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Factors Affecting the Synthesis of Penicillinase by Staphylococcus Aureus

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Abstract □ The effects of aeration, ferric ions, porphyrins, and disodium versenate were studied on the production of penicillinase (ps) by *Staphylococcus aureus* 55-C-1 in synthetic medium. The production of ps was twice as high in shaken compared to static cultures although growth ultimately was the same. Ferric ions in a concentration of 2.5×10^{-4} *M* had some stimulatory effect on ps production in static cultures but caused much greater ps stimulation in shaken cultures. This stimulatory effect could be demonstrated only if ferric ions were added prior to 3 hr. of incubation. Hemin in a concentration of 2.5×10^{-5} *M* depressed ps production in the presence of ferric ions but one-tenth the concentration of hemin did not. Hematin in the same concentration was without effect. Protoporphyrin in a concentration of 2.5×10^{-5} *M* inhibited growth in the absence of ferric ions and depressed ps production in the presence of ferric ions. One-tenth the concentration of protoporphyrin had no effect on growth but depressed ps production and only slightly depressed ps production in the presence of ferric ions. A concentration of 2.5×10^{-6} *M* hematoporphyrin inhibited ps production in the presence of ferric ions when the former was added early in the growth curve. Disodium versenate in a concentration of 1.32×10^{-5} *M* in the presence of ferric ions permitted good growth but no ps production.

Keyphrases □ Penicillinase production—*Staphylococcus aureus* □ Aeration, shaking effect—penicillinase production □ Porphyrins, ferric ions, disodium versenate, effect—penicillinase production □ Optical density—*S. aureus* growth determination □ Manometric determination—penicillinase

Abraham and Chain (1) were the first to show that certain bacteria produce an enzyme capable of hydrolyzing penicillin. Since the latter paper was published in 1940, a number of workers have demonstrated that the resistance of strains of *Staphylococcus aureus* to penicillin was related to the production of penicillinase, the enzyme which was shown to hydrolyze penicillin. Although in some studies penicillin resistance was demonstrable in the absence of penicillinase production (2, 3), there seems to be good correlation between the ability of staphylococci to produce penicillinase and to be resistant to the action of penicillin both *in vitro* and *in vivo* (4–6). Penicillinase production has been similarly implicated in penicillin resistance of *Bacteroides* sp. (7).

In some microorganisms, penicillinase has been found to be an inducible enzyme and many studies (8–12) have concentrated on the nature of the induction of penicillinase elaboration. Some strains of *S. aureus* produced penicillinase constitutively (13, 14) and a number of workers have been concerned with the conditions and factors affecting constitutive production of penicillinase. It was felt that if the factors which control or affect penicillinase production in a strain of *S. aureus* producing penicillinase constitutively could be identified, it was possible that this knowledge could be applied ultimately in the treatment of an infection caused by a penicillinase-producing strain of *S. aureus*. That is, a strain which otherwise would be resistant to penicillin could be made susceptible to the action of penicillin if penicillinase production could be inhibited by the concurrent administration of a nontoxic

