7. ———, *ibid.*, 1962, v138, 159.
8. Mott, J. C., Paintal, A. S., *Brit. J. Pharmacol.*, 1953, v8, 238.
9. Fastier, F. N., *Pharm. Rev.*, 1962, v14, 37.
10. Schneider J. A., Yonkman, F. F., *J. Pharmacol. and Exp. Therap.*, 1954, vIII, 84.
11. Armstrong, D., Dry, R. M. L., Keel, C. A., Markham, J. W., *J. Physiol.*, 1952, v117, 70P.
12. Fastier, F. N., MacDowall, M. A., Waal, H., *Brit. J. Pharmacol.*, 1959, v14, 527.
13. Burian, H. M., Fleming, W. J., Featherstone, R. M., *Fed. Proc.*, 1958, v17, 355.
14. Bradley, P. B., Kay, B. J., *Electroenceph. Clin. Neurophysiol.*, 1958, v10, 97.
15. St. Szara, Hearst, E., *Ann. N. Y. Acad. Sci.*, 1962, v96, 134.

Received February 18, 1963. P.S.E.B.M., 1963, v114.

Production of a Polysaccharide by *Staphylococcus aureus*. II. Effect of Temperature and Antibiotics. (28700)

GEORGE H. WARREN AND JANE GRAY
Research Division, Wyeth Laboratories, Inc., Radnor, Pa.

A previous study(1) has shown that various strains of *Staphylococcus aureus* possess a polysaccharide component which can be released from the staphylococcal cells into the surrounding medium by treatment with heat or with sonic disruption. Although chemical characterization of the polysaccharide is incomplete, its depolymerization by testicular hyaluronidase and not by bacterial hyaluronidases suggested a material not unlike chondroitin sulfate. However, the salient observation reported was the occurrence of a marked accumulation of the polysaccharide within cells of *S. aureus* grown in the presence of subinhibitory concentrations of penicillin G or nafcillin.

This report describes experiments which give further insight into the nature of the polysaccharide response, particularly as it influences the structural entity of the cell wall of *S. aureus*. These include the effect of lysozyme and trypsin on the cell walls of organisms grown in the presence of nafcillin and the rate of synthesis of the polysaccharide following exposure of growing cells of *S. aureus* to subinhibitory levels of a number of antibiotics. Observations were made also on the effect of subinhibitory concentrations of nafcillin on the cell structure of the organism following thermal stress.

Materials and methods. The materials and methods were for the most part described previously(1). The essential information is summarized as follows: The experiments described were performed with a penicillin-resistant strain (CHP) of *S. aureus* a penicillinase producer which is highly resistant to penicillin G, but susceptible to semi-synthetic penicillins nafcillin, oxacillin and methicillin (2,3). The organism was grown at 37°C on the surface of an agar medium in which all constituents except casein hydrolysate were chemically defined. For the studies on the effect of various antibiotics on polysaccharide production stock solutions in sterile buffered 0.85% salt solution were stored in a freezer (—20°C), thawed and final dilutions were prepared and added to previously liquefied medium immediately before use. Commercial preparations of nafcillin, oxacillin, methicillin, novobiocin, chloramphenicol, chlortetracycline, vancomycin, triacetyloleandomycin, bacitracin and erythromycin were used.

Blake bottles containing 200 ml of the agar medium were seeded with an 18-24 hour culture and incubated for 18 hours at 37°C. The highest concentration of each antibiotic incorporated into the medium which just permitted good growth to occur was selected for study. Cells were washed from the agar surface with distilled water, centrifuged and washed twice with distilled water. They were suspended in saline, standardized to contain 8.0×10^{10} cells/ml, autoclaved at 120°C for 10 minutes, cooled and centrifuged at

3000 rpm for one hour at 5°C. The supernatant fluids were then collected and assayed for polysaccharide by a turbidimetric procedure(4) in which the rate of depolymerization of the polysaccharide by hyaluronidase was used as an index of polysaccharide concentration. Bovine testicular hyaluronidase possessing 840 USP units per mg was employed.

