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Relation Between Iron Uptake, *p*H of Growth Medium, and Penicillinase Formation in *Staphylococcus aureus*

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The uptake of iron and the formation of penicillinase was examined in cultures of wild-type *Staphylococcus aureus*. Uptake of iron was about twice as great at *p*H 4.7 as at *p*H 7.4. At *p*H 4.7, increase in iron uptake in the range of 1.0 to 4.0 μg per mg of bacterial protein was associated with a progressive increase in the rate of penicillinase formation, but a direct correlation between cellular iron content and rate of enzyme formation was not demonstrated. Addition of iron to deferrated medium enhanced penicillinase formation at *p*H 6.5 to 7.4 two- to fourfold in cultures induced with benzylpenicillin and in uninduced cultures. To demonstrate an effect on the uninduced cells, it was necessary to increase iron uptake by preliminary incubation of cells with iron in buffer. Calcium and certain other ions depressed iron uptake at acidic and at neutral *p*H, and, presumably as a result of this action, depressed the formation of penicillinase. Iron did not enhance penicillinase formation at *p*H 4.7 by two penicillinase constitutive mutants nor by wild-type cells undergoing induction at *p*H 6.5 by cephalosporin C or methicillin. After removal of cephalosporin C or methicillin during an early phase of induction, residual synthesis of enzyme was increased by prior uptake of iron. The results are considered compatible with the concept that uptake of iron, especially at acidic *p*H, interferes with the formation or function of penicillinase repressor.

Staphylococcal penicillinase is an inducible enzyme (2, 6). The level of its activity in cultures of wild-type *Staphylococcus aureus* is subject to variation mainly by the action of inducers derived from penicillins, cephalosporins, or certain cyclic peptides (12, 19, 21). In addition, the amount of enzyme formed may be modified over a moderate range, without the intervention of recognized inducers, by the composition of the medium of growth (7, 8). In earlier work, we found that the rate of penicillinase synthesis was enhanced, to a range about half as great as that of fully induced cultures, by addition of 1 to 10 μM $Fe(NH_4)_2 \cdot (SO_4)_2$ to cultures of *S. aureus* undergoing limited growth at *p*H 4.7 but not when cultures were growing normally at neutral *p*H (9). The action of iron was inhibited by certain other ions, notably calcium. Furthermore, ferrous ion prolonged the synthesis of penicillinase induced by benzylpenicillin in cultures growing at *p*H 5.4 (10). We suggested that the iron salts and acidic *p*H inhibited preferentially the synthesis or activity of repressor, thus allowing penicillinase synthesis.

In subsequent studies, we have explored the relation of the uptake of iron to these observations, especially to the modifying action of *p*H, calcium, and other ions. The results indicate that some of these modifying ions may act by altering the uptake of iron, although this does not explain completely the synergistic effect of low *p*H and iron upon penicillinase formation. To test the assumption that iron acts through the repression mechanism, we have examined its effect upon cells already completely derepressed by mutation or induction. The results of these studies are reported in this paper.

MATERIALS AND METHODS

Organisms. S. aureus 55Cl was the routine test strain (6). A constitutive mutant, 55Cl-Kl, was obtained by mutagenesis with ethylmethanesulfonate and selection of colonies producing large amounts of penicillinase on plates containing the acid base indicator *N*-phenyl-1-naphthylamine-azo-*o*-carboxybenzene (14). This strain grown in broth produced 400 to 500 units of penicillinase per mg of staphylococcal protein and 600 to 700 units after induction for 3 hr with 50 μg/ml of cephalosporin C.

S. aureus 8325 α_w, a penicillinase inducible strain

and *S. aureus* 8325 αi_1^-, its constitutive derivative, were obtained from R. P. Novick (13).

Media. Synthetic medium (WMa); 9 was a modification of that described by Wright and Mundy (24), with the addition of 0.1 μM $CaCl_2$ which appeared to improve growth slightly at *p*H 4.7. The amino acids and the phosphate salts used to prepare this medium were deferrated as follows.

A solution of amino acids at *p*H 6.8 was prepared in twice the concentration specified for solution A (24), with the omission of arginine, lysine, and histidine. The solution was percolated in 2.25-liter amounts through a column (2.5 by 6 cm) of a cation-exchange resin (BioRex 70), 100 to 200 mesh (BioRad Laboratories, Richmond, Calif.) in the sodium form. Iron was removed from solutions of the basic amino acids by treatment with ascorbic acid and bathophenanthroline followed by extraction with isoamyl alcohol (22), and recrystallization. Sodium phosphate buffer, 0.1 M, *p*H 7.2, was passed in 1-liter amounts through a column (2.5 by 18 cm) of Dowex-50 in the sodium form. The remaining ingredients of WMa medium were added without special treatment.

Deferrated WMa medium contained less than 0.1 μM free iron. As used in our experiments, it ordinarily supported good growth of *S. aureus* without additional trace elements. However, it was evidently near the borderline of iron deficiency, for occasionally it was necessary to add 0.1 μM iron to insure adequate growth at *p*H 7.2.

