SACKS, L. E. & PENCE, J. W. (1958). *J. gen. Microbiol.* **19**, 542-550

The Influence of pH on the Antibacterial Action of Subtilin A

BY L. E. SACKS AND J. W. PENCE

Western Regional Research Laboratory, Agricultural Research Service, United States Department of Agriculture, Albany, California, U.S.A.

SUMMARY: The influence of pH value on the antibacterial action of subtilin A has been demonstrated by survivor counts, inhibition of respiration, and pH gradient plates. *Staphylococcus aureus* is more sensitive to subtilin as the pH value increases; *Escherichia coli* is more sensitive as the pH value decreases. The results are analogous to those obtained by other investigators working with cationic detergents, and are consistent with the hypothesis that the basic surface-active antibiotics kill bacteria by the same general mechanism as do the quaternary ammonium germicides. A survey of the growth inhibition of other organisms on pH gradient plates indicates that *Bacillus cereus* behaves like *S. aureus*, while *B. megaterium*, *Corynebacterium poinsettiae*, and *Streptococcus faecalis* behave like *E. coli*.

A pH/mobility curve for subtilin A, obtained by paper electrophoretic studies, indicates no striking changes in the charge of the molecule over the pH range 4-9, although the presence of at least one free α -amino group is indicated by inflexion points in the range of pH 6-7. The isoelectric point of subtilin A at ionic strength 0.1 is approximately 6.7.

The use of sectoried, square-shaped Petri dishes for pH-gradient plate studies with germicides is described.

The effect of pH value on the germicidal action of cationic detergents is complex: some organisms, including *Staphylococcus aureus*, are more susceptible as the pH value increases, others, including *Escherichia coli*, are more sensitive as the pH value decreases (Salton, 1950). No explanation has been offered to account for this behaviour. Since the discovery by Dubos & Hotchkiss (1942) that tyrocidine shares many of the antibacterial properties of the cationic detergents, a number of surface-active polypeptide antibiotics have been shown to belong to this group of germicides (Newton, 1953; Anderson, Villela, Hansen & Reed, 1946; Sacks, 1952*a*; Colasito, Koffler, Tetrault & Reitz, 1955). However, no extensive study has been made of the influence of pH value on the germicidal action of the surface-active antibiotics, and there has been no demonstration that these pH effects are analogous to those shown for the cationic detergents. Subtilin is a surface-active polypeptide antibiotic, and since it has shown promise as an adjunct in the preservation of both acid (Andersen & Michener, 1950; Wheaton, Burroughs & Hays, 1957) and neutral foods (Godkin & Cathcart, 1953; O'Brien *et al.* 1956) an investigation of the effect of pH value on the germicidal action of this antibiotic was deemed advisable.

METHODS

Subtilin. Subtilin was prepared at this laboratory as previously described (Fevold, Dimick & Klose 1948; Garibaldi & Feeney, 1949). The subtilin thus produced was then fractionated by column chromatography, by the method of

Alderton & Snell (1958). Subtilin A was identified by its migration angle in hanging curtain paper electrophoresis (Sacks & Pence, 1957) and by its high antibacterial activity. It was homogeneous by hanging curtain paper electrophoresis and by the countercurrent distribution methods of Alderton & Snell (to be published). Unfractionated subtilin, lot 326 (70 % potency) was used in the manometric experiments and in some of the paper electrophoretic work.

Turbidimetry. All turbidimetric measurements were carried out with a Coleman model 11 spectrophotometer, at 650 m μ , using 18 mm. test tubes.

Preparation of test organisms. Forty ml. of sterile nutrient broth in a 250 ml. nephelometer flask were inoculated with young cultures of *Staphylococcus aureus* strain H or of *Escherichia coli* strain K 12. The flasks were allowed to incubate overnight at room temperature. They were then placed on a rotary shaker at 35°, and turbidity determined periodically. The organisms were harvested by a gentle centrifugation (2400 g for 3–4 min.) before the culture reached the peak of the log growth phase (optical density = 0.85). It is extremely important to standardize the physiological age of the culture (Sacks, 1952*b*; Dawson, Lominski & Stern, 1953), and the above procedure makes it possible to obtain cultures of standardized age at a convenient time, just before running an experiment. The organisms were washed in distilled water; then the suspension was made up to o.d. = 0.40. One ml. of this suspension served as the inoculum.

