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STUDIES ON THE PROTEIN MOIETY OF ENDOTOXIN

FROM SERRATIA marcescens 08

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STUDIES ON THE PROTEIN MOIETY OF ENDOTOXIN FROM *SERRATIA marcescens* 08

CHAPTER I

INTRODUCTION

A common feature of Gram-negative bacteria is the occurrence of endotoxins or somatic O antigens in their cell walls. Much attention has been given to the chemical composition and morphological structure of Gram-negative cell walls (1-13) and the general structure is fairly well understood.

Electronmicroscopic pictures of the cell wall suggest a three-layer structure (2, 6, 9, 10). The innermost or rigid layer surrounding the cytoplasmatic membrane consists of a "bag-shaped" macromolecule murein insoluble in alkali and phenol and constituting 0.1-10% of the cell dry weight (15). This rigid layer in turn is enveloped by a so-called "soft layer" consisting of lipopolysaccharide (1-5% of the cell dry weight), phospholipid (5-15% of the cell dry weight), and protein (15). The "soft layer," according to electronmicroscopic studies (10) is actually composed of a middle layer consisting of lipopolysaccharides, and an outermost layer thought to be a lipoprotein distributed over the cell surface in patches and sausage-like structures.

It has been generally accepted, that the endotoxins of many

different species of Gram-negative bacteria represent in their intact state macromolecular entities composed of lipopolysaccharide (LPS), protein, and a loosely bound phospholipid, the so-called lipid B (16), as schematically shown below in Figure 1:

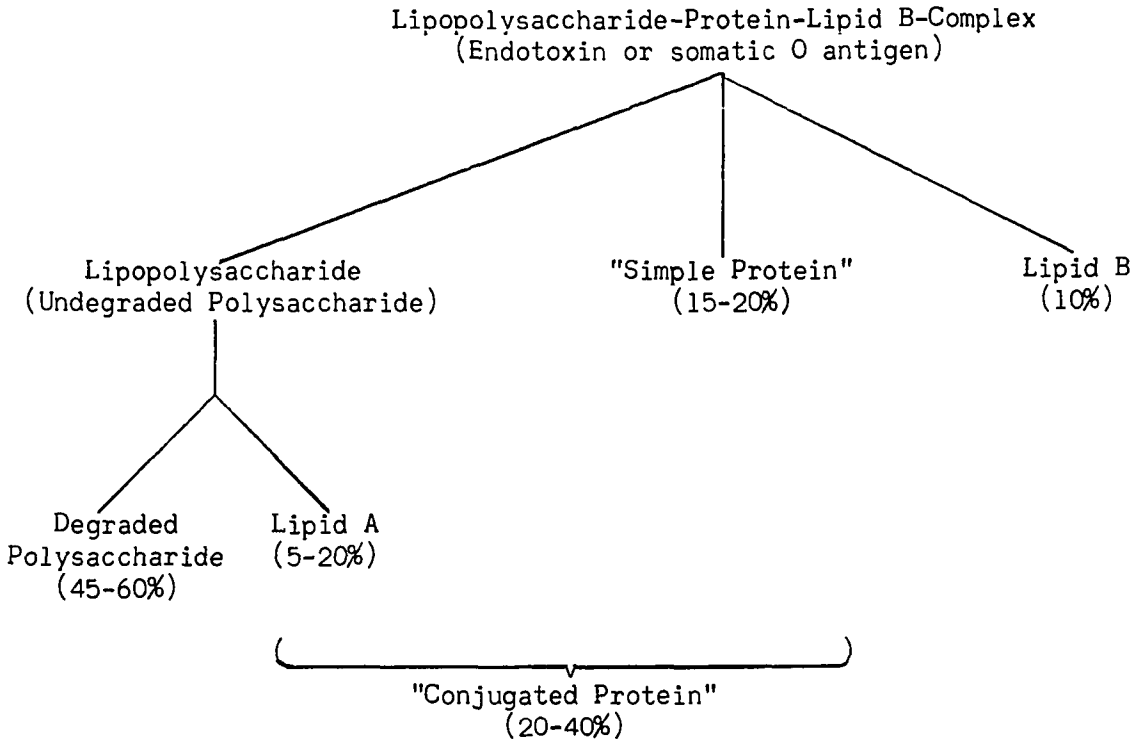


Figure 1. Components of the endotoxin complex according to Westphal and Lüderitz (16) (modified).

A variety of mainly hydrolytic procedures permits the dissociation of endotoxin into its major components.

Treatment of the endotoxin complex with hot phenol-water (17) results in the separation of the lipopolysaccharide and the protein moiety. This dissociation can be achieved also by extraction of whole cells (17) or isolated cell walls (14, 18). Mild alkali treatment, followed by ethanol precipitation yields similar results (19). The lipopolysaccharide can be further dissociated into the polysaccharide moiety and

lipid A by mild acid hydrolysis (20). Heating a 1% acetic acid solution of endotoxin complex separates the "degraded polysaccharide" from the "conjugated protein" (19, 21). Lipopolysaccharides exhibit a variety of biological effects as toxicity, antigenicity and pyrogenicity. Chemically, they can be classified as phosphorus-containing heteropolymers, consisting of a lipid moiety (lipid A), supposedly covalently linked via 2-keto-3-deoxyoctonic acid (KDO) to the core part of the polysaccharide moiety (22, 23) as schematically shown in Figure 2.

The polysaccharide moiety of a wide variety of Gram-negative bacteria has been the subject of extensive investigations by many research groups and is thus far the best known part of LPS.

Whereas the lipid and protein moieties of the O-antigens of various Gram-negative bacteria seem to be chemically similar, there is a wide variation in the sugar composition and sequence of the polysaccharide moieties (15). The polysaccharide moiety represents the carrier of serological O-specificity, and studies of the sugar composition and sequence demonstrated a correlation between structure and serological specificities. Later, it was recognized that discrete structural units exhibited in the side chain were responsible for the serological specificity (15).

Thus far, more than 20 sugars have been found as constituents of the O-specific polysaccharides, namely, hexoses, 6-deoxyhexoses, 3,6-dideoxyhexoses, heptoses, hexosamines, 2-keto-3-deoxyoctonic acid (KDO), and in a few cases also pentoses (15). In no case has neuraminic acid, a specific constituent of murein, been found as a true component of an O-specific polysaccharide.

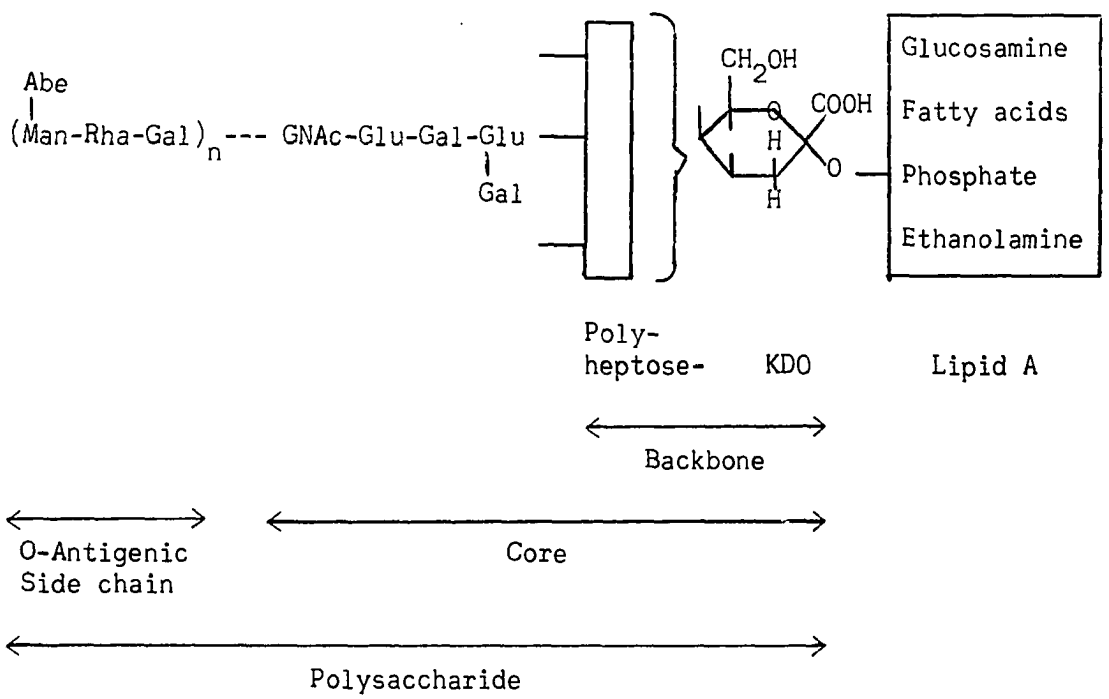
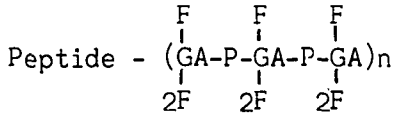


Figure 2. Postulated structure for LPS, modified according to Osborne (24) and Heath et al. (25).

Although the polysaccharide moiety exhibits the serological O-specificity, it is not immunogenic (21). Toxicity studies revealed that the isolated polysaccharide moiety is non-toxic (21).

The presence of lipid A in O-antigens was recognized independently by several groups (16, 26-29). It is generally agreed that the main constituents of lipid A are glucosamine, fatty acids and phosphate. Qualitative and quantitative gas-liquid chromatography of the methyl esters, isolated from lipid A of different bacteria revealed, among others, a relatively high concentration of β-hydroxymyristic acid (30-34). Since β-hydroxymyristic acid seems to be a rather specific constituent it is used as a marker for the detection of lipid A (15). Phosphate is linked as an ester to glucosamine in position 1 and prcbably also in position 4 or 6 (34, 35). In addition to these main constituents, ethanolamine and phosphorylethanolamine were often found in lipid A (35, 36). Although much work has been done about the structural analysis of lipid A, the exact structure has not yet been established.

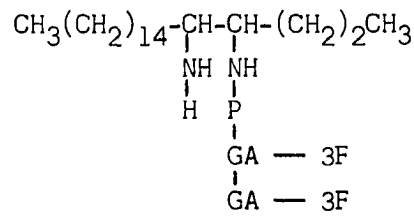
Nowotny (37) proposed an acylated poly-D-glucosamine phosphate chain as the possible structure of lipid A:



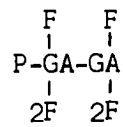
GA = glucosamine, F = fatty acid, P = phosphate

The fatty acids are linked to glucosamine via the C_3 and C_6 hydroxyl groups as esters and via the amino group as amides.

Ikawa (38) postulated necrosamine as the central component for the lipid A structure of E. coli:



However, necrosamine has been found only in lipid A preparations from an atypical strain of E. coli. Burton and Carter (30) have suggested that lipid A from E. coli consists of two glucosamine residues linked glycosidically rather than through a phosphodiester bond:



Recent studies of Gmeiner et al. (35) and Tsang (34) contributed further evidence for a glycosidically linked polyglucosamine chain and thus supported the latter proposal.

The biological role of lipid A remains controversial, although many groups have studied this problem. Goebel et al. (21, 28) found that mild acid hydrolysis of the endotoxin complex resulted in the isolation of a non-toxic degraded polysaccharide and a toxic "conjugated protein," whereas mild alkaline hydrolysis of the complex yielded toxic lipopolysaccharide and a non-toxic "simple protein." They concluded that a toxic component residing within the endotoxin complex could be isolated either linked to the protein moiety or to the polysaccharide moiety.

Westphal and Lüderitz (16) were able to couple the lipid A moi-

ety to an inert protein and the so-obtained "lipocasein" exhibited strong toxicity and pyrogenicity. However, the isolated lipid A moiety when devoid of protein and polysaccharide was less toxic than the original LPS (16). This was explained by the insolubility of lipid A in aqueous systems and by its possible partial degradation during acid hydrolysis. Ribi et al. (39) by isolating a lipid-poor lipopolysaccharide that exhibited the highest known toxicity suggested that a quantitative relationship does not exist between lipid content of lipopolysaccharide and toxicity. On the other hand, mutant strains of Salmonella produced toxic lipopolysaccharides, which contained mainly lipid A and were devoid of polysaccharide (40).

Other biological activities of endotoxin complex such as pyrogenicity (41, 42), tumor inhibitory (43) and tumor necrotizing effects (44) were also described.

The protein moiety discovered by Morgan and Partridge (45, 19) and later by Goebel et al. (46) as an integral part of the endotoxin complex, has not been studied very extensively. It has been suggested repeatedly that, except for a decreased water solubility (19) and reduced immunogenicity (15, 16), the removal of the protein moiety by chemical (17, 47, 48, 49) or enzymatic treatments (50, 51) of whole complex has little, if any, effect on the retention of characteristic serological and endotoxic properties of remaining lipopolysaccharides. The assignment of a secondary "carrier" role to the protein moiety has been probably the single most important reason for a relative lack of interest in studying its isolation, characterization and mode of linkage to the biologically active lipopolysaccharide moiety.

It has been assumed that the protein components of many Gram-negative bacteria are similar, and that the "conjugated protein" differs from the "simple protein" by containing lipid A; however, the presence of lipid A in "conjugated protein" has never been proven experimentally.

The aim of this investigation has been to isolate and characterize the protein moiety of endotoxin complex from Serratia marcescens 08 and to study the mode of linkage between the protein and lipopolysaccharide moiety.

The specific aims consisted in studying (a) the isolation and purification of "simple" and "conjugated protein," (b) physical and chemical properties of protein preparations, (c) immunogenic characteristics of intact and partially degraded proteins, and (d) antigenic similarities or differences between protein moieties from two bacterial species.

CHAPTER II

LITERATURE REVIEW

Boivin and Mesrobeanu (52, 53) first isolated in 1933 (54) a highly antigenic material by extraction of wet or acetone dried Gram-negative bacteria with 0.2 N trichloroacetic acid in the cold; this preparation became known as Boivin antigen, somatic O-antigen and, because of its toxicity, also as endotoxin. Boivin regarded these substances as lipocarbohydrates, and failed, as other early investigators, to recognize a protein or protein-like constituent. It remained for Morgan and Partridge (19, 45) to demonstrate a protein moiety as constituent of the antigenic complex of Bact. dysenteriae (Shiga) and Bact. typhosum (55). Goebel (21) studying the O-somatic antigens of the Flexner group of dysentery bacilli confirmed the presence of a protein moiety and stated:

> Our knowledge concerning the nature of antigens derived from Gram-negative bacilli was greatly advanced when Morgan and Partridge found that, in addition to the phospholipid and polysaccharide components, a third constituent was liberated on acid hydrolysis. This substance proved to be a protein and was shown to be an important component not only of the specific antigen of the Shiga bacillus, but of the typhoid bacillus as well.
>
> It has been observed that a protein-like moiety forms an integral part of the antigenic complex of each specific type of organism thus far investigated.

Morgan (19, 45, 56) introduced a mild extraction method with anhydrous diethylene glycol in the cold which, unfortunately, was found later not to be always suitable when applied to other bacteria. The ex-

tract from Shigella dysenteriae purified by centrifugation and repeated acetone and ammonium sulfate precipitation yielded the whole antigen complex. Hydrolysis with 1% acetic acid for 4 hours at 95-98° yielded the specific polysaccharide, lipid B, and a precipitate that was found to be a protein in nature and accounting for about 22% of the total antigen. Results of the elementary analysis of this protein preparation soluble in dilute alkali, but not in dilute acid, were C 44%, H 6.7%, N 11.5%. Phosphorus accounted for less than 0.2% and was at first not considered as a constituent of the protein. It was digestable by trypsin at pH 8.5 and was shown to be slightly antigenic. The protein moiety could be largely removed from the whole antigenic complex by the action of trypsin (45). It was established later that this fraction was a "conjugated protein" (19). Thereafter, a similar "conjugated protein" from Salmonella typhosa could be prepared by the same method (55). The solubility properties and analytical data (N 11.5% and P 0.47%) of this protein preparation were similar to those of Shigella protein. Nevertheless, unlike the antigenic complex of Shigella dysenteriae the O-antigen of Bact. typhosum could not be rendered free from the protein moiety by tryptic hydrolysis. Goebel (21, 46) prepared the O-antigenic complexes from different types (V, W. Z and Newcastle) of Shigella paradysenteriae (Flexner) by extraction with diethylene glycol and, since this method failed in the case of type V and Newcastle, with 50% aqueous pyridine. Hydrolysis of the Z type antigen with 1% acetic acid for 4 hrs at 100° or with picric acid dissociated the antigenic complex into a polysaccharide hapten, a phospholipid (lipid B), and a protein constituent. The results of analysis were N 11.8% and P 1.27%, and no glucosamine or re-

ducing sugars were detected. Since this "conjugated protein" was toxic, it was referred to as "toxic protein." It was shown upon electrophoresis in a Tiselius apparatus that the "conjugated protein" appeared in combination with the polysaccharide hapten and the phospholipid (lipid B). The antigen of the type Z and the protein derived from it were both readily attacked by trypsin and a considerable part of the protein was hydrolyzed. The antigen prepared by tryptic hydrolysis had a lower nitrogen and a higher carbohydrate content than the original antigen. Since tryptic digestion of the intact antigen and of "conjugated (toxic) protein" did not destroy the toxic component, it was concluded that the toxin is either not a protein or that it is a peculiar protein fraction resistant to the action of the enzyme.

