during growth at neutral than at acidic pH.

The effect of Fe^{++} on induction at acidic pH is not understood and is being studied further. Fe^{++} stimulated the constitutive formation of penicillinase in cells of strain 55-C-1 subjected to a pH of 4.7 (Leitner and Cohen, 1962). In these experiments, the specific penicillinase activity of the cells increased as much as 150-fold after exposure for 180 min to pH 4.7, and evidence was adduced to show that the increase in activity was the result of synthesis of enzyme. It is reasonable to assume that the effect of Fe^{++} on induction of penicillinase at the slightly less acidic pH of 5.4 to 5.5 is exerted also on synthetic processes. This assumption is supported by our inability to demonstrate any other function for the added Fe^{++}, such as that of a cofactor for penicillinase or alteration in the accessibility of substrate to cell-bound enzyme.

The relatively slow union of penicillin and staphylococci, as measured by the degree of induction after varying periods of contact with free inducer, contrasts with the situation in *B. cereus* where effective union is 50% complete in 1 min and virtually complete in 5 min (Pollock, 1952). Pollock demonstrated that the degree of induction of penicillin was proportional to the amount of penicillin bound to the cells. If his observation may be extended to *S. aureus*, our results would suggest that penicillin binding by exponentially multiplying strain 55-C-1 continues for at least 15 min. This is probably not due to a slow rate of union of penicillin with available sites but rather to the unmasking of previously unavailable sites during growth, in accordance with observations of Cooper (1955) on the binding of labeled penicillin by a penicillin-sensitive strain of *S. aureus*.

Other investigators have inferred a slow union between penicillin and penicillinase-producing staphylococci, based upon the relatively high concentrations of penicillin they found necessary for optimal induction (Batchelor et al., 1961). A similar observation was made by Crompton et al. (1962). They suggested that this did not necessarily mean that penicillin was intrinsically a poor inducer but rather might mean that high initial concentrations of penicillin were required to keep the induction sites saturated in spite of the action of the penicillinase produced by the large inoculum used in their experiments.

The delineation in our experiments of optimal conditions for induction reveals penicillin to be a highly effective inducer of staphylococcal penicillinase. A brief exposure to 0.15 units of penicillin/ml induced formation of the enzyme to a level one-half the maximum attainable at any penicillin concentration in experiments of this type. This contrasts with results of others under less favorable conditions, where either much larger concentrations of penicillin were required for effective induction (Steinman, 1961; Crompton et al., 1962) or little or no induction was obtained (Swallow and Sneath, 1962).

Factors other than those described above appear to play an important part in induction of staphylococcal penicillinase. In studies as yet incomplete and not included in this paper, we have found, as have others (Steinman, 1961; Knox and Smith, 1962), that the concentration of organisms at the time of induction appears to be of great importance. Induction at cell densities appreciably higher than 11 μg of protein/ml yields lower degrees of induction than at a density of 11 μg/ml, at both acid and neutral pH. This effect does not appear to be due solely to more rapid inactivation of inducer by the larger initial cell mass, since it can be overcome by the addition of 10^{-5} or 10^{-4} M Fe^{++}. Studies of these and other aspects of induction of staphylococcal penicillinase are being continued.

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