ISOLATION AND PARTIAL CHARACTERIZATION OF A CAPSULAR MATERIAL FROM *STAPHYLOCOCCUS AUREUS*[1]

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Received for publication October 16, 1961

ABSTRACT

WILEY, BILL B. (University of Saskatchewan, Saskatoon, Sask., Canada) AND JANE C. WONNACOTT. Isolation and partial characterization of a capsular material from *Staphylococcus aureus*. J. Bacteriol. **83**:1169–1176. 1962.—An encapsulated strain of *Staphylococcus aureus*, which underwent a specific capsular reaction with homologous antiserum, was grown in a semisynthetic Casamino acids-glycerol broth. A fraction containing the capsular material was isolated from the culture supernatants. The preparation was antigenic in rabbits and absorbed the antibody responsible for both the passive protection of chick embryos and the specific capsular reaction in rabbit antiserum against the homologous organism. Chemically, the material was free of nucleic acids, contained 8% nitrogen, 4.25% total phosphorus, 26% hexosamine, and 26% reducing sugar. Acid hydrolysis yielded the amino acids glycine, alanine, glutamic acid, and lysine. Glucosamine and organic phosphate compounds were also present. Organic phosphate compounds were accounted for as glycerophosphate.

Encapsulation of *Staphylococcus aureus* was reported some years ago (Gilbert, 1931; Lyons, 1937). Klieneberger-Nobel (1948) was unable to demonstrate capsules in either mucoid or nonmucoid staphylococci by the specific capsular reaction test. Price and Kneeland (1954) observed an encapsulated variant of *S. aureus* derived from a parent strain isolated from a case of staphylococcal pneumonia. Morse (1960) reported the isolation of an extracellular substance, from culture supernatants of the Smith strain of *S. aureus*, which inhibited phagocytosis of the organisms by rabbit leukocytes in antistaphylococcal serum (Cohn and Morse, 1959; Morse, 1960). Sall, Mudd, and Taubler (1961) described a pseudoencapsulation phenomenon in *S. aureus* in a carefully defined medium. Fisher (1960) gave evidence for the presence of a heat-stable protective antigen from supernatant fluids of a virulent *S. aureus* grown in semisolid agar. Vaccines consisting of a soluble fraction obtained from washed staphylococcal cells by ultrasonic disruption (Stamp, 1961) have been shown to induce specific immunity in the absence of circulating antitoxin. Wiley (1961) observed the presence of capsules on a highly mucoid variant of a strain of *S. aureus*. This communication is concerned with the chemical and serological nature of this capsular material.

MATERIALS AND METHODS

Strain. The organism was a highly mucoid variant of *S. aureus* described in a previous communication (Wiley, 1961).

Cultivation. Tubes of a semisynthetic Casamino acids-glycerol broth previously described (Wiley, 1961) were inoculated with a single colony of the strain and incubated at 37 C for 12 hr on a Model C constant speed rotary shaker (New Brunswick Scientific Co.). These cultures were tested for encapsulation by a specific capsular reaction test. They were then aseptically transferred to 250-ml flasks containing 100 ml of the medium. These flasks were incubated in an identical manner for 18 hr and then aseptically transferred to 2-liter flasks containing 1 liter of the medium. Incubation continued under the same conditions. The acid produced by the organisms was neutralized twice daily by cautious addition of sterile 10% KOH. After 5 days of incubation, formalin was added to a final concentration of 0.5%. After contact with the formalin for 48 hr, the cul-

[1] The data for this publication were taken from a thesis submitted by Jane C. Wonnacott to the University of Saskatchewan in partial fulfillment of the requirements for the degree of Master of Science.

tures were tested for sterility by inoculation onto sheep-blood agar plates.

Preparation of capsular material. The techniques were empirical, but based on those employed for the isolation of the capsular material of the pneumococcus (Heidelberger, Kendall, and Scherp, 1936) and the meningococcus (Dingle and Fothergill, 1939).