When the antigenic complex obtained from Shigella dysenteriae and Salmonella typhosa by extraction with diethylene glycol was dissolved in 90% phenol (19), it was dissociated into a loosely bound lipid (lipid B), "undegraded" polysaccharide (lipopolysaccharide consisting of a polysaccharide moiety and firmly bound lipid A) and a "simple protein." The "simple protein" was isolated and found, contrary to the "conjugated protein," to be soluble in dilute acid at pH 2.5; it contained 13% N, but was free of phosphorus. From these and other data it was concluded that the proteins isolated from the same antigenic material by two different methods (acid hydrolysis and phenol dissociation) were substantially different in character and that the product isolated from phenol was a degradation product of the "conjugated protein." To prove this a preparation of "conjugated protein" (11.2% N, 1.07% P, $[\alpha]_{5461}$ $-48 \pm 3^{\circ}$) was dissolved in 90% phenol. The isolated protein was soluble in normal

acetic acid, contained 13.8% N and no phosphorus, and showed a specific rotation $[\alpha]_{5461}$ -88 $\pm$ 2°. The analytical values and general properties of the purified protein were distinctly different from those of the "conjugated protein," but showed a close agreement with those of the protein prepared by phenol dissociation of the whole antigenic complex. Additionally, it was shown that the somatic antigen could be dissociated by ethanolic alkali (0.1 N NaOH) and a "simple protein" could be isolated by repeated precipitation with acetic acid at pH 4.5. The "simple protein" contained 13.8% N and was free of phosphorus; specific rotation was $[\alpha]_{5461}$ -71 $\pm$ 4°. The analytical data were similar to those of the "simple protein" prepared by phenol dissociation.

Goebel et al. (21, 46) were also able to isolate a "simple protein" from the antigenic complex of Z type of Shigella paradysenteriae (Flexner) or its "conjugated protein" by mild hydrolysis with ethanolic sodium hydroxide followed by isoelectric precipitation. The "simple proteins" derived from the antigenic complex and from the "conjugated protein" were indeed very similar (N 13.9 and 14.5%; P 0.18 and 0.12%, $\alpha[D]$ -65° and -66°) and resembled the "simple protein" described by Morgan. Thus, it was established that the protein component of O-antigen could be obtained in two different forms, either as "simple" or as "conjugated protein," depending on the mode of hydrolysis.

Biological studies revealed that both the "conjugated protein" and the "simple protein" gave rise to antibodies which reacted equally with the intact O-antigenic complex and the homologous antigen though the "simple protein" was a weaker antigen (21) than the "conjugated protein." Removal of the protein moiety from the whole antigenic complex

decreased the antigenicity of the "toxic carbohydrate" (LPS) in comparison with the complete antigen. The polysaccharide hapten obtained from the complete antigen was not antigenic. The intact antigen complex exhibited the highest toxicity for mice. Both "toxic carbohydrate" (lipopolysaccharide consisting of polysaccharide and lipid A) and "conjugated protein" (consisting of protein and lipid A) were toxic to about the same extent. On the other hand, "simple protein" and polysaccharide hapten exhibited no toxicity. From these results it was concluded that depending on the mode of hydrolysis, the toxic principle could be found either in lipopolysaccharide or in "conjugated protein;" Goebel called this moiety, obviously different from the protein moiety or the polysaccharide hapten, "toxic component" (21). Morgan (19) found that both the "simple" and the "conjugated" protein were weak antigens. Whereas antibodies to "conjugated protein" of Shigella dysenteriae reacted with the homologous antigen and with "simple protein" from a smooth and rough strain of Shigella, the "conjugated protein" did not react with antibodies to "simple protein." An antiserum against "simple protein" could only be prepared when material isolated from the rough strain was used for immunization. "Simple protein" from the smooth strain was not antigenic. Recombination of the isolated undegraded polysaccharide with the "conjugated protein" yielded a potent antigen and produced agglutinins against Shigella. In contrast, the "simple protein" did not form an antigenic complex when recombined with the undegraded polysaccharide in aqueous solution. These observations suggested that the "conjugated protein" contained probably a prosthetic group which was necessary for the recombination with the undegraded polysaccharide.

Subsequent studies in many laboratories have fully confirmed the lipopolysaccharide-protein nature of intact endotoxins or O-antigens isolated either by the extraction of whole cells with various organic solvents (21, 46, 47, 57, 58) or trichloroacetic acid (48, 57, 59, 60), or by precipitation from culture fluids (49, 61). Westphal et al. (17) using aqueous phenol for extraction at 68° isolated from the phenol phase the protein components of O-antigens from different bacteria and found following values for nitrogen and phosphorus: E. coli, 15.8-16.2% N, 0% P; S. abortus equi, 16.1% N, 0% P; Ent. Brestau, 15.7-15.9% N, 0% P; B. fluorescens, 16.3% N, 0% P; and Lactobac. aerogenes, 16.1% N, 0.3% P.

Homma and Suzuki (62-64) studied the protein component of endotoxin from Pseudomonas aeruginosa and compared the endotoxin protein with a protein isolated from the cell wall of the same organism. Both proteins appeared to be homogeneous by ultracentrifugation and by zone electrophoresis. Although there were some differences between the two proteins in nitrogen and phosphorus content (endotoxin-protein: 13.1% N, 1.6% P; galactosamine 2.3%) and in the sedimentation constants, the protein moieties were similar in respect to the molar ratios of amino acids. It was found that the two proteins possessed a common antigenic determinant.

Clarke et al. (65) fractionated isolated cell walls of Pseudomonas aeruginosa with aqueous phenol. Upon precipitation with methanol a protein obtained from the phenol phase was soluble in aqueous sodium dodecyl sulfate. Analytical ultracentrifugation showed a single peak, but at least two bands were discovered upon gel electrophoresis. This lipid-free fraction yielded upon hydrolysis 83.3% amino acids. Aspartic acid was present in the relatively highest concentration, glucosamine

was present only in traces. The material not precipitated from the phenol phase by methanol consisted mainly of phospholipid (44%) and fatty acids (27%), and approximately 13% protein. The aqueous fractions (66) contained both lipopolysaccharide and murein. Since the amino acids found in these fractions were those typical of murein, the aqueous phase obviously did not contain a protein. However, the residual cell walls insoluble in aqueous phenol contained approximately 50% murein and 50% protein. The total protein content of the cell wall was approximately 36% and that of lipopolysaccharide 31%. The protein fraction precipitated from the phenol phase accounted for 24.5% of the cell wall.

So far nothing is known about the nature of the linkage between the protein moiety and the lipopolysaccharide. Morgan (65) concluded from his results that "the forces that bind together the various component molecules which make up the antigenic complex are not of the covalent type and that in consequence stereochemistry of the linkages and constant molecular composition for the antigenic complex are not to be expected." Webster et al. (48) obtained by a cold trichloroacetic acid extraction of Salmonella typhosa an O-antigen complex which had an extremely low nitrogen content (2-2.5%). Fractional precipitation of this O-antigen complex either by ammonium sulfate or ethanol failed to result in separation of nitrogenous material. However, it was claimed that ethanol fractionation in combination with a high salt concentration (0.2% antigen in a solution of 35 g NaCl/100 ml) resulted in removal of 10% inert material and that the nitrogen content of remaining antigen decreased to 0.6%. This finding might indicate, in agreement with Morgan, that the protein moiety was not covalently linked. However, the same authors were

not able to remove the protein moiety of endotoxins with a higher content of protein.

Recently, Okuda and Weinbaum (67) isolated an envelope-specific glycoprotein from E. coli by extraction with aqueous phenol. The phenol soluble glycoprotein accounted for 35-45% of the total envelope protein, and 50-60% of the partially purified membrane protein. There was about 4% carbohydrate associated with the glycoprotein and N-acetyl glucosamine-^{14}C was rapidly incorporated into the glycoprotein. Amino acid analysis showed that the relative content of aspartic acid and tyrosine of glycoprotein was higher than that of the envelope protein. Pronase treatment of the glycoprotein labeled with N-acetyl glucosamine-^{14}C yielded at least one glycopeptide which contained aspartic acid and glucosamine. The exact linkage was not determined. Although it was not shown whether this glycoprotein represented actually the protein moiety of the endotoxin complex, it was suggested that the carbohydrate portion of glycoprotein might serve as the initiation site for lipopolysaccharide synthesis.

Although the "simple" protein is apparently devoid of any typical endotoxic properties, it may be the carrier of bactericidal activity in colicinogenic bacteria (68). Goebel and his co-workers (69-72) clearly demonstrated that colicine K isolated from the colicinogenic E. coli K235 is a protein-lipopolysaccharide complex identical to the whole O-antigen complex of the same organism. Dissociation of colicine K by 90% phenol resulted in the separation of a protein moiety endowed with the bactericidal activity and a lipopolysaccharide moiety displaying the typical endotoxic properties. A comparative study of O-antigens of the colicine-producing organism and its non-colicinogenic variant revealed no sero-

logical or chemical differences between the corresponding lipopolysaccharide moieties; it was, therefore, suggested (71) that a study of the protein moieties should reveal the chemical basis for this difference in bactericidal activity. However, the possibility that colicinogenic strains contain a phenol-soluble protein different from the protein moiety of endotoxin has not been excluded by these experiments.

Homma and Suzuki (62, 63) showed in similar studies with Pseudomonas aeruginosa that the protein moiety of endotoxin isolated from the autolysate and a protein preparation (protein A) obtained from the cell wall had very similar amino acid composition, possessed a common specific antigen and displayed the same pyocine or bactericidal activities.

Mesrobeanu and her co-workers (73) isolated, in addition to the usual thermostable endotoxin, a toxic thermolabile fraction (neurotoxin) from chloroform autolysate of S. typhimurium S and demonstrated that its protein moiety endowed with bactericidal activity was identical to that of thermostable endotoxin.

The endotoxin complex of Serratia marcescens has been the subject of extensive investigations in many respects. Shear and Turner (74) isolated from culture filtrates of S. marcescens a lipopolysaccharide which caused necrosis and hemmorrhage in mouse sarcomas. Antitumor activity of polysaccharides obtained from different strains of S. marcescens were investigated by Creech et al. (75). The determination of nitrogen, phosphorus and carbohydrate values for fractions obtained from different strains of bacteria showed that they varied considerably depending on the growth conditions and composition of the medium (75). Rathgeb and Sylvan (60) first investigated the chemical composition of the polysaccharides.

Lipopolysaccharide-protein complexes were isolated and their chemical composition determined (75, 76). Comparative studies on the chemical composition and biological properties of lipopolysaccharides isolated from S. marcescens 08 and from the non-chromogenic strain S. marcescens Bizio were carried out by Alaupovic, Olson and Tsang (33). Tsang (34) also studied the composition of lipid A, isolated from the lipopolysaccharide of S. marcescens 08.

CHAPTER III

MATERIAL AND METHODS

Preparative Methods

Bacteria

Cells of Serratia marcescens 08 were obtained from General Biochemicals, Chagrin Falls, Ohio. Cells grown in a medium consisting of casein hydrolysate (2%), glycerin (0.1%), nutrient broth (0.5%) and NaCl (0.2%) were harvested in the late log-phase by centrifugation in a Sharpless continuous-flow centrifuge, and frozen for storage and shipment.

The dialyzed and lyophilized phenol phases of E. coli 08:K42(A) and E. coli 0141:K85(B) were supplied by Dr. K. Jann, Max-Planck Institut für Immunbiologie, Freiburg, Germany.

Extraction of Bacteria

Extraction of the chromogenic cells of Serratia marcescens 08. Water-washed wet cells were extracted (Figure 3) twice with trichloroacetic acid (100 g cells/200 ml TCA) according to a modification of the method of Boivin et al. (52). The combined extracts were dialyzed against distilled water for 48 hours, concentrated in vacuo to a small volume (approximately 50 ml), and centrifuged in a Spinco Model L ultra-

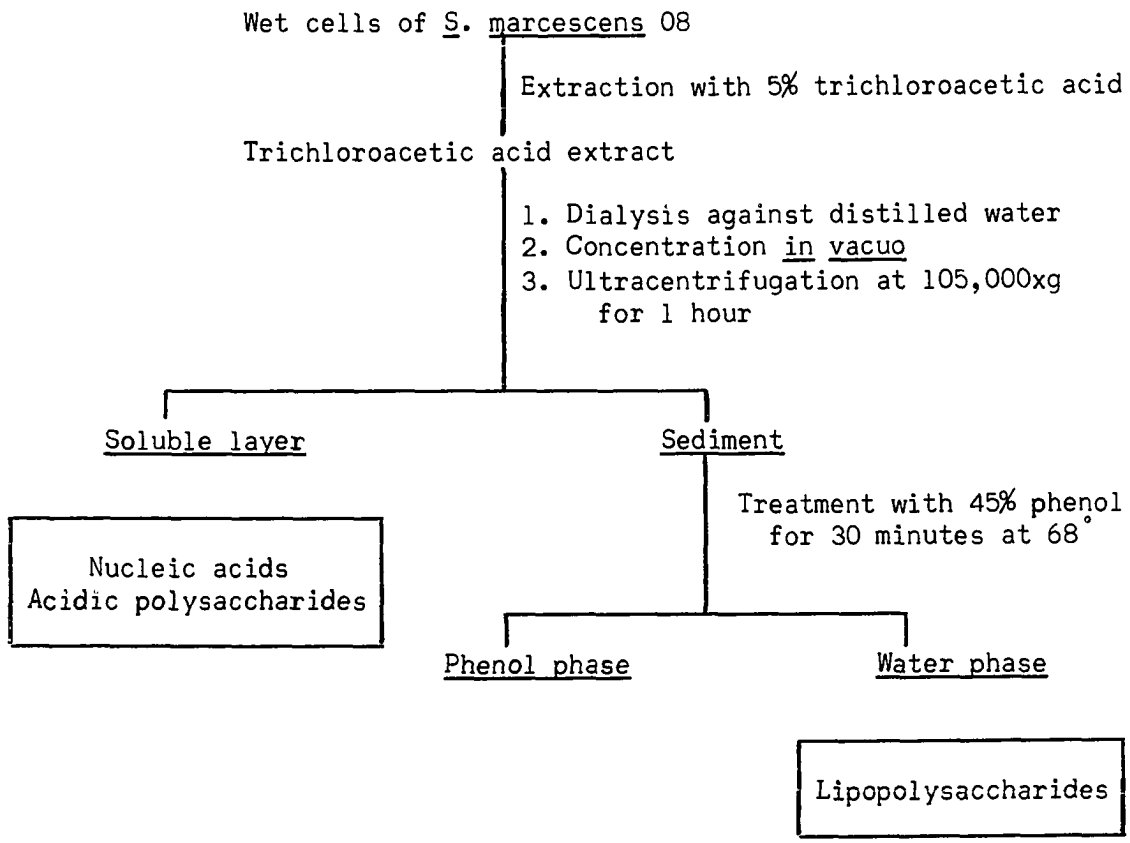


Figure 3. Isolation of the phenol phase.

centrifuge at 40,000 rpm (105,000 x g). The soluble layer contained nucleic acids and acidic polysaccharides. The lyophilized nucleic acid-free sediment was treated with 45% aqueous phenol for 30 minutes at 68° according to the method of Westphal et al. (17). The cooled water and phenol phases were separated. The aqueous phase contained lipopolysaccharides; the phenol phase was used for the isolation of the protein moiety.

Extraction of E. coli O8:K42(A) or E. coli O141:K85(B). Twenty grams of dry cells were extracted with 45% aqueous phenol for 10-15 minutes at 65° without previous trichloroacetic acid treatment. After cooling, the phenol and the water phase were separated by centrifugation. The water phase contained lipopolysaccharides, acidic polysaccharides and nucleic acids. Addition of 3-4 volumes of methanol to the phenol phase yielded a precipitate which was isolated by centrifugation. This precipitate was resuspended in distilled water, dialyzed and lyophilized. To isolate protein moieties of S. marcescens and E. coli under identical experimental conditions, 4-5 g of E. coli precipitate were dissolved in 500 ml 90% phenol by stirring for 12 hours at 35°.

Isolation of "Simple Protein" (PX-S and PX-E) from Phenol Phases of S. marcescens and E. coli

The phenol phases of S. marcescens or E. coli were washed several times with distilled water to remove trace amounts of lipopolysaccharides and peptides (Figure 4). The aqueous fractions were lyophilized and used for peptide mapping and immunological testing. The disappearance of peptide spots and immunoprecipitin lines of lipopolysaccharides was used as a criterion for the purity of the phenol phase. The addition

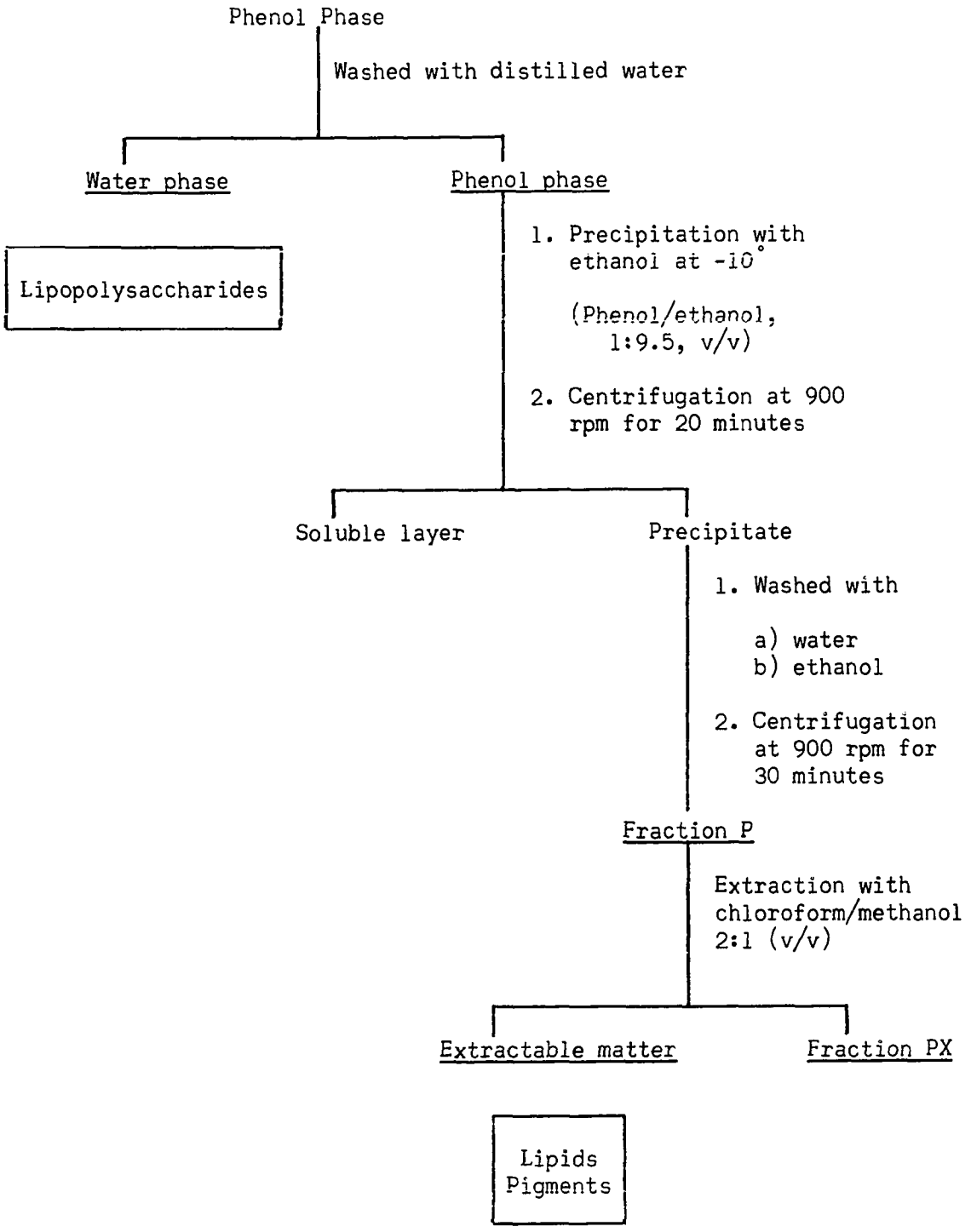


Figure 4. Isolation of fractions P-S, P-E, PX-S, and PX-E.

of 9.5 volumes ethanol to the phenol phases and the storage of mixtures at -10° for 7-10 days yielded a dark bluish-red precipitate from S. marcescens and a white precipitate from E. coli. Precipitates removed by centrifugation for 20 minutes at 900 rpm were washed twice with ethanol and several times with distilled water; after repeated centrifugation and lyophilization these precipitates represented fractions P-S and P-E, respectively.