The lytic action of crystalline egg white lysozyme (Armour Laboratories) and crystalline trypsin (Worthington Laboratories) was studied with the following types of preparations: (1) untreated bacteria suspended in 0.1 M phosphate buffer pH 7.0; (2) cell suspensions which had previously been grown in subinhibitory levels of nafcillin or novobiocin. Cells grown in the presence of antibiotics were harvested from the medium with distilled water and were centrifuged; the deposits were washed twice with distilled water and were finally suspended in 0.1 M phosphate buffer, pH 7.0.

The Klett-Summerson photoelectric colorimeter with a red filter (No. 66) was used throughout the investigation for measuring turbidities. For the turbidimetric study of lysis, suspension densities of nafcillin-treated and untreated bacteria were adjusted to give final Klett readings of 400 and were then placed in a water bath at 37°C. To these tubes 1.0 ml of lysozyme or trypsin in buffer or 1.0 ml of buffer was added and the contents thoroughly mixed. The tubes were incubated for various time intervals in a 37°C water bath and were then read for changes in optical density.

Electron micrographs were taken with an RCA EMU-3G Electron Microscope equipped with an objective aperture. Bacterial suspensions were washed twice with distilled water, suspended in saline and treated as previously described. A small drop of the suspensions, suitably diluted with distilled water was mounted on formvar coated grids and allowed to dry. Preparations were made at the initial magnification of either 8,500 × or 32,000 ×.

Results. It has been shown previously(1) that stress due to heat-treatment was required for release of the polysaccharide from normal cells of *S. aureus* and that cell concentration was critical in detecting significant levels. The fact that levels of polysaccharide were markedly increased upon growth of the organism in subinhibitory concentrations of nafcillin prompted further investigation of temperature as it influences the rate of release of the polysaccharide from cells exposed to this penicillin. *S. aureus* strain CHP was grown for 18 hours at 37°C in the presence of 0.1 μg/ml nafcillin and the release of the polysaccharide was followed with a standardized cell suspension, *i.e.*, 8×10^{10} cells/ml at 37°C, 60°C, 80°C, 100°C and 120°C. This concentration of cells gave no significant levels of polysaccharide when *S. aureus* was grown in the absence of nafcillin. The data illustrated in Fig. 1 show only small amounts of polysaccharide in the supernates of cell suspensions treated at 37°C and 60°C, whereas appreciable leakage from cells occurred at 80°C. The yields of polysaccharide progressively increased with increasing temperature.

The effect of subinhibitory concentrations of various antibiotics on release of polysaccharide from autoclaved suspensions of *S. aureus* was determined by extensive titration of the antibiotics at concentrations ranging from 0.001 to 0.6 μg/ml. (Figures in parentheses indicate the highest concentration of each antibiotic incorporated into the medium which permitted the accumulation of the highest concentration of polysaccharide under study.) The bacteria which had been grown in the presence of nafcillin (0.1 μg/ml), oxacillin (0.25 μg/ml), methicillin (0.5 μg/ml) and erythromycin (0.15 μg/ml) produced comparable polysaccharide responses which were of considerable magnitude (Fig. 2). Vancomycin (0.6 μg/ml), however, supported only a slight synthesis of the polysaccharide and chloramphenicol (0.5 μg/ml), bacitracin (0.6 μg/ml), chlortetracycline (0.5 μg/ml), novobiocin (0.025 μg/ml) and triacetyloleandomycin (0.3 μg/ml) had little or no effect.

Since the data presented suggested that subinhibitory concentrations of penicillin disorganized the structure of the bacterial cell wall to the extent where an increase in the release of the polysaccharide occurred, investigations were undertaken to determine the

influence of lysozyme and trypsin on the cells of *S. aureus* grown in presence of either nafcillin or novobiocin. Turbidity measurements performed on phosphate buffered (pH 7.0) control suspensions of *S. aureus* incubated for 3 hours at 37°C with either crystalline trypsin (100 μg/ml) or lysozyme (20 μg/ml) showed no significant differences from suspensions in buffer alone. Cells of *S. aureus* which were previously grown in the presence of nafcillin (0.1 μg/ml) showed no significant lysis on incubation with trypsin (100 μg/ml). However, partial lysis was observed following treatment with lysozyme (20 μg/ml). The lysis of nafcillin and novobiocin-treated cells was also investigated after incubation with