Serological procedures. Hyperimmune antistaphylococcal serum was prepared by the method of Alexander (1940). The antibody titer was estimated by performing specific capsular-reaction tests on saline dilutions of the serum, using a 6-hr broth culture of the encapsulated *Staphylococcus.* The titer was taken as the highest dilution of antiserum capable of producing a specific capsular reaction.

Evidence that the isolated fraction contained the capsular material was supplied by the in vitro removal of the anticapsular antibody from rabbit serum. Antiserum (diluted 1:25 with saline) was absorbed with 60 and 100 μg of capsular material per ml of serum for 1 hr at 37 C, and then at 6 C for 48 hr. After centrifugation in the cold at 1,085 $\times$ *g*, the supernatant was titrated in the manner described for the determination of a specific capsular-reaction titer.

The serological activity of the purified material was measured by performing ring precipitin tests on saline dilutions of the antigen and undiluted homologous antiserum.

The antigenicity of the material was investigated in rabbits. Daily 1-ml injections (5.0 μg/ml in saline) were administered intravenously on four consecutive days per week.

The relationship between anticapsular antibodies and protection was studied by utilizing a passive protection test in chick embryos (Wiley, 1961). With this technique, it was possible to observe the removal of the protective antibody by small amounts of the purified capsular material. Samples of antiserum were absorbed with 10 to 50 μg of capsular material per ml of antiserum. Saline was added to the controls to compensate for any dilution of antibody. The capsular material and serum were allowed to interact for 1 hr at 37 C and stored for 7 days at 4 C. The serum was centrifuged in the cold at 1,085 $\times$ *g* for 20 min; the supernatant was removed and treated as described (Wiley, 1961) for intravenous injection into chick embryos (10 to 13 days old).

Agar diffusion. The double-diffusion technique of Ouchterlony described by Kabat and Meyer (1961) was employed to ascertain the minimal number of antigens in the partially purified capsular material.

Analytical procedures. Nitrogen was determined on 1-mg samples by a modification of the micro-Kjeldahl technique (Kabat and Meyer, 1961) using the distillation apparatus of Markham (1942).

Total and inorganic phosphorus were determined on 1-mg samples by a modification of the method of Fiske and SubbaRow (1925).

Nucleic acids were estimated by comparing the ultraviolet-absorption spectrum in the region of 260 mμ of a 0.005% aqueous solution of the unhydrolyzed material with that produced by a 0.001% aqueous solution of yeast nucleic acid (Nutritional Biochemicals Corp.). A Beckman DU spectrophotometer was used.

Reducing substances were estimated on acid hydrolyzates (1.5 N HCl, 100 C) of 1-mg samples of the material by a modification of the method of Hagedorn and Jensen (1923). Readings were made on a Bausch and Lomb Spectronic "20" spectrophotometer.

Chromatographic procedures. Samples (10 mg) of the material were hydrolyzed in 1.5 N HCl at 100 C for 5 hr. The acid was removed by distillation in vacuo in an all-glass system. The hydrolyzates were reconstituted to 10 mg/ml with distilled water and stored at 4 C.

Strips of Whatman no. 1 filter paper were

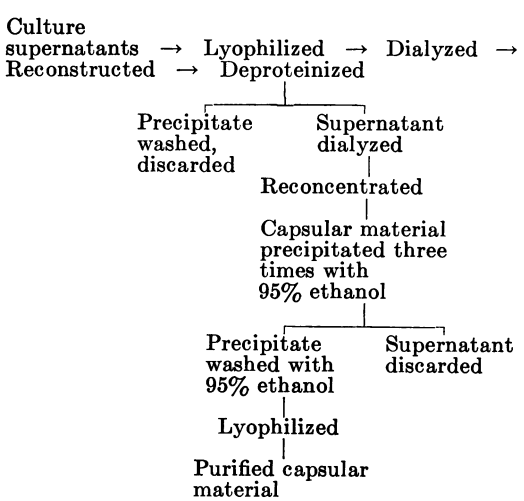

FIG. 1. *Procedure used for the isolation of a capsular material from Staphylococcus aureus.*