To remove "free lipids," fractions P-S and P-E were extracted by chloroform/methanol (2:1, v/v) in a Soxhlet extractor for 12 hours. The residual fractions, PX-S and PX-E, were used as parent substances for further investigation. To detect peptide or amino acid impurities, PX-S and PX-E were submitted to high voltage paper electrophoresis (3000 V, 30 minutes, pyridine-acetate buffer, pH 3.65, ninhydrin stain; Savant Instruments, Inc., Hicksville, N. Y.). If ninhydrin-positive spots were detected, washing with distilled water for 2-3 days removed impurities.

Oxidation of PX-S and PX-E

Fifty milligrams of PX-S or PX-E were oxidized with 25 ml freshly prepared performic acid, by stirring the reaction mixture continuously for 24 hours at 4° according to a slightly modified method of Mueller et al. (77). The resulting solution was either lyophilized or evaporated in a vacuum dessicator over KOH and diluted with distilled water. The aqueous solution was concentrated to dryness in a rotary evaporator at 36° The crystalloid, slightly yellow material was either dissolved in boric acid buffer, pH 8.9 (50 ml of a mixture 0.1 M H_3BO_3/KCl + 20.8 ml of 0.1 M NaOH, diluted to 100 ml with distilled water) or suspended in distilled water and dialyzed for 7 days against distilled water at 4°

and then lyophilized. The oxidized proteins were designated PXOD-S and PXOD-E, respectively.

Isolation of the "Conjugated Protein" from Endotoxin Complex of *Serratia marcescens* 08

To remove free lipids, nucleic acid-free endotoxin complex obtained by trichloroacetic acid extraction of wet cells of *S. marcescens* (Figure 3) was extracted by chloroform/methanol (2:1, v/v) in a Soxhlet extractor for 12 hours. About 500 mg of this material (LPS-Ud) was dissolved in 250 ml preheated 1% acetic acid and hydrolyzed (Figure 5) for 4 hours at 90°C according to the method of Morgan (19, 45). The solution was constantly stirred. After cooling for 1 hour at 4°C, the flocculent slightly yellow precipitate was separated from the clear supernate by centrifugation at 12,000 rpm for 20 minutes at 10°C. The protein was dissolved in 0.02 M ammonium carbonate buffer, pH 8.6, and after three isoelectric precipitations at pH 3.5 washed for 24 hours with distilled water, and finally lyophilized. The "conjugated protein" was tested for impurities by high voltage electrophoresis as described above for PX-S.

Isolation of the Polysaccharide Moiety

After removal of the "conjugated protein," the clear supernate was extracted three times by chloroform to remove lipids. The lyophilized aqueous phase, which contained the "degraded polysaccharide," was dissolved in distilled water and dialyzed against distilled water for 7 days. The outer and the inner dialysates were lyophilized. The lyophilized "inner dialysate" was identified as the O-specific side chain of the polysaccharide moiety.

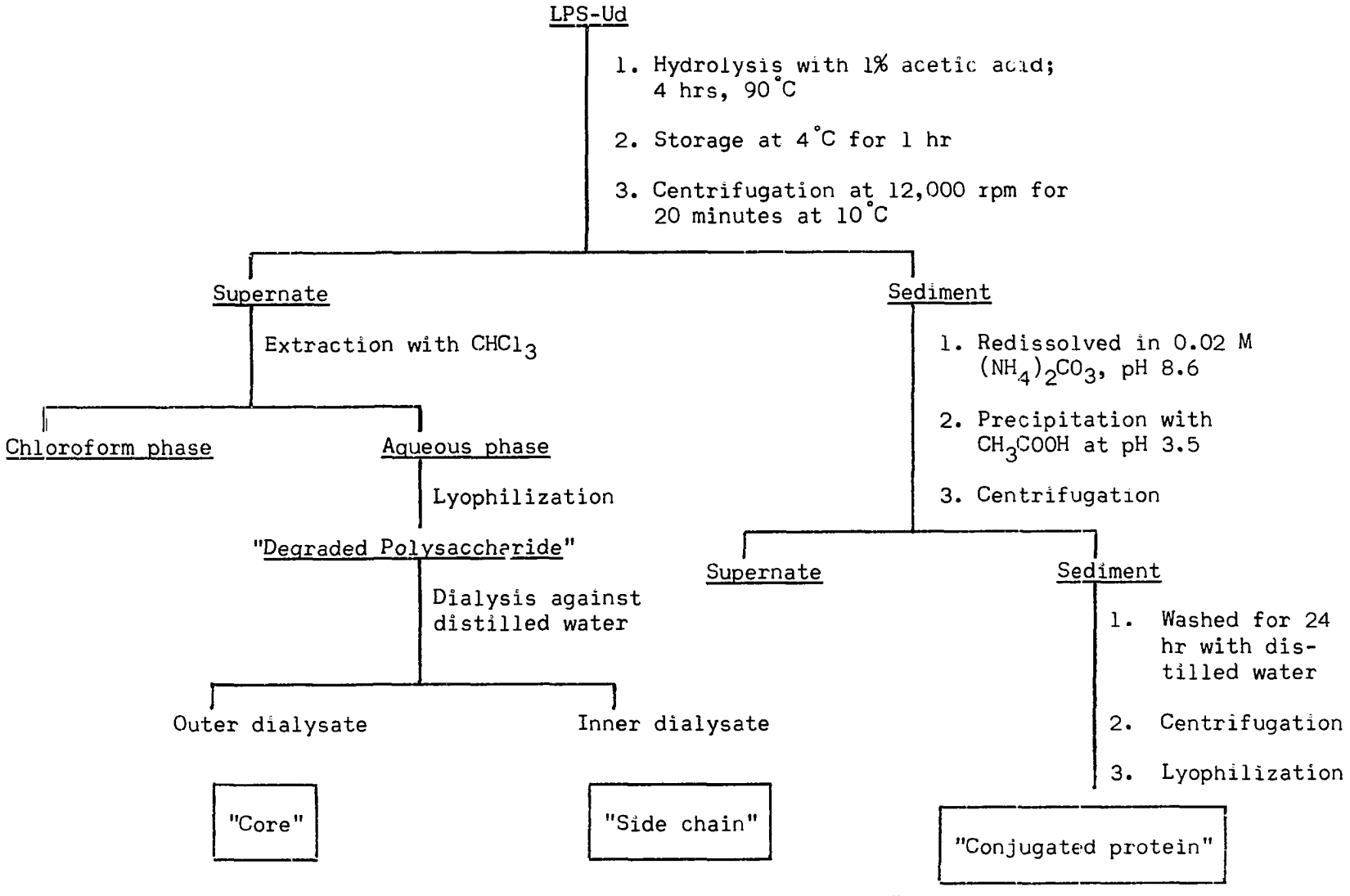


Figure 5. Isolation of "conjugated protein" from LPS-Ud.

Preparation of the Tryptic and Pronase Cores of PX-S and "Conjugated Protein" from *Serratia marcescens* 08

Fifty milligrams of PX-S or "conjugated protein" were suspended in 15 ml 0.02 M ammonium carbonate buffer, pH 8.6 (Figure 6), heated for 30 seconds in a boiling water bath, and cooled in an ice bath to 37°C. DFP-treated trypsin (Worthington Biochemical Corp., Freehold, N. J.), dissolved in a small volume of the same buffer was added (protein preparation/trypsin 100:1 (w/w)). The reaction mixture was gently stirred. The hydrolysis was performed for 12 hours at 37°C (the digestion was completed after 8-10 hours, as determined photometrically by the reaction with ninhydrin). During hydrolysis the reaction mixture became completely clear and no residue was observed. The solution was again heated for 30 seconds in a boiling water bath, cooled to room temperature and acidified with acetic acid. At pH 3.5-3.8 a precipitate was formed and separated on standing overnight at 4°C. The precipitate, separated from the clear supernate by filtration through membrane filter AM7 (Gelman Instrument Company, Chelsea, Michigan) or by centrifugation at 12,000 rpm for 20 minutes at 10°, was redissolved in ammonium carbonate buffer and again precipitated at pH 3.5-3.8. After washing three times with acetic acid and two times with distilled water, the precipitate was lyophilized and designated tryptic core of PX-S or tryptic core of "conjugated protein" (C.P.). The combined, lyophilized supernatants represented the tryptic peptides. The tryptic cores of PX-S and "conjugated protein" were digested with pronase (Pronase, B grade, Calbiochem, Los Angeles, California) according to a similar procedure; since maximum release of amino acids was reached after 20-21 hours, the hydrolysis time was pro-

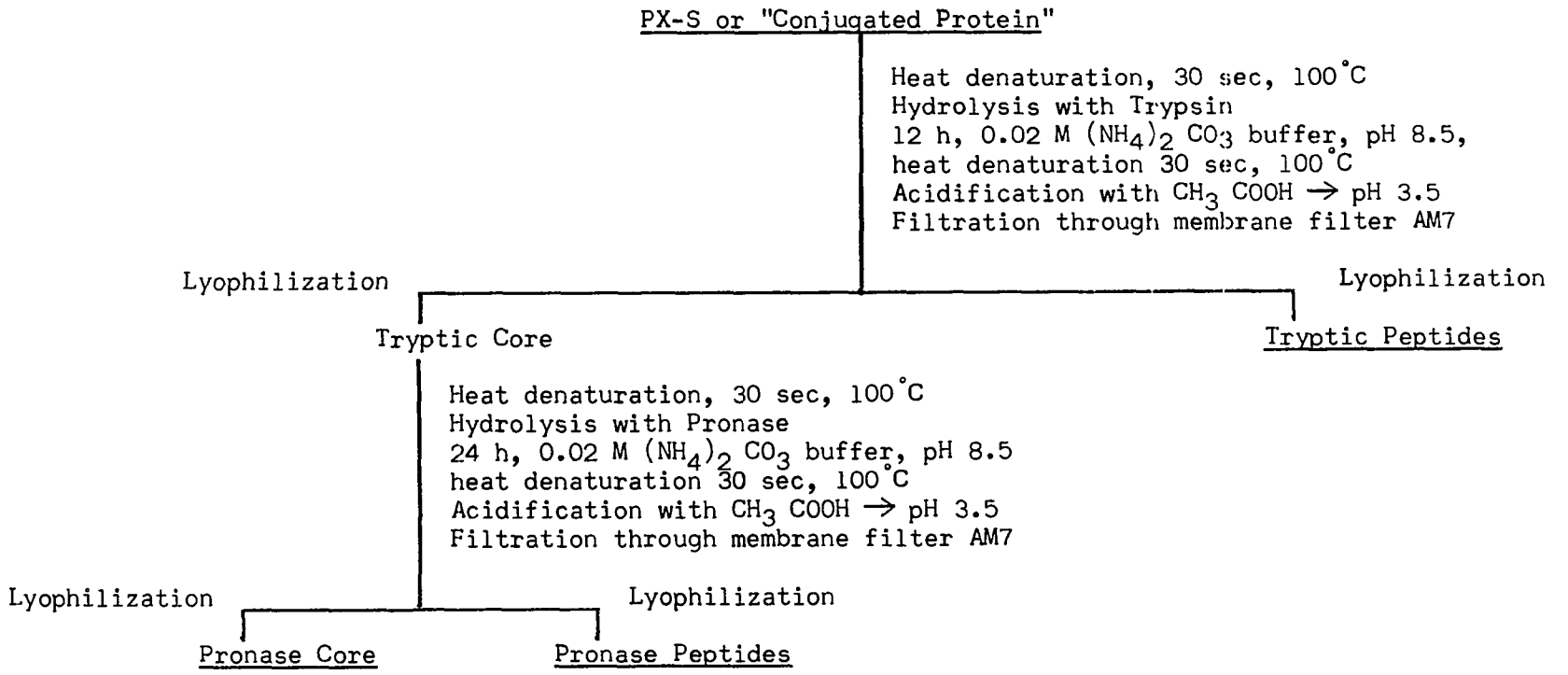


Figure 6. Preparation of the tryptic and the pronase cores of PX-S and "conjugated protein."

longed to 24 hours. Fractions were called Pronase core of PX-S and "conjugated protein," and Pronase peptides. The isoelectric points of both cores were at pH 3.5-3.8. The tryptic and pronase cores were checked for ninhydrin-positive impurities on high voltage electrophoresis. If impurities were detected, the cores were washed with distilled water until free of impurities.

Preparation of carboxypeptidase A core. Two milligrams of pronase core of PX-S were dissolved in a small volume of 0.05 M Tris buffer containing 0.5% SDS, pH 7.6. After dissolving substrate, 0.2 M N-ethylmorpholine buffer, pH 8.5, was added to a final volume of 1 ml. The final concentration of sodium dodecyl sulfate was 0.2%.

Carboxypeptidase A (Carboxypeptidase A, DFP-treated, Worthington Biochemical Corp., Freehold, N. J.) was added in a substrate/enzyme ratio 80:1. Aliquots were taken at different time intervals within 72 hours, and the undigested material was precipitated with 0.1 N HCl. The supernates evaporated to dryness and dissolved in citrate buffer, pH 2.2, were used for the amino acid analysis. The final precipitate washed with distilled water and lyophilized represented the carboxypeptidase core.

Isolation of Lipid A from PX-S and "Conjugated Protein" of Serratia marcescens 08

PX-S and "conjugated protein" were hydrolyzed with 0.1 N HCl (mg protein preparation/0.2 ml acid). The sample was added to the preheated acid and hydrolyzed for 30 minutes at 85-90°. After cooling in an ice bath, the hydrolysate was centrifuged at 12,000 rpm for 10 minutes at 4°. The supernate and the unhydrolyzed sediment were separately extracted three times by chloroform. The combined chloroform extracts

were washed exhaustively with deionized water to remove traces of acid, and concentrated to dryness *in vacuo*. The lipid residue was treated several times with boiling acetone. The acetone insoluble material (lipid A) was separated by filtration. The unhydrolyzed sediment (protein) was washed with ice-cold water and lyophilized.

Hydrolysis of the Pronase Core of "Simple" and "Conjugated Protein" by 2 N HCl

Eighteen to twenty milligrams of pronase core of PX-S and "conjugated protein" were hydrolyzed in an evacuated sealed tube with 2 N HCl for 30 minutes at 105°C. The unhydrolyzed portion was removed by centrifugation at 12,000 rpm for 20 minutes at 5°C. The supernate was extracted three times by chloroform. The chloroform phase was exhaustively washed with water and concentrated *in vacuo* to dryness. The aqueous phase was evaporated *in vacuo* to dryness, dissolved in distilled water, evaporated, and finally lyophilized. The unhydrolyzed residue was washed with ice-cold distilled water and lyophilized.

Preparation of Antisera

Antigens and the homologous antibodies are symbolized by the same letters; the capital letters designate antigens and the small letters antibodies. Antibodies were prepared against the following fractions:

Antigens	Symbol	Antibodies	Symbol
PX-S	A	Anti PX-S	a
PX-E	B	-	-
PXOD-S	C	Anti PXOD-S	c

Antigens	Symbol	Antibodies	Symbol
PXOD-E	D	Anti PXOD-E	d
PX-S tryptic core	E	Anti PX-S tryptic core	e
PX-S pronase core	F	Anti PX-S pronase core	f
Conjugated protein	G	Anti C.P.	g
C.P. tryptic core	H	Anti C.P. tryptic core	h
C.P. pronase core	I	Anti C.P. pronase core	i
LPS-Ud	K	Anti LPS-Ud	k
Polysaccharide (I.D.)	L	-	-

Additionally, antibodies were prepared also against whole cells of S. marcescens.