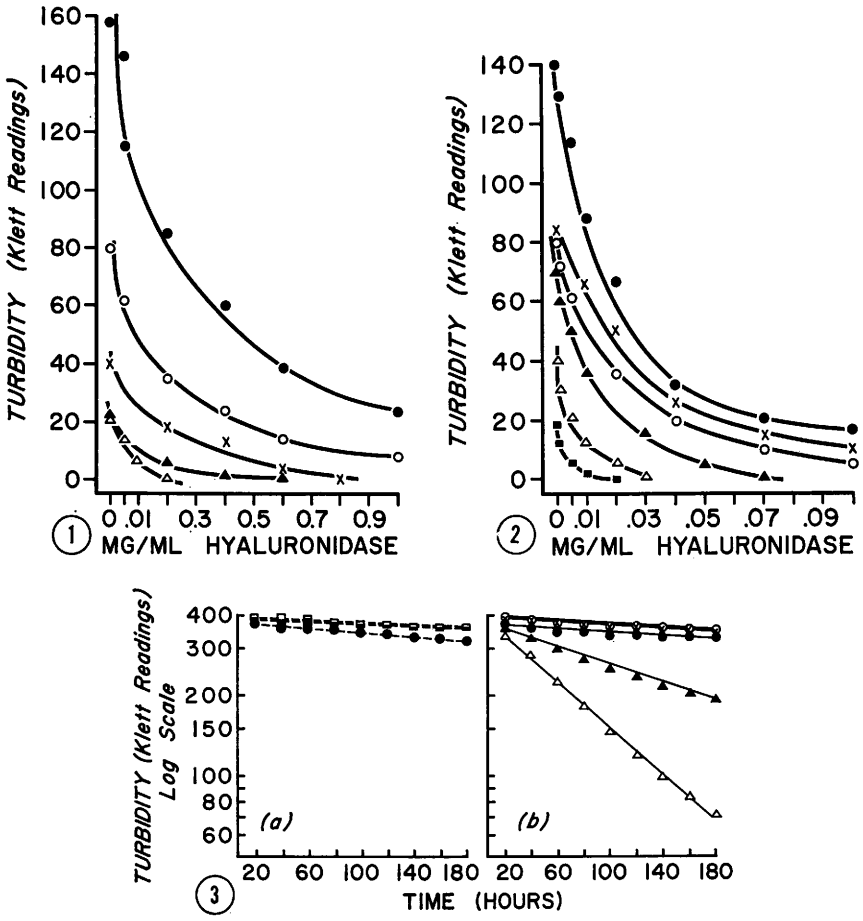

FIG. 1. Effect of temperature on concentration of polysaccharide released from *S. aureus* strain CHP grown in medium containing nafcillin (0.1 μg/ml). ●——●, 120°C for 10 min.; ○——○, 100°C for 30 min.; ×——×, 80°C for 30 min.; ▲——▲, 60°C for 30 min.; △——△, 37°C for 30 min.

FIG. 2. Concentration of polysaccharide in 18 hr culture of *S. aureus* strain CHP grown in medium containing subinhibitory concentrations of various antibiotics. ●——●, nafcillin (0.1 μg/ml); ×——×, methicillin (0.5 μg/ml); ○——○, oxacillin (0.25 μg/ml); ▲——▲, erythromycin (0.15 μg/ml); △——△, vancomycin (0.6 μg/ml); ■——■, polysaccharide control.

FIG. 3a, 3b. Enzymic lysis of nafcillin-treated cells of *S. aureus* strain CHP. ●------●, control cells incubated with lysozyme (20 μg/ml); □——□, control cells suspended in buffer (pH 7.0); ■——■, control cells incubated with trypsin (100 μg/ml); ×——×, novobiocin-treated cells incubated with lysozyme and trypsin; ○——○, nafcillin-treated cells suspended in buffer; ●——●, nafcillin-treated cells suspended in trypsin; ▲——▲, nafcillin-treated cells incubated with lysozyme; △——△, nafcillin-treated cells incubated with lysozyme and trypsin.