TABLE 1. *In vitro absorption of antibody from rabbit antiserum by purified capsular material*

Specific capsular reaction	Reciprocal of the dilution of antiserum					
	25	50	100	150	200	250
Using unabsorbed antiserum	+	+	+	+	−	−
After absorption using 60 μg/ml	+	−	−	−	−	−
After absorption using 100 μg/ml	−	−	−	−	−	−

washed in 0.05 N NH_4OH for analysis of the nitrogen-containing components. Sheets of the same paper, washed in 2 N acetic acid (Hanes and Isherwood, 1949), were used for investigation of the phosphorus-containing components. Unidirectional descending chromatography was employed.

RESULTS

Preparation of capsular material. Bacterial cells were removed from the pooled, formalin-killed cultures by centrifugation in a type KSA-1 Szent-Gyorgi and Blum 8-tube continuous-flow system with a Serval type SS1A superspeed centrifuge. The system was operated at 22,940 × *g*. The cell-free supernatants were treated as shown in Fig. 1.

The preliminary lyophilization was carried out in a Proctor-Schwartz freeze-drying unit with a 16-liter capacity. The material was dialyzed against distilled water, using dialysis tubing (1¾ in.). The dialyzed material was reconcentrated to 250 ml in a Rinco rotating vacuum-type evaporator. This reconcentrated solution was made 4% with respect to sodium acetate, and sufficient glacial acetic acid was added to lower the pH to 5.0. Chloroform and *n*-butanol were used for deproteinization (Heidelberger, Kendall, and Scherp, 1936). Addition of eight volumes of 95% ethanol to the upper layer precipitated the bulk of the material. Lyophilization of this material yielded an amorphous, buff-colored powder. A 1% aqueous solution of the product had a pH of 6.9.

In vitro inhibition of the specific capsular reaction. The results of the absorption experiments employing purified capsular material are shown in Table 1. The values represent pooled results from three separate experiments. The evidence supported the contention that the empirically isolated material contained the antigen specific to the antibody producing a specific capsular reaction, i.e., the capsular material of the organism (Fig. 2 and 3).

Serological activity. Ring precipitin tests were performed on unhydrolyzed and hydrolyzed (1.5 N HCl, 100 C) material. After observing the rings, the tubes were inverted several times, kept at 4 C for 12 hr, and then centrifuged for 15 min in the cold. Evidence of a precipitate was recorded. Table 2 shows the unhydrolyzed material to be serologically active to a concentration of 0.25 μg/ml. Acid hydrolysis for 2 hr resulted in a 400-fold decrease in serological activity.

Antigenicity. Intravenous injection of dilute solutions of the purified material produced a significant antibody response. Specific capsular reaction tests performed on the serum showed the titer to rise from 0 to 1:16 over a 2-month immunization period.

Removal of chick embryo-protective activity. Previous work (Wiley, 1961) showed that rabbit antistaphylococcal serum protects chick embryos (10 to 13 days old) against 10 to 100 LD_{50} of the encapsulated *S. aureus*. Absorption of this antiserum with minute amounts of purified capsular material, prior to injection into the embryo, lowered the protective activity of the serum (Table 3).

A statistical analysis of these and other results has been made to quantitate their significance. By the Chi-square method (Documenta Geigy, 1956), the removal of protection by 0.010 mg of capsular material is significant at the 1% level. Wiley (1961) showed that normal serum provided no significant protection against infection with this organism. Because the removal of protection at no time approached 100% of that afforded by the unabsorbed serum, the removal of the protective effect by whole cells of *Staphylococcus* was investigated in a similar manner (Table 4).