Measurement of bactericidal action of subtilin. A series of test tubes, each containing 6 ml. McIlvaine buffer (diluted 1/3) at graded pH values were placed in a water bath at 36°. One ml. of subtilin A solution (500 p.p.m. for *Escherichia coli*; 200 p.p.m. for *Staphylococcus aureus*) was added to each tube in the series. After temperature equilibration, the tubes were inoculated with the test organism at regular time intervals. After 1 hr. the first tube was plated on nutrient agar, and succeeding tubes were plated at appropriate intervals. Plates were counted after 24 or 48 hr. of incubation.

Manometric experiments. *Staphylococcus aureus* H and *Escherichia coli* 451B were cultivated in medium II*b* (Lewis *et al.* 1947) on a shaking machine at 35°. Organisms were harvested before the culture reached the peak of the log growth phase, washed twice in 0.3 % NaCl, and resuspended in this diluent to an optical density of *c.* 0.65. The Warburg vessels contained 1.4 ml. McIlvaine buffer, 0.01 M-glucose, and 0.2 ml. organism suspension in a total volume of 2.0 ml. Subtilin concentrations were 1.0 p.p.m. for *S. aureus*, and 20 p.p.m. for *E. coli*. The subtilin used in these experiments was lot 326. A control vessel from which subtilin was omitted was run at each pH value. Results were calculated by dividing the oxygen uptake of the subtilin-treated organisms (in the time interval of 10–50 min. after tipping the side-arm contents) by the oxygen uptake of the untreated organisms at the same pH values. Temperature was 37°. Control vessels, from which glucose was omitted, showed that there was no appreciable oxidation of the citrate present in the buffer.

pH-gradient plates. A modification of the method previously described

(Sacks, 1956) was used. Square plastic Petri dishes (100 mm.) made it possible to achieve a uniform pH gradient over the entire plate, and also made possible equal streaking distances for all test organisms. The pH gradient was obtained with two 25 ml. layers of Bacto Penassay seed agar (Difco), one supplemented with $\text{m-K}_2\text{HPO}_4$, the other with $\text{m-KH}_2\text{PO}_4$ (1/10, v/v). Eugonagar (Baltimore Biological Laboratory, Inc.) was substituted for Penassay seed agar in experiments involving *Corynebacterium poinsettiae*. After the complementary layers had solidified, three pieces of 2 mm. solid glass rod were placed in parallel positions on the surface of the agar in the direction of the gradient, thus creating four compartments in which the pH effect could be demonstrated on the same plate. Two ml. of melted agar containing appropriate amounts of subtilin A were then carefully poured into each compartment. (These subtilin-agar mixtures were prepared slightly in advance and were kept at 45° in 12 mm. screwcap tubes until needed.) After solidification of the surface layer, the test organisms were streaked over each compartment with a sterile swab stick. Suspensions of test organisms were prepared from 24 hr. slopes on Bacto Penassay Base agar, and were made up to an optical density of 0.2–0.4. When the subtilin concentrations were appropriately selected, the plates, after incubation, generally resembled a bar graph, in which pH was the ordinate and subtilin concentration the abscissa. The pH values of uninoculated plates were determined by inserting a glass electrode into the agar.

