Studies on the Carbohydrate–Peptide Fraction of the Centrifugal Supernatants of *Staphylococcus aureus* Cultures*

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ABSTRACT: The carbohydrate–peptide fraction was obtained by repeated treatment, with cold trichloracetic acid and ethanol, of the condensed centrifugal supernatants of cultures of the viscid Wiley wound strain of *Staphylococcus aureus*. The partially purified starting material contained four immunologically active components, P1, P2, P3, and P4, which were distinguishable by immunoelectrophoresis with homologous hyperimmune antistaphylococcal rabbit serum. The positively charged components, P1 and P2, were separated from the negatively charged components, P3 and P4, in a DEAE-cellulose column (equilibrated with 0.02 M phosphate buffer, pH 7.2) by elution with the same buffer.

The P3 and P4 components remaining in the column were eluted with 0.4 M $(NH_4)_2CO_3$, or P3 alone was obtained by linear molarity gradient elution with NaCl in the same buffer. P3 and P4 were teichoic acid like substances, with positive reactions for organic phosphorus, reducing sugar activity, amino sugar, and free NH_2 groups. P4 was isolated from a mixture of P1, P2, and P4, obtained from a DEAE-cellulose column (equilibrated with 0.1 N NaOH and 0.1 M $NaHCO_3$) by linear molarity gradient elution with $(NH_4)_2CO_3$ in water. P1, which was positive only for the reaction for the free NH_2 group but not for the other three reactions, was isolated from the mixture with P2 by Sephadex G-50 gel filtration, in which P1 was eluted in the imbibed volume while P2 was eluted in the void volume. Each isolated component showed its corresponding single precipitin band in immunoelectrophoresis.

The extracellular carbohydrates of *Staphylococcus aureus* fall into two categories (Mudd, 1965): (1) the true capsular substance, characteristic of the Smith-type encapsulated strains (Fisher, 1960; Morse, 1962; Lenhart *et al.*, 1962; Koenig and Melly, 1965), and (2) the substances which yield the extracellular peripheral precipitation reaction[1] (Mudd and De Courcy, 1965) characteristic of the Wiley viscid "wound" strain (Wiley, 1961, 1963, 1964; Wiley and Wonnacott, 1962). The true capsular substance has been reported by Morse as the Smith surface antigen (SSA) (Morse, 1962), and also by Fisher as the staphylococcal polysaccharide antigen (SPA) (Fisher *et al.*, 1963). SPA has recently been characterized as consisting of D-glucosaminuronic acid and L-alanine with *O*- and *N*-acetyl substitution (Haskell and Hanessian, 1963, 1964; Hanessian and Haskell, 1964).

The extracellular substance of the wound strain has been found in our work using the double-diffusion technique (Hisatsune *et al.*, 1965) to contain at least four components. The further fractionation and isolation of these four components are presented in this paper.

Materials and Methods

Bacterial Strain Used. The strain of *S. aureus* used throughout this investigation was the Wiley wound strain (Wiley, 1961; Mudd and De Courcy, 1965). In practice, single colonies are transferred from stock agar plates of glycerol–nutrient agar (Wiley, 1961) to several bottles containing 200 ml of the corresponding broth, agitated at 37° for 24 hr, and then each one exhibiting the proper aggregated growth is transferred *in toto* to 6 l. of casamino broth (Wiley, 1961). These 6-l. suspensions of growing organisms are subsequently maintained under constant agitation at 37° and the pH is kept at approximately 7.0 by cautious periodic addition of 10% KOH. At the end of 72 hr of cultivation, formalin is added to a final concentration of 0.5%, and the formalinized cultures are allowed to stand for 72 hr at room temperature. They are then centrifuged at 2° and dialyzed against running tap water for 24 hr, followed by dialysis against polyvinylpyrrolidone (PVP), reducing the volume to approximately 150 ml (from 6 l.). The condensed fluid is centrifuged and the supernatant is lyophilized to dryness. This material is henceforth referred to in this report as the "crude" material. Average yield of the crude material from 6-l. cultures was 6.2 g.

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[1] Abbreviations used: DNFB, dinitrofluorobenzene; EPPR, extracellular peripheral precipitation reaction; SPA, staphylococcal polysaccharide antigen; PVP, polyvinylpyrrolidone; TCA, trichloroacetic acid.