A defect of unsupplemented WMa medium was that staphylococcal penicillinase was not regularly stable in it. This was tested by adding 100 μg of chloramphenicol per ml to an experimental culture and assaying penicillinase during a further 30-min period of shaking at 37 C. Earlier work with cultures in tryptic digest broth had shown that chloramphenicol stopped further increase in enzyme activity and that less than 10% of the activity was lost during the next 60 min (10). In the present studies, cultures of *S. aureus* 55Cl in WMa medium at *p*H 4.8 lost 11% or less (usually 3 to 4%) of penicillinase activity in 30 min, but cultures induced at *p*H 7.2 lost as much as 30%. This was reduced to 10% or less in WMa medium supplemented with 0.1% lactalbumin hydrolysate (WMaL; Nutritional Biochemicals Corp., Cleveland, Ohio). The lactalbumin hydrolysate deferrated according to Theodore and Schade (22) contributed 0.2 μM free iron to the complete medium, making the free iron content of WMaL medium about 0.3 μM. The rates of inactivation of penicillinase in WMaL without added iron were either the same as or slightly less than those with added iron. All experiments cited were performed in WMaL medium except where otherwise noted.

Amino acids (A grade) were purchased from Nutritional Biochemicals Corp. or Calbiochem (Los Angeles, Calif.). $Fe(NH_4)_2(SO_4)_2$, which was the source of added iron, other metal salts, and glucose were reagent grade. Glassware was cleaned in chromic sulfuric acid and rinsed with deionized water.

Experimental conditions. Staphylococci were grown in shaken culture overnight at 37 C in tryptic digest broth (BBL) buffered by the addition of 0.05 M phosphate (*p*H 7.2). The broth contained 6.4 to 8.5 μM total iron. The cells contained 0.2 to 0.3 μg of total iron per mg of bacterial protein. They were centrifuged and washed with the low iron medium to be used in the experiment and were inoculated at an initial cell density of about 25 μg of protein per ml into 60 to 80 ml of experimental medium at *p*H 7.2. The cocci were then grown through 2.5 to 3 generations of log-phase growth with vigorous rotary shaking. The experiments proper were begun by dilution of this culture into the same medium with the desired modifications of *p*H, concentration of iron (labeled with $^{59}FeSO_4$), etc. At this point, the cocci contained about 0.05 μg of iron per mg of protein, and this was diluted further to the extent of growth during the experiment. This initial iron content was not detected by the tracer and is not included in the results.

Details of induction, sampling, and measurement of staphylococcal cell mass have been reported (10). Penicillinase was assayed by the iodometric method of Perret (15), as modified by Novick (12). Iron uptake by staphylococci was assayed with ^{59}Fe. Amounts of 5 to 10 mμc/ml were added to cultures containing various amounts of stable iron as $Fe(NH_4)_2(SO_4)_2$. Experimental samples were chilled promptly and centrifuged at 4 C. We confirmed that the low temperature stopped further uptake of iron. Samples (2 ml) of supernatant fluid were collected in disposable plastic tubes and counted in a gamma well counter for at least 1,000 net counts. Iron was determined chemically either as total iron by digestion with nitric and perchloric acids, followed by the *o*-phenanthroline method (20), or as free iron, by the bathophenanthroline method without prior digestion (5).

RESULTS

Iron uptake and penicillinase formation. Figure 1 shows the time curve of ^{59}Fe uptake in strain 55Cl at *p*H 4.7 and the related changes in cell mass and constitutive penicillinase activity. These experiments were performed in WMa medium. It was not considered necessary to repeat them in WMaL medium in view of the demonstrated stability of penicillinase at *p*H 4.7. Iron was taken up relatively quickly. The cellular iron content reached a maximum at about 2 hr and then declined in consequence of continued increase in cell mass. Net uptake stopped at about 2.5 hr when 90 to 94% of the label was removed from the supernatant fluid. Within the range of concentrations employed, the amount of iron taken up per unit of cell mass rose with increasing concentrations of iron, and was accompanied by increased rates of penicillinase synthesis. It is apparent, however, that the synthesis of the enzyme was not determined by the concentration of cell-bound iron alone, since enzyme formation continued at a rapid rate, whereas cell-bound iron was declining during the period 2 to 4 hr after acidification. With increasing iron concentration in the medium there was a progressive diminution