pH/mobility curve. Paper electrophoresis was employed in these studies because the relative insolubility of subtilin even at low ionic strength renders such a determination difficult by the conventional Tiselius techniques. The low concentrations of polypeptide required for paper electrophoresis made this a satisfactory technique. The subtilin was applied to the paper as unbuffered solutions of 0.35–0.50 % (w/v) concentration. Migrations at various pH values were determined as described previously (Pence, 1953), by techniques based on those of Kunkel & Tiselius (1952). Buffer solutions were used at an ionic strength of 0.1 and consisted of acetate, phosphate, cacodylate (0.02 M-sodium cacodylate + cacodylic acid + 0.08 M-sodium chloride), or barbital (0.01 M-sodium diethyl barbiturate + barbituric acid + 0.09 M-sodium chloride) systems. Whatman 3 MM filter papers were used exclusively. After completion of a run (18 hr., 4.5–5.0 v./cm.) the papers were dried and subsequently stained by the modification of the Rydon & Smith technique developed by Pan & Dutcher (1956). Most of the subtilin used for these experiments was lot 326, which contains about 20 % subtilin B, but since subtilin B has a considerably lower isoelectric point, no difficulties were encountered in interpreting the electrophorograms.

RESULTS

The influence of pH values on the anti-bacterial action of subtilin A was demonstrated by determining plate counts on washed organisms exposed to the antibiotic for 1 hr. in the presence of citrate + phosphate buffers. The results of two typical experiments are shown graphically as survivor/pH curves in

Figs. 1 and 2. Two conclusions emerge from these studies. The effect of pH value is opposite for the two test organisms, and the effect of pH is much more marked for *Escherichia coli* than it is for *Staphylococcus aureus*. Microscopic examination showed no evidence of clumping induced by subtilin.

Manometric experiments were carried out with *Staphylococcus aureus* H and *Escherichia coli* 451 B in which the effect of pH value on the inhibition of respiration was determined. The results of two experiments are shown in

Fig. 1

Fig. 2

Fig. 1. pH/survivor curve for *Staphylococcus aureus* H. McIlvaine buffers, diluted 1 to 4. Exposure to subtilin, 60 min., 36°; ○ = 25 p.p.m. subtilin A; □ = control.

Fig. 2. pH/survivor curve for *Escherichia coli* K12. McIlvaine buffers, diluted 1 to 4. Exposure to subtilin, 80 min., 36°; ○ = 63 p.p.m. subtilin A; □ = control.

Fig. 3. The respiration in the controls was not greatly affected by pH value and generally about 200 μ l. oxygen/hr. were consumed, although there was a slight diminution of oxygen uptake (c. 10–30 %) at the highest pH values used. The results obtained with *E. coli* 451 B showed a marked increase in sensitivity as the pH value decreased, particularly in the region of pH 7.6–6.4. *S. aureus* H, however, showed no definite change in sensitivity at different pH values in these experiments.

In order to extend and verify these observations, the pH sensitivity to subtilin of several other bacterial species was investigated. It was possible to survey a greater number of species rapidly by use of the pH-gradient agar plate (Sacks, 1956). Many organisms proved unsuitable because they were incapable of growing well throughout the entire pH range of the plate (c. 5.6–7.9). However, six subtilin-sensitive organisms were quickly found which were capable of eugonic growth throughout the entire pH range. For purposes of recording the results photographically, sector square Petri dishes were used, making it possible to use several concentrations of subtilin A on the same plate. Results are shown in Pl. 1, in which the opposing effects of pH value on the germicidal action of subtilin A are once more illustrated. *Staphylococcus aureus* H, *S. aureus* S30 b, and *Bacillus cereus* v. *terminalis* showed increasing sensitivity to subtilin as the pH value rose. *Corynebacterium poinsettiae*, *B. megaterium* and *Streptococcus faecalis* showed increasing

sensitivity as the pH value decreased. The pH-gradient plates have the disadvantage that bacteriostatic action can also affect the results obtained. Nevertheless, it seems evident that the pH value can affect the germicidal action of subtilin in opposing ways. Some organisms show a slightly increasing sensitivity as the pH value increases, others show a markedly increasing sensitivity as the pH value decreases. The overall picture is analogous to that obtained by Salton (1950) and by Soike, Miller & Elliker (1952) with cationic detergents.

It should be noted that *Escherichia coli* proved extremely resistant to this type of test, growing over the entire pH range, even at very high subtilin concentrations. Perhaps the simplest way to account for this anomaly is to assume the presence of an appreciable number of highly resistant organisms in any clone of *E. coli*.

