EFFECT OF pH AND INORGANIC SALTS ON PENICILLINASE FORMATION IN *STAPHYLOCOCCUS AUREUS*

FELIX LEITNER AND SIDNEY COHEN

Department of Microbiology, Michael Reese Hospital and Medical Center, Chicago, Illinois

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ABSTRACT

LEITNER, FELIX (Michael Reese Hospital, Chicago, Ill.) AND SIDNEY COHEN. Effect of pH and inorganic salts on penicillinase formation in *Staphylococcus aureus*. J. Bacteriol. **83**:314–323. 1962.—*Staphylococcus aureus* strain 55-C-1 exhibited a progressive increase in penicillinase activity as the pH of its growth medium dropped from 6.0 to 4.7. At pH 4.7, the increase in enzymic activity was inhibited markedly by Ca^{++}, somewhat less by Mg^{++}, and moderately by NaCl, KCl, and NH_4Cl. Inhibition by NaH_2PO_4 was greater than by NaCl. Growth at the same pH, in small concentrations of $Fe(NH_4)_2(SO_4)_2$ (10^{-5} to 10^{-6} M), elevated penicillinase activity strikingly, an effect shared to a lesser degree with $FeCl_3$, $CoCl_2$, and, very slightly, $MnCl_2$. The available evidence indicated that the changes in enzymic activity were the result of changes in rate of formation of the enzyme.

In earlier studies on staphylococcal penicillinase, a marked increase in constitutive penicillinase activity of *Staphylococcus aureus* strain 55-C-1 was noted as the culture passed from the exponential to the stationary phase (Geronimus and Cohen, *unpublished data*). The mechanism of this change in enzymic activity was considered to merit further investigation as possibly bearing upon the broader problems of the control of microbial enzymic biosynthesis and the degree of resistance of pathogenic staphylococci to penicillin. In this paper we report the results of further studies which indicate that the observed rise in constitutive penicillinase activity was secondary to a drop in pH of the growth medium. In strains of *S. aureus* tested, penicillinase activity increased markedly in media of low pH that still allowed a slow rate of growth. This effect of pH was found to be modified further by changes in the concentration of certain inorganic ions.

MATERIALS AND METHODS

The routine test organism was *S. aureus* strain 55-C-1, used in previous studies (Geronimus and Cohen, 1957*a*). Portions of overnight cultures desiccated on porcelain beads over silica gel were used as inocula, except for experiments with synthetic media for which larger inocula were found desirable. These were taken from stock cultures on slants of synthetic medium solidified with 2% agar. At the outset the routine medium was tryptic digest broth (Baltimore Biological Laboratory, Inc.). Two lots of this medium were used. An effect of phosphate concentration was first noted with the second lot (No. 003607), which was found to contain 0.002 M total phosphorus and 0.001 M inorganic phosphate when prepared as broth. The only material available for analysis from the first lot (No. 11956) was a few milliliters in an old flask; this contained 0.01 M inorganic phosphate. Other lots of tryptic digest broth, purchased after these first two, were unsatisfactory for this study, since they failed to support growth at pH 5.4 or lower. At this point, however, it was found advantageous, for other reasons, to continue the investigation with a synthetic medium prepared according to Wright and Mundy (1960), with the following modifications. Solution A was modified by the use of L-methionine, L-histidine hydrochloride, L-isoleucine, and L-tryptophan, instead of the DL compounds, in concentrations one-half that of the latter. L-Glutamic acid was used instead of its hydrochloride. Solution B was modified by the omission of NaCl, KH_2PO_4, and Na_2HPO_4. Sodium phosphate buffer (pH 7.2) was added to give 0.002 M phosphate in solution B.

The medium, minus glucose, was autoclaved at neutral pH for 15 min at 12 to 14 lb. Glucose was autoclaved similarly in 10% solution and added sterilely. The resultant synthetic medium, referred to hereafter as WMa medium, contained an amino acid mixture, thiamine, nicotinamide,

glucose, and the following salts: KCl, 2.7×10^{-3} M; $MgSO_4$, 4×10^{-4} M; and NaH_2PO_4-Na_2HPO_4, 1×10^{-3} M. In addition, it contained 0.01 M sodium and 0.008 M chloride (derived from the sodium hydroxide used to neutralize the amino acids and their hydrochlorides). No provision was made for trace-metal requirements, which we assume were met from contaminants in the reagents used. The pH was adjusted as necessary with 1 N HCl. Experimental modifications of this basic formula are indicated in the Results.