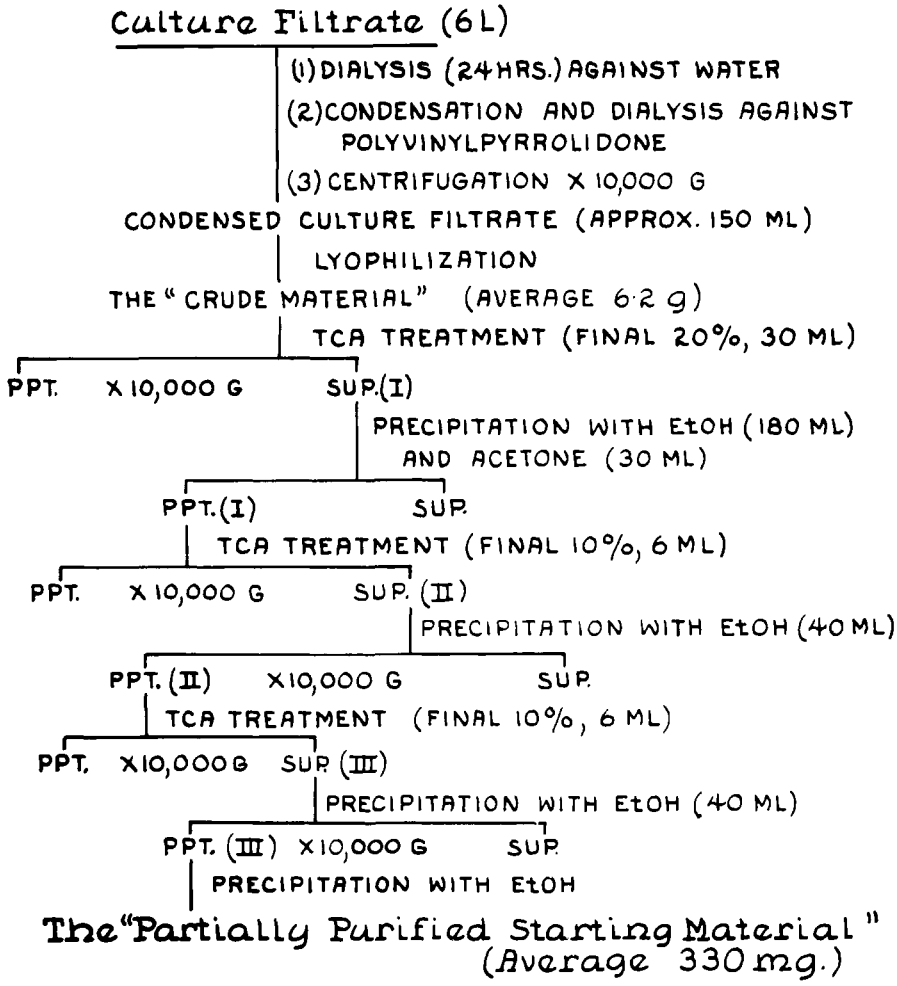

FIGURE 1: Flow diagram for the partial purification of the carbohydrate–peptide fraction of culture filtrates of *S. aureus*.

Immunization Program. Four-pound white male New Zealand rabbits were hyperimmunized *via* ear vein according to the protocol of Alexander *et al.* (1946) with formalin-killed Wiley strain cells. Following four full courses of immunization, the animals were trial bled (5 ml) by cardiac puncture and the sera were titrated against Wiley strain organisms as to their ability to evoke the EPPR (Mudd and De Courcy, 1965). A strong positive reaction in this test signaled the readiness of the animals for bleeding out.

Immunoelectrophoresis. In immunoelectrophoresis, 25 × 75 mm microscope slides overlayed with 2 ml of 2% Noble agar buffered with 0.1 M phosphate, pH 7.2, were routinely subjected to current for 30–60 min at 200 v, 25–35 ma, in 0.1 M phosphate buffer, pH 7.2.

Fractionation. All fractionations were made at 2°. DEAE-cellulose and Sephadex G-25 (medium grade) and G-50 (fine grade, bead type) were purchased from Pharmacia Inc., Rochester, Minn., and Bio-Gel P-2 (100–200 mesh) from Bio-Rad Laboratories, Richmond, Calif.

Chemical Analysis. TOTAL PHOSPHORUS was determined according to a modification of the method of Allen (1940). The test sample (1 or 0.2 ml) was heated with 0.8 ml of 60% perchloric acid at 150–160° for 3 hr. After the addition of 2 drops of 30% hydrogen peroxide, it was reheated for 90 min. After cooling, 1 ml of water was added and the mixture was heated in a water bath for 10 min to hydrolyze pyrophosphate which might have been formed during heating with the perchloric acid. The inorganic phosphorus estimation was made without any further addition of perchloric acid, and the appropriate amount of each reagent was adjusted to render the total volume of reaction mixture to 10 ml. Absorption was measured at 660 mμ or by using the Klett–Sommerson colorimeter (filter no. 660).

SUGAR ESTIMATIONS. All sugar estimation was done after hydrolysis with 2 N HCl at 100–102° for 2 hr

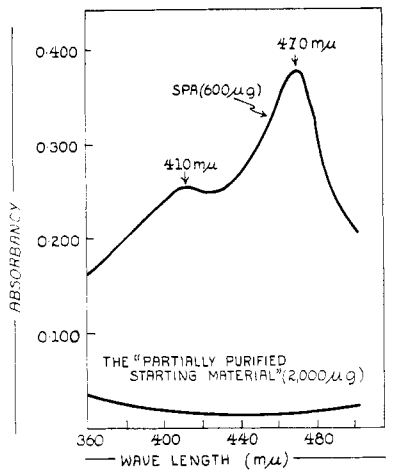

FIGURE 2: Comparison of absorption spectra of partially purified starting material and staphylococcal polysaccharide antigen by means of the specific color reaction for SPA. To the partially purified starting material (2000 μg) or SPA (600 μg) in 2 ml of water was added 4 ml of glacial acetic acid containing 10% concentrated H_2SO_4 (v/v); the solution was heated in boiling water for 30 min and cooled to room temperature. Absorption was measured against a blank solution which contained 2 ml of water and 4 ml of acid reagent and which was treated in the same way as the sample solutions.