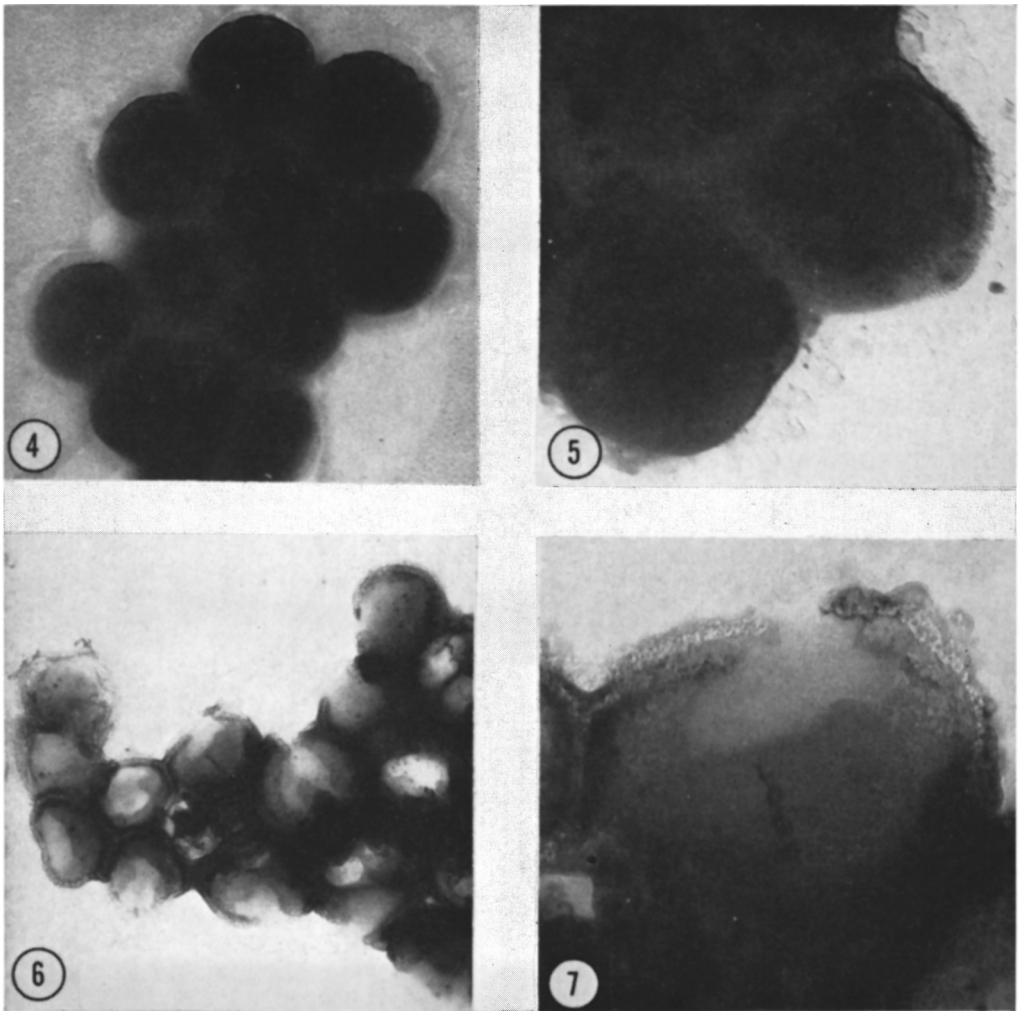

FIG. 4. Nafcillin-treated cells of *S. aureus* strain CHP. Magnification × 17,000.
FIG. 5. Heated cells of *S. aureus* strain CHP. Magnification × 64,000.
FIG. 6-7. The appearance of nafcillin-treated cells of *S. aureus* strain CHP after heat treatment. Fig. 6, × 17,000; Fig. 7, × 64,000.

lysozyme (20 μg/ml) and trypsin (100 μg/ml) for 3 hours. Addition of trypsin to suspensions of *S. aureus* and lysozyme resulted in rapid lysis of nafcillin-treated cells and a further decrease in turbidity. On the other hand, no lysis was obtained with lysozyme (20 μg/ml) and trypsin (100 μg/ml) when the cells were grown in the presence of novobiocin (0.025 μg/ml). The lysis curves are shown in Fig. 3a, b.