Experiments were begun from cultures incubated with slow shaking for approximately 16 hr in tryptic digest or WMa medium at an initial pH of 7.2. The final pH of these poorly buffered cultures was 5.5 to 6.5, dependent upon the amount of growth. One volume of culture was added to 10 volumes of fresh medium, pH 7.2, in 125- or 300-ml Erlenmeyer flasks (final volume 50 to 150 ml) equipped with cuvette sidearms for absorbancy determinations. These flasks were shaken vigorously in a rotatory shaking water bath for two to four generations. The experiment proper was then begun by diluting portions of these cultures two- to fivefold in experimental medium to final volumes of 35 to 150 ml in flasks of the same type. The pH was adjusted to the desired range with 1 N HCl and incubation continued with vigorous shaking. In typical experiments the initial absorbancy was 0.1 at 540 mμ in a 19-mm cuvette. We verified that the pH of acidified cultures prepared in this way did not change more than ±0.1 unit during typical experiments.

The absorbancy of cultures at 540 mμ in a Coleman Junior spectrophotometer was related to bacterial protein content of the bacterial residue insoluble in 5% trichloracetic acid after 6 min at 90 C, determined by the assay method of Lowry et al. (1951). The residue was dissolved by heating at 100 C for 10 min in 1 N NaOH. Crystalline bovine albumin was used as a standard. The ratio of the protein content of staphylococci to their absorbancy was found to vary between 425 and 650 μg protein per unit absorbancy, depending on the pH of the culture, the duration of growth at acidic pH, and the composition of the medium. Calculations of penicillinase activity were based upon experimentally determined values of this ratio applicable within ±10% to the experimental conditions. In all experiments to be cited, the significant changes in enzymic activity were much greater than could be attributed to any analytic uncertainties about this ratio.

Penicillinase was assayed manometrically at 37 C and pH 7.46 by the method of Henry and Housewright (1947). The main compartment of the Warburg vessels contained washed organisms and 200 μg chloramphenicol in 3.2 ml of 0.017 M $NaHCO_3$. Sodium penicillin G (12 mg in 0.2 ml of 0.017 M $NaHCO_3$) was added to the sidearms. The gas phase was 5% CO_2 and 95% N_2. Occasional assays of supernatant culture fluid from representative experiments at neutral and acidic pH indicated that 90% or more of the enzyme was associated with the bacterial cells. For the assay of disrupted cells, penicillin was added to the main compartment and the enzyme to the sidearm to avoid inactivation of the free enzyme by dilution in bicarbonate solution. A unit of penicillinase is the amount of enzyme that hydrolyzes 1 μmole of penicillin per hr at 37 C and pH 7.46 (Pollock and Torriani, 1953). Specific activity is expressed as units per mg of protein.

Amino acids and crystalline bovine albumin were purchased from Nutritional Biochemicals Corp. Salts and glucose were reagent grade. Distilled water was passed through a mixed bed resin deionizing column before use. The pH levels were determined with a Beckman pH meter, model G. Sodium penicillin G and chloramphenicol were gifts of Chas. Pfizer and Co., Inc. and Parke, Davis and Co., respectively.

RESULTS

Our initial observations of the effect of pH on staphylococcal penicillinase were made with static cultures of *S. aureus* strain 55-C-1. In well-buffered media in which the pH remained between 5.8 and 7.4 throughout the growth period, the penicillinase activities were relatively constant between 2.4 and 5.7 units. However, in the same media acidified to an initial pH of 5.1 to 5.4, or in a poorly buffered medium such as tryptic digest broth in which the pH fell as low as 4.7 during static growth, the penicillinase activity of strain 55-C-1 increased many fold (Table 1). Similar results were obtained with either sulfuric or hydrochloric acids as acidifying agents and four strains of penicillinase-producing staphylococci other than strain 55-C-1. Accordingly, the results do not appear to be peculiar to any

TABLE 1. *Effect of pH of medium on penicillinase activity**

Culture medium		Initial pH	Final pH	Penicillinase
				units/mg protein
Heart infusion broth	Neutral	7.41	7.02	3.6
	Acidified	5.07	5.14	18.4
Trypticase soy broth	Neutral	7.27	5.81	3.7
	Acidified	5.21	5.01	13.6
Tryptic digest broth	Neutral	7.25	4.80	35

* Into 125-ml Erlenmyer flasks containing 50 ml broth were inoculated overnight cultures of strain 55-C-1 in the same broth, incubated statically 18 hr, and their penicillinase activity assayed. The pH of acidified cultures was adjusted with 1 N HCl.