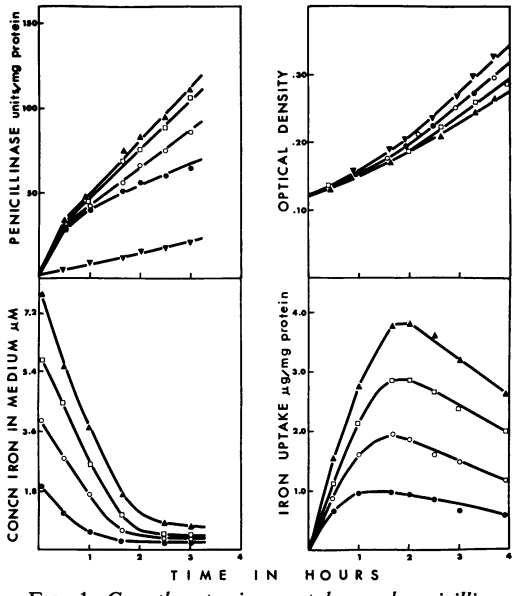

FIG. 1. *Growth rate, iron uptake, and penicillinase activity of Staphylococcus aureus 55C1 in WMa medium (pH 4.7) containing* $Fe(NH_4)_2(SO_4)_2$. *Symbols:* ▲, *8 μM;* □, *6 μM;* ○, *4 μM;* ●, *2 μM;* ▼, *0 μM.*

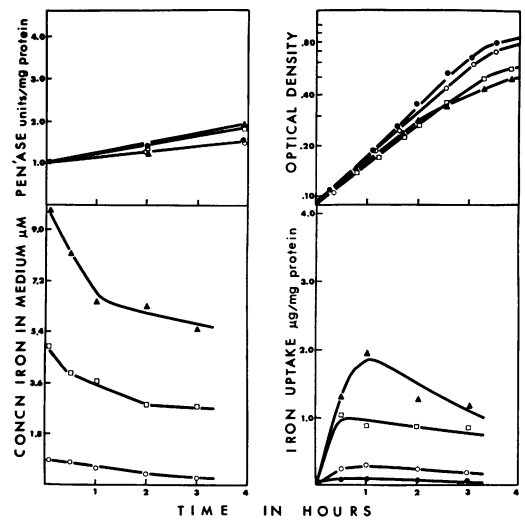

FIG. 2. *Growth rate, iron uptake, and penicillinase activity of Staphylococcus aureus 55C1 in WMa medium (pH 7.4) containing* $Fe(NH_4)_2(SO_4)_2$. *Symbols:* ▲, *10 μM;* □, *5 μM;* ○, *1 μM;* ●, *0.1 μM.*

in the rate of linear growth at *p*H 4.7, as measured by optical density. With 10 μM iron, growth was often inhibited completely.

In experiments at progressively less acidic *p*H, the addition of iron had similar but less marked effects on penicillinase synthesis and growth. These experiments were also performed with WMa medium to permit direct comparison of iron uptake with those in Fig 1. At *p*H 7.4, added iron had little or no effect on penicillinase formation except for a slight increase as the cells entered the late log and declining phase of growth. At this *p*H, the cocci took up less iron than at *p*H 4.7, but the process continued at a slow rate throughout the experiment (Fig. 2). The cellular iron content remained fairly constant after the first 0.5 hr, except for a moderate drop in cells grown in 10 μM Fe, the highest concentration tested. Appreciable amounts of iron remained in the supernatant fluid even after 3 hr of incubation.

At any concentration of cell-bound iron, the rate of penicillinase synthesis was much higher in cells grown at acidic than at neutral *p*H (Fig. 1 and 2), additional evidence that the concentration of cell-bound iron was not the sole determinant of the rate of penicillinase synthesis.

Attempts to define a fraction of cell-bound iron more directly related to the rate of penicillinase synthesis were unsuccessful. At any *p*H of growth, the iron remaining in cocci washed with 0.1 M citrate buffer (*p*H 4.2) or extracted with cold 5%

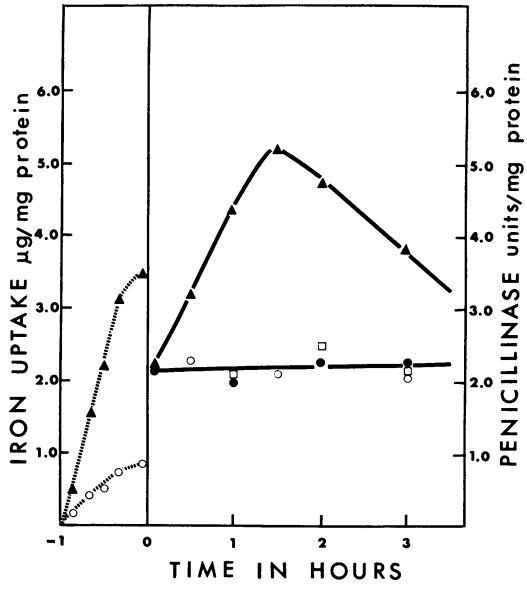

FIG. 3. *Penicillinase formation by Staphylococcus aureus 55 C1 at pH 7.4 after uptake of iron from buffer. Cells (83 μg of protein per ml) from a log-phase culture were suspended in buffer (10 mM KCl, 1.7 mM* $MgSO_4$, *4 mM sodium phosphate, pH 7.2) containing:* □, *0.05 M* $CaCl_2$; ▲, *10 μM* $Fe(NH_4)_2(SO_4)_2$; ○, *0.05 M* $CaCl_2$ *and 10 μM* $Fe(NH_4)_2(SO_4)_2$; ●, *no addition. Uptake of iron is recorded in the left panel (interrupted lines). After 1 hr, the cells were centrifuged, washed with buffer, and resuspended in WMaL medium. Penicillinase activities during the following 3 hr are recorded in the right panel (continuous lines).*

trichloroacetic acid tended to be proportional to the total iron taken up by the cocci. Iron taken up by cells grown in acidic cultures was more firmly bound than that taken up in neutral cultures. As an example, in one experiment, 48 to 63% of ^{59}Fe taken up at neutral *p*H was extracted from the staphylococci with cold trichloroacetic acid, whereas only 19 to 29% was removed from cells grown at *p*H 4.7.