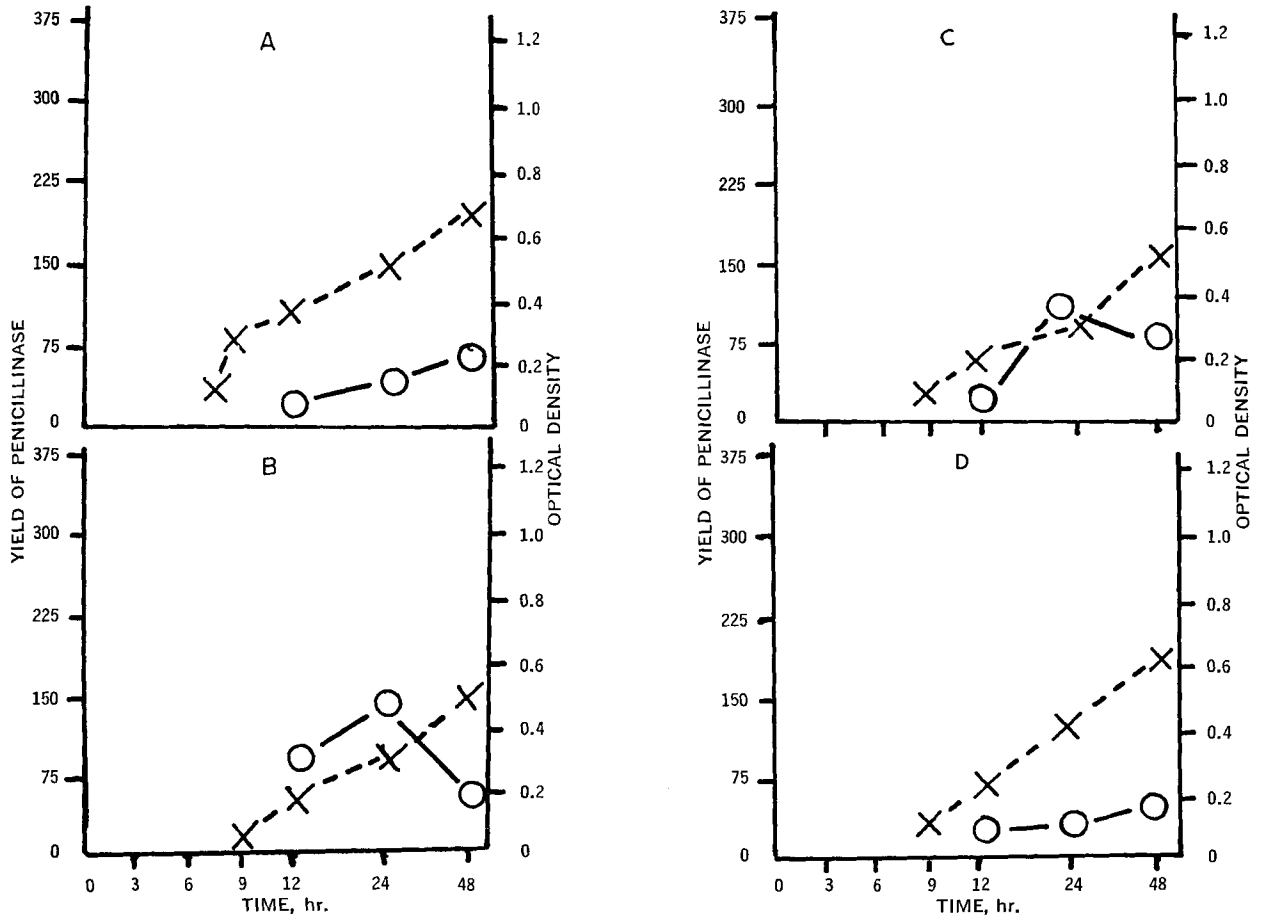

Figure 1—*The effect of ferric ions on the production of penicillinase in static cultures of* S. aureus. (A) *no added ferric ions.* (B) *2.5 × 10^{-4} M final concentration of ferric ions.* (C) *2.5 × 10^{-5} M final concentration of ferric ions.* (D) *2.5 × 10^{-6} M final concentration of ferric ions. Key:* ○, *penicillinase;* ×, *growth.*

substance inhibiting the production of penicillinase by these microorganisms. A study was, therefore, undertaken to examine the effects of several conditions and substances on the production of penicillinase by a strain of *S. aureus* producing this enzyme constitutively.

MATERIALS AND METHODS[1]

Materials—The culture media used in all experiments was that used by Wright and Mundy (15) with certain modifications made by Leitner and Cohen (14). The medium was made up in two separate portions. Solution 1 contained the following: L-leucine, 0.8 g.; L-tryptophan, 0.025 g.; L-proline, 0.05 g.; DL-phenylalanine, 0.26 g.; DL-threonine, 0.5 g.; L-histidine monohydrochloride, 0.15 g.; L-tyrosine, 0.21 g.; DL-valine, 1.00 g.; L-arginine monohydrochloride, 0.4 g.; L-glutamic acid, 0.65 g.; DL-serine, 0.61 g.; glycine, 0.06 g.; DL-methionine, 0.05 g.; DL-alanine, 0.43 g.; DL-lysine monohydrochloride, 1.70 g.; L-aspartic acid, 0.45 g.; DL-isoleucine, 0.44 g.; L-cystine, 0.05 g. The above amino acids were dissolved in a solution consisting of 482 ml. of distilled water and 18 ml. of 4% NaOH. Solution 2 contained the following minerals and vitamins; KCl, 0.2 g.; $MgSO_4.7H_2O$, 0.1 g.; thiamine hydrochloride, 0.01 g., and nicotinamide, 0.01 g. These chemicals were dissolved in 500 ml. of distilled water and then mixed with Solution 1, cleared by filtration, and the pH adjusted to 7.2 using 1 *N* HCl. The medium was autoclaved for 15 min. at 15 pounds of pressure. The culture was maintained on nutrient agar and transferred monthly.

