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Production of a Polysaccharide by *Staphylococcus aureus*. I. Enhancement by Penicillins. (28333)

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

Previous investigations from this laboratory have demonstrated that various bacteria other than mucoid strains of Groups A and C streptococci synthesized mucopolysaccharides which were depolymerized by hyaluronidase(1-4). The results of these studies showed that mucopolysaccharides were not limited to the capsular structure of the organism as in streptococci but that substrates depolymerized by hyaluronidase may be synthesized by gram negative bacteria as endocellular and exocellular metabolic products.

Studies aimed at determining the role of polysaccharides of *Staphylococcus aureus* in resistance to penicillin revealed that a polysaccharide extracted from washed suspensions of staphylococci was depolymerized by bovine testicular but not by bacterial hyaluronidases. In addition, the production of this staphylococcal constituent was markedly increased when the organisms were grown in a medium containing sub-bacteriostatic amounts of potassium penicillin G. It seemed of interest, therefore, to study some of the properties of this polysaccharide and to ascertain the effect of potassium penicillin G and sodium nafcillin* on the accumulation of the polysaccharide in strains of *S. aureus*.

Materials and methods. Bacterial cells. The experiments described were performed with a penicillin-sensitive strain (GF7) and a penicillin-resistant strain (CHP) of *S. aureus*. The latter, a penicillinase producer, was highly resistant to potassium penicillin G but susceptible to sodium nafcillin(5). Steinman(6) found sodium nafcillin to be markedly resistant to destruction by staphylococcal penicillinase. Organisms were grown on the surface of agar in Blake bottles; each bottle contained the following medium: casein hydrolysate (enzymatic) 50 g; $Na_2HPO_4 \cdot 12H_2O$, 11.2 g; KH_2PO_4, 0.96 g; $MgSO_4$, 0.04 g; Ca-d-pantothenate, 1 mg; pyridoxine HCl, 1 mg; riboflavin, 0.1 mg; glycerol, 2.0 ml; agar, 3.0 g; distilled water, 200 ml. The medium was adjusted to pH 7.6-7.8 with 5N NaOH and sterilized by autoclaving at 120°C for 20 minutes. Following sterilization, the bottles were heavily seeded with an 18-hour nutrient agar slant culture of the organism taken up in 10 ml of saline and incubated for 18-24 hours at 37°C. *Quantitative determinations.* Since the polysaccharide gave a fairly stable colloidal suspension with dilute acidified horse serum (pH 3.1) similar to that produced by hyaluronic acid, a turbidimetric procedure(7) was employed in quantitating the polysaccharide. As a standard it was found convenient to dilute the polysaccharide with acetate buffer so that a turbidity of 100-150 scale divisions on the Klett colorimeter (red filter No. 66) was produced by the interaction of 1.0 ml of polysaccharide, 0.5 ml of 0.1 M acetate buffer pH 6.0, and 4.0 ml of a 1:40 dilution of acidified horse serum pH 3.1. The depolymerization of crude and partially purified polysaccharide by hyaluronidase was deter-

* Wy-3271 [6-(2-ethoxy-1-naphthamido)penicillanic acid].