Although iron had no discernible effect on the penicillinase content of cells grown at neutral *p*H, the concentration of cell-bound iron did not exceed 2 μg per mg of protein, even with 10 μM iron, the highest concentration employed. In the medium employed, iron in higher concentration formed a precipitate, presumably with phosphate. Higher cellular iron concentrations (up to 3.5 μg of Fe per mg of protein), and a reproducible, but limited, effect upon penicillinase formation were obtained by incubating cells with iron in buffer for 30 to 90 min (Fig. 3). After being washed and resuspended in complete deferrated medium, the organisms grew promptly with little or no lag and at the same rate as control cells similarly incubated in buffer without iron. During the growth period, cells enriched with iron in this way synthesized penicillinase at initial rates two to four times that of control cells. Again, there was a rough parallel between amount of iron taken up and the rate of penicillinase formation.

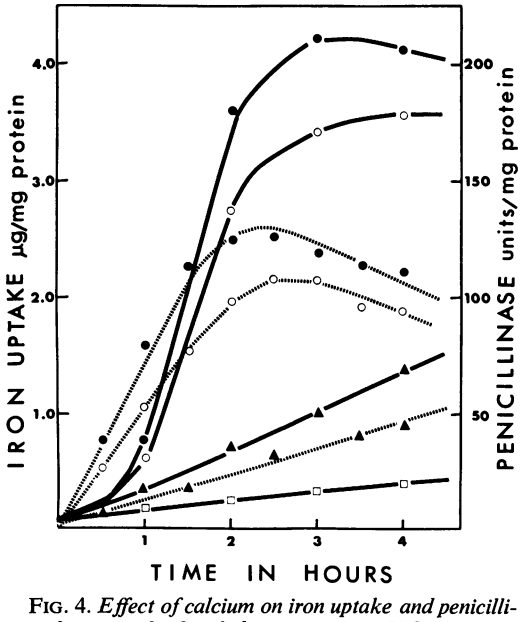

FIG. 4. *Effect of calcium on iron uptake and penicillinase formation by Staphylococcus aureus 55C1 in WMa medium at pH 4.7. The continuous lines record penicillinase activity; the interrupted lines, iron uptake in the presence of* (●) *6 μM* $Fe(NH_4)_2(SO_4)_2$, (○) *6 μM* $Fe(NH_4)_2(SO_4)_2$ *+ 0.01 M* $CaCl_2$, (▲) *6 μM* $Fe(NH_4)_2(SO_4)_2$ *+ 0.1 M* $CaCl_2$, (□) *no addition. Results with medium containing 6 μM* $Fe(NH_4)_2(SO_4)_2$ *were not changed by the addition of 0.1 or 1 mM* $CaCl_2$.

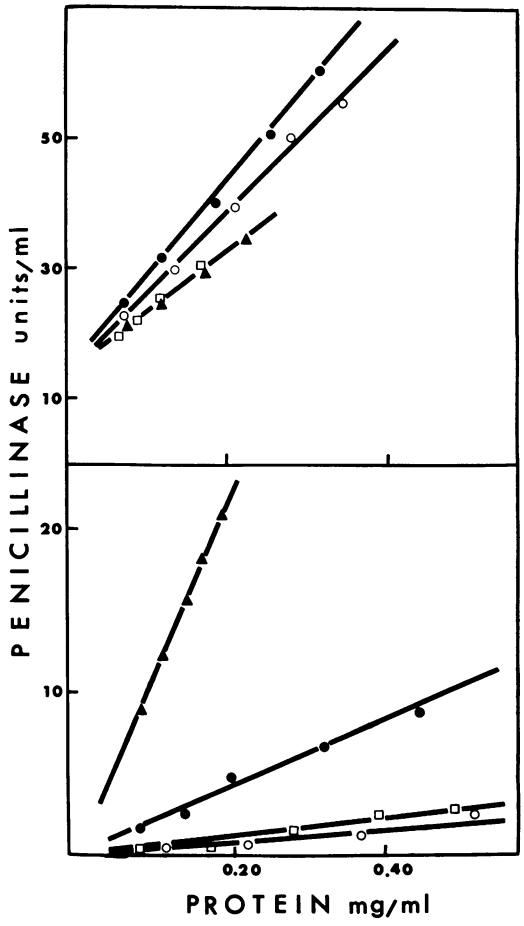

FIG. 5. *Comparison of the effect of iron and calcium on penicillinase formation by Staphylococcus aureus 55C1 (lower panel) and its constitutive derivative 55C1-K1 (upper panel) in WMaL medium (pH 4.7). Symbols:* ●, *no addition;* ○, *0.1 M* $CaCl_2$; □, *0.1 M* $CaCl_2$ *and 5 μM* $Fe(NH_4)_2(SO_4)_2$; ▲, *5 μM* $Fe(NH_4)_2(SO_4)_2$.