White rabbits were immunized by three intraperitoneal injections of 1 mg of each substance dissolved in 2 ml 0.05 M Tris buffer containing 0.5% SDS and emulsified with 2 ml of Freund's complete adjuvant. The injections were administered in one week intervals. Identical antibodies were also obtained against PX-S, PXOD-S and PXOD-E, when these substances were suspended in 0.9% NaCl. Subcutaneous injections were avoided, because they elicited strong local necroses (Schwartzman phenomenon). Blood was obtained by cardiac puncture. Antibodies were first demonstrated after the second injection. SDS-Tris buffer was used to solubilize rather than suspend the antigenic preparations. This buffer system did not give rise to antibodies.

Analytical Methods

Ultracentrifugal Analyses

Ultracentrifugal analyses were carried out in a Spinco Model E

ultracentrifuge equipped with a phase plate schlieren diaphragm and an automatic temperature control unit. Plate measurements were made with a Nikon microcomparator (Nikon Co., Japan) having a sensitivity of 0.001 mm. Sedimentation coefficients were determined at constant temperature (25-26°) employing rotor speeds of 56,100 rpm. Solutions of PX-S and "conjugated protein" in 0.05 M Tris-Cl buffer containing 0.1-0.2% sodium dodecyl sulfate, and of PXOD-S and PXOD-E in boric acid buffer, pH 8.9, density 1.004, were spun in single sector of synthetic boundary cells. The observed sedimentation coefficients were corrected to values in water at 20° by the usual methods (78). The apparent diffusion coefficients of PXOD-S and PXOD-E dissolved in the same buffer solutions were determined in synthetic boundary cells at a rotor speed of 12,590 rpm by applying the height-area method. The diffusion coefficient, corrected to zero time, was converted to standard conditions, $D_{20,w}$, in the usual manner (78).

Infrared Spectroscopy

Infrared spectra were obtained with the Beckman infrared spectrophotometer JR 10. The KBr pellets were made from 1.0-1.8 mg of substance and 250 mg KBr.

Amino Acid Analysis

About 2 mg of each protein sample in duplicate were hydrolyzed with 5.7 N HCl in evacuated sealed tubes at 110°C for 24 hr and 72 hr. To each sample 0.05 μM of norleucine and 56.8 μg of heptadecanoic acid were added as internal standards. The hydrolysates were extracted three times with chloroform to remove fatty acids. The chloroform extracts

were washed three times with distilled water to remove traces of amino acids. The amino acid hydrolysates and washings were evaporated to dryness in vacuo and redissolved in 2 ml of 0.2 N Na-citrate buffer, pH 2.2, filtered through a fine frit sintered glass filter and the aliquots were used for the determination of the amino acids.

The chromatography of the neutral and acidic amino acids was performed on a long column (52 cm) packed with Beckman ion exchange resin PA 28. Aminex citrate buffers (Bio-Rad Laboratories, Richmond, California), pH 3.25 (about 65 min) and pH 4.25 (about 105 min), were used for the elution. The basic amino acids were separated on a short (6 cm) column packed with Beckman ion exchange resin PA 35, and developed with Aminex citrate buffer, pH 5.28, for 60 minutes. The Beckman Model 120C amino acid analyzer was calibrated with an amino acid calibration mixture. An oxidized amino acid calibration mixture was used in the case of oxidized proteins, and methionine and half-cystine were determined as methionine sulfoxide and cysteic acid, respectively. Tryphophan was estimated according to the method of Spies and Chambers (92). The values obtained for 24 and 72 hr hydrolysis were extrapolated to zero time.

Fatty Acid Analysis

Chloroform extracts containing heptadecanoic acid (Supelco, Inc., Bellefonte, Pa.) as an interval standard were evaporated to dryness in vacuo, redissolved in 0.5 N methanolic KOH and boiled for 1 hr under reflux. Unsaponifiable material was extracted by n-heptane. After acidification, the free fatty acids were extracted by chloroform and esterified by boron trifluoride methanol reagent (Applied Science Laboratories, Inc., State College, Pa.). The fatty acid methyl esters were analyzed

qualitatively and quantitatively on a Barber-Colman gas chromatograph, Series 5000, equipped with flame detector. The glass column was packed with 15% diethylene glycol succinate on chromosorb W, 80-100 mesh (Applied Science Laboratories, Inc.). The chromatography was carried out at 172-175°.

The hydrogenation of the fatty acid methyl esters was performed in a Brown Hydro Analyzer (79) (Delmar Scientific Laboratories, Inc., Maywood, Ill.). Acetylation of fatty acid methyl esters by freshly distilled acetic acid anhydride in distilled pyridine was used to convert hydroxy fatty acids into acetoxy fatty acids (80). Fatty acid methyl ester standards were obtained from Applied Science Laboratories. A sample of β-hydroxy myristic acid was kindly supplied by Professor J. Asselineau, Faculte de Sciences, Toulouse, France.

Carbohydrate Analyses

Glucosamine was determined by the method of Rondel and Morgan (81) and by the amino acid analyzer as described above. Protein samples were hydrolyzed with 5.7 N HCl in evacuated sealed tubes at 110° for 16, 24 or 72 hours, and the obtained values were extrapolated to zero time.

Anthrone-positive carbohydrates were determined according to the method of Koehler (82).

Elementary Analyses

Samples were dried in high vacuum over P_2O_5. The analyses for carbon, hydrogen, nitrogen and ash were performed by Galbraith Laboratories, Knoxville, Tennessee. Phosphorus was determined according to the method of Gerlach and Deuticke (83).

Paper and Thin-layer Chromatography

Separation of carbohydrates was performed by descending chromatography on Whatman No. 1 paper according to the procedure described by Colombo *et al.* (84). The solvent systems used were (a) ethyl acetate/pyridine/water (3.5:1:1.5, v/v) or (b) n-butanol/pyridine/water (1092:728:546, v/v). The spots were detected by spraying with alkaline silver nitrate or by ninhydrin, and identified by comparison with known standards.

Lipids were separated by thin-layer chromatography according to the method of Mangold *et al.* (85). Glass plates (20 x 20 cm) were coated with Silica gel G (E. Merck, Darmstadt, Germany) and developed with n-hexane/diethyl ether (90:10, v/v) as a solvent for neutral lipids, and chloroform/methanol/water (85:19:2, v/v) or diisobutyl ketone/formic acid/water (40:15:2, v/v) (86) as solvent systems for phospholipids. Glycerides, fatty acids, and phospholipids were detected by spraying individual plates with 50% sulfuric acid and charring, and with bromothymol blue (87), and the phospholipids with molybdenum blue (88) and ninhydrin.

Agarose electrophoresis was carried out in 1% agarose gel by the method of Grabar and Williams (89) employing barbital buffer, pH 8.6, ionic strength 0.05, or 0.05 M Tris buffer containing 0.5% SDS, pH 7.6. Samples were dissolved in 0.05 M Tris buffer containing 0.5% SDS, pH 8.6. The plates were fixed for 60 minutes in a solution of acetic acid-ethanol-water (5:70:25, v/v), washed for 8 hours in distilled water and dried at room temperature. Amido Black 10B was used for protein staining.

Preparative Agarose Electrophoresis

Preparative agarose electrophoresis of PX-S was carried out on a Fractophoretor (Buchler Instruments, Fort Lee, N. J.). Ten to twenty mg PX-S dissolved in 1 ml 0.05 M Tris buffer containing 0.5% SDS, pH 7.6, were mixed with 0.5 ml 2% agarose in veronal buffer, pH 8.6, ionic strength 0.1, at 85°. This stacking gel was poured on top of a column of separating gel of 2% agarose in veronal buffer. The separating gel column was 10 cm long and 1.3 cm in diameter. The electrophoresis was performed with veronal buffer, pH 8.7, ionic strength 0.1, at 25 mA. Eluates were monitored by measuring the absorption at 280 mμ and 470 mμ. The main fraction was dialyzed and lyophilized.

Polyacrylamide gel disc electrophoresis was performed in Canalco Model 6 unit (Canal Industrial Corporation, Bethesda, Md.) according to the procedure of Davis (90). Electrophoresis was carried out at 5 mA/tube until the tracking dye had migrated approximately 4.2 cm into the separating gel. The acrylamide monomer concentration was 7.5% and a continuous buffer system of Tris-glycine, pH 8.8, was used.

Immunodiffusion and Immunoelectrophoresis

The immunological properties of protein preparations were studied by Ouchterlony's (91) double diffusion technique and by immunoelectrophoresis (7 volts/cm) in 1% agar gel plates employing veronal buffer, pH 8.6, ionic strength 0.1. Plates, allowed to develop for 24-30 hours, were washed several times with 0.15 M NaCl and distilled water, and dried at room temperature. They were stained for protein with Amido Black 10B.

Gel Filtration Column Chromatography

Gel filtrations of fractions PXOD-S and PXOD-E were performed on Sephadex G-100 columns. Boric acid buffer, pH 8.9, was used for elution. Absorption at 280 mμ was automatically recorded. The filtration of the pronase cores of PX-S and "conjugated protein" was performed on Sephadex G-50 column; ammonium carbonate buffer, pH 8.6, was used as eluant. The column was 100 cm long and 1.2 cm in diameter. Flow rate 0.2 ml/min. Absorption at 280 mμ was recorded automatically.

Peptide Mapping

The two-dimensional separation of peptides was carried out according to the procedure by Katz et al. (93). A mixture of tryptic peptides (2-3.5 mg) of PX-S, PX-E and "conjugated protein" were applied to Whatman No. 3 paper and subjected, in the first dimension, to descending chromatography in n-butanol/acetic acid/water (4:1:5, v/v). After drying, high voltage electrophoresis was performed in the second dimension in a buffer composed of pyridine/acetic acid/water (1:10:189, v/v), pH 3.65, at 2000 Volts for 1 hour. The peptides were stained with 0.05% ninhydrin in ethanol and heating to 70° for 20 minutes.

Lethality for Mice (LD_{50})

Two different strains of white male mice (Swiss-register, random bred, Pel-Freeze, Bio-Animals, Inc., Rogers, Ark., and Balb-C inbred mice, Texas Inbred Mouse Co., Houston, Texas) weighing 17-21 g were used for toxicity studies. Each group contained 6 mice. Each substance was tested at least two times or more. The substances were either suspended in 0.9% NaCl or dissolved in 0.05 M Tris buffer containing 0.5% SDS, pH

7.6, and injected intraperitoneally. Control groups were injected with this buffer system. Deaths occurring within 7 days were registered.

The LD_{50} was calculated according to Cornfield *et al.* (94):

$$M = h - d\left(\frac{A}{n} - 0.5\right),$$

where h = log max. dose,

d = log interval equals 0.3,

A = number of dead animals,

n = number of animals in each group.

The following categories of toxicity were used (95):

(1) Extremely toxic	1 mg/kg or less
(2) Highly toxic	1 - 50 mg/kg
(3) Moderately toxic	50-500 mg/kg
(4) Slightly toxic	500-5000 mg/kg
(5) Practically nontoxic	5-15 gm/kg

CHAPTER IV

RESULTS

The protein moiety of the endotoxin complex can principally be obtained in two forms, namely, either as a "simple protein" or as a "conjugated protein." To isolate the "simple protein" (PX-S), the trichloroacetic acid extracted endotoxin complex of S. marcescens 08 was treated with 45% aqueous phenol (Figure 3). The purple colored fraction (P-S) (Figure 4) obtained from phenol phase by precipitation with ethanol contained after lyophilization about 20% free lipids (w/w) extractable by chloroform/methanol (2:1). The thin-layer chromatography of the extractable matter showed the presence of unesterified fatty acids, glycerides and phospholipids. Triglycerides were the major component of glycerides and phosphatidyl ethanolamine was the principal component of phospholipids. Some of the spots stained by bromothymol blue reacted also with alkaline $AgNO_3$, indicating possibly the presence of glycolipids. Surprisingly, only a very small amount of pigment, supposedly prodigiosin or its derivatives, could be extracted from P-S.

After separation of P-S from the phenol phase, no further precipitation of protein material by addition of ethanol and storage at -10° was observed. The phenol phase was submitted to steam distillation or dialysis against distilled water to remove the phenol. After lyophiliza-

tion, the residual material representing less than 10% of the material originally present in the phenol phase was soluble in several organic solvents. Column chromatography of this pigment fraction on neutral aluminum oxide as adsorbent yielded upon elution with chloroform, chloroform/methanol (5:1, 3:1, 1:1) and methanol, seven major pigment fractions of differing color tonalities. Thus, the phenol phase consisted of approximately 70% "simple protein" (Px-S), 20% free lipids, and 10% free pigments.

Since it has been assumed without rigorous experimental evidence that the protein moieties of endotoxins of many Gram-negative bacteria are similar (15), the "simple protein" (PX-E) was isolated also from a strain of E. coli according to the procedure outlined in Figure 4; the phenol phase was obtained by direct extraction of cells rather than by treatment of trichloroacetic acid extracted endotoxin complex. The lyophilized fraction P-E was extracted by chloroform/methanol (2:1, v/v) and, similarly to P-S, about 20% free lipids were obtained. The "simple protein" (PX-E) accounted for only 40-50% of the material originally dissolved in phenol.

Physical-Chemical Properties of PX-S and PX-E

PX-S and PX-E were insoluble in water, various buffer systems, dilute alkali, dilute acid, and in many organic solvents such as formamide, dimethylformamide, etc. Both compounds were easily soluble in dimethylsulfoxide and PX-S, but not PX-E, was soluble in 0.05 M Tris-Cl buffer, pH 8.6, containing 0.2% sodium dodecyl sulfate. PX-S, dissolved in Tris-Cl buffer containing 0.1-0.2% SDS exhibited a single symmetrical

peak upon ultracentrifugation (Figure 7) with s_{obs} = 2.4S.

On agarose electrophoresis both PX-S and PX-E (Figure 8, patterns a and b) showed single bands of approximately same mobility. The preparative agarose gel electrophoresis of PX-S yielded a single fraction characterized by a symmetrical absorption curve with two maxima at 280 mμ and 470 mμ (absorption of prodigiosin). Additionally, PX-S showed a single band on polyacrylamide gel disc electrophoresis (Figure 9). Since the purple colored band of PX-S was identical with the band obtained upon staining with Amido Black, it was concluded that prodigiosin or one of its derivatives, was bound to PX-S.

There was a striking similarity between the infrared spectra of PX-S and PX-E (Figure 10). In addition to the broad absorption at 3400-3280 cm^{-1} (ν OH ass. or ν NH ass.) and 2960-2850 cm^{-1} (ν CH_3 + CH_2) and the amide I and amide II bands at 1650 cm^{-1} and 1520 cm^{-1}, there were also two bands at 1230 cm^{-1} and 1060 cm^{-1}. Whereas the latter absorption could possibly be assigned to ν P=O ass., the band at 1060 cm^{-1} might be due to δ OH and ν C–O. The chemical analysis of PX-S and PX-E supports this interpretation. That a small shoulder on the amide I absorption band at 1740 cm^{-1} may have been caused by ν C=O ester absorption was confirmed later by other means.

Immunological Properties of PX-S and PX-E

PX-S (A) gave a single immunodiffusion (Figure 11, pattern 1) or immunoelectrophoretic line (Figure 12, pattern 1) with antibodies to PX-S (a). PX-E (B) showed a single precipitin line with antibodies to PX-S (a) as well as a line of complete fusion with PX-S (A) (Figure 11, pattern 2). PX-S (A) and PX-E (B) displayed very similar mobilities on

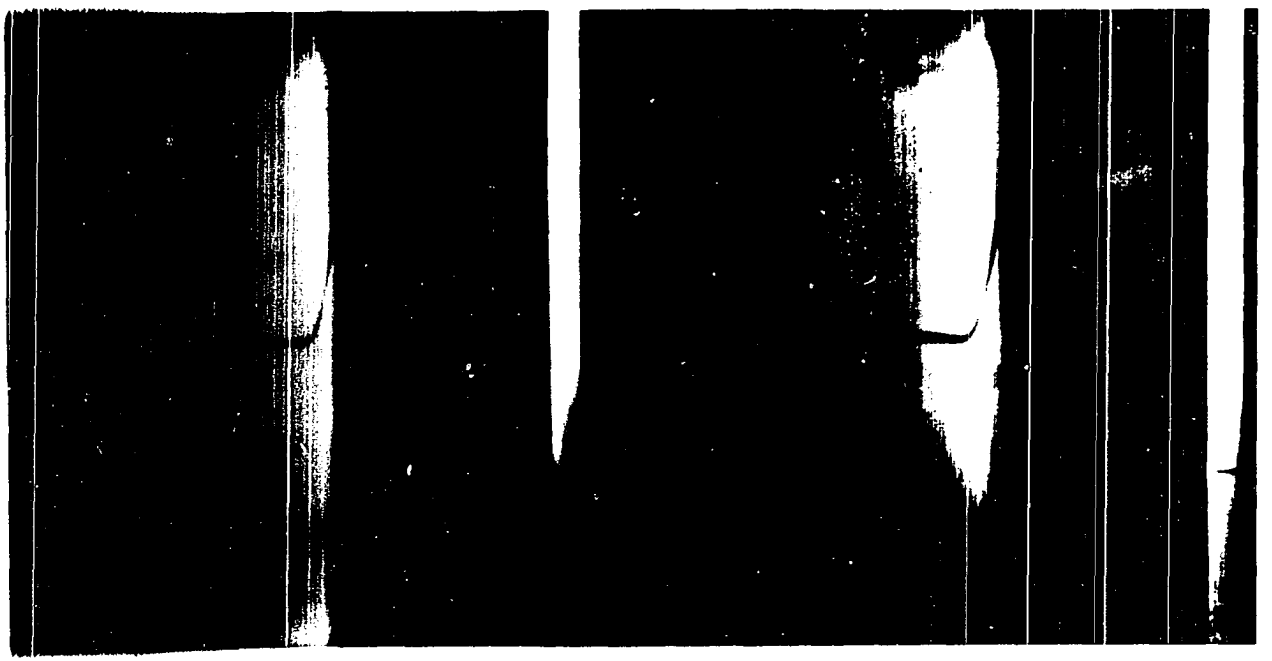


Figure 7. Ultracentrifugal sedimentation pattern of PX-S.