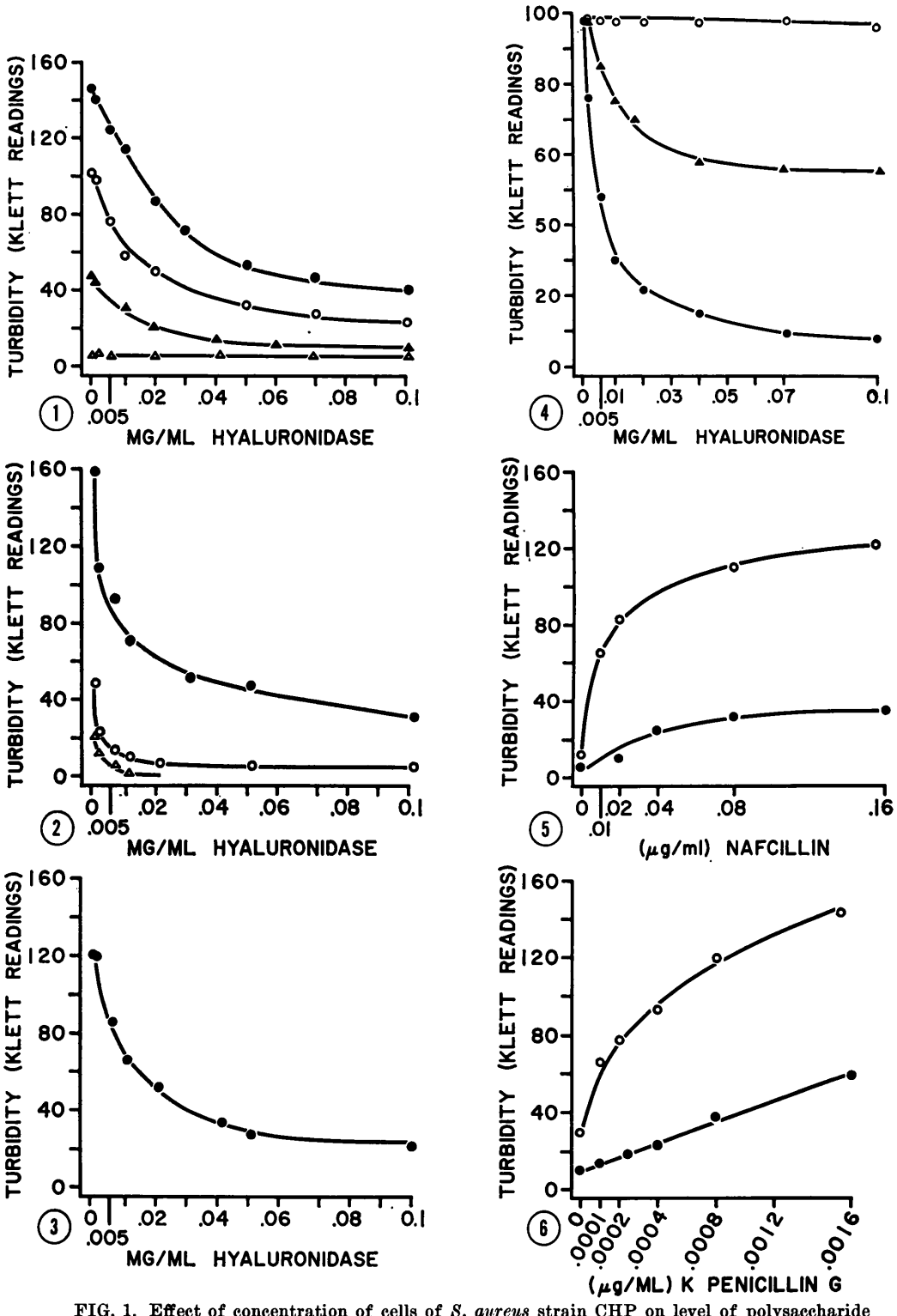

FIG. 1. Effect of concentration of cells of *S. aureus* strain CHP on level of polysaccharide released. ●——●, 1.4 × 10^{11} cells/ml; ○——○, 1.1 × 10^{11} cells/ml; ▲——▲, 8.7 × 10^{10}

mined by a turbidimetric method described previously(7). *Hyaluronidases.* Bovine testicular hyaluronidase and crude Group A *Streptococcus pyogenes* and *S. aureus* hyaluronidase were used in the depolymerization studies. The testicular, streptococcal and staphylococcal enzymes possessed 840, 110 and 65 USP units per mg, respectively. *Drugs.* Potassium penicillin G (Wyeth) and the semi-synthetic penicillin sodium nafcillin (Wyeth) were used. *Production of polysaccharide.* After 18 to 24 hours growth at 37°C the cells were harvested by washing from the agar surface with distilled water. Following centrifugation they were washed twice with distilled water. Cells were finally suspended in saline, standardized to contain 1.4×10^{11} cells/ml, autoclaved at 120°C for 10 minutes and centrifuged at 3000 rpm for 1 hour at 5°C. The deposit of cell debris was discarded, the supernatant fluid collected and treated with 3 volumes of acetone. A white precipitate which formed immediately was dissolved with distilled water, dialyzed against frequent changes of distilled water for 48 hours and then freeze-dried. The yield of polysaccharide from 100 bottles of culture medium averaged 1.5 g.