Electron microscopical observations of nafcillin-treated and autoclaved suspensions of *S. aureus* are shown in Fig. 4 and 5 respectively. The influence of thermal stress on the cell structure of nafcillin-treated cells can be readily seen in Fig. 6-7. Electron micrographs showed the cells that were treated independently with nafcillin and heat were dense and homogeneous. The pictures of the cells of *S. aureus* grown in the presence of nafcillin and subsequently subjected to heat indicate that such treatment resulted in cells with irregularities and destruction of the cell wall structure and with loss of electron-dense material.

Discussion. The presence of appreciable amounts of a polysaccharide in the supernates of suspensions of *S. aureus* grown in media

in the presence of subinhibitory concentrations of various penicillins and erythromycin demonstrate that they can serve in some manner in enhancing the polysaccharide content of the cell and making this constituent available for release when the cells are subjected to heat. That this is a metabolic activity of some significance is shown by the fact that insignificant levels of the polysaccharide were released from cells grown on antibiotic-free media and a progressive increase in accumulation of the polysaccharide occurred when nafcillin-treated cells were subjected to increasing temperatures. From these data it can be concluded that this penicillin produces a defect in the construction of the cell wall fabric of *S. aureus* causing an increase in permeability so that the polysaccharide is released following the stress due to heat.

The simplest hypothesis at the moment for the polysaccharide-enhancing properties of penicillins and other antibiotics is that this polysaccharide is normally incorporated into the mucopolysaccharide-peptide component of the cell wall and that these agents interfere with the cell wall synthesizing mechanism by preventing incorporation or transfer of the polysaccharide into the wall. Although Park and his co-workers(5-8) demonstrated that penicillin inhibits cell wall synthesis with concomitant accumulation of nucleotides and intermediates that are apparently essential for biosynthesis of cell wall material, the accumulation of these constituents has been shown only with inhibitory levels of penicillin far above minimal inhibitory concentrations.

It would seem that not every antibiotic with a specificity for inhibiting the strain of *S. aureus* studied enhances the production of polysaccharide. Although erythromycin and vancomycin were effective to a lesser extent than the penicillins, other antibacterial antibiotics, *e.g.*, bacitracin, chloramphenicol, novobiocin, chlortetracycline and triacetyloleandomycin were without a significant effect. These results demonstrate that the mechanism of action of certain antibiotics is not favorable for accumulation of the polysaccharide and that, in general, there appears to be a parallel between the ability of an antibiotic to interfere with bacterial cell wall synthesis *per se* and release of elevated concentrations of the polysaccharide from the cell. This explanation for the activity of penicillins is substantiated by electron microscopical studies of nafcillin in which *S. aureus* showed distinct damage to cell wall structures when the organism was grown in media containing subinhibitory concentrations and when these cells were then subjected to thermal stress.

Shockman and Lampen(9), employing *Streptococcus faecalis,* demonstrated a clear differentiation between penicillin which inhibited cell wall synthesis without preventing protoplast growth, and other antibiotics (*e.g.*, novobiocin) which inhibited protoplasts and intact organisms at the same concentrations. These authors concluded that the "primary attack of novobiocin and other antibiotics cannot be bacterial cell wall synthesis *per se,* but must be reaction(s) that is at least equally important to the growth of the protoplasts." It is interesting that with the exception of erythromycin, the same antibiotics (chloramphenicol, novobiocin, chlortetracycline, bacitracin, vancomycin, triacetyloleandomycin) which were effective in inhibiting protoplast growth within the range of concentrations that was effective against streptococci were unable to support the accumulation of significant concentrations of the staphylococcal polysaccharide.