In considering the effect of pH values on the germicidal action of the synthetic detergents, the charge of the detergent has always been considered to be of paramount importance, the anionic detergents showing completely different pH effects from cationic detergents (Baker, Harrison & Miller 1941 *a*; Gershenfeld & Milanick, 1941; Gershenfeld & Ibsen, 1942).

Fig. 3

Fig. 4

Fig. 3. Inhibition of O_2 uptake (substrate glucose) by subtilin. McIlvaine buffers, diluted 1 to 1.43. $\circ$ = *Escherichia coli* 451 B; subtilin 20 p.p.m.; $\square$ = *Staphylococcus aureus* H; subtilin 1 p.p.m.; (subtilin, lot 326).

Fig. 4. Effect of pH value on the electrophoretic mobility of subtilin in acetate, cacodylate, phosphate, and barbital buffers at an ionic strength of 0.1 on filter paper, with 4.7 v./cm. for 24 hr. at 25–28°. Results obtained with crystalline bovine serum albumin under similar conditions are included for comparison. $\circ$ = subtilin; Δ bovine serum albumin.

Earlier studies on high-potency subtilin indicated an excess of free amino groups (Lewis & Snell, 1951) over free carboxyls, and subtilin has generally been considered a basic polypeptide. However, the isoelectric point of subtilin has never been determined, nor have there been any published electrophoretic mobility studies or titration curves for this polypeptide. Because of its obvious relationship to the present study, a pH/mobility curve was drawn from values obtained by horizontal paper electrophoresis (Fig. 4). The curve obtained clearly reveals inflexion points that suggest the presence of at least one α -amino group ($pK=8$) which would be assumed to be part of a lanthio-

nine or β -methyl lanthionine residue (Carson, 1952). Since this article was written, A. Stracher and L. C. Craig indicated in a personal communication to J. C. Lewis of this laboratory that sarcosine very probably furnished the only free amino group in subtilin, other than ϵ -amino groups. The inflexion points are somewhat lower than anticipated, but this very likely results from the effects of bound buffer ions at the ionic strength used (0.1; cf. Velick, 1949; Zittle & Custer, 1957). Considering the excess of amino groups present in subtilin, the relatively low isoelectric point (6.7) is also rather surprising, but this too may probably be explained as the result of bound buffer ions. A similar curve for serum albumin is used to illustrate the low mobility of subtilin at this ionic strength. The heavy adsorption on paper has been previously noted with subtilin (Sacks & Pence, 1957) and with cationic proteins (Monty, Morrison, Alling & Stotz, 1956).

DISCUSSION

This study indicates that the pH value will affect subtilin A activity differently, depending upon the test organism used. In the range pH 4.0–8.0, subtilin A is slightly more effective against *Staphylococcus aureus* and *Bacillus cereus* as the pH rises. It is markedly more effective against *Escherichia coli*, *Corynebacterium poinsettiae*, *Streptococcus faecalis*, and *B. megaterium* as the pH value decreases. These results are in reasonably good agreement with those of Salton (1950) and Soike *et al.* (1952) for cationic detergents, and give added weight to the belief that the surface-active basic polypeptide antibiotics have the same mode of action (Dubos & Hotchkiss, 1942; Newton, 1953). The older theories that cationic detergents are more effective in alkaline solution (Baker *et al.* 1941 *a*; Gershenfeld & Milanick, 1941; Gershenfeld & Ibsen, 1942) have recently been modified in the light of newer evidence which shows that such pH effects are largely dependent upon the test organism (Salton, 1950; Soike *et al.* 1952). For example, it seems clear that *S. aureus* is more sensitive to the quaternary ammonium compounds in alkaline solution, while the pseudomonads are more sensitive in acid solution. Results reported for *E. coli* indicate increasing sensitivity to cationic surface-active agents as the pH value decreases (Salton, 1950; Soike *et al.* 1952) although this trend is not always clear-cut (Quisno & Foter, 1946; Soike *et al.* 1952).