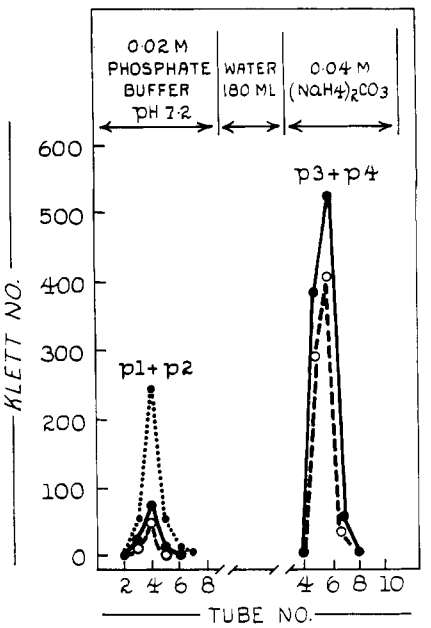

FIGURE 3: The separation of the positively charged components, P1 and P2, from the negatively charged components, P3 and P4, by the DEAE-cellulose column separation method. The partially purified starting material (330 mg in 2 ml of 0.02 M phosphate buffer, pH 7.2) was placed on the DEAE-cellulose column (1.5 × 22 cm, equilibrated with 0.02 M phosphate buffer, pH 7.2) and eluted successively with the same buffer (100 ml), water (180 ml), and 0.4 M $(NH_4)_2CO_3$ (100 ml) adjusted to pH 8.6 with HCl. Fractions (6.6 ml) were collected automatically at a flow rate of 0.22 ml/min. (●—●—●), reducing sugar activity. (O- - -O- - -O), amino sugar. (●····●····●), free NH_2 group.

in sealed tubes and evaporation of the acid *in vacuo*. *Amino sugar* was determined by the method of Belcher *et al.* (1954). Absorption was measured at 512 mμ or by using the Klett color filter no. 520. *Reducing sugar activity* was measured by the method of Momose *et al.* (1960a,b) using 3,5-dinitrophthalic acid monopyridium salt. To 0.5 ml of the sample solution, 0.5 ml of the alkaline solution, consisting of a mixture of 500 ml of 0.3% 3,5-dinitrophthalic acid pyridium and 500 ml of alkaline solution containing 125 g of sodium carbonate and 25 g of thiosulfate, was added, followed by heating in boiling water for 10 min. After the addition of water to a total volume of 5 ml, absorption was measured at 450 mμ or by using the Klett color filter no. 440. The 3,5-dinitrophthalic acid pyridium salt was supplied through the courtesy of Dr. Momose, Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmaceutical Science, University of Kyushu, Japan.

FREE NH_2 GROUPS were measured according to Ghuysen and Strominger (1963) using dinitrofluorobenzene (DNFB) with a slight modification. Absorption was measured at 420 mμ or by using the Klett color filter no. 420. The $(NH_4)_2CO_3$ contained in the sample was removed by repeated evaporation at 37° *in vacuo* over concentrated H_2SO_4 before estimation. Absorbancy measured by means of the Klett–Sommerson colorimeter is presented using "Klett number" in the figures.

THE EPPR was performed according to the method described by Mudd and De Courcy (1965).

THE "SPECIFIC COLOR REACTION" for SPA was done according to the method described by Haskell and Hanessian (1964). Standard SPA was supplied through the courtesy of Dr. Haskell, Research Division, Parke Davis and Co., Ann Arbor, Mich.

Results

Partial Purification of the Crude Material

Procedures including centrifugation were carried out at 2° or lower to avoid any possible degradation which might be caused by trichloracetic acid (TCA). The crude material, approximately 6.2 g/6 l., was dissolved in 20 ml of water and cooled in an ice bath. To this, 10 ml of cold 60% TCA was added dropwise with vigorous mixing. Heavy gummy precipitates resulted and were removed by centrifugation. Cold ethanol (180 ml) and acetone (30 ml) were added to the supernatant (supernatant I) and left for 30 min in an ice bath. The resultant white precipitate was collected by centrifugation and successively washed