The experimental cultures were grown in side-arm, 300-ml. flasks (Bellco No. 10-514, 14 × 130 mm.) containing a total volume of 40 ml. of medium including all other additions. The flasks were inoculated with 0.3 ml. of a 24-hr. statically grown culture which had reached an optical density of 0.30 (measured in 16 × 150-mm. screw-cap test tubes at 625 mμ in a Bausch & Lomb Spectronic 20 spectrophotometer). Also added was 0.2 ml. of 50% dextrose. Any pH adjustments were made with 1 *N* HCl. During typical growth experiments, samples were removed at various time intervals and frozen until assayed for penicillinase activity. The period of storage at −10° never exceeded 24 hr. and in preliminary experiments, it was found that the penicillinase activity of samples stored for various periods of time up to 24 hr. under these frozen conditions did not vary by more than 10% from the values of the samples prior to freezing. Thawing of the samples up to three times within a 24-hr. period of storage in the frozen state yielded similar results, that is, penicillinase activities varying by no more than 10% from that of the fresh sample prior to freezing. Optical density measurements of cell suspensions were made at 625 mμ in a Bausch & Lomb Spectronic 20 spectrophotometer in all cases and optical density measurements were related to viable cell counts as had been determined by Steinman (16) and confirmed by the present authors. An optical density of 0.102 in a 14 × 130-mm. side-arm flask was equivalent to 1.7 × 10^8 cells/ml.

Methods—Penicillinase was measured manometrically at 37° and pH 7.46 by the method of Henry and Housewright (17) because this has been the most commonly used method by other investigators especially with whole cells. The main compartment of the Warburg flasks contained 2.0 ml. of chloramphenicol succinate

[1] The culture used in all experiments was *Staphylococcus aureus* strain 55-C-1 obtained from Drs. Felix Leitner and Sidney Cohen, Department of Microbiology, Michael Reese Hospital and Medical Center, Chicago, Ill. (14).