Studies designed to elucidate the damage to the cell wall of *S. aureus* showed that the cells at subinhibitory levels of nafcillin were partially lysed following addition of lysozyme and that the concomitant action of trypsin with this enzyme further enhanced the lytic activity. Although, the enhanced trypsin lysis of cells of *S. aureus,* exposed to lysozyme, was demonstrated by other investigators(10,11), heated suspensions (100°C) were required to produce this activity. Since in the present experiments neither nafcillin, lysozyme, nor trypsin acting independently on the cells resulted in a significant lysis compared with the control suspensions, further evidence is thus available which substantiates the conclusion that nafcillin produced a disorganization of the cells rendering the cell walls susceptible to the lytic action of

lysozyme and thus further exposes the protoplasmic contents of the cell to the direct action of the proteolytic enzyme.

Summary. It has been shown that *S. aureus* grown in the presence of subinhibitory concentrations of nafcillin produced a disturbance or disorganization of the cell wall fabric resulting in a marked accumulation of a polysaccharide within the cell. An appreciable polysaccharide response was also found for oxacillin, methicillin and erythromycin. This conclusion is based on data which demonstrate a clear differentiation between such an inhibitor of bacterial wall synthesis as penicillin which exerted a pronounced effect on polysaccharide synthesis and other antibacterial antibiotics which failed to increase the quantity of polysaccharide formed. In addition, treatment with nafcillin rendered the normally lysozyme resistant cells of *S. aureus* susceptible to partial lysis by lysozyme and the concomitant addition of trypsin resulted in further dissolution of the cell bodies. Electron micrographs of such treated cells subjected to thermal stress provide further evidence of the relationship between accumulation of polysaccharide and actual interference with cell wall synthesis. The significance of the results relating to the mechanism of action of the semi-synthetic penicillin nafcillin and possibly other penicillins is discussed.

The author is indebted to Mr. James J. Byrd, RCA Laboratories, Camden, N. J., for electron micrographs.

1. Warren, G. H., Gray, J., PROC. SOC. EXP. BIOL. AND MED., 1963, v113, 251.

2. Rosenman, S. B., Warren, G. H., *Antimicrobial Agents and Chemotherapy,* 1961, *Am. Soc. Microb.,* 1962, p611.

3. ———, *ibid.,* 1962; 1963, p369.

4. Warren, G. H., Durso, J. G., Levin, N. R., *Endocrinol.,* 1948, v43, 48.

5. Park, J. T., Johnson, M. J., *J. Biol. Chem.,* 1949, v179, 585.

6. Park, J. T., *ibid.,* 1952, v194, 877, 897.

7. Park, J. T., Strominger, J. L., *Science,* 1957, v125, 99.

8. Park, J. T., *Biochem. J.,* 1958, v70, 2.

9. Shockman, G. D., Lampen, J. O., *J. Bact.,* 1962, v84, 508.

10. Webb, M., *J. Gen. Microbiol.,* 1948, v2, 260.

11. Salton, M. R. J., *ibid.,* 1953, v9, 512.

Received July 3, 1963. P.S.E.B.M., 1963, v114.

Asymmetrical Incorporation of C^{14} Acetate into β-Carotene Biosynthesized by *Phycomyces blakesleeanus.** (28701)

F. J. LOTSPEICH, R. F. KRAUSE, V. G. LILLY AND H. L. BARNETT

Departments of Biochemistry and Plant Pathology, West Virginia University Medical Center, Morgantown

Lotspeich *et al*(1) reported on the partial incorporation of C-1 and C-2 labeled acetate into the side chain of β-carotene by *Phycomyces blakesleeanus.* The evidence at that time indicated that the carbons of the ring system were more radioactive than those of the side chain. Furthermore, when C-2 acetate was utilized, radioactivity was found in the positions expected if carotene was formed by a head to tail condensation of acetate, as well as in the other positions. This was in contrast to the finding of Grob and Butler (2) that β-carotene synthesized by *Mucor hiemalis* from C-2 acetate contained activity only in positions expected if β-carotene was formed by a head to tail condensation of acetate. Goodwin's results(3) with *Phycomyces blakesleeanus* were similar to those of Grob and Butler(2).

In this paper we describe the incorporation of C-1 and C-2 labeled acetate into the ring system of β-carotene.

Materials and methods. Radioactive substrates 1-C^{14} acetate and 2-C^{14} acetate were obtained from commercial sources.

* This work was accomplished under A.E.C. contract and with the aid of a grant from Rockefeller Foundation.