Adsorption of the cationic detergents increases with increasing pH value regardless of the test organism (Salton, 1950). Thus, it seems likely that some organisms must be able to bind the surface-active agent in a non-toxic way as well as in a toxic way. Indeed, such a concept was enunciated long ago (Baker *et al.* 1941 *b*). By assuming the presence of at least two different dissociable receptor sites in or near the cytoplasmic membrane, it might be possible to explain the opposing effects of surface-active agents which occur with different microorganisms. If the relative amount of protective binding component varies with different species, such opposing effects may be readily accounted for.

The site of action of the cationic surface-active agents has been a subject of much speculation. Lipoproteins (Dawson *et al.* 1953) phospholipids (Gilby & Few, 1957) polyphosphates (Newton, 1954; Armstrong, 1957) and ribonucleic

acids (Stacey, 1955) have been suggested as the site of action. Any theory purporting to explain the mode of action of the cationic surface-active agents must harmonize with the striking pH effects upon the action of these germicides, and these pH effects may be useful in ruling out certain groups as possible sites of action.

The studies of Carson (1952) and of Lewis & Snell (1951) indicate that subtilin contains some lanthionine or β -methyl lanthionine with free α -amino groups. The pH/mobility curve obtained in this study reveals inflexion points which are a probable reflexion of the presence of these free α -amino groups. The relatively high ionic strengths (0.1) employed account for the apparently low pK indicated. However, the three free ϵ -amino groups of lysine (Carson, 1952; Lewis & Snell, 1951) undoubtedly remain positively charged throughout the range pH 4.0–8.0 used in these experiments, and it seems likely that these ϵ -amino groups play an essential role in the antibacterial action of subtilin (Bichowsky-Slomnicki, Berger, Kurtz & Katchalski, 1956). There is no indication that dissociation of the α -amino groups in subtilin influences its antibacterial action. Somewhat more surprising is the fact that subtilin A exhibits a relatively low isoelectric point for a molecule possessing an excess of free amino groups (Lewis & Snell, 1951). A shift of isoelectric points of proteins as a result of buffer-ion binding has been demonstrated in several instances (cf. Velick, 1949; Zittle & Custer, 1957). At lower ionic strengths effective charge could be much greater, giving greater mobility and a higher isoelectric point.

The use of square Petri dishes to demonstrate the influence of pH value on the antibacterial action of germicides has several advantages over the round plates originally suggested (Sacks, 1956). The square plate provides for a uniform pH gradient in all parallel lines across the plate, it allows an equal streaking distance for all test organisms, it permits the use of divided sections, demonstrated above, and it prevents the slipping of the agar layers in the preparation of the plate. However, the pH-gradient plates are subject to a common defect, viz. they do not distinguish between bactericidal and bacteriostatic effects.

The influence of pH on the action of subtilin as an adjunct in food preservation cannot be predicted on the basis of these results; it will depend on the particular spoilage organisms. Moreover, the use of subtilin to decrease the processing time required to destroy bacterial spores involves several factors not considered here, e.g. the influence of pH value on the adsorption of subtilin by heat-treated spores (Michener, 1955). The work of O'Brien *et al.* (1956) indicated that pH value did not affect the reduction of D values for PA 3679 spores in pea puree.

The influence of pH on the action of impure subtilin has been briefly touched upon in two other publications. Krasnow, Jann & Salle (1953) reported that subtilin exhibited maximum effectiveness in preventing outgrowth of *Clostridium botulinum* spores in two pH ranges, 5.5–6.5, and 8.5–9.0. Housewright, Henry & Berkman (1948) showed that cup plate assays for subtilin had larger inhibition zones in acid media, when *Bacillus cereus* was the test organism.