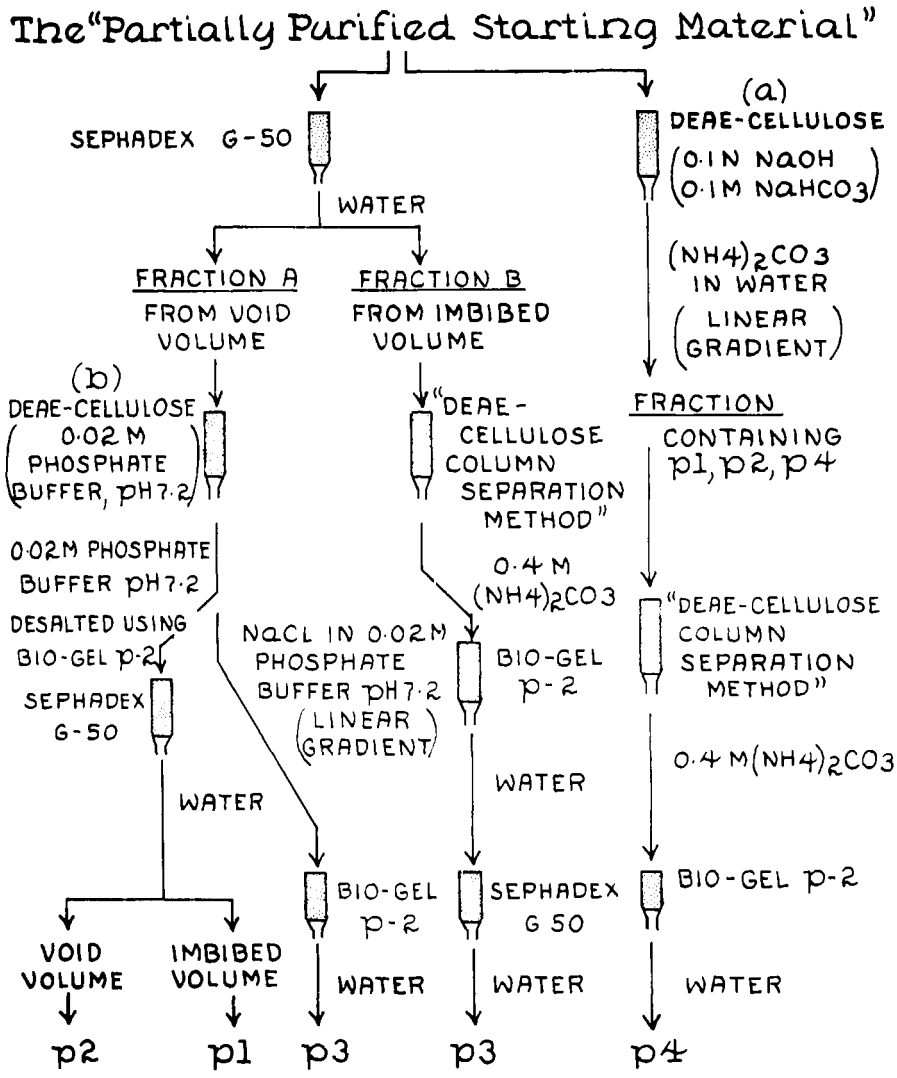

FIGURE 4: Flow diagram for the fractionation procedures for the partially purified starting material. (a) DEAE-cellulose column prepared by using 0.1 N NaOH and 0.1 M $NaHCO_3$. (b) DEAE-cellulose column equilibrated with 0.02 M phosphate buffer, pH 7.2.

with ethanol and ether to dryness. This precipitate (precipitate I) was dissolved in 3 ml of water, mixed with 3 ml of cold 20% TCA and left in the ice bath for 30 min. After centrifugation, the supernatant (supernatant II) was placed in the ice bath with 40 ml of cold ethanol and left standing for 30 min. The resultant white precipitate was collected and dried using ethanol and ether (precipitate II). This precipitate was dissolved in water, retreated with cold 20% TCA, and centrifuged. Cold ethanol (40 ml) was added to the supernatant (supernatant III). The resultant precipitate (precipitate III) was redissolved in a minimal amount of water and reprecipitated with cold ethanol. The harvested precipitate ("partially purified starting material") was dried and fractionated. The yield averaged approximately 330 mg. The summarized scheme of partial purification of the crude material is shown in Figure 1.

In immunoelectrophoresis, as shown in Figure 11a, the partially purified starting material interacted with antistaphylococcal (Wiley) serum showing at least four precipitin bands; two positively charged components were observed at the minus pole and two negatively charged components at the plus pole. They were designated as P1, P2, P3, and P4, respectively, as shown in Figure 11a. The positively charged component, P2, showed a rather heavy band near the central well.

The specific color reaction for SPA was tested on this starting material. As shown in Figure 2, the standard SPA (600 μg) gave the characteristic absorption spectrum, showing two peaks at 410 and 470 mμ (Haskell and Hanessian, 1964), whereas no corresponding peaks were observed using 2000 μg of the partially purified starting material.

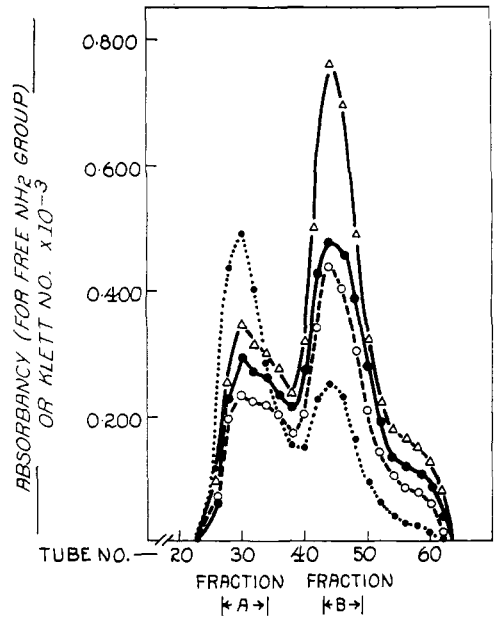

FIGURE 5: The elution pattern of Sephadex G-50 gel filtration of the partially purified starting material. The sample (330 mg in 3 ml of water) was placed on a Sephadex G-50 column (1.8 × 68 cm) and eluted with water. Fractions (approximately 3.0 ml) were collected at a flow rate of 0.3 ml/min. Symbols of Figures 5–10 inclusive: (△—△—△), organic phosphorus. (●—●—●), reducing sugar activity. (○- - - -○- - - -○), amino sugar. (●·····●·····●), free NH_2 group.