Calcium and other cations. Salts of calcium or certain other metal cations, or NaH_2PO_4 depress the rate of penicillinase synthesis by *S. aureus* strain 55Cl at acidic *p*H and counteract the effect of added iron (9). This may now be explained by the observation that these salts depress the uptake of iron by strain 55Cl. Figure 4 shows that 6 μM iron caused a marked increase in penicillinase formation at *p*H 4.7, associated with uptake of iron to a maximum of 2.6 μg per mg of protein. In the presence of 0.1 M $CaCl_2$, iron uptake was

TABLE 1. *Effect of salts on penicillinase and iron uptake by strain 55C1 at pH 4.7*[a]

Salts added	Penicillinase (units/mg of protein)	Fe^{++} taken up (μg/mg protein)
$Fe(NH_4)_2(SO_4)_2$, 5 μM	100	0.95
NaCl, 0.5 M + $Fe(NH_4)_2(SO_4)_2$, 5 μM	13	0.52
KCl, 0.5 M + $Fe(NH_4)_2(SO_4)_2$, 5 μM	16	0.51
NH_4Cl, 0.5 M + $Fe(NH_4)_2(SO_4)_2$, 5 μM	11	0.36
NaH_2PO_4, 0.05 M + $Fe(NH_4)_2(SO_4)_2$, 5 μM	22	0.28
$CaCl_2$, 0.1 M + $Fe(NH_4)_2(SO_4)_2$, 5 μM	9	0.32
$MgSO_4$, 0.1 M + $Fe(NH_4)_2(SO_4)_2$, 5 μM	15	0.23
None	22	—

[a] Penicillinase activity and iron uptake were determined after 3 hr of growth in deferrated WMaL medium at *p*H 4.7.

TABLE 2. *Effect of Co^{++} on penicillinase and iron uptake by strain 55C1 at pH 4.7*[a]

Salts added	Penicillinase (units/mg of protein)	Fe^{++} taken up (μg/mg of protein)
None	28	—
$CoCl_2$, 20 μM	55	—
$Fe(NH_4)_2(SO_4)_2$, 5 μM	136	1.1
$CoCl_2$, 20 μM + $Fe(NH_4)_2(SO_4)_2$, 5 μM	131	0.88

[a] Penicillinase activity and iron uptake were determined after 3 hr of growth in deferrated WMaL at *p*H 4.7.

reduced to a maximum of 1.0 μg per mg of protein, and penicillinase was formed at a much lower rate. A considerable excess of calcium is evidently necessary, since its effectiveness was much reduced at a concentration of 0.01 M and was undetectable at 0.001 M. Addition of $CaCl_2$ to cultures growing in deferrated media either had no effect on penicillinase formation or depressed