TABLE 2. *Effect of phosphate concentration on penicillinase activity of Staphylococcus aureus grown in tryptic digest broth at various pH levels**

Phosphate concn	pH						
	5.0	5.5	6.0	6.5	7.0–7.5	8.0	8.5
M							
0.05	4.8	3.4	3.8	2.7	2.7	2.9	2.9
0.001	31	6.9	4.5	2.8	2.7	2.5	2.9

* Cells of strain 55-C-1 grown in tryptic digest broth at initial pH 7.2 with vigorous shaking for 4.5 generations were inoculated into tryptic digest broth incubated with shaking at 37 C with continuous control of pH by a glass electrode. The pH was maintained in the desired range ±0.2 unit by addition of small amounts of 1 N HCl or 1 N NaOH. Broth containing 0.001 M phosphate received 0.05 M NaCl as a supplement. Repeated samples were taken over a period of 2.5 to 5.5 hr until the equilibrium values (penicillinase units/mg protein) listed in the table were attained.

single acidifying agent or test strain. Anaerobiosis reduced the formation of penicillinase. Thus, staphylococci grown in static culture in 50 ml of broth in 50-ml flasks had only 0.3 to 0.5 times as much penicillinase activity as when grown in the same amount of broth in 250-ml flasks, although the final pH in each case was the same (4.7 to 5.0).

Phosphate concentration and penicillinase at pH 4.7 in tryptic digest broth. The effect of growth under acidic conditions was analyzed further with strain 55-C-1 grown in shaken culture in tryptic digest broth. Growth was rapid in either lot of broth at pH 7.2, with generation times of about 25 min. Broth acidified with HCl to a pH as low as 5.4 and seeded with a large inoculum (e.g., 0.5 ml of an overnight culture into 50 ml of broth) supported good growth. By successive dilution, apparently exponential growth of cultures with bacterial densities as high as 0.15 to 0.2 mg dry wt/ml could be maintained at pH 5.4 for three to five generations, with generation times from 26 to 34 min. Growth was not completely balanced, for on continued dilution with fresh broth the growth rate eventually declined progressively. At pH 5.4 or lower, growth in lot 003607 fell below that in lot 11956, evidently due to the low phosphate concentraton of the former; when its phosphate was increased to 0.01 M, growth was the same in either broth. At appreciably lower pH (e.g., 4.7 to 5.0), growth was retarded, linear, and of short duration, even with phosphate supplementation.

Table 2 lists the specific penicillinase activity of strain 55-C-1 after growth in tryptic digest broth (lot 003607) over a range of pH and in high and low phosphate concentrations. The cultures at pH 5.4 to 8.5 grew exponentially. Evidently, the penicillinase activity of the organisms was unaffected by pH or phosphate concentration in the pH range 6.5 to 8.5. In cultures at pH 6 or lower, the penicillinase activity rose, the increase being especially marked in the medium containing 0.001 M phosphate.

The effects of phosphate concentration and pH were studied further in cultures at pH 4.6 to 4.8, where the increase in penicillinase activity was greatest. Figure 1 contains results of experiments at pH 4.6 and in 0.001 and 0.05 M phosphate. Growth, as measured by absorbancy of the cultures, was initially linear but declined progressively after 1 to 2 hr, the decline being slightly more rapid in 0.001 M phosphate. The increase in absorbancy was due to an increase in bacterial protoplasm, as demonstrated by analysis for bacterial protein. The differential rate of penicillinase synthesis rose in both media, more quickly in the low phosphate broth, to new values that remained constant during the remainder of the experiment. The final rate in