PX-S (5 mg/ml) dissolved in 0.05 M Tris-Cl buffer, pH 7.6, containing 0.1-0.2% SDS, density 1.004 g/ml, was centrifuged in a synthetic boundary cell at 52,640 rpm at 25°. Exposures were taken from left to right at 4 minute intervals for a total run of 64 minutes. Photographs were obtained at 8 and 12 minutes after reaching speed.

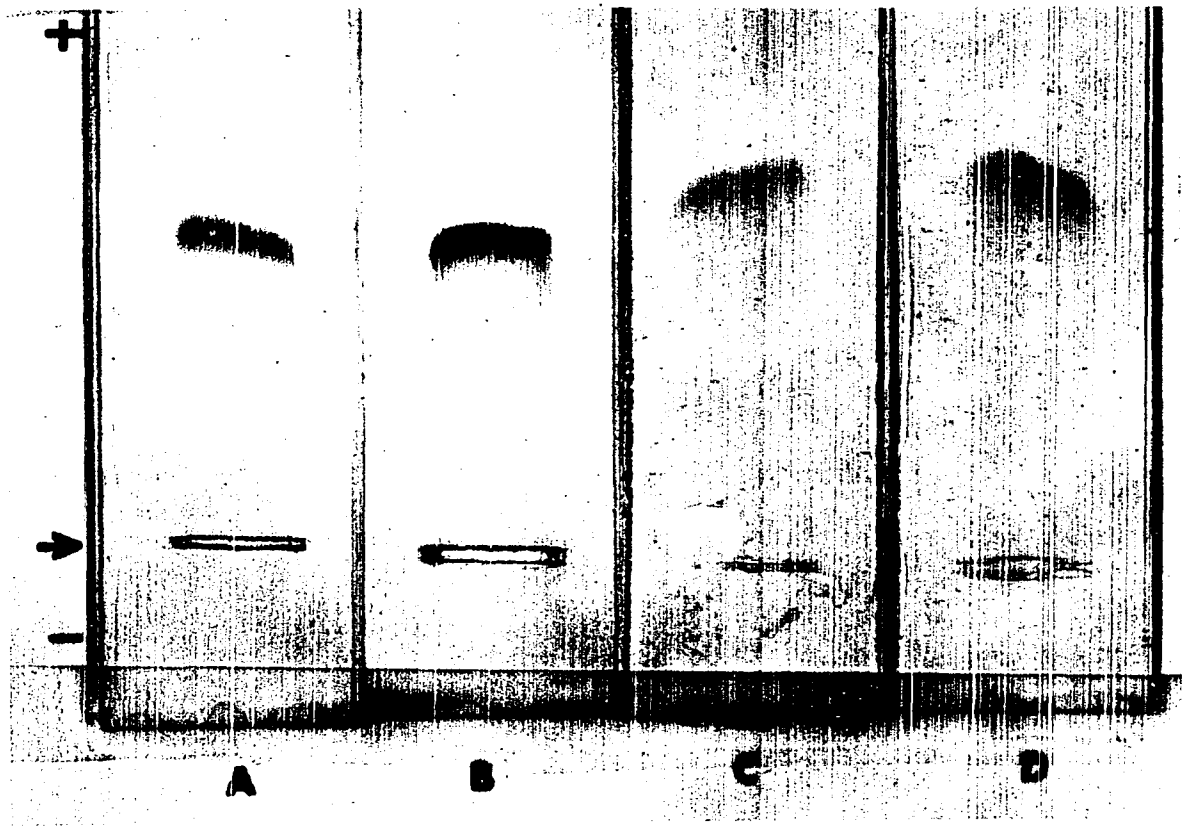


Figure 8. Agarose electrophoresis of PX-S (A), PX-E (B), PXOD-S (C), and PXOD-E (D).

Protein preparations (20 mg/ml) were dissolved in 0.05 M Tris-Cl buffer, pH 7.6, containing 0.5% SDS and diluted to a final concentration of 10 mg/ml with 1% agarose in barbital buffer. 1% agarose gel; barbital buffer, pH 8.6; ionic strength 0.05; 40 minutes.

Figure 9. Polyacrylamide gel disc electrophoresis of PX-S.

PX-S (10 mg/ml) was dissolved in 0.05 M Tris buffer, pH 7.6, containing 0.5% SDS. Acrylamide monomer concentration 7.5%; continuous buffer system of Tris-glycine, pH 8.8. Not stained; color of prodiogiosin.

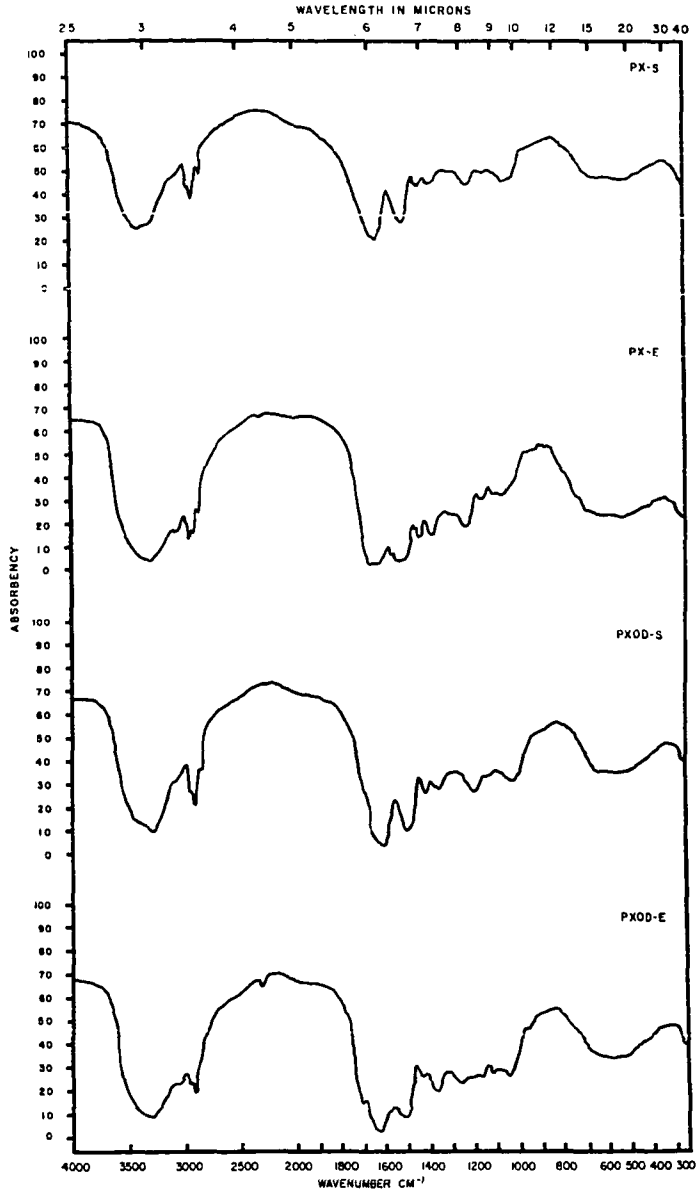


Figure 10. Solid infrared spectra of PX-S, PX-E, PXOD-S, and PXOD-E (1.5-1.8 mg substance/250 mg KBr).

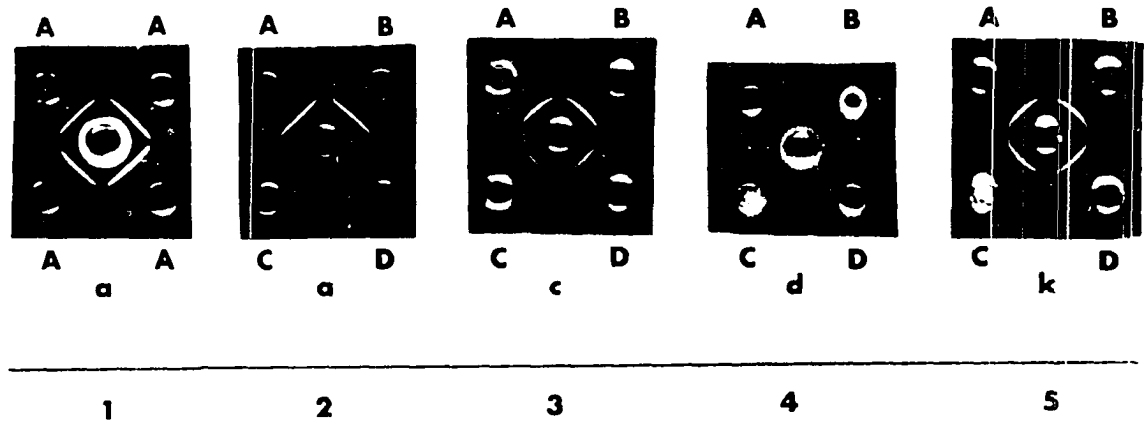


Figure 11. Immunodiffusion patterns of PX-S (A), PX-E (B), PXOD-S (C), and PXOD-E (D). a, anti PX-S serum; c, anti PXOD-S serum; d, anti PXOD-E serum; k, anti LPS-U-S serum.

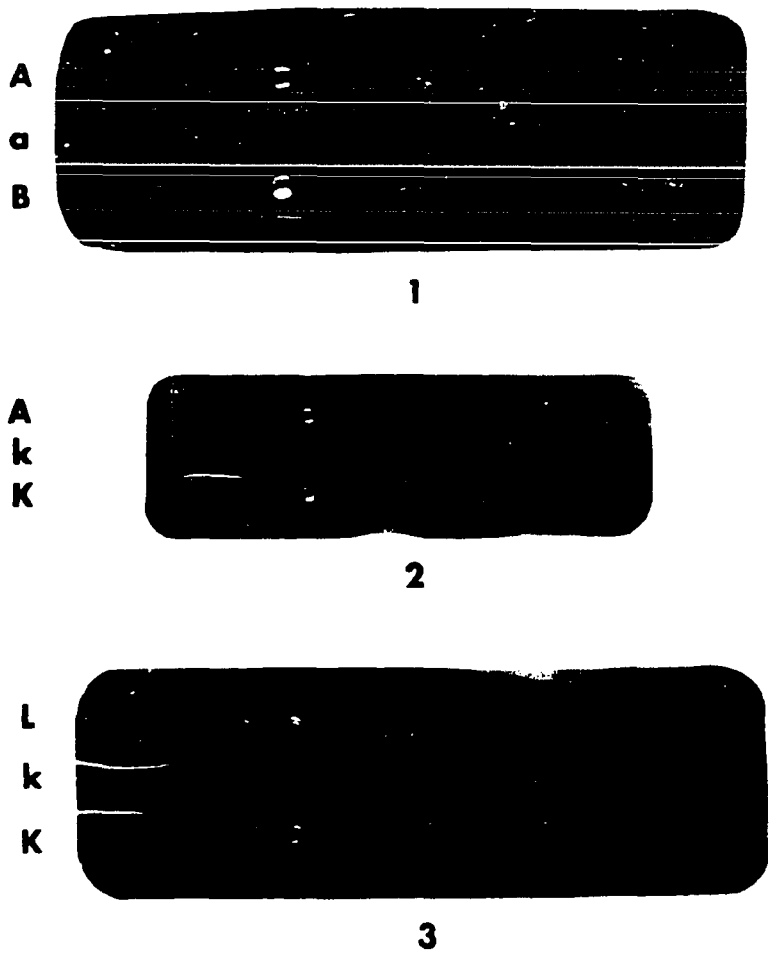


Figure 12. Immunoelectrophoresis patterns of PX-S (A), PX-E (B), LPS-U-S (K), and polysaccharide side chain (L).

Pattern 1, anti PX-S serum (a) and patterns 2 and 3, anti LPS-U-S (k).

agar immunoelectrophoresis (Figure 12, pattern 1). A single identical precipitin line of PX-S (A) and PX-E (B) against the endotoxin complex anti-LPS-U-S (k) is shown in Figure 11, pattern 5. Immunoelectrophoretic pattern of LPS-U-S (K) with antibodies to LPS-U-S (k) revealed three precipitin bands, one of which seemed to be identical with the band exhibited by PX-S (A) upon reaction with anti-LPS-U-S (k) (Figure 12, pattern 2). This band disappeared upon absorption of the anti-LPS-U-S serum with PX-S (Figure 13). The band migrating fastest towards the cathode was identified as polysaccharide side chain (L) of the endotoxin complex (Figure 12, pattern 3). The third band represents most probably the intact endotoxin complex. This result indicates, that the endotoxin complex (LPS-U-S) isolated by trichloroacetic acid extraction and used for the preparation of antisera contained the free polysaccharide side chain and the free protein moiety. On the other hand, Figure 12, pattern 2, also indicates that PX-S was neither contaminated with the polysaccharide moiety nor with the entire endotoxin complex. Additionally, PX-S and PX-E exhibited a single identical precipitin line against anti-whole cell (Serratia marcescens) serum.

Chemical Characterization

The results of the elementary analysis and chemical composition of PX-S and PX-E are shown in Table 1. Hydrolysates consisted not only of amino acids, but also of glucosamine, fatty acids and phosphorus. The nitrogen and sulfur content was slightly higher in PX-E, whereas carbon, glucosamine, fatty acids, and phosphorus were found in slightly higher concentrations in PX-S. Although the analysis indicated presence of some anthrone-positive carbohydrate in both protein preparations, no sugars

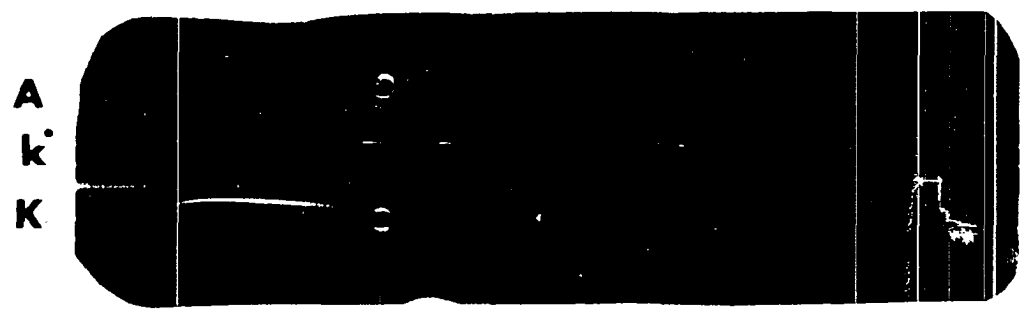


Figure 13. Immunoelectrophoresis pattern of PX-S (A) and LPS-U-S (K). k', anti LPS-U-S serum absorbed with PX-S (A).

TABLE 1

ELEMENTARY ANALYSIS AND CHEMICAL COMPOSITION OF
PX-S, PX-E, PXOD-S AND PXOD-E

	C	H	N	S	PO_4	Anthrone-positive carbohydrates	Glucosamine	Fatty Acids	Amino Acids	Recovery*	Ash
	%	%	%	%	%	%	%	%	%	%	%
PX-S	51.23	6.92	13.43	0.32	2.01	1.88	2.87	10.10	72.89	89.75	4.9
PX-E	48.44	7.66	15.18	1.03	1.59	1.22	1.31	5.98	83.23	93.33	3.2
PXOD-S	45.10	6.26	10.94	0.74	1.44	1.78	2.63	6.60	66.20	78.65	7.01
PXOD-E	43.31	6.31	14.19	1.24	1.17	1.11	1.13	3.71	72.64	79.76	6.00

*Recovery was calculated from anthrone-positive carbohydrates, PO_4, glucosamine, fatty acids and amino acids.

other than glucosamine could be detected by paper and thin-layer chromatography.

The amino acid composition is shown in Table 2. Both fractions, PX-S and PX-E, have a relatively high content of acidic amino acids. Sulfur is present in the form of methionine, whereas cystine is absent or found only in traces.

Gas liquid chromatography of the fatty acid methyl esters revealed the presence of β-hydroxy myristic acid (Figure 14) in addition to other fatty acids of a chain length from C_{12}-C_{18}. The distribution of the fatty acids is shown in Table 3. The β-hydroxy myristic acid recognized as the characteristic marker of lipid A was identified as the principal fatty acid of PX-S (44.5%) but not of PX-E (15.9%).

Distribution and Comparison of PX-S and PX-E

Mild alkaline hydrolysis of PX-S and PX-E with 0.5 N methanolic KOH released about 40-50% of the total fatty acids. This result indicated that the released acids were bound most probably through ester linkages; whereas the remaining fatty acids released by strong acid hydrolysis were bound through amide linkages.