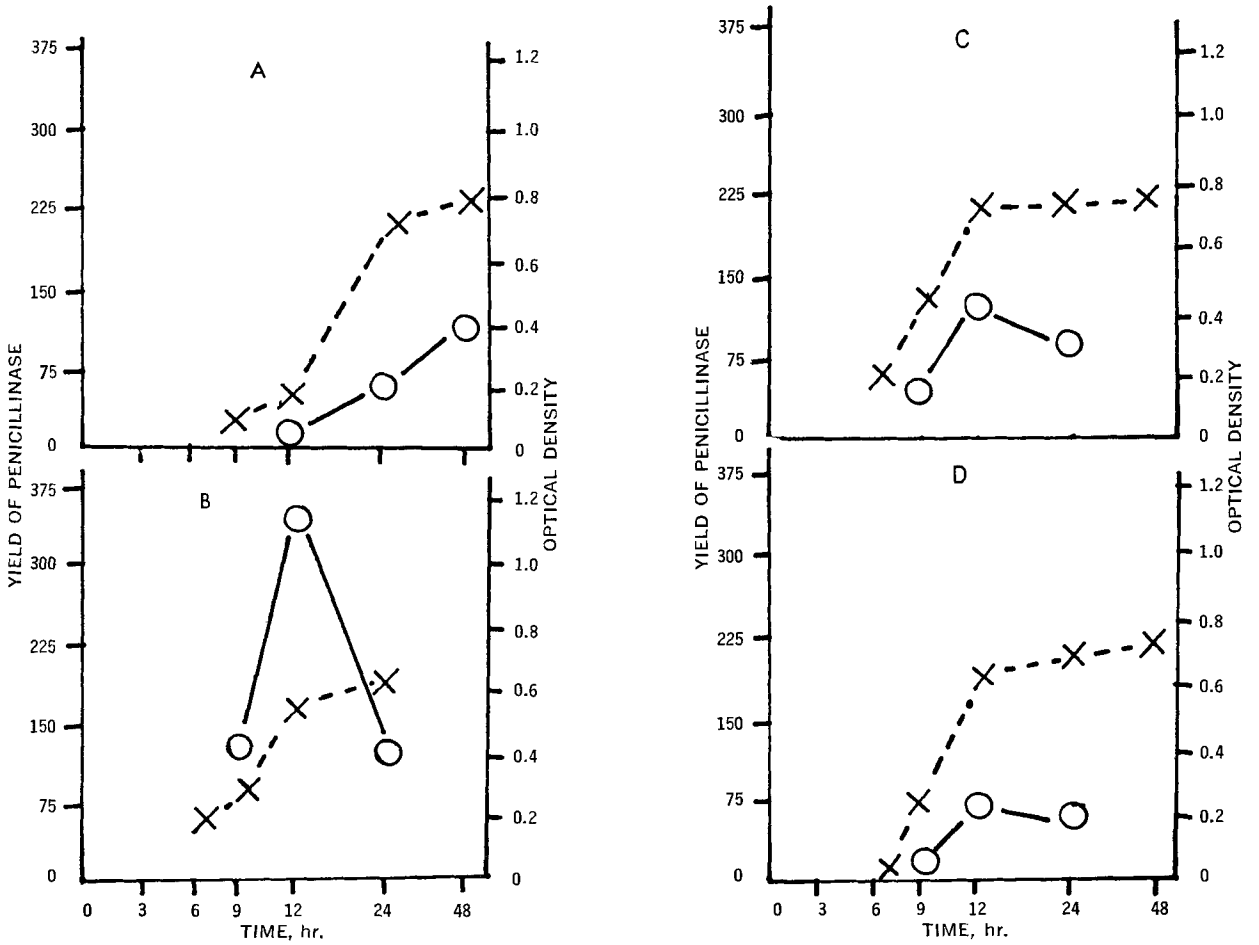

Figure 2—*The effect of ferric ions on the production of penicillinase in cultures of* S. aureus *shaken at an amplitude of 0.8 cm.* (a) *no added ferric ions.* (b) *2.5* × *10^{-4}* M *final concentration of ferric ions.* (c) *2.5* × *10^{-5}* M *final concentration of ferric ions.* (d) *2.5* × *10^{-6}* M *final concentration of ferric ions. Key:* ○, *penicillinase;* ×, *growth.*

solution (200 mcg./2 ml. of 0.017 *M* $NaHCO_3$) and 0.5 ml. of distilled water. One side-arm contained 0.5 ml. of bacterial suspension. Bacterial cultures were centrifuged and the cells resuspended in the same volume of distilled water. The other side-arm of the two side-arm Warburg flasks contains 0.2 ml. sodium penicillin G (12 mg./0.2 ml. of 0.017 *M* $NaHCO_3$). The flasks were flushed with 5% carbon dioxide and 95% nitrogen for 15 min. Following equilibration, the contents of the side-arms were tipped into the main compartments and measurements taken every 5 min. Occasional assays of the supernatant were made, and it was found that 95% of the enzyme was associated with the bacterial cells. Activity was expressed as microliters of carbon dioxide per hour per 1.7 × 10^8 cells/ml.

Culture flasks were shaken on a Burrel Wrist Action shaker to effect aeration. Ferric chloride ($6H_2O$) was dissolved in distilled water and autoclaved. The porphyrins were dissolved in distilled water, the pH adjusted to 7.6, and the solution sterilized by Seitz filtration. Adjustment of pH was necessary because the porphyrins tend to precipitate out of solution at lower pH's.

Biochemicals (Nutritional Biochemicals Corp. or the California Corporation for Biochemical Research) and chloramphenicol succinate (Parke, Davis & Co.) were used and all other chemicals were of reagent grade.

Table I—Penicillinase Production in Shaken and Static Cultures

Cultural Conditions	Time of Incubation, hr.	Optical Density	Yield of Penicillinase[a]
Shaken, 0.8 cm. stroke amplitude	24	0.677	50
	30	0.745	109
	48	0.796	147
Shaken, 1.5 cm. stroke amplitude	12	0.221	0
	24	0.770	100
	48	0.854	123
Static	12	0.337	20
	24	0.523	37
	48	0.658	50
	72	0.745	50
	96	0.824	65

[a] The units of penicillinase activity are microliters of CO_2/hr/1.7 × 10^8 cells/ml.

RESULTS AND DISCUSSION

The effect of the degree of aeration of the cultures on penicillinase production can be seen from the data presented in Table I. Although more vigorous aeration as shown by a 1.5-cm. stroke did not give greater yield than more moderate aeration, penicillinase production in aerated cultures was clearly greater than in static cultures. This effect was observed even though the final cell density reached in static cultures was equivalent to that reached in shaken cultures.

The addition of ferric ions was studied on penicillinase production in both static and shaken cultures (Figs. 1–3). Penicillinase production in static cultures was only slightly affected by the addition of ferric ions. However, these ions were clearly stimulatory to penicillinase production in shaken cultures at either of the two stroke amplitudes used. In order to gain some clue as to the mechanism by which ferric ions may be stimulatory to penicillinase production, the time of addition of the ferric ion and the age of the cultures were studied. From Fig. 4, it can be seen that if ferric ions