The *S. epidermidis* pool was a suspension of washed cells of three strains of *S. epidermidis*. Whole cells of the encapsulated strain of *S. aureus* removed over 80% of the protection from an antiserum, and cells of *S. epidermidis* failed to remove a significant amount. It is evident that the anticapsular antibody contributes to the

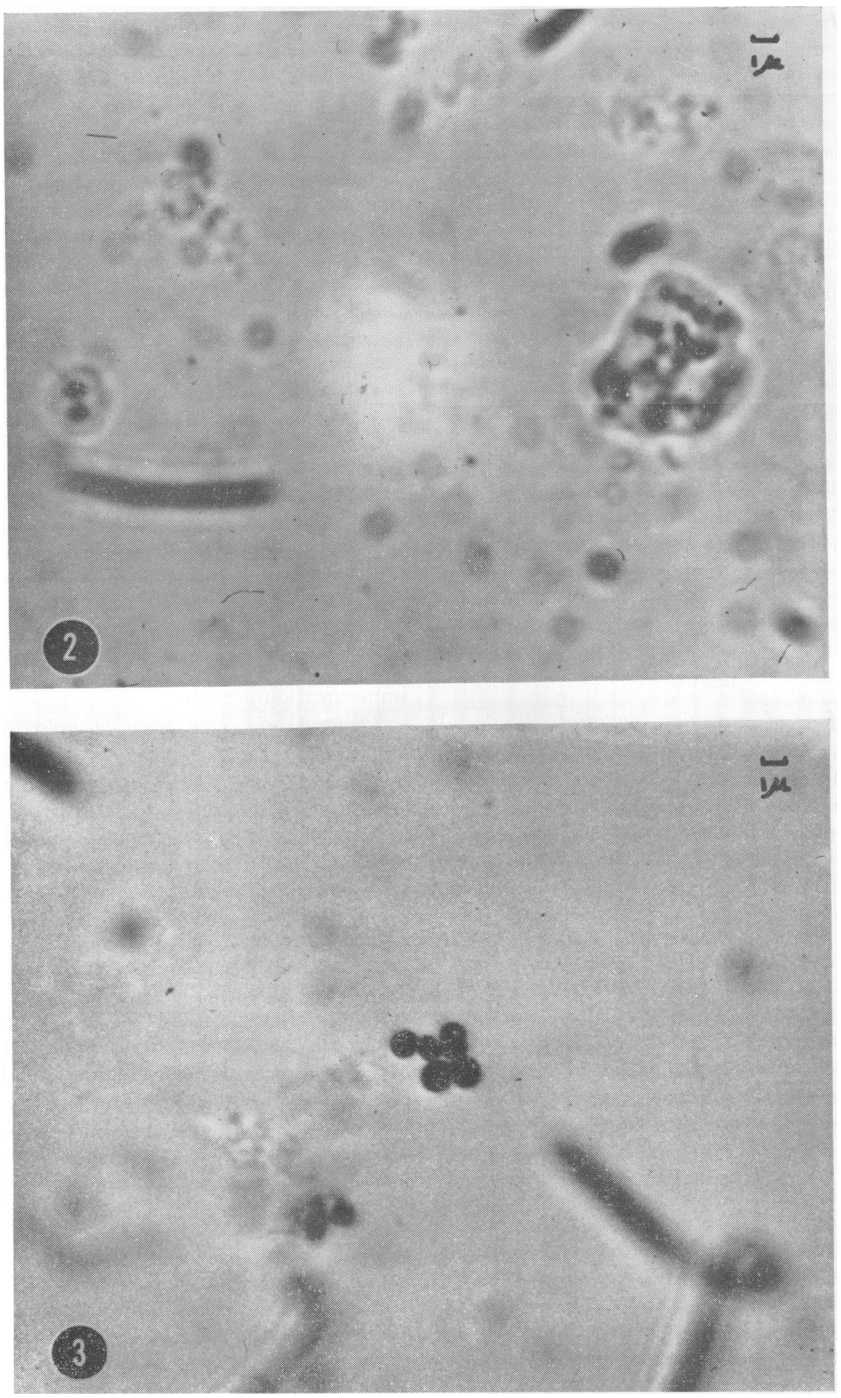

FIG. 2. *Encapsulated Staphylococcus aureus strain in the presence of immune serum*

FIG. 3. *Encapsulated Staphylococcus aureus strain in the presence of normal serum (previously absorbed with 0.5 ml of washed encapsulated S. aureus cells per milliliter).*

protection of the chick embryo against infection by an encapsulated *S. aureus* and that this protection may be significantly lowered by absorbing the serum with microgram quantities of purified capsular material.