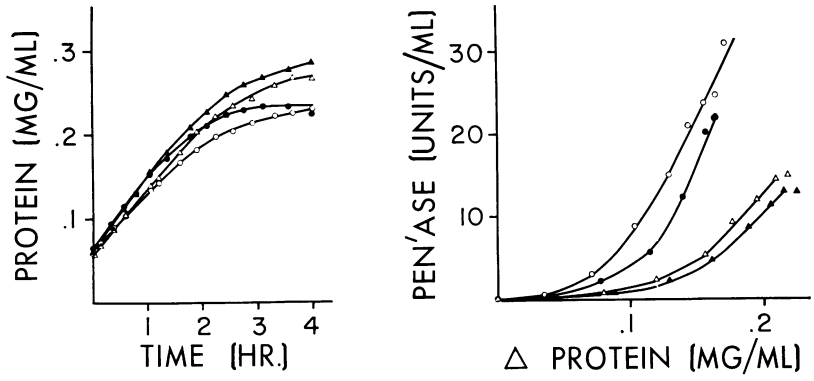

FIG. 1. *Effect of phosphate concentration on penicillinase synthesis in tryptic digest broth at pH 4.6. The left hand plot illustrates bacterial growth during the experiment. The right hand plot relates penicillinase content of the culture to bacterial protein content.* ○ = *broth* + *0.05* M *NaCl inoculated with strain 55-C-1 grown in the same medium;* ● = *broth* + *0.05* M *NaCl inoculated with strain 55-C-1 grown in broth* + *0.05* M *sodium phosphate;* △ = *broth* + *0.05* M *sodium phosphate inoculated with strain 55-C-1 grown in broth* + *0.05* M *NaCl;* ▲ = *broth* + *0.05* M *sodium phosphate inoculated with strain 55-C-1 grown in the same medium. Strain 55-C-1 was grown exponentially in tryptic digest broth supplemented with 0.05* M *NaCl or 0.05* M *sodium phosphate at pH 7.2 for three generations; 25-ml portions of each were added to 110-ml portions of each of the two modifications of the broth in 300-ml flasks and the pH adjusted to 4.6. The flasks were shaken at 37 C and samples taken every 30 min for penicillinase assay.*

TABLE 3. *Effect of sodium, ammonium, and potassium chlorides on penicillinase activity of Staphylococcus aureus grown at pH 4.7**

Salt added	NaCl	NH_4Cl		KCl	
		A	C	B	C
M					
0	140	140	150	110	150
0.01	120	110	—	87	—
0.02	100	90	—	79	—
0.05	74	60	110	44	—
0.1	35	—	44	—	53
0.2	9.9	—	13	—	20
0.5	—	—	4	—	8.8

* The table lists the specific penicillinase activities (penicillinase units/mg protein) of strain 55-C-1 grown for 3 hr at pH 4.7 in WMa medium supplemented with graded concentrations of NaCl, NH_4Cl, or KCl. A, B, and C designate different experiments. The inocula had penicillinase activities between 2.4 and 4.7 units/mg protein.

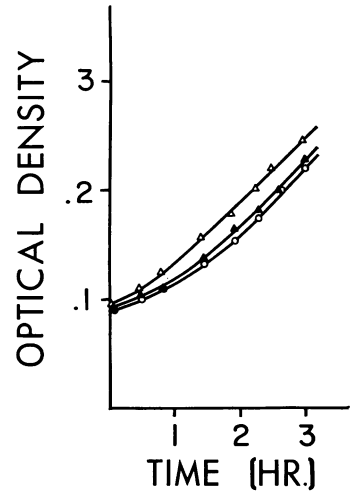

FIG. 2. *Effect of sodium chloride on the growth of strain 55-C-1 in WMa medium at pH 4.7. (Penicillinase values given in Table 3.)* ○ = *WMa;* △ = *WMa* + *0.02* M *NaCl;* ▲ = *WMa* + *0.2* M *NaCl.*

low phosphate medium was twice that in high phosphate and was, in fact, 50% of the maximal rate we have achieved by induction of the enzyme with penicillin in other experiments. The range of specific penicillinase activities at the end of this experiment was from 130 units in 0.001 M phosphate to 45 units in 0.05 M phosphate. The corresponding activities before acidification were 3.1 and 2.9. Evidently, the low pH stimulated penicillinase synthesis more effectively in low than in high phosphate medium.

TABLE 4. *Effect of calcium chloride and magnesium sulfate on penicillinase activity of Staphylococcus aureus grown at pH 4.7**

Salt added	$CaCl_2$		$MgSO_4$	
	A	B	A	C
M				
0	120	150	120	170
0.00125	86	—	68	—
0.0025	49	15	66	—
0.005	21	9.1	51	58
0.01	8.3	—	39	—
0.0125	—	6.0	—	—
0.02	6.2	—	25	38

* Results under heading A were obtained with WMa medium supplemented with 0.05 M NaCl, those under B and C with unsupplemented WMa. The inocula were grown in corresponding media at pH 7.2. Samples were taken for assay after growth for 3 hr in the test media at pH 4.7. The specific penicillinase activities of the inocula were 2.4 units for experiment A, 1.6 units for experiment B, and 3.3 for experiment C. Results are expressed as penicillinase units/mg protein.