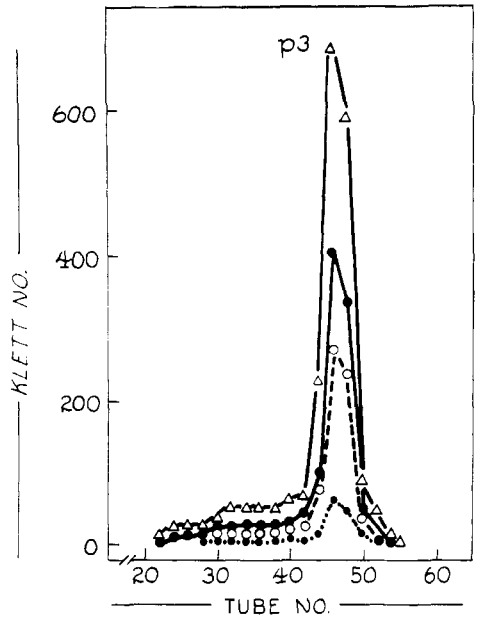

FIGURE 6: The elution pattern of Sephadex G-50 gel filtration of the P3 component. The sample (50 mg in 3 ml) was placed on a Sephadex G-50 column (1.8 × 68) and eluted with water. Fractions (3.0 ml) were collected automatically at a flow rate of 0.3 ml/min. Symbols are as in Figure 5.

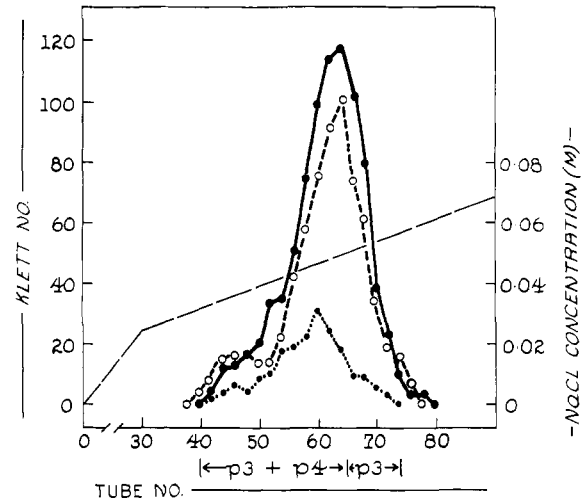

FIGURE 7: The elution pattern of DEAE-cellulose column chromatography of the negatively charged components, P3 and P4. After fraction A (172 mg) was placed on a DEAE-cellulose column (1.5 × 22 cm, equilibrated with 0.02 M phosphate buffer, pH 7.2) and eluted with the same buffer (100 ml), elution was effected with linear molarity gradient of NaCl by using 0.02 M phosphate buffer, pH 7.2 (500 ml), in the mixing vessel and 0.8 M NaCl in the same buffer (500 ml) in the reservoir. Fractions (4.0 ml) were collected automatically at a flow rate of 0.3 ml/min. Symbols are as in Figure 5. Diagonal line shows concentration of NaCl.

Separation of Oppositely Charged Groups

The negatively and positively charged groups were separated using a DEAE-cellulose column which was equilibrated with 0.02 M phosphate buffer, pH 7.2. The first step of the elution, done with equilibrating buffer, produced a fraction positive for the free NH_2 group, reducing sugar, and amino sugar (Figure 3). Immunoelectrophoresis showed two precipitin bands (the P1 and P2 components) at the negative pole (Figure 11b). After washing the column with approximately 180 ml of water, the second step of elution, using 0.4 M $(NH_4)_2CO_3$ adjusted to pH 8.6 with HCl, gave a fraction which was positive for the reducing sugar and amino sugar reactions. This fraction showed two bands (P3 and P4 components) at the plus pole with immunoelectrophoresis (Figure 11c). Desalting of this fraction was accomplished with Bio-Gel P-2 or with Sephadex G-25 gel filtration. This DEAE-cellulose column chromatography was used routinely for the separation of the oppositely charged groups throughout the following fractionating procedure, termed "DEAE-cellulose column separation method." Figure 4 summarizes the fractionation procedures described in the following text.