Chemical investigations. Chemical determinations made on the purified material are summarized in Table 5.

The stability of the phosphate bonds was investigated by hydrolyzing 1-mg samples of the purified material in 1 N H_2SO_4 at 100 C. The release of inorganic phosphate is shown in Fig. 4. After 15 hr of hydrolysis, 61% of the total phosphorus content of the material was present as inorganic phosphate. Further hydrolysis failed to produce a significant alteration in this value.

Reducing sugar and hexosamine estimations (Boas, 1953; Elson and Morgan, 1933) gave optimal values after acid hydrolysis for 1 hr (Fig. 5).

Chromatographic analyses. 1) Ninhydrin-reacting components:—Three solvent systems were employed: *n*-butanol–acetic acid–water (Brimley and Barret, 1952); *n*-butanol–pyridine–water (Morrison, 1953); and phenol–ethanol–water–ammonia (Morrison, 1953). Spots were located with a 0.1% ethanolic solution of ninhydrin.

Unhydrolyzed material failed to react with ninhydrin after development in any of the solvent systems. Hydrolysis of the material for 5 hr

TABLE 2. *Serological reactivity of purified capsular material in the presence of antistaphylococcal serum*

Capsular material	Precipitates		
	Unhydrolyzed material	After 1 hr hydrolysis	After 2 hr hydrolysis
μg/ml			
1000	+	+	+
100	+	+	+
10	+	+	+
1	+	+	−
0.5	+	−	−
0.33	+	−	−
0.25	+	−	−
0.2	−	−	−

TABLE 3. *Protection and its removal in chick embryos by purified capsular material*

LD_{50} *S. aureus*	Capsular material/antiserum added	Total eggs	Deaths	Survivals
no.	*mg/ml*			%
10	0	14	4	71.7
10	0.010	15	10	33.3

TABLE 4. *Protection and its removal in chick embryos by whole cells of Staphylococcus*

LD_{50} *S. aureus*	Amount absorbing material per ml serum	Total eggs	Deaths	Survivals
no.				%
10	0	14	4	71.5
10	0.2 ml/ml*	10	9	10.0
10	40 mg/ml†	10	9	10.0
19	pool 0.2 ml/ml‡	14	4	71.5

* Wet cells of *S. aureus*.
† Dried cells of *S. aureus*.
‡ Wet cells of *S. epidermidis*.

TABLE 5. *Chemical characteristics of a capsular material isolated from a mucoid Staphylococcus*

Determination	Average value
	%
Nitrogen (micro-Kjeldahl)	8.02
Total phosphorus	4.25
Inorganic phosphorus	0.27
Organic phosphorus	3.98
Reducing sugar activity	26.2
Hexosamine	26.9
Nucleic acids	None

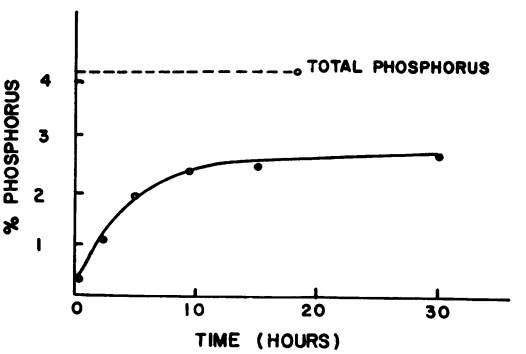

FIG. 4. *Rate of release of inorganic phosphate during acid hydrolysis of a capsular material from Staphylococcus aureus.*

released approximately 100% of the ninhydrin-reacting material. The calculated R_F values (cm) of the spots located by the spray are shown in Table 6.