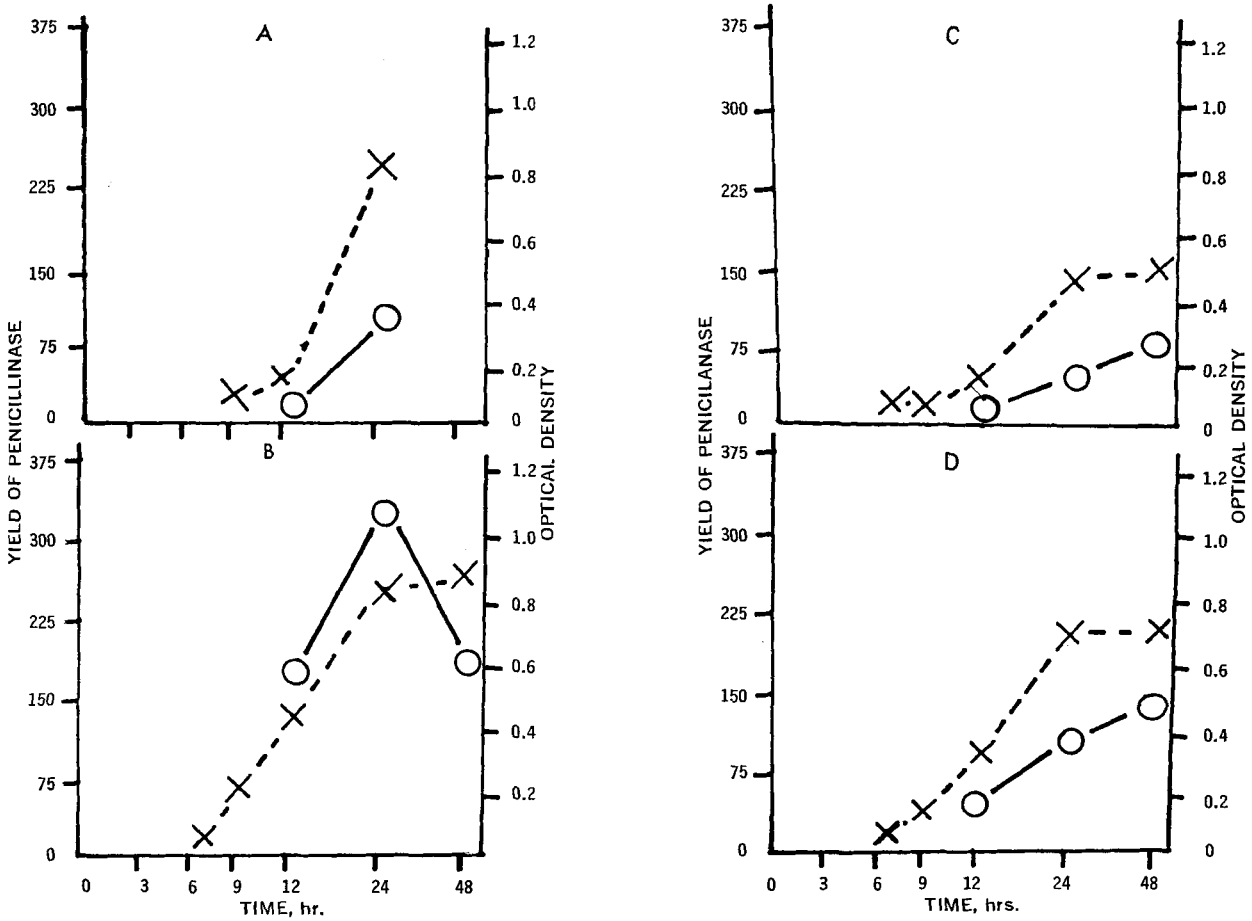

Figure 3—*The effect of ferric ions on the production of penicillinase in cultures of* S. aureus *shaken at an amplitude of 1.5 cm.* (a) *no added ferric ions.* (b) *2.5 × 10^{-4} M final concentration of ferric ions.* (c) *2.5 × 10^{-5} M final concentration of ferric ions.* (d) *2.5 × 10^{-6} M final concentration of ferric ions. Key:* ○, *penicillinase;* ×, *growth.*

are added at 0 or 3 hr. of incubation, the stimulatory effects are seen but if added after that time, there is a decreasing effect on penicillinase production.

Sack (18) and Sack and Judis (19) showed that certain porphyrin compounds affected the production of coagulase by *S. aureus* and it was of interest to determine whether such compounds would affect penicillinase production by this organism. Hemin (Fig. 5) in a concentration of 2.5 × 10^{-5} *M* depressed penicillinase production in the presence of ferric ions, but in a concentration of 2.5 × 10^{-6} *M* this depression did not appear. Hematin, in the presence of ferric ions, had no effect on penicillinase production as stimulated by ferric ions (Fig. 6). Protoporphyrin, in a concentration of 2.5 ×