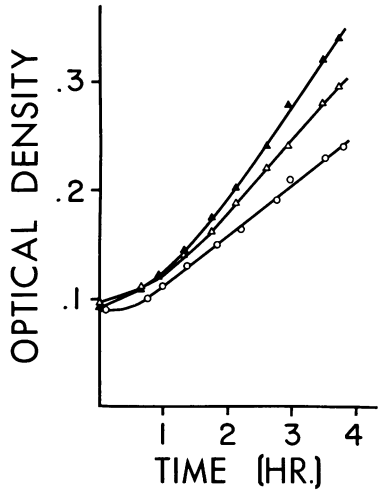

FIG. 3. *Effect of calcium chloride on the growth of strain 55-C-1 in WMa medium containing 0.05 M NaCl at pH 4.7. (Penicillinase values given in Table 4.)* ○ = *WMa + 0.05 M NaCl;* △ = *WMa + 0.05 M NaCl + 0.005 M* $CaCl_2$; ▲ = *WMa + 0.05 M NaCl + 0.02 M* $CaCl_2$.

Penicillinase in synthetic medium at pH 4.7.. As indicated in Fig. 1, the inhibitory effect of sodium phosphate on penicillinase formation at pH 4.7 was not duplicated by 0.05 M NaCl or, in other experiments, Na_2SO_4. Thus, in these experiments the effective moiety was the $H_2PO_4^-$ ion. Nevertheless, some effect of other ions was suggested by the fact that organisms grown in unsupplemented broth had higher penicillinase

TABLE 5. *Effect of sodium chloride and ferrous ammonium sulfate on penicillinase activity on Staphylococcus aureus grown at pH 4.7**

Salt added	Penicillinase
	units/mg protein
None	150
NaCl, 0.2 M	7
$Fe(NH_4)_2(SO_4)_2$, 2.5×10^{-6} M	260
NaCl, 0.2 M plus $Fe(NH_4)_2(SO_4)_2$, 2.5×10^{-6} M	84

* The specific penicillinase activity of the inoculum grown in WMa medium at pH 7.2 was 1.8 units. Samples were taken for assay after growth for 3 hr in the test media at pH 4.7.

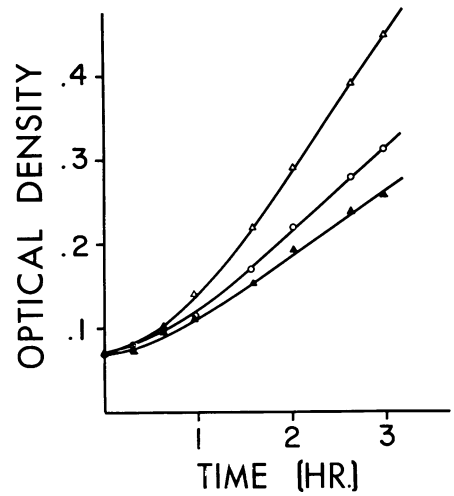

FIG. 4. *Effect of* $Fe(NH_4)_2(SO_4)_2$ *and* $CaCl_2$ *on the growth of strain 55-C-1 in WMa medium at pH 4.7.* ○ = *WMa;* △ = *WMa + 0.005 M* $CaCl_2$; ▲ = *WMa + 0.005 M* $CaCl_2 + 5 \times 10^{-6}$ *M* $Fe(NH_4)_2(SO_4)_2$. *Strain 55-C-1 was grown in WMa medium at pH 7.2 for three generations. A 100-ml portion was centrifuged, the packed cells suspended in 2 ml* H_2O, *and added to 360 ml of WMa at pH 4.7. Portions of 36 ml were placed in 125-ml flasks.* $CaCl_2$ *and* $Fe(NH_4)_2(SO_4)_2$ *were added in concentrations listed above. The cultures were shaken at 37 C for 3 hr. The initial specific penicillinase activity was 2.9 units. The final values were:* ○ = *69 units;* △ = *9.5;* ▲ = *160.*

TABLE 6. *Effect of metal ions on penicillinase in WMa medium with 0.01 M $CaCl_2$ at pH 4.7**

Metal salt added	Penicillinase
	units/mg protein
None	34
$Fe(NH_4)_2(SO_4)_2$, 10^{-5} M	310
$FeCl_3$, 10^{-5} M	100
$CoCl_2$, 10^{-5} M	79
$MnSO_4$, 10^{-5} M	48
$NiCl_2$, 10^{-5} M	42

* Strain 55-C-1, grown exponentially in WMa at pH 7.2 for two generations, was centrifuged and the packed cells resuspended in 2 ml WMa, pH 4.8. The latter was added as inoculum in 0.25-ml portions to flasks containing 0.01 M $CaCl_2$ and the indicated concentrations of metal salts in 30 ml of WMa medium, pH 4.8. After 3 hr shaking at 37 C, samples were taken for penicillinase assay.