REFERENCES

- ALDERTON, G. & SNELL, N. (1958). The isolation of subtilin A. *J. Amer. chem. Soc.* (in the Press).
- ANDERSEN, A. A. & MICHENER, H. D. (1950). Preservation of foods with antibiotics. I. The complementary action of subtilin and mild heat. *Food Tech.* 4, 188.
- ANDERSON, H. H., VILLELA, G. G., HANSEN, E. L. & REED, R. K. (1946). Some physical and biological properties of subtilin and other antibiotics. *Science*, 103, 419.
- ARMSTRONG, W. McD. (1957). Surface active agents and cellular metabolism. I. The effect of cationic detergents on the production of acid and of carbon dioxide by baker's yeast. *Arch. Biochem. Biophys.* 71, 137.
- BAKER, Z., HARRISON, R. W. & MILLER, B. F. (1941a). Action of synthetic detergents on the metabolism of bacteria. *J. exp. Med.* 73, 249.
- BAKER, Z., HARRISON, R. W. & MILLER, B. F. (1941b). Inhibition by phospholipids of the action of synthetic detergents on bacteria. *J. exp. Med.* 74, 621.
- BICHOWSKY-SLOMNICKI, L., BERGER, A., KURTZ, J. & KATCHALSKI, E. (1956). The antibacterial action of some basic amino acid copolymers. *Arch. Biochem. Biophys.* 65, 400.
- CARSON, J. F. (1952). The free amino groups of subtilin. *J. Amer. Chem. Soc.* 74, 1480.
- COLASITO, D. J., KOFFLER, H., TETRAULT, P. A. & REITZ, H. C. (1955). Release of cellular constituents as basis of sensitive assay for circulin. *Canad. J. Microbiol.* 1, 685.
- DAWSON, I. M., LOMINSKI, I. & STERN, H. (1953). An electron-microscope study of the action of cetyl-trimethyl-ammonium bromide on *Staphylococcus aureus*. *J. Path. Bact.* 66, 513.
- DUBOS, R. J. & HOTCHKISS, R. D. (1942). Origin, nature and properties of gramicidin and tyrocidine. *Trans. Coll. Physicns Philad.* 10 (Series 4), 11.
- FEVOLD, H. L., DIMICK, K. P. & KLOSE, A. A. (1948). Isolation of subtilin from submerged cultures. *Arch. Biochem. Biophys.* 18, 27.
- GARIBALDI, J. A. & FEENEY, R. E. (1949). Subtilin production. *Industr. Engng Chem.* 41, 432.
- GERSHENFELD, L. & IBSEN, M. (1942). The inhibitory effect of lipoids on detergents with disinfectants acting against *S. aureus* and *E. typhosa* at different pH values. *Amer. J. Pharm.* 114, 281.
- GERSHENFELD, L. & MILANICK, V. E. (1941). Bactericidal and bacteriostatic properties of surface tension depressants. *Amer. J. Pharm.* 113, 306.
- GILBY, A. R. & FEW, A. V. (1957). Reactivity of ionic detergents with *Micrococcus lysodeikticus*. *Nature, Lond.* 179, 422.
- GODKIN, W. J. & CATHCART, W. H. (1953). The complimentary action on subtilin and terramycin in preserving custard fillings. *Food Tech.* 7, 282.
- HOUSEWRIGHT, R. D., HENRY, R. J. & BERKMAN, S. (1948). A microbiological method for the assay of subtilin. *J. Bact.* 55, 545.
- KRASNOW, I., JANN, G. J. & SALLE, A. J. (1953). The effect of pH and heat on the activity of subtilin against spores of *Clostridium botulinum*. *Bact. Proc.* 28.
- KUNKEL, H. G. & TISELIUS, A. (1952). Electrophoresis of proteins on filter paper. *J. gen. Physiol.* 35, 89.
- LEWIS, J. C., HUMPHREYS, E. M., THOMPSON, P. A., DIMICK, K. P., BENEDICT, R. G., LANGLYKKE, A. E. & LIGHTBODY, H. D. (1947). The microbiological assay of subtilin. *Arch. Biochem.* 14, 437.
- LEWIS, J. C. & SNELL, N. S. (1951). The amino acid composition of subtilin. *J. Amer. Chem. Soc.* 73, 4812.
- MICHENER, H. D. (1955). The action of subtilin on heated bacterial spores. *J. Bact.* 70, 192.