A semiquantitative estimation of the molecular proportions of the amino acids present in the material was made using a Spinco Analatrol with a green filter. Chromatograms of hydrolyzed material (50 μg) were compared with 10 and 5 μg samples of the previously identified amino acids (Nutritional Biochemical Corp.) treated in an identical manner. Designating glutamic acid as 1, the amino acids glutamic acid, lysine, alanine, and glycine were found to be present in a molar ratio of 1:1:2:6.

2) Phosphorus-containing components:—Ring precipitin tests run on dilutions of the unhydrolyzed material and an antiserum known to contain antibodies to polyglycerophosphate (McCarty, 1959) showed the material to be serologically active at a concentration of 1 mg/ml. This led to the assumption that a polyglycerophosphate was present in our material.

Chromatographic studies were undertaken, using the ethyl acetate–pyridine–water solvent of Hanes and Isherwood (1949) and the propanol–ammonia–water solvent of Baddiley, Buchanan, and Carss (1958). Papers were sprayed with the ammonium molybdate spray described by Burrows, Gryees, and Harrison (1952). Confirmation of glycerol as a breakdown product of the polymer was made using ammoniacal $AgNO_3$ (Houghs, 1950). Treatment of the acid hydrolyzates with alkaline phosphatase (McCarty, 1959) was

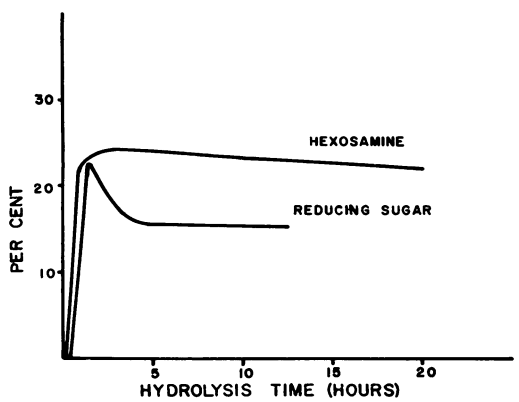

FIG. 5. *Reducing sugar activity and presence of hexosamine in acid hydrolyzates of a capsular material from Staphylococcus aureus.*

TABLE 6. *R_F values obtained from chromatographic studies on the ninhydrin-reacting components of a capsular material from Staphylococcus*

Chromatogram	Solvent system		
	n-Butanol: acetic acid: water	*n*-Butanol: pyridine: water	Phenol: ethanol: water: ammonia
Spot A	0.138	0.025	0.544
Lysine	0.138	0.025	0.558
Spot B	0.272	0.119	0.109
Glutamic acid	0.272	0.113	0.108
Aspartic acid	0.168	0.061	0.074
Spot C	0.219	0.142	0.274
Glycine	0.215	0.146	0.279
Spot D	0.288	0.238	0.425
Alanine	0.285	0.226	0.418
Spot E	0.303	0.421	0.430
Glucosamine	0.305	0.422	0.429

TABLE 7. *R_F values obtained from chromatographic studies on the phosphorus-containing components of a capsular material from Staphylococcus*

Chromatogram	Solvent system	
	Propanol: ammonia: water	Ethyl acetate: pyridine: water
Spot A	0.171	0.062
Phosphoric acid (H_3PO_4)	0.173	0.061
Spot B	0.303	0.028
α-Glycerophosphate	0.299	0.032
β-Glycerophosphate	0.298	0.030
Spot C	0.608	0.219

necessary prior to chromatographic examination. The release of glycerophosphate, inorganic phosphate, and an unidentified phosphorus-containing compound is illustrated in Table 7. Glycerol and ribitol compounds have been identified in our material through the kind cooperation of J. Baddiley, Newcastle upon Tyne, England (*personal communication*).

An indication of the minimal number of antigens present in the capsular material isolated was obtained by a double-diffusion technique in agar, described by Kabat and Meyer (1961). Undiluted antiserum was used in the center well, and capsular material, dissolved in saline at concentrations of 10, 1, 0.1, and 0.01 mg/ml, was used in the circumferential wells. Four bands

appeared in the agar between the central well and the wells containing 10 and 1 mg/ml of antigen. Only a single band formed when antigen was employed at a concentration of 0.1 and 0.01 mg/ml, respectively.