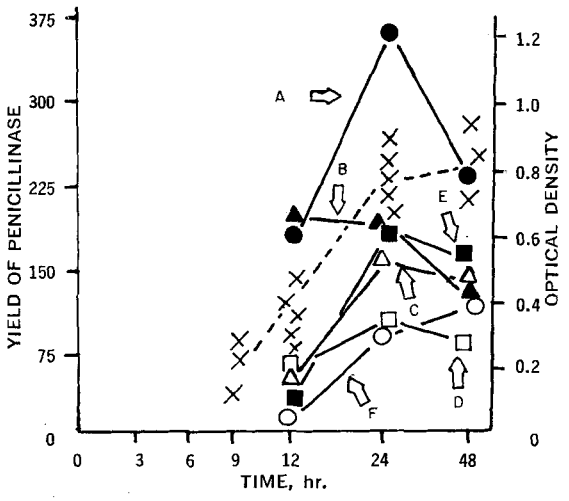

Figure 4—*Influence of time of addition of ferric ions on the stimulation of penicillinase production in shaken cultures of* S. aureus. *Ferric ions, in a final concentration of 2.5 × 10^{-4} M were added at the following times of incubation:* (a) *0 hr.;* (b) *3 hr.;* (c) *6 hr.;* (d) *9 hr.;* (e) *12 hr.;* (f) *no added ferric ions. The growth curve shown is a composite of the individual growth curves for* (a) *through* (f). *Key:* ●, A; ▲, B; △, C; □, D; ■, E; ○, F; ×, *growth.*

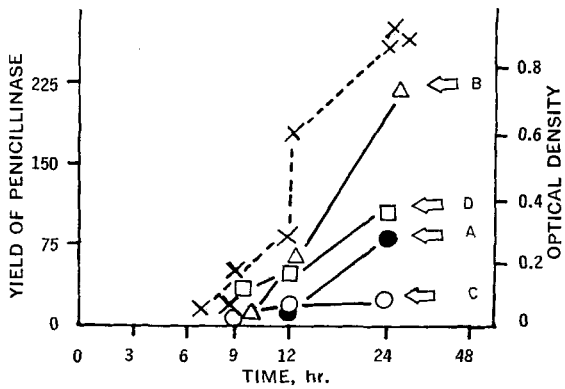

Figure 5—*Effect of hemin on the production of penicillinase by* S. aureus *in shaken cultures. The concentration of ferric ions, where added was 2.5 × 10^{-4} M.* (a) *No added hemin or ferric ions.* (b) *Ferric ions added to a final concentration of 2.5 × 10^{-4} M.* (c) *hemin added at a final concentration of 2.5 × 10^{-5} M.* (d) *Both hemin and ferric ions added. The growth curve shown is a composite of the individual curve for* (a) *through* (d). *Key:* ●, *neither hemin or ferric ions added;* △, *ferric ions added;* ○, *hemin added;* □, *both hemin and ferric ions added;* ×, *growth.*

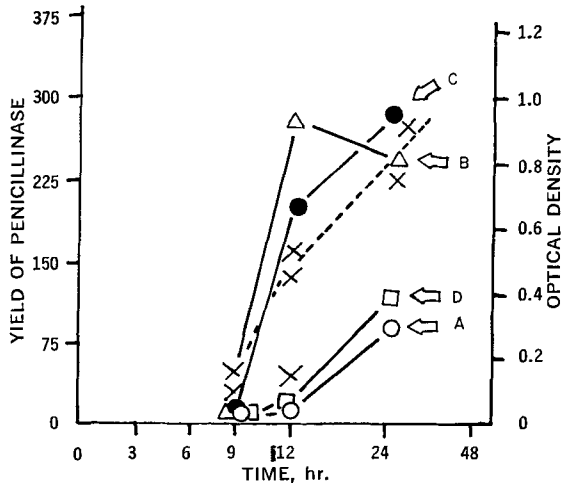

Figure 6—*Effect of hematin on the production of penicillinase by* S. aureus *in shaken cultures. The concentration of ferric ions, where added, was 2.5 × 10⁻⁴* M. (a) *Hematin added at a concentration of 2.5 × 10⁻⁵* M. (b) *Ferric ions added only.* (c) *Hematin (at same concentration as in* a) *and ferric ions added.* (d) *Neither hematin nor ferric ions added. Key:* ○, *hematin added;* △, *ferric ions added only;* ●, *hematin and ferric ions added;* □, *neither hematin nor ferric ions added;* ×, *growth.*