- MONTY, K. J., MORRISON, M., ALLING, E. & STOTZ, E. (1956). Electrophoresis of cationic proteins on filter paper. *J. biol. Chem.* **220**, 295.
- NEWTON, B. A. (1953). The release of soluble constituents from washed cells of *Pseudomonas aeruginosa* by the action of polymyxin. *J. gen. Microbiol.* **9**, 54.
- NEWTON, B. A. (1954). Site of action of polymyxin on *Pseudomonas aeruginosa*: antagonism by cations. *J. gen. Microbiol.* **10**, 491.
- O'BRIEN, R. T., TITUS, D. S., DEVLIN, K. A., STUMBO, C. R. & LEWIS, J. C. (1956). Antibiotics in food preservation. II. Studies on the influence of subtilin and nisin on the thermal resistance of food spoilage bacteria. *Food Tech.* **10**, 352.
- PAN, S. C. & DUTCHER, J. D. (1956). Separation of acetylated neomycins B and C by paper chromatography. *Analyt. Chem.* **28**, 836.
- PENCE, J. W. (1953). Approximate isoelectric pH's of albumins from wheat flour. *Cereal Chem.* **30**, 328.
- QUISNO, R. & FOTER, M. J. (1946). Cetyl pyridinium chloride. I. Germicidal properties. *J. Bact.* **52**, 111.
- SACKS, L. E. (1952a). Subtilin considered as a germicidal surface-active agent. *Antibiot. & Chemother.* **2**, 79.
- SACKS, L. E. (1952b). The subtilin sensitivity of bacteria as a function of culture age. *Antibiot. & Chemother.* **2**, 411.
- SACKS, L. E. (1956). A pH gradient agar plate. *Nature, Lond.* **178**, 269.
- SACKS, L. E. & PENCE, J. W. (1957). Characterization and purification of subtilin by paper electrophoresis. *Analyt. Chem.* **29**, 1802.
- SALTON, M. R. J. (1950). The bactericidal properties of certain cationic detergents. *Aust. J. Sci. Res. (B)*, **3**, 45.
- SOIKE, K. I., MILLER, D. D. & ELLIKER, P. R. (1952). Effect of pH of solution on germicidal activity of quaternary ammonium compounds. *J. Dairy Sci.* **35**, 764.
- STACEY, M. (1955). Chemistry of the Gram-staining process. *Nature, Lond.* **176**, 1145.
- VELICK, S. F. (1949). The interaction of enzymes with small ions. I. An electrophoretic and equilibrium analysis of aldolase in phosphate and acetate buffer. *J. Phys. Coll. Chem.* **53**, 135.
- WHEATON, E., BURROUGHS, J. D. & HAYS, G. L. (1957). Flat sour spoilage of tomato juice and its control with subtilin. *Food Tech.* **11**, 286.
- ZITTLE, C. A. & CUSTER, J. H. (1957). Electrophoresis of β -lactoglobulin in the presence of CaCl_2 . *Arch. Biochem. Biophys.* **71**, 229.

EXPLANATION OF PLATE 1

pH-gradient plates. Penassay seed agar, potassium phosphate buffers. pH values at right were determined at 24 hr. by inserting a glass electrode into the agar of uninoculated plates. Test organisms, and subtilin A concentration in surface layer are, from left to right. Upper row: *Staphylococcus aureus* H (8, 10, 12, 14 p.p.m.); *Bacillus cereus* var. *terminalis* (50, 60, 70, 80 p.p.m.); *S. aureus* S30b (3, 6, 10, 15 p.p.m.). Lower row: *Corynebacterium poinsettiae* (1, 2, 3, 4 p.p.m.); *B. megaterium* B-938 (1, 2, 3, 4 p.p.m.); *Streptococcus faecalis* (30, 40, 50, 60 p.p.m.).

(Received 6 June 1958)

L. E. SACKS & J. W. PENCE—pH AND ACTIVITY OF SUBTILIN A. PLATE 1

(Facing p. 550)