Isolation of the P3 Component

The partially purified starting material (330 mg) was

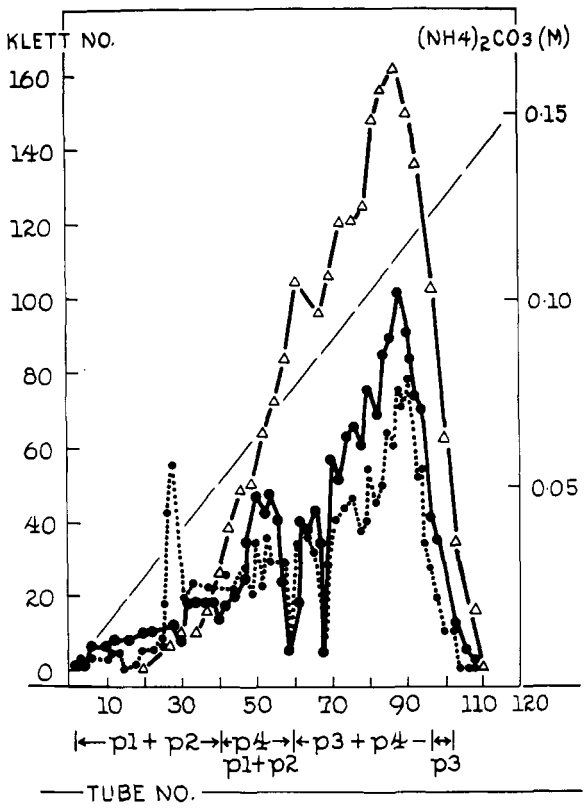

FIGURE 8: The elution pattern of DEAE-cellulose column chromatography of the partially purified starting material. The sample (350 mg in 2.0 ml of water) was placed on a DEAE-cellulose column (1.5 × 22 cm, prepared by using 0.1 N NaOH and 0.1 M $NaHCO_3$). After washing with water (100 ml), elution was effected with a linear molarity gradient of $(NH_4)_2CO_3$ by using water (500 ml) in the mixing vessel and 0.5 M $(NH_4)_2CO_3$ (500 ml) in the reservoir. Fractions (5.0 ml) were collected automatically at a flow rate of 0.6 ml/min. Symbols are as in Figure 5. Diagonal line shows concentrations of $(NH_4)_2CO_3$.

filtered on a Sephadex G-50 column (1.8 × 68 cm) using water as the effluent. Two fractions were eluted (Figure 5): "fraction A" in the void volume, and "fraction B" in the imbibed volume. Each of the fractions showed the same peaks for the chemical assays performed. Fraction A (172 mg) contains the four precipitin bands seen in the partially purified starting material, whereas fraction B (120 mg), containing the P3 component collected from tubes near the top of the peak, shows a single precipitin band which migrates rapidly toward the plus pole in immunoelectrophoresis. Fraction B (118 mg) was subjected to the DEAE-cellulose column separation method to remove the positively charged component which might possibly

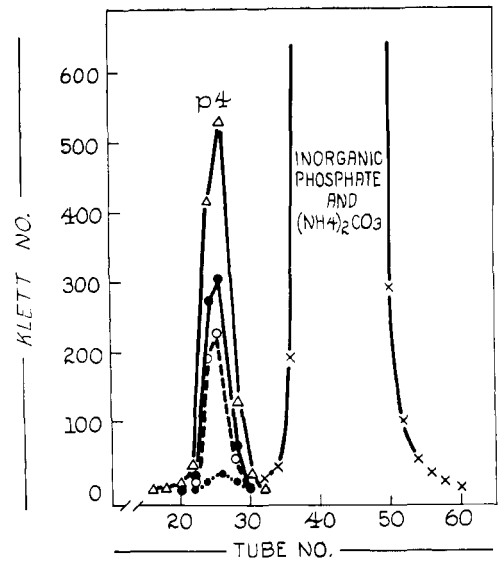

FIGURE 9: The elution pattern of Bio-Gel P-2 gel filtration of the P4 component. Fraction containing the P4 component, inorganic phosphate, and $(NH_4)_2CO_3$ (total 83 mg in 1.5 ml of water) was placed on a Bio-Gel P-2 column (1.5 × 44 cm) and eluted with water. Fractions (1.5 ml) were collected automatically at a flow rate of 0.36 ml/min. Symbols are as in Figure 5.

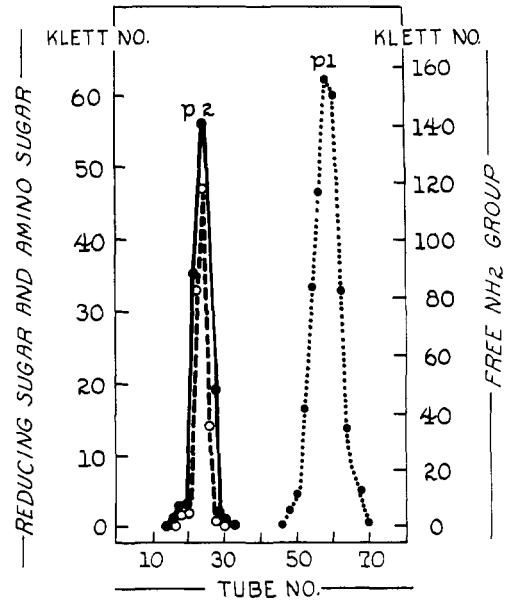

FIGURE 10: The elution pattern of Sephadex G-50 gel filtration of the positively charged group, a mixture of the P1 and P2 components. The sample (68 mg in 3 ml of water) was placed on a Sephadex G-50 column (1.8 × 66 cm) and eluted with water. Fractions (2.8 ml) were collected automatically at a flow rate of 0.3 ml/min. Symbols are as in Figure 5.