DISCUSSION

Although there are several reports in the literature on the occurrence of capsular material in *S. aureus* (Gilbert, 1931; Lyons, 1937; Price and Kneeland, 1954), there are no reports of any characterization of this substance. Our demonstration, that the material could absorb antibody producing the specific capsular reaction as well as significantly lower the passive protective properties of an antistaphylococcal serum, supports the hypothesis that our isolated material contained the capsular substance.

The chemical properties of the fraction varied slightly from those reported by Morse (1960) for a mucopeptide he isolated from culture supernatants of the Smith strain of *S. aureus*. His fraction contained seven amino acids, a hexosamine, and one or more unidentified nonreducing sugars. The phosphorus content of his fraction was 0.18%. By contrast, our material contained four amino acids, glucosamine, glycerophosphate, and an unidentified phosphate ester. The total phosphorus content of our fraction was 4.25%. The material isolated by Morse possessed antiphagocytic properties when tested in a system employing staphylococci, antistaphylococcal serum, and rabbit polymorphonuclear leukocytes. Our fraction was antigenic in rabbits, although that described by Morse failed to show antigenicity.

The amino acid content of our material expressed as molar ratios against glutamic acid is similar to that reported by Rogers and Perkins (1959) for the cell-wall amino acids of the Oxford strain of *S. aureus*.

The high phosphorus content of our material led to attempts to identify the phosphorus-containing component. An antiserum, known to react with polyglycerophosphate, reacted with our material at a concentration of 1 mg/ml. Chromatographic investigation of the hydrolyzed material confirmed the presence of glycerophosphate and also produced evidence for the presence of an unidentified phosphorus-containing compound, glycerophosphate, glycerol, and inorganic phosphate. Glycerol and inorganic phosphate are presumed to be hydrolysis products from the breakdown of polyglycerophosphate. Polyglycerophosphate has already been shown to be a common component of a variety of gram-positive organisms, including staphylococci (McCarty, 1939). Although McCarty presented evidence for the occurrence of polyglycerophosphate in the protoplasm of the cell, Baddiley (1958) has shown polyribitol phosphate or polyglycerophosphate to be present in the cell wall. There is evidence to suggest that the mucopeptide-polyglycerophosphate compound we have isolated is located in the cell wall since it inhibited the specific capsular reaction and diminished the passive protective activity of an antiserum against an encapsulated strain of *S. aureus*. Because the method of preparation was mild and no nucleic acids were detected in the product, autolysis of the cells was considered to be minimal. All the evidence obtained suggests a surface location for the mucopeptide-polyglycerophosphate fraction.

Evidence was obtained that the capsular material isolated may contain multiple antigens. At least four antigens may be present in the fraction isolated. The bands appeared only after the wells were refilled once and only where the capsular material was employed in a concentrated solution. Attempts will be made to further purify the capsular fraction, and these studies will be reported in future publications.

The purified material was not toxic for chick embryos 11 to 13 days old. If the material were found to be antigenic in humans, it might prove to be a successful immunizing agent in the stimulation of active immunity to staphylococcal infection. Fisher (1960) isolated a soluble fraction from culture supernatants of *S. aureus*. This fraction stimulated active immunity in mice to challenge injections of virulent *S. aureus*; only microgram quantities were required. Stamp (1961), working with rabbits, showed that soluble fractions prepared from weak alpha-toxigenic strains of *S. aureus* stimulated active immunity to challenge inoculations of the strains. At the time of challenge, the anti-alpha toxin content of the serum of the immunized rabbits was very low, indicating the protection observed was partially due to antibacterial factors.

ACKNOWLEDGMENTS

This work was supported by Canadian Public Health grant no. 607-7-19.

The authors wish to thank R. D. Comtois,

Ottawa, Ont., Canada, for bacteriophage typing the strain under study and Maclyn McCarty of the Rockefeller Institute for his generous gift of antiserum.

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