Mild acid (0.01 or 0.1 n HCl) or alkaline (0.01 or 0.1 n NaOH) hydrolysis of PX-S in aqueous acetone or ethanol at 4° failed to split off prodigiosin or its derivative. On the other hand, acid hydrolysis with 0.5 N HCl or alkaline hydrolysis with 0.5 N methanolic KOH for 1 hour at 105° destroyed the pigment and caused partial hydrolysis of the protein moiety. It has been reported that prodigiosin cannot be extracted completely from cells by organic solvents (96), and that the unextractable fraction is linked to a protein (97). Since Purkayastha and Williams

TABLE 2

AMINO ACID COMPOSITION OF PX-S, PX-E, PXOD-S, AND PXOD-E

	PX-S		PX-E		PXOD-S		PXOD-E	
	μMoles/g	mg/100	μMoles/g	mg/100	μMoles/g	mg/100	μMoles/g	mg/100
Lysine	245	3.58	234	3.42	160	2.41	232	3.39
Histidine	74	1.15	87	1.34	48	0.78	62	0.96
Arginine	181	3.15	212	3.65	151	2.61	242	4.22
Aspartic acid	824	10.97	786	10.42	813	12.39	656	8.73
Threonine	422	5.03	384	4.57	384	5.21	295	3.51
Serine	442	4.64	354	3.71	385	4.48	319	3.35
Glutamic acid	537	7.90	910	13.32	479	8.02	879	12.93
Proline	137	1.58	253	2.91	100	1.29	198	2.28
Glycine	657	4.93	653	4.90	607	5.29	466	3.50
Alanine	657	5.85	798	7.10	635	6.54	964	8.59
1/2 Cystine	-	-	-	-	-	-	-	-
Valine	378	4.43	454	5.31	344	4.39	423	4.97
Methionine	42	0.63	145	2.16	88*	1.72*	137*	2.04*
Isoleucine	238	3.12	329	4.30	182	2.49	298	3.91
Leucine	478	6.27	623	8.16	375	5.71	527	6.91
Tyrosine	240	4.35	209	3.79	Traces	Traces	Traces	Traces
Phenylalanine	228	3.77	220	3.62	175	3.29	203	3.35
Tryptophan	75	1.54	27	0.55	-	-	-	-

*Methionine sulfoxide

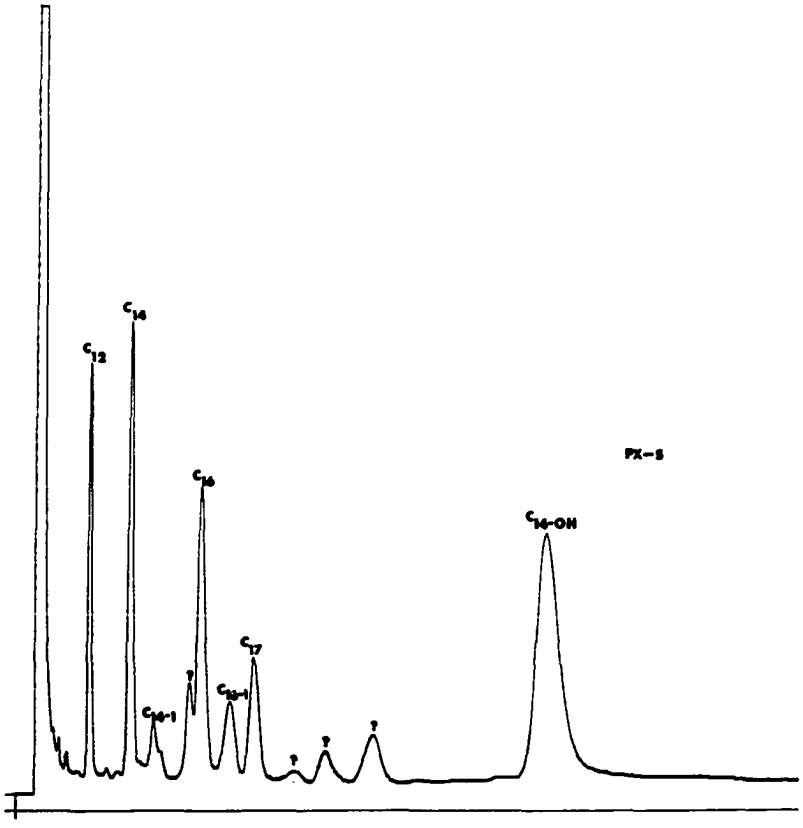


Figure 14. Gas-liquid chromatogram of fatty acid methyl esters of PX-S.

TABLE 3

PERCENT FATTY ACID DISTRIBUTION OF PX-S, PX-E, PXOD-S AND PXOD-E

	PX-S %	PX-E %	PXOD-S %	PXOD-E %
C_{12}	2.6	3.85	5.23	-
C_{14}	9.5	5.52	5.23	14.0
C_{14-1}	Trace	-	2.53	-
Unknown	12.9	-	12.75	-
C_{16}	16.5	26.42	10.46	30.0
C_{16-1}	5.5	-	-	-
C_{18}	-	38.79	-	10.0
C_{18-1}	Trace	4.34	-	15.0
Unknown	Trace	-	-	-
Unknown	3.9	-	-	-
Unknown	4.5	-	-	-
C_{14-OH}	44.5	15.88	63.79	29.0

(98) found the pigment associated with a cell envelope fraction of S. marcescens and suggested its linkage with hexosamine, we assumed that prodigiosin may be bound to glucosamine through a C-N linkage. However, mild alkaline photolysis (0.1 N NaOH, UV irradiation for 6 hours) as employed by Kuhn et al. (99) to split ribose from riboflavin, produced a colorless solution. Acetone extraction, followed by air irradiation and acidification, did not result in recovery of the pigment. Acidification of the remaining alkaline solution yielded a precipitate at pH 3.5 and a non-precipitable fraction in the supernate. Amino acid analysis of these two protein fractions revealed that histidine had been completely destroyed in the course of irradiation. Thus far, the attempts to isolate the intact pigment have failed. Nevertheless, the preliminary results indicate that the pigment is covalently linked to PX-S.

Oxidation of PX-S and PX-E

In an attempt to obtain soluble products, PX-S and PX-E were submitted to performic acid oxidation; a prolonged treatment for 24 hours was essential for the oxidative decomposition of the pigment. After a prolonged dialysis against distilled water, the resulting lyophilized and dried oxidation products PXOD-S and PXOD-E were obtained in a 90% yield. Both fractions were readily soluble in boric acid buffer, pH 8.9 and exhibited single symmetrical peaks in the analytical ultracentrifuge (Figures 15 and 16). Values for the sedimentation and diffusion coefficients of these two oxidized protein preparations are presented in Table 4.

PXOD-S and PXOD-E moved on agarose electrophoresis as single bands of approximately the same mobility (Figure 8, patterns c and d).

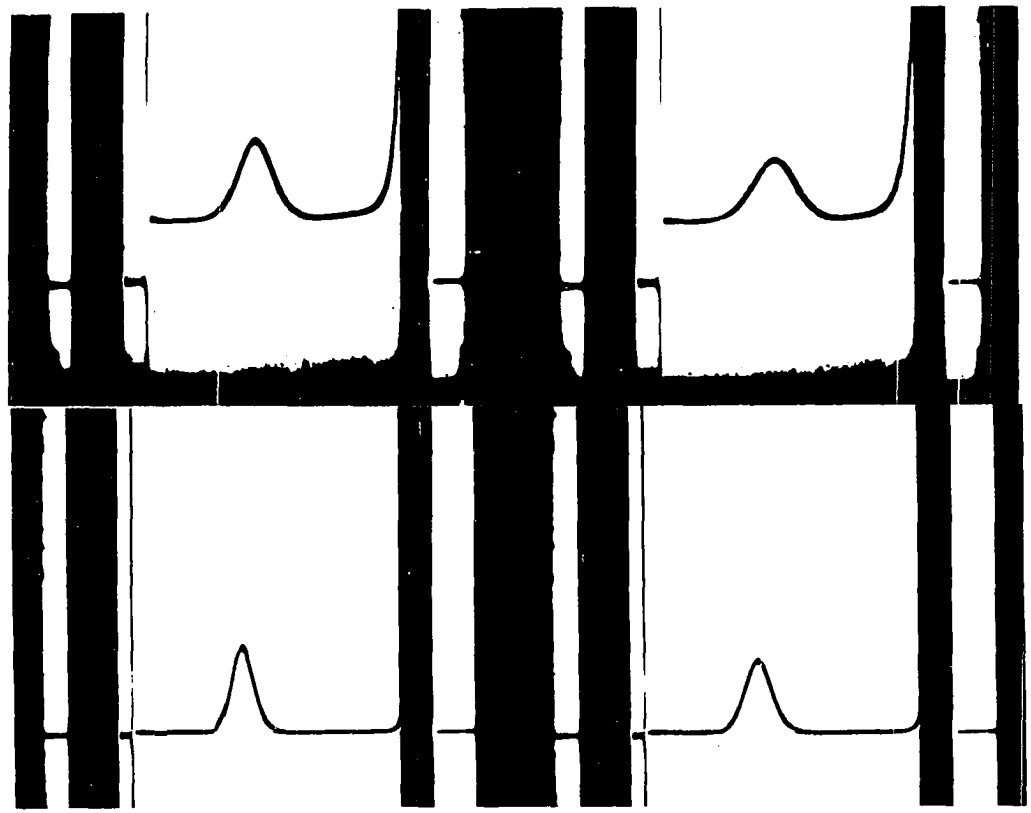


Figure 15. Ultracentrifugal sedimentation pattern of PXOD-S (top) and PXOD-E (bottom).

Protein preparations (10 mg/ml) dissolved in boric acid buffer, pH 8.9, density 1.004 g/ml, were centrifuged in synthetic boundary cells at 52,640 rpm at 25°. Exposures were taken from left to right at 4 minute intervals for a total run of 64 minutes. Photographs were obtained at 8 and 12 minutes after reaching speed.

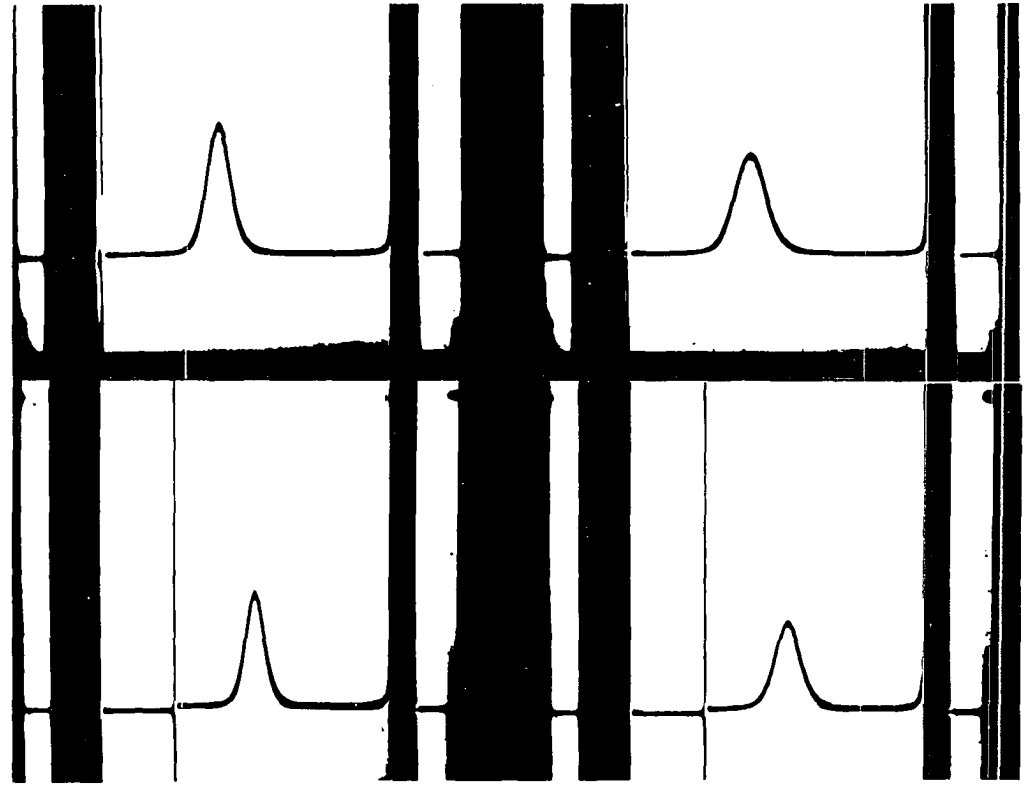


Figure 16. Ultracentrifugal diffusion pattern of PXOD-S (top) and PXOD-E (bottom).

Protein preparations (10 mg/ml) dissolved in boric acid buffer, pH 8.9, density 1.004 g/ml, were centrifuged in synthetic boundary cells at 12,590 rpm at 25°. Exposures were taken from left to right at 4 minute intervals for a total run of 64 minutes. Photographs were obtained at 8 and 12 minutes after reaching speed.

TABLE 4

SEDIMENTATION AND DIFFUSION COEFFICIENTS OF PXOD-S AND PXOD-E

	$s^{o}_{20,w}$ (S)	$D^{o}_{20,w} \times 10^{-7}$ (cm^2/sec)
PXOD-S	0.71	14.90
PXOD-E	1.23	8.66

The oxidized products migrated slightly faster than the corresponding parent substances and exhibited at the same concentration a weaker reaction with Amido Black upon staining.

The infrared spectra of PXOD-S and PXOD-E were essentially the same as those obtained for PX-S and PX-E (Figure 10).

Immunological Properties of PXOD-S and PXOD-E

Both oxidized proteins were immunogenic and antisera against PXOD-S (C) and PXOD-E (D) were obtained (c and d). PX-S (A), PX-E (B), PXOD-S (C) and PXOD-E (D) gave lines of complete fusion with antibodies to PXOD-S (C) (Figure 11, pattern 3), to PXOD-E (d) (Figure 11, pattern 4), to PX-S (a) (Figure 11, pattern 2) and LPS-U-S (k) (Figure 11, pattern 5). Immunoelectrophoresis revealed single precipitin bands for PXOD-S and PXOD-E against anti-PX-S and anti-PXOD-S serum.

Thus, it could be demonstrated that the oxidized proteins were immunologically identical with the parent fractions PX-S and PX-E, and that antisera developed against fractions from S. marcescens reacted with material from E. coli and vice versa in an identical reaction.

Chemical Characterization of PXOD-S and PXOD-E

The relative content of carbon, nitrogen, phosphate, glucosamine, amino acids and fatty acids of PXOD-S and PXOD-E was lower than that of PX-S and PX-E (Table 1). Partial hydrolytic cleavage of PX-S and PX-E by performic acid resulted in release of some glucosamine, a phosphorus-containing compound (most probably a glucosamine-phosphate), approximately 30-40% of fatty acids and some amino acids detected in the outer dialysates of PXOD-S and PXOD-E. The amino acid analysis (Table 2) and the fatty acid distribution (Table 3) of PXOD-S and PXOD-E confirmed these results. Increase in the relative content of β-hydroxy myristic acid of PXOD-S (64%) and PXOD-E (29%), in comparison with PX-S (45%) and PX-E (16%), was similar to that obtained by mild alkaline hydrolysis indicating that the hydrolytic action of performic acid affected primarily the ester bound fatty acids. Sephadex G-100 column chromatography of PXOD-S and PXOD-E showed that the remaining glucosamine, phosphate and fatty acids were firmly linked to the protein moiety. Although oxidized fractions PXOD-S and PXOD-E represent somewhat artificial products, their solubility properties could be utilized advantageously for immunochemical and physical studies.

Isolation and Characterization of the Tryptic and Pronase Cores of PX-S

The chemical analysis of the "simple proteins" PX-S and PX-E, and of the oxidized proteins PXOD-S and PXOD-E revealed unexpectedly the presence of the typical components of lipid A, namely, glucosamine, fatty acids and phosphorus, not separated from the protein moiety by preparative agarose electrophoresis or by gel filtration of the oxidized pro-

teins. Therefore, the removal of the protein moiety from lipid A was attempted by proteolytic enzymes. The purple colored tryptic core of PX-S, isolated from the hydrolysate (Figure 6) by isoelectric precipitation at pH 3.5-3.8 accounted for 64.4% of PX-S. The peptide fraction was colorless. The pronase core, derived from the tryptic core, was precipitated from the hydrolysate in the same pH range (3.5-3.8) as the tryptic core. The purple pronase core accounted for 28.7% of the tryptic core of 18.5% PX-S. To remove an additional amount of protein moiety the pronase core was subsequently subjected to the action of carboxypeptidase A and B; only carboxypeptidase A released a very small amount of amino acids yielding the carboxypeptidase core of PX-S. Tryptic and pronase digestion of PX-E resulted in the isolation of a tryptic core (60.3% of PX-E) and a pronase core (20.4% of PX-E). Isoelectrical precipitation occurred in the same pH range as that for the PX-S cores.

Physical-Chemical Characterization of the Tryptic and Pronase Cores of PX-S

Both cores were readily soluble in 0.02 M ammonium carbonate buffer, pH 8.6, but were insoluble at pH lower than 3.8, in water, and in saline. However, after repeated lyophilizations solubilization of PX-S cores in the same buffer was difficult without addition of 0.1-0.2% SDS.

Agarose electrophoresis of the tryptic and pronase cores exhibited single bands similar in electrophoretic mobility to that of PX-S (Figure 17). Gel filtration (Sephadex G-50) of the pronase core yielded a single fraction, as demonstrated by a nearly symmetrical absorption curve at 280 mμ. The amino acid and glucosamine analysis showed the same

Figure 17. Agarose electrophoresis of PX-S (A), tryptic core (B), and pronase core (C).

Protein preparations (20 mg/ml) were dissolved in 0.05 M Tris buffer, pH 7.6, containing 0.5% SDS, and diluted to a final concentration of 10 mg/ml with 1% agarose in barbital buffer. 1% agarose gel; barbital buffer, pH 8.6, ionic strength 0.05; 40 minutes.

values before and after column chromatography.

The infrared spectra of the tryptic and pronase cores were similar to that of PX-S (Figure 18). With shortening of the protein moiety (PX-S → pronase core) there was a decrease in the ν OH ass. and the ν NH ass. absorption at 3400-3280 cm^{-1} and an increase in the ν CH_3 + CH_2 absorption at 2960-2850 cm^{-1} and the carbohydrate absorption at approximately 1060 cm^{-1} (γ OH and ν C-O). The most striking feature in the spectrum of pronase core was the appearance of a strong ester absorption ν C=O at 1740 cm^{-1} which could be recognized in the spectra of PX-S and the tryptic core only as a shoulder on the amide I absorption band at 1650 cm^{-1}. The ester absorption was obviously caused by a decreased protein and increased fatty acid ester content in accordance with the simultaneous increase of CH_3 and CH_2 absorption at 2960-2850 cm^{-1} as well as with the results chemical analysis.

Only the cores, but not the peptide fractions were found to contain prodiogiosin or its derivative. The increased pigment absorption of cores is shown in Table 5.