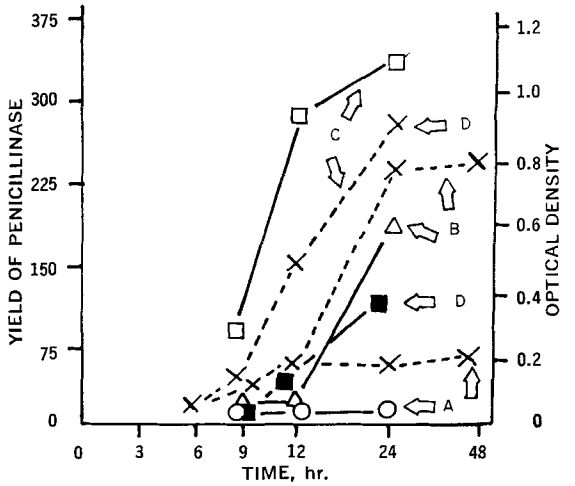

Figure 8—*Effect of protoporphyrin on the production of penicillinase by* S. aureus *in shaken cultures. The concentration of ferric ions, where added, was 2.5 × 10⁻⁴* M. (a) *Protoporphyrin added at a concentration of 2.5 × 10⁻⁵* M. (b) *Protoporphyrin (same concentration as in* a) *and ferric ions added.* (c) *Ferric ions only added.* (d) *Neither protoporphyrin nor ferric ions added. Key:* ○, *protoporphyrin added;* △, *protoporphyrin and ferric ions added;* □, *ferric ions only added;* ■, *neither protoporphyrin nor ferric ions added;* ×, *growth.*

10^{-6} *M* inhibited the production of penicillinase but had no effect on growth (Fig. 7). In the presence of ferric ions, there was observed a slight decrease in penicillinase production when added at 0, 3, or 6 hr. of incubation but had no effect after 6 hr. of incubation. At a concentration of 2.5×10^{-5} *M*, protoprophyrin inhibited both growth and penicillinase production but not in the presence of ferric ions (Fig. 8).

Hematoporphyrin, at a concentration of 2.5×10^{-5} *M*, inhibited both growth and penicillinase production in the absence of ferric ions and was capable of inhibiting growth in the presence of ferric ions to a considerable extent. When the concentration of hematoporphyrin was reduced to 2.5×10^{-6} *M*, growth was not affected although penicillinase production was reduced (Fig. 9). The addition of ferric ions overcame the inhibition of growth and penicillinase production if the hematoporphyrin was added as late as after 9 hr. of incubation but penicillinase production was inhibited if the porphyrin was added at 0, 3, or 6 hr. of incubation. The data obtained in the studies on the effects of disodium versenate are represented in Fig. 10. A concentration of disodium versenate of 1.32×10^{-5} *M* permitted good growth but no penicillinase production. Reduction in concentration of the disodium versenate to 6.7×10^{-6} *M* also permitted good growth and only moderate production of penicillinase and a further reduction of the concentration of disodium versenate to 3.35×10^{-6} *M* permitted both good growth and good penicillinase production. In all of these studies, ferric ions were present and whether the disodium versenate was added at 0 hr. of incubation or 12 hr., the results obtained were the same.

It is interesting to speculate on the mechanism by which ferric ions stimulate the production of penicillinase. In experiments in

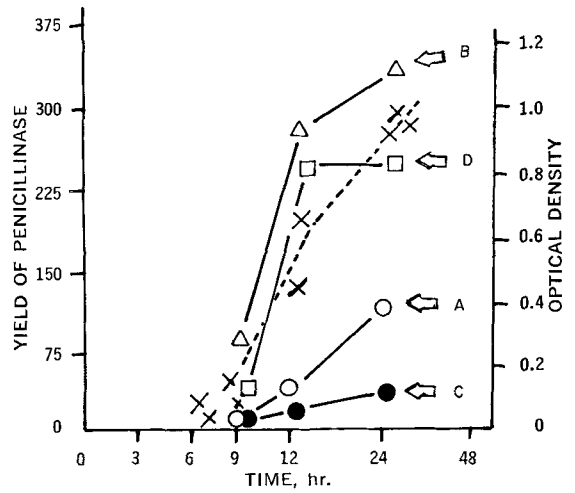

Figure 7—*Effect of protoporphyrin on the production of penicillinase by* S. aureus *in shaken cultures. The concentration of ferric ions, where added, was 2.5 × 10⁻⁴* M. (a) *Neither protoporphyrin nor ferric ions added.* (b) *Ferric ions only added.* (c) *Protoporphyrin added at a concentration of 2.5 × 10⁻⁶* M. (d) *Protoporphyrin (same concentration as in* c) *and ferric ions added. Key:* ○, *neither protoporphyrin nor ferric ions added;* △, *ferric ions only added;* ●, *protoporphyrin added;* □, *protoporphyrin and ferric ions added;* ×, *growth (composite of individual growth curves).*