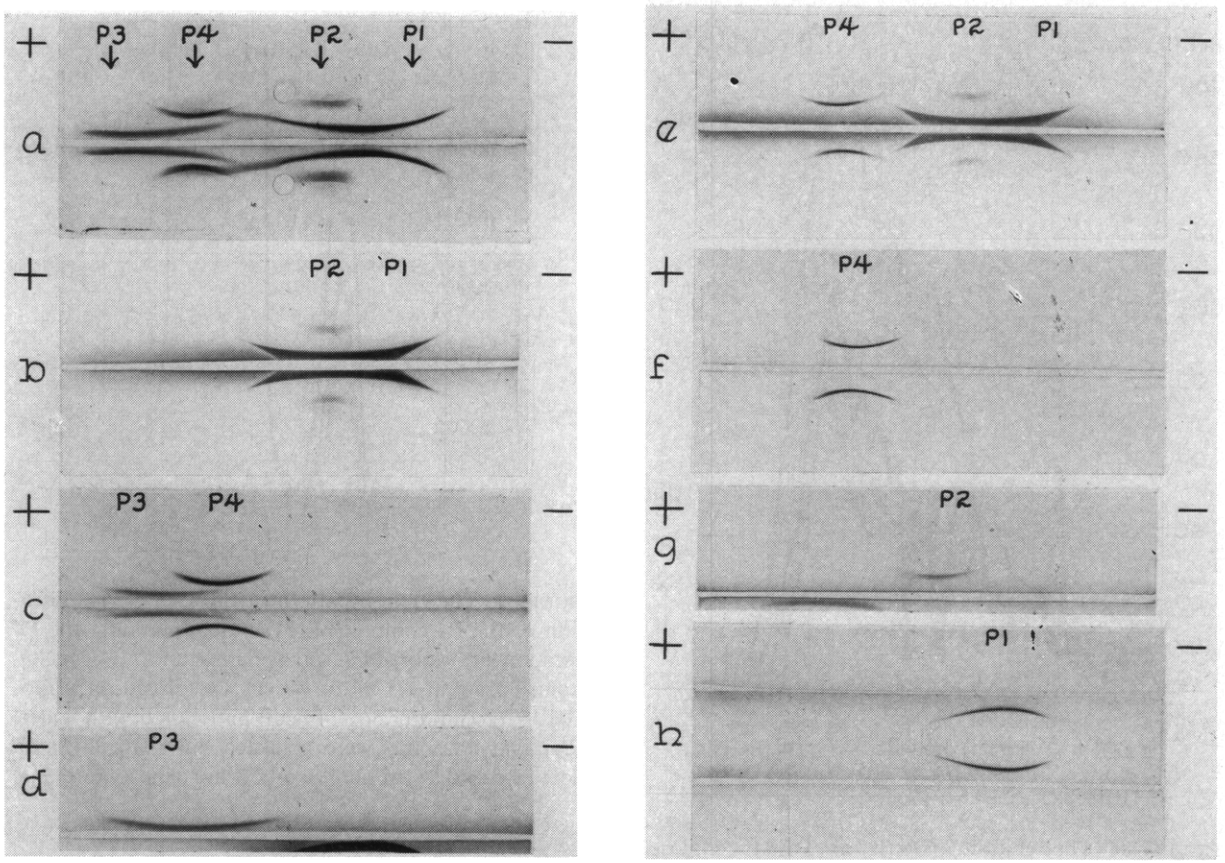

FIGURE 11: Immunoelectrophoretic patterns of the components with antistaphylococcal (Wiley) serum. The following antigen solutions (1 mg/ml) were placed in the wells: (a) The partially purified starting material. (b) The positively charged group (P1 and P2). (c) The negatively charged group (P3 and P4). (d) P3 (the band appears above the trough). (e) A mixture of P1, P2, and P4. (f) P4. (g) P2 (the band appears above the trough). (h) After completion of electrophoresis (0.1 M phosphate buffer, pH 7.2, 200 v, 35 ma for 1 hr), undiluted antistaphylococcal (Wiley) serum was added to each trough.

have been present, and further refiltered through Sephadex G-50 (Figure 6) after desalting using Bio-Gel P-2. In chromatograms, this fraction showed the same peak for all four chemical assays, and a single precipitin band was obtained with immunoelectrophoresis (Figure 11d) (yield, 18 mg).

The P3 component was also isolated from fraction A using the DEAE-cellulose column separation method. The positively charged components were at first removed by elution with 0.02 M phosphate buffer, pH 7.2. The negatively charged components remaining in the column were eluted by a linear molarity gradient elution of NaCl (0–0.08 M) in the same buffer (Figure 7). The fraction collected from tubes 40 to 65 contained a mixture of components, P3 and P4, while the fraction from tubes 66 to 74 contained only the P3 components (Figure 11d). The P4 component could also be isolated by DEAE-column chromatography using a linear molarity gradient of $(NH_4)_2CO_3$ as described below.