TABLE 5

ABSORPTION OF PRODIGIOSIN IN PX-S AND ITS CORES
(0.5 mg/1 ml buffer)

	pH	537 mμ	470 mμ
PX-S	7.6	0.170	0.163
Tryptic core	8.5	0.181	0.196
Pronase core	8.5	0.342	0.369

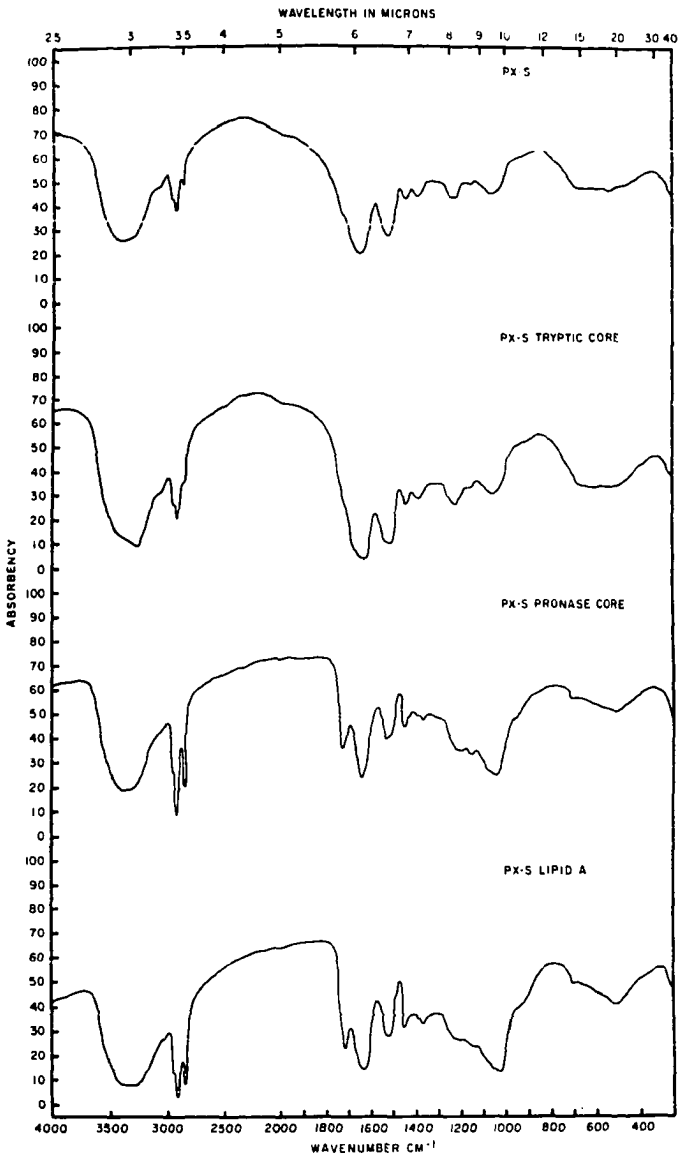
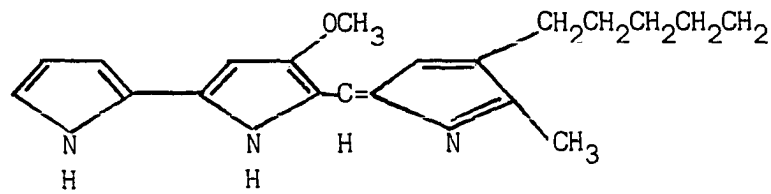


Figure 18. Solid infrared spectra of PX-S, tryptic core, pronase core, and lipid A (1.5-1.8 mg substance/250 mg KBr).

Prodigiosin could not be removed by the extraction of pronase core with organic solvents. Thus, it appears very likely that the pigment is covalently linked to the lipid A moiety or to the residual protein of pronase core.



Prodigiosin

Immunological Properties of the Tryptic and Pronase Cores of PX-S

The tryptic core (E) and the pronase core (F) gave a single identical precipitin line with antibodies to PX-S (Figure 19, pattern 1) and exhibited immunological identity with PX-S (A). Upon immunoelectrophoresis both cores formed single precipitin bands with antibodies to PX-S. The tryptic and the pronase cores were immunogenic. PX-S (A), tryptic core (E) and pronase core (F) showed a total coalescence of precipitin lines with antibodies to tryptic core (Figure 19, pattern 2) and with antibodies to pronase core (Figure 19, pattern 3).

Chemical Characterization of the Tryptic and Pronase Cores of PX-S

Results of the elementary analysis and chemical composition of cores are compared with those of PX-S (Table 6). Shortening of the protein moiety (PX-S $\rightarrow$ pronase core) caused an increase in relative content of carbon, hydrogen, phosphate, glucosamine and fatty acids, and a de-

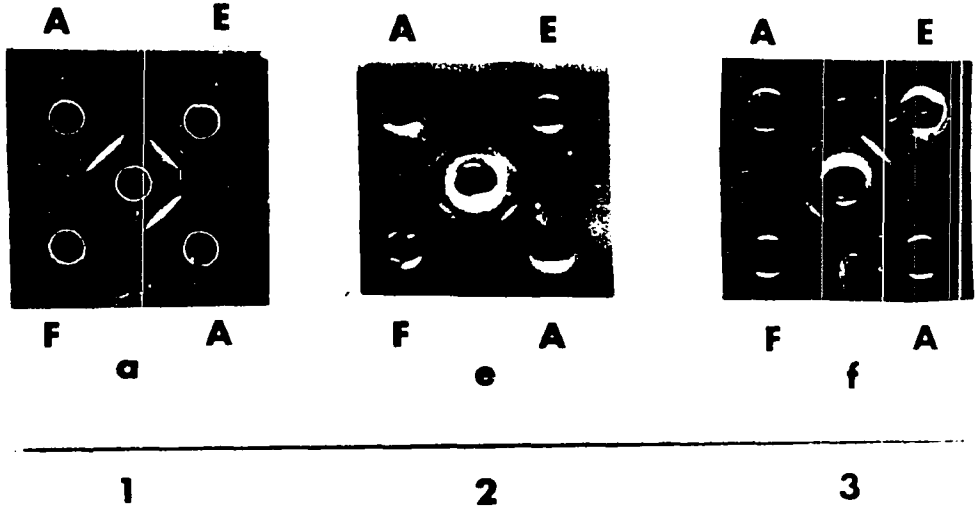


Figure 19. Immunodiffusion patterns of PX-S (A), tryptic core (E) and pronase core (F). a, anti PX-S serum; e, anti PX-S tryptic core serum; f, anti PX-S pronase core serum.

TABLE 6

ELEMENTARY ANALYSIS AND CHEMICAL COMPOSITION OF PX-S, TRYPTIC CORE AND PRONASE CORE

	C %	H %	N %	PO_4 %	Glucosamine %	Fatty Acids %	Amino Acids %	Recovery* %	Ash %
PX-S	51.23	6.92	13.43	2.01	2.87	10.1	72.89	89.75	4.9
PX-S Tryptic core	51.29	7.11	11.07	3.06	4.81	11.4	68.99	88.26	4.3
PX-S Pronase core	52.30	8.00	4.01	5.79	12.09	14.6	11.45	43.93	3.6

*Recovery was calculated from phosphate, glucosamine, fatty acid and amino acids.

creased content of nitrogen and amino acids. Proteolysis of tryptic core by pronase resulted in a more drastic composition change than that of PX-S by trypsin in accordance with the observed infrared spectra and yields of tryptic (64% of PX-S) and pronase (18% of PX-S) cores. Cores contained the characteristic components of the lipid A moiety with a molar ratio of fatty acids/phosphate/glucosamine 2:1:1 for tryptic core and 1:1:1 for pronase core. Recovery of the pronase core was very poor. The more likely molar ratio of 2:1:1, as determined for PX-S and the tryptic core, suggests that about 50% of the fatty acids were lost. A relatively low recovery of pronase core may be explained also by a loss of amino acids known to occur when hydrolysis is carried out in the presence of carbohydrates (98) and by the fact that the absolute amount of prodigiosin, present in the pronase core in the highest concentration, is not known. The molar ratio fatty acids/phosphate/glucosamine of PXOD-S was also found to be 2:1:1.

Comparison of the results of amino acid and glucosamine analyses between tryptic and pronase cores, their corresponding peptide fractions and PX-S is shown in Table 7. Upon hydrolysis neither glucosamine nor fatty acids could be detected in the peptide fractions. Aspartic acid was the major amino acid of pronase and carboxypeptidase cores. The percent content of glucosamine and the approximate molar ratios of aspartic acid and glucosamine of PX-S and corresponding cores are shown in Table 8.

The approximate molar ratio of glucosamine and major amino acids present in the pronase and carboxypeptidase core is shown in Table 9. From these data it became obvious that aspartic acid, glutamic acid or

TABLE 7

AMINO ACID COMPOSITION OF PX-S, TRYPTIC CORE, PRONASE CORE, AND PEPTIDES

	PX-S		Tryptic peptides		Tryptic core		Pronase peptides		Pronase core	
	μMoles/g	mg/100	μMoles/g	mg/100	μMoles/g	mg/100	μMoles/g	mg/100	μMoles/g	mg/100
Lysine	245	3.58	246	3.60	142	2.08	165	2.41	52	0.76
Histidine	74	1.15	56	0.87	80	1.24	75	1.16	13	0.20
Arginine	181	3.15	156	2.72	95	1.65	117	2.04	27	0.47
Aspartic acid	824	10.97	811	10.79	876	11.66	1640	21.83	142	1.89
Threonine	422	5.03	394	4.69	402	4.79	570	6.78	50	0.60
Serine	442	4.64	360	3.78	490	5.15	610	6.41	122	1.28
Glutamic acid	537	7.90	471	6.93	477	7.02	632	9.30	69	1.02
Proline	137	1.58	130	1.50	219	2.52	223	2.57	31	0.36
Glycine	657	4.93	598	4.49	600	4.50	867	6.51	89	0.67
Alanine	657	5.85	657	5.85	680	6.05	862	7.68	109	0.97
1/2 Cystine	-	-	-	-	-	-	-	-	-	-
Valine	378	4.43	419	4.91	307	3.60	482	5.65	60	0.70
Methionine	42	0.63	57	0.85	60	0.90	89	1.33	15	0.22
Isoleucine	238	3.12	227	2.98	182	2.39	268	3.52	45	0.59
Leucine	478	6.27	385	5.05	535	7.02	695	9.12	66	0.87
Tyrosine	240	4.35	222	4.02	235	4.26	350	6.34	17	0.31
Phenylalanine	228	3.77	188	3.11	235	3.88	322	5.32	32	0.53

TABLE 8

PERCENT CONTENT OF GLUCOSAMINE AND MOLAR RATIOS OF ASPARTIC ACID/GLUCOSAMINE IN PX-S AND ITS CORES

	Glucosamine in %	Molar Ratio Aspartic Acid:Glucosamine
PX-S	2.87	6.4:1
Tryptic core	4.81	3.2:1
Pronase core	12.09	1.0:5
Carboxypeptidase core	16.12	1.0:10

TABLE 9

MOLAR RATIO OF GLUCOSAMINE AND MAJOR AMINO ACIDS IN THE PRONASE AND CARBOXYPEPTIDASE

	Pronase Core	Carboxypeptidase Core
Glucosamine	10	10
Aspartic acid	2	1
Serine	2	1
Glycine	1	1
Alanine	2	1
Glutamic acid	1	1

serine could be most likely involved in a possible covalent linkage between lipid A and the protein moiety.

The fatty acid composition of PX-S and its cores was characterized by the presence of β-hydroxy myristic acid, a marker for lipid A, in a concentration higher than any other saturated and unsaturated fatty acid of a chain length from C_{12}-C_{18} (Table 10).

Isolation of Lipid A from the "Simple Protein" PX-S

The analysis of the "simple proteins" PX-S, PX-E, PXOD-S, and PXOD-E and the tryptic and pronase cores indicated the presence of lipid A, which could not be isolated from the protein moiety by extraction with chloroform, a characteristic solvent for lipid A. Upon hydrolysis of PX-S with 0.1 N HCl, a standard procedure for the isolation of lipid A from the lipopolysaccharide, a chloroform soluble fraction was obtained; like lipid A, this fraction was insoluble in acetone. The infrared spectrum (Figure 18) of this fraction was identical with the spectrum obtained for lipid A isolated from lipopolysaccharide and, on the other hand, was very similar to the spectrum of the pronase core. The ester/amide I ratio of this fraction was 0.86, a slightly higher value than that of pronase core (0.79). This could be explained by a reduced amide absorption, caused by a decreased protein content of lipid A. The glucosamine content of this preparation was 27.68%. Thus the hydrolysis of "simple protein" resulted in the separation of lipid A, which could not be extracted without prior hydrolysis and indicated that the lipid moiety may be covalently linked to the protein.

TABLE 10

PERCENT FATTY ACID DISTRIBUTION OF PX-S, TRYPTIC CORE AND PRONASE CORE

	PX-S %	Tryptic core %	Pronase core %
C_{12}	2.6	3.7	5.4
C_{14}	9.5	9.8	10.7
C_{14-1}	Trace	-	-
Unknown	12.9	14.6	14.4
C_{16}	16.5	18.2	13.3
C_{16-1}	5.5	3.4	2.8
Unknown	Trace	3.1	5.7
Unknown	3.9	4.7	-
Unknown	4.5	3.7	5.8
C_{14-OH}	44.5	38.7	41.8

Isolation and Characterization of "Conjugated Protein" and Polysaccharide Moiety from LPX-Ud

It has been tacitly surmised, but never proven, that the presence of lipid A in "conjugated protein" represents the only difference between this protein and "simple protein." To test this assumption, the physical, chemical and immunological properties of "conjugated protein" isolated from endotoxin complex were compared with those of "simple protein" (PX-S).

The endotoxin complex (LPS-U) isolated by trichloroacetic acid extraction of S. marcescens (Figure 5) contained approximately 20% free lipids extractable by chloroform-methanol (2:1, v/v). The "conjugated protein" isolated from the defatted endotoxin complex (LPS-Ud) by hydrolysis with 0.1% acetic acid represented 36% of the endotoxin complex. Upon hydrolysis of a small amount of material extractable by chloroform from the acetic acid hydrolysate, only traces of fatty acids were detected. The unsaponifiable material was not investigated. The polysaccharide moiety, accounting for 60% of the endotoxin complex, was separated by dialysis into an inner and outer dialysate. The non-dialyzable fraction (inner dialysate) represented about 52% of the polysaccharide fraction and was characterized by 41.78% C, 6.24% H, 3.23% N and less than 0.43% P. Amino acid analysis revealed trace amounts of amino acids (less than 0.48%) and 38.39% glucosamine. The results of quantitative gas-liquid chromatography (this analysis was carried out by Mr. Chi-Sun Wang) indicated that inner dialysate contained glucose, galactose and glucosamine in a molar ratio 2:1:2. This fraction, representing most probably the so-called side chain of the polysaccharide moiety, gave a

single precipitin line with antibodies to LPS-Ud (Figure 12). The outer dialysate contained 2.18% P, 5.6% N, and heptose as the most characteristic sugar component (this result was provided by Mr. Chi-Sun Wang). Since heptose or a heptose phosphate is a typical component of the polysaccharide "core," the outer dialysate represents a partially degraded "core" portion of the polysaccharide moiety. Both fractions were easily soluble in distilled water.

The tryptic and pronase cores of "conjugated protein" were obtained according to the procedure utilized for the preparation of cores from the "simple protein." The tryptic core represented 75% of "conjugated protein," and the pronase core 60.5% of tryptic core or approximately 45% of "conjugated protein."

Physical-Chemical Characterization of "Conjugated Protein" and Corresponding Tryptic and Pronase Cores

The "conjugated protein" and both cores were soluble in ammonium carbonate buffer at pH 8.6 and precipitable by addition of acetic acid at pH 3.5-3.8. However, after repeated lyophilizations these protein preparations were soluble only in Tris buffer, pH 7.6, containing 0.2% SDS.

"Conjugated protein" dissolved in Tris buffer containing 0.1-0.2% SDS exhibited a single symmetrical peak in the analytical ultracentrifuge (Figure 20) with a $s_{obs.} = 2.4S$. Electrophoresis on agarose prepared with veronal buffer, pH 8.7, and Tris buffer, pH 7.6, containing 0.5% SDS exhibited a single band for LPS-Ud, "conjugated protein" and the tryptic and pronase cores (Figure 21). Infrared spectra of "conjugated protein" and both cores are shown in Figure 22. Although spectra

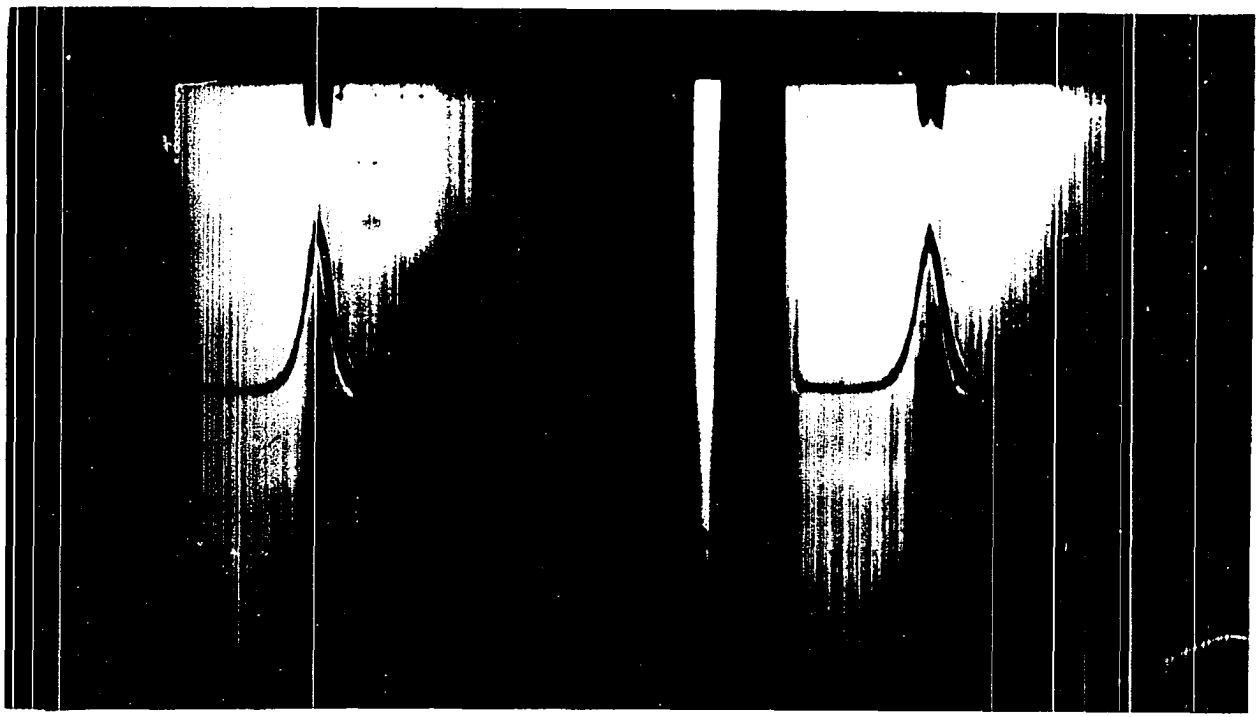


Figure 20. Ultracentrifugal sedimentation pattern of "conjugated protein."