TABLE 7. *Penicillinase activity of disrupted staphylococci after growth at pH 7.2**

Time of sonic oscillation	Penicillinase
min	*units/mg protein*
0	3.9
15	4.0
30	3.2
45	3.2

* Late log-phase organisms in shaken culture in tryptic digest broth, pH 7.2, buffered with 0.05 M phosphate were washed, resuspended in water, and subjected to sonic oscillation. Samples were taken at the indicated times.

activities for the most part than those grown in broth supplemented with 0.05 M NaCl or Na_2SO_4. The results, however, were rather irregular. Since a systematic study of the effects of inorganic salts in the broth medium would have been impracticable, further investigations were made with the synthetic WMa medium. Results with this medium are given in terms of specific penicillinase activities, since it would have been unduly burdensome to obtain enough experimental data to plot reasonably accurate differential rates of enzyme synthesis.

The inhibitory effect of sodium phosphate on acid-induced penicillinase synthesis was confirmed in WMa medium. Thus, organisms grown for 3 hr at pH 4.7 in WMa medium supplemented with 0.05 M NaCl had a specific penicillinase activity of 170 units, and those grown in the same medium supplemented with 0.05 M NaH_2PO_4 had a penicillinase activity of 54 units.

In WMa medium the concentration of NaCl

TABLE 8. *Penicillinase activity of strain 55-C-1 after incubation in amino acid-deficient medium at pH 4.7**

Time	Penicillinase, units/mg bacterial protein		
	Threonine deficient	Valine deficient	Complete medium
min			
90	2.4	17	45
120	2.2	22	78

* The inoculum, grown in WMa medium plus 0.05 M NaCl at pH 7.2, was added to two flasks of medium at pH 4.7 identical with WMa except for the omission of threonine in one and valine in the other. Samples were taken as indicated for penicillinase assay. For comparison, the value at zero time was recorded as 1.8 units.

TABLE 9. *Penicillinase activity of strain 55-C-1 after incubation in chloramphenicol-containing medium at pH 4.7**

Time	Penicillinase					
	WMa		WMa + 0.02 M $CaCl_2$			
min	*units/mg protein*		*units/mg protein*			
0	10		10			
70	39		10			
160	100		11			
					+ $Fe(NH_4)_2(SO_4)_2$, 5×10^{-6} M	
	Control	Chloramphenicol	Control	Chloramphenicol	Control	Chloramphenicol
220	130	78	13	9.5	77	10
280	150	78	18	8.7	130	9.4

* The inoculum was added to two flasks of WMa medium at pH 4.7, one supplemented with 0.02 M $CaCl_2$. After a growth period of 160 min at 37 C with shaking, the unsupplemented culture was divided into two, one receiving 100 μg/ml of chloramphenicol. At the same time, the other culture was divided into two pairs of flasks, of which one pair received in addition $Fe(NH_4)_2(SO_4)_2$, 5×10^{-6} M. Chloramphenicol, 100 μg/ml, was added to one flask of each pair. Incubation was continued for 120 min.

TABLE 10. *Effect of salts on penicillinase activity of disrupted strain 55-C-1**

Salt added	Penicillinase
	units/mg protein
None	99
NaCl, 0.2 M	84
KCl, 0.2 M	85
NH_4Cl, 0.2 M	53
$MgSO_4$, 0.02 M	77
$CaCl_2$, 0.01 M	92
$Fe(NH_4)_2(SO_4)_2$, 10^{-5} M	95

* Cells of strain 55-C-1 were shaken in WMa medium at pH 4.7 for 3.5 hr, harvested, washed once with H_2O, and treated in a 10-kc Raytheon sonic oscillator for 30 min. Neutral solutions of the salts were added to the main compartment of Warburg vessels in the indicated final concentrations in 0.017 M $NaHCO_3$. The disrupted cell preparation (0.2 ml) was tipped in from the sidearm. During 35-min assay periods, the penicillinase activities were constant as listed above.

could be shown clearly to affect the penicillinase activity. Increasing concentrations of NaCl were accompanied by decreased penicillinase activities, from a control value of 140 units for cells grown without added salt, that is, in approximately 0.01 M NaCl, to 9.9 units for cells grown in 0.2 M NaCl (Table 3). These changes in enzymic activity were not accompanied by significant changes in growth rates (Fig. 2). Table 3 demonstrates also that these results were in no way specific for NaCl, since similar depression of enzymic activity was observed in cultures grown with added KCl or NH_4Cl.