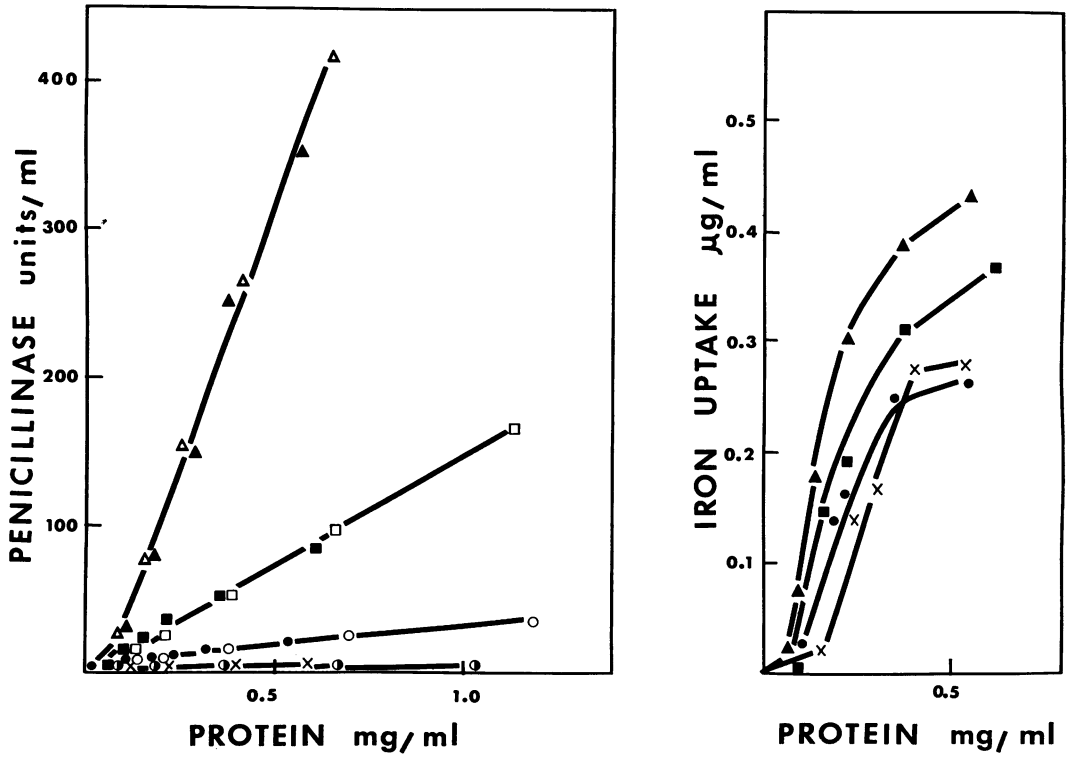

FIG. 6. *Iron uptake and penicillinase induction by varying amounts of cephalosporin C. Staphylococcus aureus 55C1 growing in WMaL at pH 6.5 was induced with cephalosporin C:* (△) *50 μg/ml,* (□) *10μg/ml,* (○) *2 μg/ml. The corresponding closed symbols indicate cultures similarly induced in the presence of 10 μM Fe $(NH_4)_2(SO_4)_2$;* × *indicates uninduced control with 10 μM Fe $(NH_4)_2(SO_4)_2$ and* ◑ *indicates absence of iron. Incremental values are plotted from the beginning of the experiment.*

it slightly (Fig. 5). We assume that the latter effect was the result of suppression of the uptake of traces of iron remaining in the medium.

The rate of growth of the cocci at *p*H 4.7 was increased by 0.1 M $CaCl_2$. This too was a result of the suppression of iron uptake, since added iron at this *p*H inhibited growth.

Calcium had a similar effect on iron uptake and penicillinase formation in cells exposed to iron in neutral buffer followed by growth in complete medium (Fig. 3). $MgSO_4$, NaCl, KCl, NH_4Cl, or NaH_2PO_4, which inhibit the effect of iron on penicillinase formation at acidic *p*H (9), also suppressed its uptake to a greater or lesser degree (Table 1). Cobalt chloride (20 μM) stimulated penicillinase formation slightly at *p*H 4.7 in the absence of added iron, and depressed the uptake of iron added at 5 μM. Therefore, it appears that the action of cobalt is similar to that of iron and is independent of it (Table 2).

Effect of iron on derepressed cells. We have suggested that the enhancement of penicillinase formation by iron at acidic *p*H might be due to inhibition of the synthesis or action of repressor. It would follow that synthesis of penicillinase in maximally derepressed cells should not be increased by iron. This expectation was confirmed in two constitutive mutants. Addition of iron to acidified cultures of strain 55Cl-K1 did not enhance penicillinase synthesis, but rather depressed it moderately (Fig. 5) even though the cells took up iron normally. Similarly, calcium or sodium salts depressed enzyme formation only slightly, much less than in experiments with the parental inducible strain. Strain 8325α_w and its constitutive derivative, αi_1^-, gave results qualitatively similar to those with the corresponding 55Cl strains, although the stimulation of penicillinase formation by iron at *p*H 4.7 was less marked with 8325α_w than with 55Cl.

A similar result was sought in cells maximally derepressed by induction at neutral *p*H. In strain 55Cl growing in deferrated medium at *p*H 6.5 to 7.2, the course and degree of penicillinase formation during induction with cephalosporin C was unaffected by added iron, whether induction was maximal with 50 μg of inducer per ml or submaximal with 5 to 10 μg/ml, even though the cells exposed to iron took it up (Fig. 6). Under similar circumstances, penicillinase formation induced by benzylpenicillin was approximately doubled by addition of iron (Fig. 7). This effect of iron was much reduced, but was still discernible, at *p*H 7.2. [We have reported that added iron did not affect induction with benzylpenicillin of strain 55Cl growing in tryptic digest broth at neutral *p*H (10). Evidently, the broth contained enough iron to produce the full effect of iron at this *p*H.

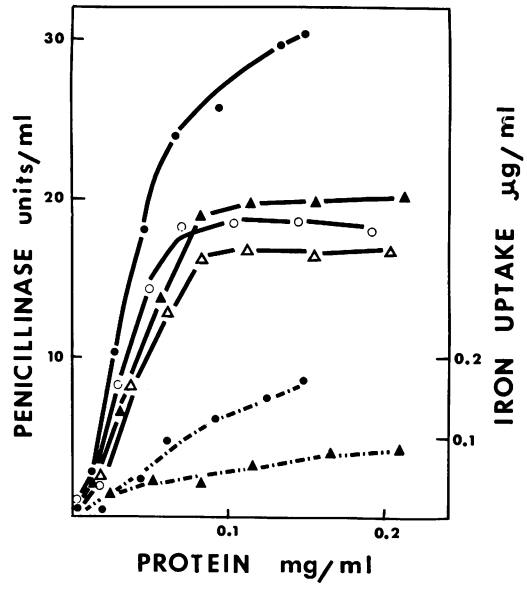

FIG. 7. *Penicillinase formation and iron uptake of Staphylococcus aureus 55Cl after induction by benzylpenicillin (2 units/ml) in WMaL medium (pH 6.5) containing:* (●) *10 µM* $Fe(NH_4)_2(SO_4)_2$; ▲, *10 µM* $Fe(NH_4)_2(SO_4)_2$ *and 0.1 M* $CaCl_2$; △, *0.1 M* $CaCl_2$; ○, *no addition. Penicillinase activities are indicated by continuous lines; iron uptake, by interrupted lines. Incremental values are plotted from the beginning of the experiment.*