Comparable yields of the polysaccharide were readily obtained from washed bacteria treated in 5 ml batches in the Raytheon sonic oscillator operating at 250 W and at 10 kcyc/sec for 1 hour. The sonically-treated bacteria were then centrifuged at 10,000 rpm for 30 minutes, and the polysaccharide extracted from the supernatant fluids by the method described. However, since the sonic disintegration method did not lend itself to isolation of the polysaccharide on a production basis, no attempt was made to make a quantitative recovery of the polysaccharide by this procedure. *Chemical analysis.* The partially purified material was white, amorphous and readily soluble in water and gave a positive Molisch test. Nitrogen was determined by micro-Kjeldahl method and values of 12.4% were obtained. Glucosamine was determined by the method of Elson and Morgan(8) as modified by Rimington(9). Glucosamine content for the polysaccharide was 2.03%; phosphorus, 5.1%; sulfur, 2.6%. For acetyl determinations the polysaccharide was hydrolyzed for 1½ hours in 25 N sulfuric acid at 100° under a reflux condenser. The distillate was titrated against N/100 sodium hydroxide(10). This method gave acetyl values of 3.71%.

Results. Depolymerization of crude polysaccharide by hyaluronidase. During the early investigations with *S. aureus* polysaccharide, it became apparent that both penicillin-sensitive and penicillin-resistant strains produced the polysaccharide in presence of glycerol. However, comparable yields were obtained from glucose, galactose and sucrose. Media to which no carbohydrate was added also supported the synthesis of the polysaccharide but not to the same extent as in the presence of the carbohydrates. In addition only concentrated suspensions of autoclaved or sonically disrupted cells released detectable levels of the polysaccharide.

Fig. 1 shows the relationship between cell concentration and levels of polysaccharide as

cells/ml; △——△, 5.8×10^{10} cells/ml. Each system consisted of 1.0 ml of supernatant of autoclaved suspensions and 0.5 ml of various dilutions of testicular hyaluronidase. Incubated 30 min at 37°C. Reaction stopped and turbidity developed by addition of 4 ml of 1:40 acidified horse serum, pH 3.1.

FIG. 2. Effect of time of incubation of *S. aureus* strain CHP on synthesis of polysaccharide. ●——●, 24 hr; ○——○, 48 hr; △——△, 72 hr.

FIG. 3. Depolymerization of sonic released substrate by testicular hyaluronidase (1.4×10^{11} cells/ml).

FIG. 4. Depolymerization of partially purified staphylococcal polysaccharide by testicular hyaluronidase. The system consisted of 0.5 ml of 1.0 mg/ml polysaccharide and 0.5 ml of various dilutions of enzyme. ●——●, active enzyme; ▲——▲, enzyme + heparin (.01 mg/ml); ○——○, enzyme inactivated by heat.

FIG. 5. Concentration of polysaccharide in 48 hr culture of *S. aureus* strain CHP grown in medium containing various subinhibitory concentrations of nafcillin. ●——●, testicular hyaluronidase, 0.1 mg/ml; ○——○, polysaccharide control.

FIG. 6. Concentration of polysaccharide in 48 hr culture of *S. aureus* strain GF7 grown in medium containing various subinhibitory concentrations of K penicillin G. ●——●, testicular hyaluronidase, 0.1 mg/ml; ○——○, polysaccharide control.

revealed by depolymerization studies with testicular hyaluronidase. Results indicate no detectable levels of the polysaccharide at cell concentrations under 8.7×10^{10} cells/ml. There was a progressive increase in yield of polysaccharide with increase in cell concentration. Moreover, as the staphylococci grew, the polysaccharide recoverable from the supernatant fluid of autoclaved suspensions decreased and after 72 hours incubation no significant amounts of the polysaccharide were evident (Fig. 2).

In experiments where the staphylococcal cells were sonically disrupted, the yield of the polysaccharide and the pattern of depolymerization with hyaluronidase were similar to those reported for the autoclaved suspensions (Fig. 3).

Depolymerization of partially purified polysaccharide. Depolymerization of the partially purified polysaccharide by crude bacterial and purified testicular hyaluronidases was studied. Repeated attempts to demonstrate depolymerization in the turbidimetric assay procedure with streptococcal and staphylococcal enzymes were unsuccessful. However, the polysaccharide was readily depolymerized by testicular hyaluronidase. Fig. 4 illustrates the depolymerization of the polysaccharide by active and heat-inactivated (100°C- 20 minutes) testicular hyaluronidase. It is evident from this experiment that heat-inactivated hyaluronidase as shown by the inability of the enzyme to reduce the turbidity of vitreous humor potassium hyaluronate, did not depolymerize the substrate. Since these studies showed that the concentrations of hyaluronidase required to depolymerize the staphylococcal polysaccharide were greater than that required to depolymerize hyaluronic acid, it was of special interest to determine whether heparin,† a potent hyaluronidase inhibitor, would affect the rate of depolymerization of the bacterial polysaccharide. Fig. 4 shows that the enzyme reacts with the specific inhibitor and that there is marked decrease in extent of depolymerization.