$CaCl_2$ reduced penicillinase activity strikingly in concentrations much lower than the salts of the alkali metals (Table 4). Thus, cells grown in 0.02 M $CaCl_2$ had penicillinase activities almost as low as after growth at neutral pH. $CaCl_2$ was similarly effective in WMa medium supplemented with 0.05 M NaCl, confirming the calcium ion as the effective portion of the molecule. Growth at pH 4.7 was markedly stimulated by $CaCl_2$ (Fig. 3). An effect of acidity in the presence of added calcium could be demonstrated by further reduction of pH. At pH 4.2 in WMa medium with added 0.05 M NaCl and 0.02 M $CaCl_2$, strain 55-C-1 grew more slowly than at pH 4.7 and penicillinase rose to 179 units compared with 11 units at the higher pH. An effect qualitatively similar to that of $CaCl_2$ but less marked was given by $MgSO_4$ (Table 4). $MgCl_2$ was comparably effective.

Addition of a small amount of iron in the form of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ enhanced penicillinase formation strikingly (Tables 5 and 9) and concomitantly depressed growth at pH 4.7 (Fig. 4). When added to unmodified WMa medium, in which the penicillinase activity was already high, the iron salt moderately stimulated further formation of penicillinase. When added to cultures in which penicillinase formation was depressed by calcium, magnesium, sodium, or potassium salts, 2.5×10^{-6} M $Fe(NH_4)_2(SO_4)_2$ enhanced penicillinase activity markedly. Table 5 cites a representative experiment with NaCl. In similar concentrations, $FeCl_3$ increased penicillinase activity moderately but much less than the ferrous salt; $CoCl_2$, $MnSO_4$, and $NiCl_2$ were decreasingly effective (Table 6). In neutral WMa medium, $Fe(NH_4)_2(SO_4)_2$ had no effect on growth or penicillinase activity.

Evidence for increased enzyme protein formation at pH 4.7. A number of experiments were performed to explore some aspects of the mechanism of the foregoing observations. The increase in enzymic activity appeared to be adaptive and not due to selection of mutants, since it was detectable as quickly as 70 min under conditions of very slow growth (Table 9).

The possibility that organisms grown at neutral pH contained undetected cryptic enzyme was excluded by the observation that penicillinase activities of cells before and after sonic disruption were nearly identical (Table 7).

Protein synthesis, albeit at a slow rate, appeared to be a prerequisite for the acid-stimulated increase in penicillinase activity. Deletion of an amino acid essential for growth (e.g., threonine or valine) inhibited the increase in penicillinase found in the acidified complete medium (Table 8). With threonine deficiency, inhibition was complete; with valine deficiency, inhibition was incomplete.

The addition of chloramphenicol blocked any further increase in penicillinase activity of strain 55-C-1 growing at pH 4.7. The inhibition was equally effective in unsupplemented WMa medium and in medium supplemented with $CaCl_2$ and $Fe(NH_4)_2(SO_4)_2$ (Table 9). Comparable results were achieved in experiments of this type by using omission of threonine instead of addition of chloramphenicol to inhibit protein syn-

thesis. These observations suggest, therefore, that the stimulating effect of the ferrous salt on the enzymic activity is similarly dependent on protein synthesis.

Further evidence of a lack of a direct effect of the various salts on the activity of preformed enzyme was obtained by direct testing with sonically disrupted staphylococci. With the exception of NH_4Cl, none of the salts affected the enzymic activity of the preparation appreciably, although in the concentrations employed they produced marked changes in cells growing at pH 4.7 (Table 10).

DISCUSSION

We may summarize the results as follows. (i) A pH below 6.0 leads to an increase in penicillinase activity of strain 55-C-1 and other staphylococci, along with a reduction in growth rate. (ii) The effect of a low pH can be modified extensively by certain inorganic ions which in similar concentrations have no effect during growth at more nearly physiological pH. (iii) Our evidence suggests that the concentration of H^+ and other ions affects the rate of synthesis of penicillinase under the conditions described.

These experimental results with staphylococcal penicillinase present analogies to older observations of an increase in concentration of certain bacterial enzymes as the result of growth in acidified cultures. This subject was studied extensively by Gale (1943), who found the synthesis of several enzymes to vary with the pH of growth in *Escherichia coli* and other bacterial species. In particular, amino acid decarboxylases were formed in appreciable amount only in cells grown at a pH less than 6.0. The synthesis of these enzymes was inducible, with the exception of glutamic acid decarboxylase. In certain strains of *E. coli*, this enzyme, like staphylococcal penicillinase, was formed constitutively, but in greater concentration at low pH.