Induction of cells in deferrated tryptic digest broth was enhanced by added iron.]

An obvious difference in the two experiments was that cephalosporin C, which is very poorly hydrolyzed by staphylococcal penicillinase, was present as free inducer throughout, whereas the benzylpenicillin must have been inactivated quickly (10). Therefore, with benzylpenicillin as inducer, one could not distinguish between more efficient induction while free inducer was available and more prolonged residual synthesis of enzyme after free inducer was inactivated. This question could be tested with cephalosporin C. When this inducer was removed by membrane (Millipore Filter Corp., Bedford, Mass.) filtration after 15 to 30 min of induction at *p*H 6.5 and the washed cells were resuspended in fresh medium with or without added iron, the rate of penicillinase synthesis declined more slowly in the cells induced with added iron (Fig. 8). Their net induced enzyme synthesis was about 1.5 to 2.0 times greater than that in cells without added iron. Similar results were obtained with methicillin (1 μg/ml) as inducer. The effect of iron was maximal when it was present during induction. Addition of iron

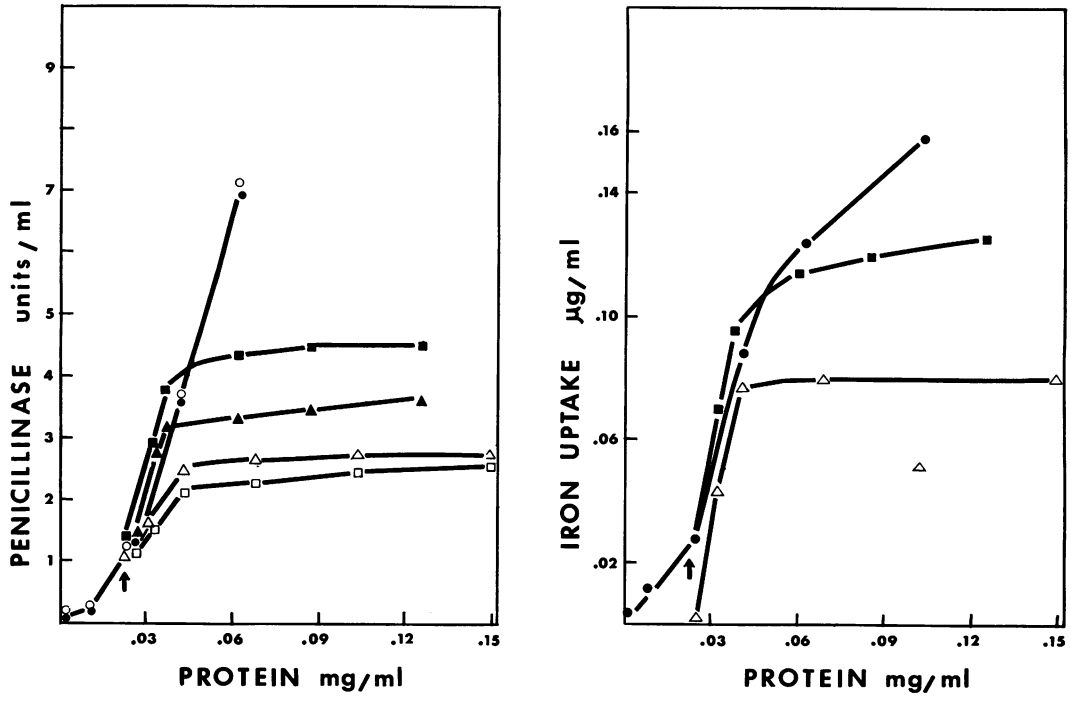

FIG. 8. *Effect of iron on penicillinase synthesis after removal of free inducer (cephalosporin C). Staphylococcus aureus 55C1 growing in WMaL (pH 6.5) with or without 10 µM Fe $(NH_4)_2(SO_4)_2$ was induced with 50 µg of cephalosporin C per ml. After 30 min, indicated by arrows, portions of each culture were filtered. The cells were washed with iron-free WMaL medium and transferred to fresh medium with or without 10 µM Fe $(NH_4)_2(SO_4)_2$. Symbols: ■, iron present both during and after induction; ▲, iron present during induction only; △, iron present after induction only; □, filtered control without addition of iron; ●, unfiltered control with iron; ○, unfiltered control without iron. The results were normalized to the same optical density for all cultures. Incremental values are plotted from the beginning of the experiment.*

after removal of cephalosporin C increased penicillinase formation only slightly.