† Liquaemin® sodium, Organon, Inc., West Orange, N. J.

Effect of penicillins on production of polysaccharide. It has previously been demonstrated that addition of subinhibitory concentrations of penicillin G to a growing culture of a hyaluronic acid-hyaluronidase producing strain of *Streptococcus pyogenes* resulted in a significant increase in hyaluronic acid content of the culture(11). It seemed of interest, therefore, to ascertain whether potassium penicillin G and nafcillin were similarly capable of increasing the synthesis of the staphylococcal polysaccharide.

The results of typical experiments designed to determine whether accumulation of the polysaccharide occurs following addition of various subinhibitory concentrations of the antibiotics are shown in Fig. 5 and 6. Cultures of *S. aureus* were incubated at 37°C for 48 hours and growth appeared as abundantly in the antibiotic-containing medium as in the antibiotic-free medium. The concentration of bacterial cells was standardized (4.4×10^{10}/ml) so that 1 ml of the supernatant of autoclaved suspensions gave no significant levels of polysaccharide. Polysaccharide production was markedly increased when a penicillin-sensitive strain of *S. aureus* was grown in the presence of penicillin G (Fig. 6). Similar results were found for the semi-synthetic sodium nafcillin and a penicillin-resistant strain (CHP) of *S. aureus*.

Discussion. It has been shown that a polysaccharide is released from heat-treated or sonically disrupted cells of *Staphylococcus aureus*. The fact that the polysaccharide may be detected only when concentrated suspensions of the organism are employed suggests that it is present in the cell in relatively small amounts. In experiments designed to characterize the polysaccharide it was found that the latter served well as a substrate for bovine testicular hyaluronidase. Further study revealed the inability of bacterial hyaluronidases to attack the crude or partially purified polysaccharide. This observation together with the fact that relatively large concentrations of testicular enzyme were needed to effect depolymerization of the polysaccharide would suggest a material not unlike chondroitinsulfate. However, evidence con-

sistent with this possibility must be treated with reserve until a more rigorously purified polysaccharide has been obtained and chemical characterization is more complete.

The experiments in which *S. aureus* was grown in the presence of subinhibitory concentrations of penicillin G and nafcillin gave results that indicated a marked enhancement of polysaccharide synthesis by the cells. This influence of penicillin on staphylococcal polysaccharide may be compared with the results obtained by Rosendal and Faber(11) on the effect of penicillin on production of hyaluronic acid by hemolytic streptococci. These investigators demonstrated that penicillin significantly increased the synthesis of hyaluronic acid. It was postulated that the antibiotic inhibited release of hyaluronidase from the cells or decreased production of the enzyme. Although the mechanism by which penicillin increased the level of staphylococcal polysaccharide is not known, the inability of staphylococcal hyaluronidase to depolymerize the polysaccharide might preclude the role of this enzyme in establishing the steady state of 2 opposing rates, *i.e.*, rate of synthesis and rate of depolymerization. On the other hand prolonged incubation of *S. aureus* in antibiotic-free media showed a progressive decrease in polysaccharide content of the cell and would appear to suggest that an enzyme system is involved in the breakdown of the polysaccharide. The characterization of such an enzyme and the biological significance of the polysaccharide await further study.

After the completion of this study, Clausen and Rosenkast(12), in a paper on acid polysaccharides in *S. aureus*, showed by chemical procedures the existence of hyaluronic acid and chondroitinsulfates in some groups of staphylococci. The polysaccharide described here may be similar or identical to the chondroitinsulfate described by these authors.

Summary. A polysaccharide has been isolated from heat-treated or sonically disrupted cells of *S. aureus*. The polysaccharide is readily attacked by bovine testicular but not by bacterial hyaluronidase. Under the influence of subinhibitory concentrations of penicillin G and nafcillin, the production of the polysaccharide is greatly increased. The possible influence of penicillin on a fundamental pathway or enzyme system involved in the breakdown of the polysaccharide is discussed.

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