Another example is the marked increase in catalase activity in the late exponential and retardation phases of cultures of *Bacterium lactis aerogenes* (McCarthy and Hinshelwood, 1959). The increase in the enzyme was related to acidification of the medium by the growing bacteria. Maintenance of the pH at 7.0 by repeated neutralization prevented any rise in catalase.

The modifying effects of inorganic salts on acid-induced formation of penicillinase are unexplained at this time. They seem to be due in part both to the cation and the anion. Of the cations studied, Na^+, K^+, NH_4^+, Ca^{++}, and Mg^{++} depress penicillinase formation, an effect most marked with Ca^{++}. On the other hand, Fe^{++} stimulated penicillinase formation, most strikingly when the latter had been inhibited previously by depressive cations, such as Na^+ or Ca^{++}. Fe^{+++}, Co^{++}, and, to a lesser extent, Mn^{++} were stimulatory but much less effective than Fe^{++}. Ni^{++} had little or no effect in comparable concentrations. Some of these ions, especially Fe^{++}, exert an effect in concentrations so low that traces of contaminants in reagents or in glassware would be expected to present an experimental hazard. In fact, we observed from time to time appreciable variations in penicillinase activity of the cells grown in WMa medium, ascribed tentatively to such unrecognized contamination. Nevertheless, it is important to note that the added salts always displayed their characteristic actions regardless of variations in the level of enzyme in the unsupplemented WMa medium.

Among the anions, phosphate was distinctly more inhibitory to penicillinase formation at pH 4.7 than chloride or sulfate. This effect of phosphate is not due to ionic strength alone, since at pH 4.7 phosphate occurs almost entirely as $H_2PO_4^-$, with ionic strength equal to that of Cl^- at corresponding concentrations. Attempts to assess any effect of organic anions (e.g., citrate or succinate) were unsuccessful, since they inhibited growth completely at pH 4.7.

Depression of penicillinase formation was often accompanied by more rapid growth at pH 4.7, most strikingly with added Ca^{++}. Contrariwise, enhancement of penicillinase formation by Fe^{++} was accompanied by retardation of growth. This pattern was not completely consistent, however, for Na^+, K^+, and NH_4^+ depressed penicillinase synthesis in concentrations having little or no effect on growth.

The low concentrations in which Fe^{++} and Ca^{++} were effective excluded a primary role of osmotic pressure or ionic strength in these events. It remains to be determined how the effects of the various salts are related to the intracellular concentrations of the corresponding ions or to changes in the internal pH. The uptake of a given ionic species by microbial cells may be influenced complexly by the over-all ionic composition of the medium (Rothstein, 1960;

Mitchell, 1953, 1954). Accordingly, the various salts may act by controlling the absorption of a single substance, for example, H^+.

A possible clue to the mechanism of stimulation of penicillinase formation by growth at low pH may be discerned in observations that other unfavorable growth conditions also may stimulate the formation of certain enzymes. Kjeldgaard, Maaløe, and Schaechter (1958) found that the later in the growth cycle that *Salmonella typhimurium* was transferred from a rich to a poor medium, the shorter was the subsequent lag. They suggested that the "... viable cells of an outgrown broth culture can be shown to possess a more complete spectrum of enzymic activities than the exponentially growing 'broth cells'."

An increase in a bacterial enzymic activity was induced by an exogenous inhibitor of growth in a uracil-requiring mutant of *E. coli* which, in the presence of 5-fluorouracil, exhibited a fivefold increase in succinic dehydrogenase activity, while bacterial protein increased only threefold (Horowitz, Saukkonen, and Chargaff, 1960).

These observations of increased synthesis of certain enzymes under conditions highly unfavorable for general protoplasmic synthesis can be understood in the light of current concepts of the control of enzyme synthesis (Pardee, 1959) by the assumption that the inhibitory environmental factors depress preferentially the synthesis or the activity of compounds acting as repressors of the enzyme in question, while still permitting some degree of protein synthesis. In these circumstances a relatively specific increase in the corresponding enzyme would be expected. If this assumption is correct, it is possible that study of staphylococci during growth at acid pH may furnish leads to the mechanisms of the control of enzyme formation.

The degree of resistance of penicillinase-producing staphylococci to penicillin is proportional, in general, to their penicillinase content. Admittedly, the highly acidic pH of our experiments is not likely to be duplicated in infections in an animal host, although it may be approached in urine. Nevertheless, these results taken together with earlier findings of in vivo induction of penicillinase in experimental murine infections emphasize that it may not be safe to assume that a given strain of staphylococcus in different anatomic sites and pathologic lesions has necessarily an identical degree of resistance to penicillin (Geronimus and Cohen, 1957*b*).

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