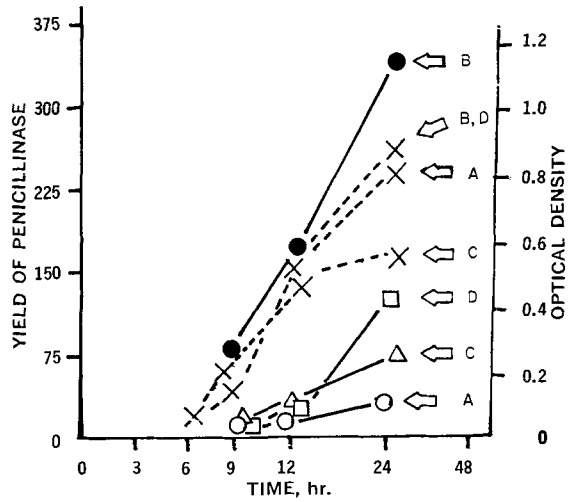

Figure 9—*Effect of hematoporphyrin on the production of penicillinase by* S. aureus *in shaken cultures. The concentration of ferric ions, where added, was 2.5 × 10⁻⁴* M. (a) *Hematoporphyrin added at a concentration of 2.5 × 10⁻⁶* M. (b) *Ferric ions only added.* (c) *Both ferric ions and hematoporphyrin added.* (d) *Neither ferric ions nor hematoporphyrin added. Key:* ○, *hematoporphyrin added;* ●, *ferric ions only added;* △, *both ferric ions and hematoporphyrin added;* □, *neither ferric ions nor hematoporphyrin added;* ×, *growth.*

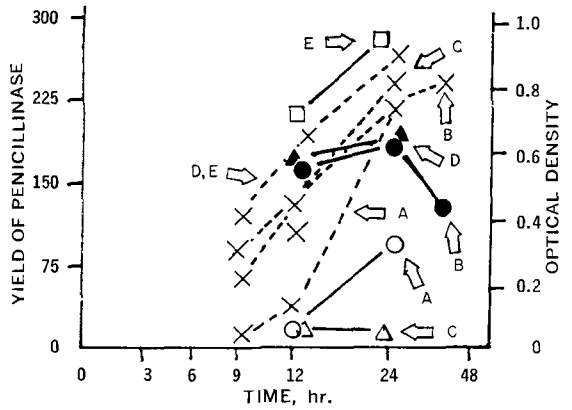

Figure 10—*Effect of disodium versenate on the production of penicillinase by* S. aureus *in shaken cultures. The concentration of ferric ions, where added, was 2.5 × 10⁻⁴* M. (a) *Neither ferric ions nor disodium versenate added.* (b) *Ferric ions only added.* (c) *Disodium versenate added in a concentration of 1.32 × 10⁻⁵* M *and ferric ions.* (d) *Disodium versenate added in a concentration of 6.7 × 10⁻⁶* M *and ferric ions.* (e) *Disodium versenate added in a concentration of 3.35 × 10⁻⁶* M *and ferric ions. Key:* ○, *neither ferric ions nor disodium versenate added;* ●, *ferric ions only added;* △, *disodium versenate added—1.32 × 10⁻⁵* M *and ferric ions;* ▲, *disodium versenate added—6.7 × 10⁻⁶* M *and ferric ions;* □, *disodium versenate added—3.35 × 10⁻⁶* M *and ferric ions;* ×, *growth.*

which the time of addition of ferric ions to the culture was varied, it was seen that maximal stimulation of penicillinase production by ferric ions occurred if the latter were added early in the growth period. It is during this time that RNA synthesis and protein synthesis are most active and possibly some inhibitor either in the medium or produced by the microorganism is present and it is the latter which ferric ions are inactivating. Also, perhaps ferric ions may be inhibiting the synthesis of a repressor substance as suggested by Cohen *et al.* (20). For example, Rogers (21) who studied the relationship between growth of *S. aureus* and hyaluronidase production found that early in the growth curve, cellular growth was more rapid than enzyme production and at a certain time, the reverse was true. The initial lag in the formation of the enzyme was found to be primarily due to accumulation of α-amino butyric acid, which was subsequently (22) found to inhibit the formation of the enzyme. In the authors' studies with penicillinase, it was found that in the presence of ferric ions, penicillinase is produced rapidly and subsequently drops. This might lead one to believe that the penicillinase formed in the presence of ferric ions is an unstable one but further studies comparing the stability of the staphylococcal penicillinase and commercially purchased penicillinase indicated no difference in stability. It is possible that the role of ferric ions is to inhibit the production of something analogous to the α-amino butyric acid found by Rogers to affect the production of hyaluronidase.

Perhaps the inhibitory effects shown by porphyrins can be related to their ability to coordinate with inorganic ions (23). Disodium versenate is known to form a strong metal complex with ferric ions and perhaps inhibit the production of penicillinase by making the stimulatory effect of ferric ions impossible. It should be of interest to explore the effect of disodium versenate on the course of staphylococcal infections in experimental animals, especially the ability of penicillin to eradicate infections caused by penicillin resistant staphylococci. If, indeed, disodium versenate is able to inhibit the production of penicillinase by a penicillin resistant staphylococcus, the latter should become amenable to penicillin therapy.

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