Cells exposed to cephalosporin C for 30 min were just attaining their maximal differential rate of penicillinase formation. If the inducer was removed after 60 min, there was no difference in residual formation of enzyme with or without iron. Results at 45 min were intermediate. Thus, added iron prolonged the synthesis of penicillinase after removal of free inducer during a relatively early phase of induction, but had no effect in cells induced for a longer time. As yet, this observation is unexplained.

DISCUSSION

The levels of activity of some bacterial enzymes may be affected by the inorganic ionic composition of the medium in which the organisms were grown (23). The mechanism is straightforward when the inorganic ion is a cofactor of the enzyme. This does not appear to be the case with staphylococcal penicillinase. The activity of the enzyme in broken cell preparations of strain 55C1 is not altered by addition of iron or chelating agents (10). Furthermore, staphylococcal exopenicillinase has been shown to be a simple protein. Its activity is unaffected by metals or chelating agents (16). The situation may be different for other β-lactamases, for zinc appears to be a cofactor for cephalosporinase activity in *Bacillus cereus* (17).

The effect of iron upon penicillinase activity in staphylococci appears to be mediated by changes in the rate of formation of the enzyme protein itself (9). The present data indicate that, under appropriate conditions, uptake of iron increased penicillinase synthesis in wild-type, inducible *S. aureus* 55C1, either in the absence of inducer or in induced cells after removal of free inducer. This effect of iron increased as the *p*H of the growth medium was made more acidic. On the other hand, iron did not stimulate penicillinase formation in two constitutive mutants or in cells undergoing induction with cephalosporin C or methicillin. It appears that iron may enhance staphylococcal penicillinase formation only in cells with a

functional repressor mechanism; that is, it acts by release of repression. We have suggested that iron uptake, under conditions for the most part inhibitory to cellular multiplication, may have as its ultimate consequence the preferential inhibition of repressor synthesis (9). The present observations are not discordant, although other models are possible, such as an increased rate of inactivation of repressor or interference with its interaction with an operator site.

The failure of iron to increase penicillinase formation in cultures partially induced by suboptimal amounts of cephalosporin C is not explained adequately. If the cultures contained a homogeneous population of partially induced cells, one would have expected iron to shift the equilibrium between the formation of repressor and its inactivation by inducer in the direction of increased enzyme synthesis. Possibly, the partially induced cultures were a mixture of more or less fully induced cells, which are not susceptible to any further action of iron, and uninduced cells, which, as we have shown, are not appreciably affected by iron when growing at neutral *p*H. The distribution of β-galactoside permease and, in turn β-galactosidase itself, was found to be heterogeneous in *Escherichia coli* induced with low amounts of β-methylthiogalactoside (3, 11). However, these results may not be relevant to the penicillinase system in which there is no evidence for a permease. The distribution of penicillinase among the cells of a mass culture has been studied in *B. licheniformis* (4). A wide range of enzyme activities was found among individual cells of an uninduced culture and a more restricted range was found among fully induced constitutive cells. The results reported did not include data on partially induced cultures.

Recently, evidence has been obtained bearing on the mechanism of control by iron concentration of the synthesis of another specific bacterial protein, diphtheria toxin (18). The synthesis of this protein is inhibited by iron present above an optimally low concentration. Sato and Kato (18) showed that toxin was synthesized in a cell-free system of ribosomes and soluble extract prepared from diphtheria bacilli which had been actively synthesizing toxin as a result of growth in a medium low in iron. An extract from the same organisms grown in a medium high in iron, and therefore synthesizing little toxin, inhibited toxin formation, but not the general synthesis of protein in the subcellular system. The inhibitor, apparently a protein, stopped toxin formation much more quickly than did actinomycin D. This suggests to us an action on some step in the translation phase of protein synthesis. This mechanism would differ from the effect of iron on staphylococcal penicillinase formation where the postulated action upon the repression system would result in changes in the rate of transcription of the penicillinase structural gene, i.e., messenger ribonucleic acid formation.

The antagonistic effect of calcium and other cations on the enhancement of penicillinase formation by addition of iron appears explicable on the basis of the inhibition of iron uptake. It is apparently another example of antagonism between ions for uptake by bacteria (1). Nevertheless, it must be conceded that it is impossible to exclude completely some action more specific than inhibition of iron uptake, since the rate of penicillinase synthesis was not equally affected by similar degrees of inhibition of iron uptake caused by different cations. Furthermore, since we have not measured uptake of calcium or other ions as affected by iron concentration, it is not possible to assign a primary role to the uptake of iron rather than that of some of the other ions present in the medium, although the low concentrations in which iron was effective suggest its importance. The uptake of calcium by staphylococci in a medium containing 0.1 to 0.4 μM iron was found (25) to be undetectably low, an observation which makes it unlikely that, in our experiments, iron acted by inhibiting calcium uptake.

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