Isolation of the P4 Component

The DEAE-cellulose column (1.5 × 22 cm) was prepared according to the procedure of Tenner *et al.* (1958) using 0.1 N NaOH and 0.1 M $NaHCO_3$ solutions. The partially purified starting material (350 mg in 2 ml of water) was applied to the column. After washing with water (100 ml), elution was effected with a linear molarity gradient of $(NH_4)_2CO_3$ in water (0–0.15 M). As seen in Figure 8, many irregular peaks were obtained in the chromatogram. Immunoelectrophoresis gave the following results. Pooled fractions 1–40 were found to show precipitin bands for P1 and P2 components, pooled fractions 41–58 the P1, P2, and P4 components (Figure 11e), pooled fractions 59–95 the P3 and P4, and pooled fractions 96–101 only the P3 component. After repeated evaporation *in vacuo* over concentrated H_2SO_4 at 37° to remove $(NH_4)_2CO_3$, pooled fractions 41–58 were subjected to the DEAE-cellulose column separation method to separate the P1 and P2 components from the P4 component (Figure 11f). The isolated P4 component was desalted by filtration through a Bio-Gel P2 column giving a peak which was positive for organic phosphorus, free NH_2 group, reducing sugar activity, and amino sugar reactions in the chromatogram (Figure 9) (yield,

5.5 mg). In immunoelectrophoresis, the P4 component showed a single sharp precipitin band which moved toward the positive pole, but its mobility was less than that of the P3 component (Figure 11f).

Isolation of the P1 and P2 Component

As described above, the P1 and P2 components could be separated from the P3 and P4 components on DEAE-cellulose (Figure 3). After desalting, using Bio-Gel P-2, the mixture of the P1 and P2 components (68 mg) was again filtered through Sephadex G-50 under the conditions described above. The two positively charged components were completely dissociated (Figure 10). The first fraction, which was predominantly positive for reducing sugar activity and amino sugar reactions, was eluted in the void volume. This fraction showed the precipitin band of the P2 component near the center well in immunoelectrophoresis (Figure 11g) (yield, 1 mg).

The second fraction, which was positive only for the free NH_2 group reaction, was eluted in the imbibed volume. Refiltration using the same column gave a single sharp peak identical with that of the first gel filtration. In immunoelectrophoresis, the fraction also showed a single sharp precipitin band of the P1 component which moved rapidly toward the negative pole (Figure 11h).

Extracellular Peripheral Precipitation Reaction

It was found that each of the isolated components alone failed to show complete absorption when anti-staphylococcal (Wiley) serum was absorbed with them to test the EPPR (extracellular peripheral precipitation reaction), whereas absorption with the crude material inhibited the EPPR. The EPPR seems, therefore, not to be attributable to any one component alone, but to the components acting together.

Discussion

Since the partially purified starting material was obtained after repeated treatment with cold TCA, it is considered to be virtually free of protein and is termed the carbohydrate–peptide fraction. It is likely that the molecular size of the P1 component is small, judging from its chromatographic properties on Sephadex G-50 (exclusion limit molecular weight 10,000). Originally, it was eluted in the void volume with fraction A (Figure 5). This behavior suggests the possibility that the P1 component forms a complex with the oppositely charged P3 or P4 components.

Evidence as to the purity of the P1 and P3 components is based on the results of immunoelectrophoresis and gel filtration. The electrophoretic mobilities of the P1, P3, and P4 components indicate a considerable net charge.

A considerable amount of organic phosphorus was found in the P3 and P4 components. It is likely that the net negative charge of these components is attributable to this organic phosphorus. A preliminary chemical analysis of the acid hydrolysate of the P3 component revealed the presence of organic phosphorus, glucosamine, alanine, and a polyol component which is not identical with ribitol, but, rather, more likely with glycerol. These results are compatible with the interpretation that the P3 component is a type of teichoic acid, conceivably a glycerol type.

Although a complete separation of the two negatively charged components has been affected, the yield of the P4 component was very small. Preliminary chemical analysis suggests that the P4 component is also a type of teichoic acid. A positive reaction to the free NH_2 group of the P3 and P4 components with DNFB is probably attributable to the free NH_2 group of alanine combined through an ester linkage with teichoic acid.

The heavy, thick precipitin band of the P2 component in immunoelectrophoresis for the partially purified starting material suggests heterogeneity. The hydrolysate of this component showed reducing sugar activity which could be accounted for by the amino sugar content.

A considerable yield of the P1 component was obtained in this fractionation. In the acid hydrolysate of this component, neither organic phosphorus nor sugar components were detected. In a preliminary communication (Hisatsune *et al.*, 1966), it was reported to be a peptide composed of only four amino acids: D- and L-alanine, D-glutamic acid, lysine, glycine, with NH_3 (molar ratios, 2:1:1:5:1). Based on the amino acid composition and the optical configuration study of the component amino acids, it is concluded that the P1 component is a cell wall peptide polymer of *S. aureus* which possesses immunological activity. The positive reaction to the free NH_2 group test was found to be attributable to the free NH_2 group of the N-terminal alanine residue of this peptide (Hisatsune *et al.*, 1967).

In the present study, a peptide component, the P1, has been isolated with three other components from the culture filtrate of the viscid Wiley wound strain of *S. aureus* and shown to be immunologically active. A detailed study of this immunologically active peptide, P1, is reported in the following paper (Hisatsune *et al.*, 1967). Further chemical and immunological characterizations of the other isolated components are also under investigation.

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