"Conjugated protein" (6 mg/ml) dissolved in 0.05 M Tris-Cl buffer, pH 7.6, containing 0.1-0.2% SDS, density 1.004 g/ml, was centrifuged in a synthetic boundary cell at 52,640 rpm at 25°. Exposures were taken from left to right at 4 minute intervals for a total run of 64 minutes. Photographs were obtained at 4 and 8 minutes after reaching speed.

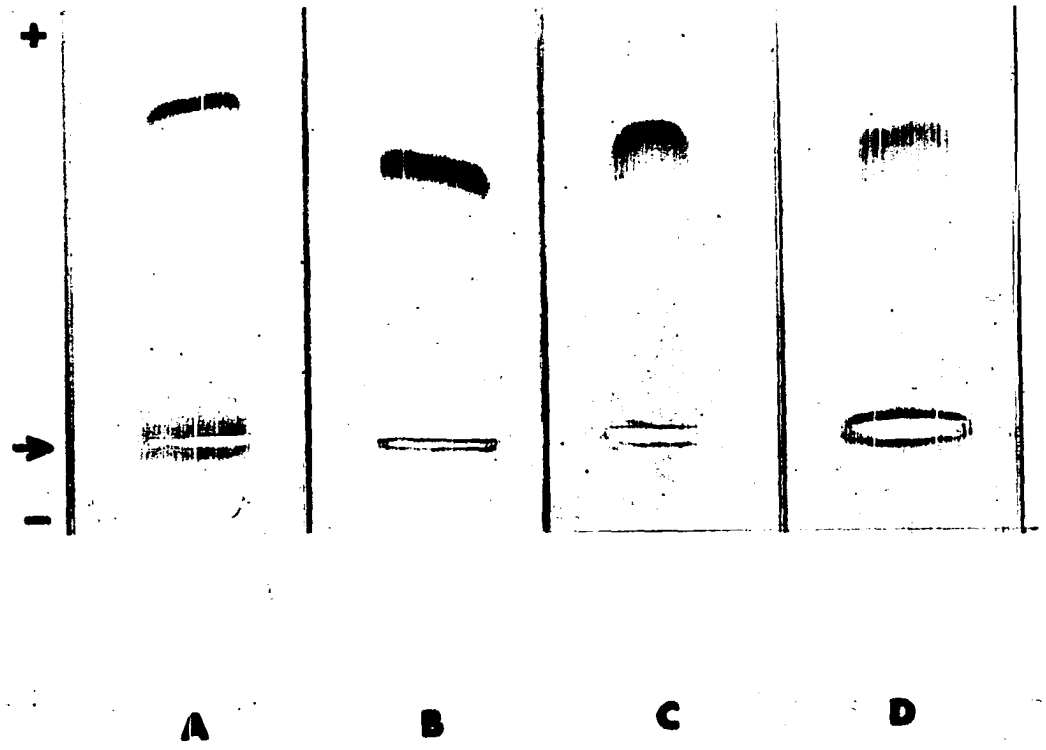


Figure 21. Agarose electrophoresis of LPS-U-S (pattern A), "conjugated protein" (pattern B), tryptic core (pattern C) and pronase core (pattern D).

Protein preparations (20 mg/ml) were dissolved in 0.05 M Tris buffer, pH 7.6, containing 0.5% SDS and diluted to a final concentration of 10 mg/ml with 1% agarose in barbital buffer. 1% agarose gel; barbital buffer, pH 8.6, ionic strength 0.05; 40 minutes.

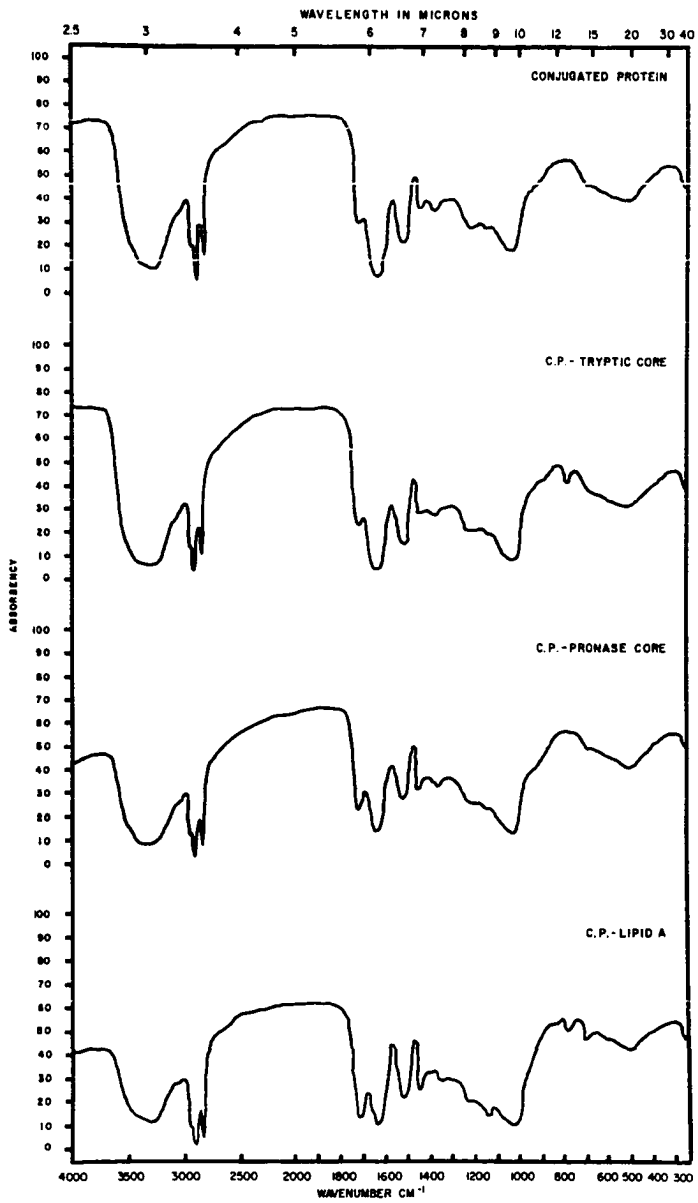


Figure 22. Solid infrared spectra of "conjugated protein," tryptic core, pronase core and lipid A (1.5-1.8 mg substance/250 mg KBr).

resembled those of the "simple protein" and corresponding cores (Figure 18), the ν CH_3 and CH_2 absorption at 2960-2850 cm^{-1}, the ν C–O and γ OH carbohydrate absorption at approximately 1060 cm^{-1} and the ν P=O band at approximately 1230 cm^{-1} are more distinct in the spectra of the "conjugated protein" and its cores. In contrast to "simple protein," the "conjugated protein" exhibited a distinct ν C=O ester absorption which gradually increased in the tryptic and pronase cores. The ester/amide I ratios are shown in Table 11.

TABLE 11

ESTER/AMIDE I RATIOS OF "CONJUGATED PROTEIN" CORES AND LIPID A

	Length of Amide I Band in mm	Length of Ester Band in mm	Ester/Amide I Ratio
"Conjugated Protein"	84	57	0.67
Tryptic core	82	61	0.74
Pronase core	64	55	0.86
Lipid A	65	63	0.96

Immunological Properties of "Conjugated Protein" and Its Tryptic and Pronase Cores

Similarly to "simple protein" and its cores, the "conjugated protein" and corresponding tryptic and pronase cores were found to be immunogenic. The "conjugated protein" (G) exhibited a single precipitin line against anti "conjugated protein" serum (g) (Figure 23, pattern 1). The tryptic core (H) and pronase core (I) gave single precipitin lines with antibodies to "conjugated protein" (g) (Figure 23, pattern 2),

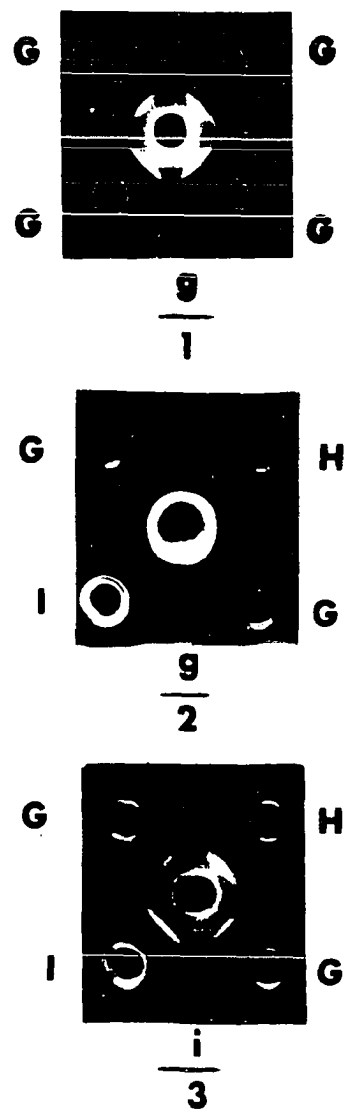


Figure 23. Immunodiffusion patterns of "conjugated protein" (G), tryptic core (H) and pronase core (I).

g, anti "conjugated protein" serum; i, anti "conjugated protein" pronase core serum.

tryptic core (h) and pronase core (i) (Figure 23, pattern 3). The immunoelectrophoretic patterns of tryptic and pronase core are shown in Figure 24. "Conjugated protein" (G), PX-S (A) and PX-E (B) gave a line of total coalescence with antibodies to PX-S (a) (Figure 25, pattern 1). A similar line of total fusion was also obtained in the reaction of "conjugated protein" (G) with antibodies to "conjugated protein" (g), PX-S (a), C.P. tryptic core (h) and pronase core (i) (Figure 25, pattern 2). However, the reaction of "conjugated protein" with antibodies to LPS-Ud (k) reveals partial identity with other precipitin lines.

Chemical Characterization of "Conjugated Protein" and Corresponding Tryptic and Pronase Cores

"Conjugated protein" and both cores contained fatty acids, phosphate and glucosamine, the characteristic constituents of lipid A, but no other sugars or lipids (Table 12). The percent content of fatty acids, phosphorus and glucosamine was higher and that of amino acids lower in "conjugated protein" than in "simple protein," indicating a higher content of lipid A in "conjugated protein." Shortening of the protein part of "conjugated protein" resulted in a decreased content of amino acids and nitrogen and an increased content of carbon, hydrogen, phosphorus, glucosamine and the fatty acids. As with the "simple protein," the change of data was more pronounced from the tryptic core into the pronase core. The molar ratio of fatty acids/phosphate/glucosamine was approximately 3:1:1 for "conjugated protein" and its tryptic and pronase core in comparison with a molar ratio of 2:1:1 for the "simple protein" and its derivatives. Thus, the fatty acid content of the lipid A moiety of "conjugated protein" was higher than that of "simple protein."

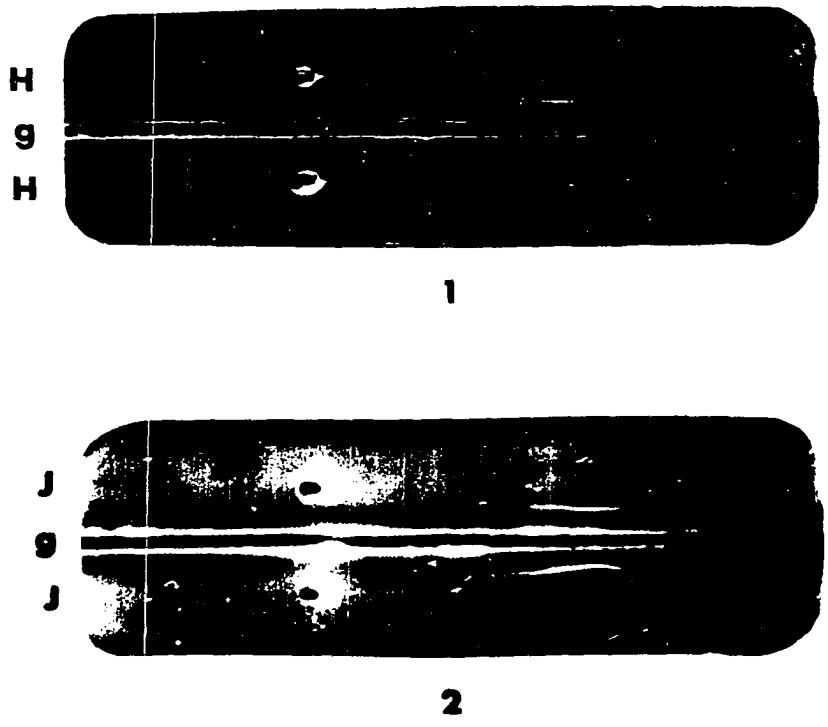


Figure 24. Immunoelectrophoresis patterns of "conjugated protein" tryptic core (H) and pronase core (I).

g, anti "conjugated protein" serum.

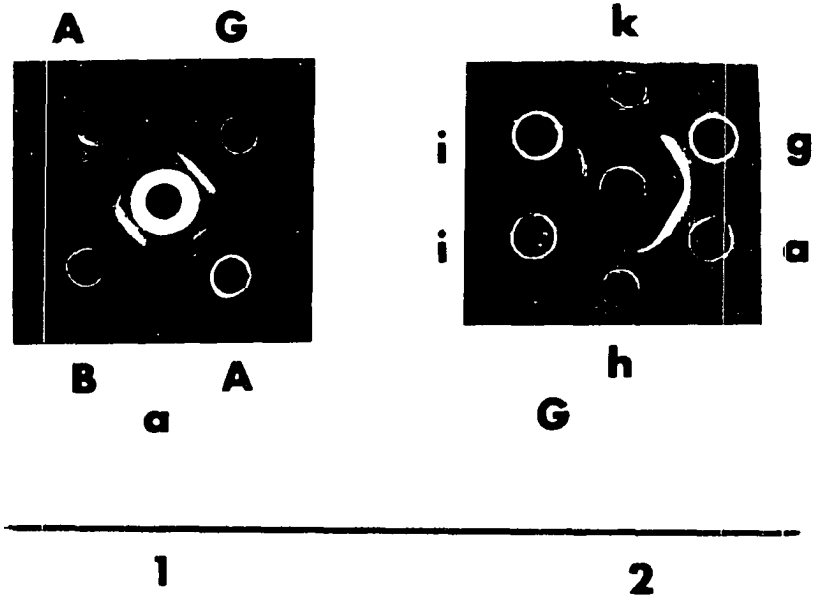


Figure 25. Immunodiffusion patterns of PX-S (A), PX-E (B), "conjugated protein" (G), pattern 1, and "conjugated protein" (G), pattern 2.

a, anti PX-S serum; k, anti LPS-U-S serum; g, anti "conjugated protein" serum; h, anti "conjugated protein" tryptic core serum; i, anti "conjugated protein" pronase core serum.

TABLE 12

ELEMENTARY ANALYSIS AND CHEMICAL COMPOSITION OF "CONJUGATED PROTEIN," TRYPTIC CORE AND PRONASE CORE

	C %	H %	N %	PO_4 %	Glucosamine %	Fatty Acids %	Amino Acids %	Recovery* %	Ash %
"Conjugated Protein"	54.74	8.26	6.30	4.98	9.35	46.08	35.26	95.67	0.46
CP tryptic core	54.86	8.37	5.26	5.52	11.71	49.60	30.72	97.55	None or Trace
CP pronase core	57.39	9.23	3.45	6.48	18.06	53.20	12.90	90.64	None or Trace

*Recovery is calculated from phosphate, glucosamine, fatty acids and amino acids.

The amino acid composition of "conjugated protein" and both cores is shown in Table 13. As with the "simple protein," aspartic acid was found in the relatively highest concentration and cystine was absent. Neither glucosamine nor fatty acids were found upon hydrolysis of the tryptic and pronase peptides.

Gas-liquid chromatography of the fatty acids isolated from "conjugated protein" (Figure 26) and its cores revealed again that the β-hydroxy myristic acid was present in relatively highest concentration among saturated and unsaturated fatty acids of a chain length of C_{12}-C_{18} (Table 14). Hydrogenation and acetylation of the fatty acid methyl esters proved the presence of unsaturated and hydroxy acids, as determined by comparison with standard fatty acid mixtures.

Isolation of Lipid A

Lipid A was isolated from "conjugated protein" by the same hydrolytic procedure used for its isolation from "simple protein." The infrared spectrum of lipid A is shown in Figure 22. It was again identical with the spectrum of lipid A isolated from lipopolysaccharide. The ester/amide I ratio (0.96) of lipid A was higher than that of the pronase core (0.86). The glucosamine content of the lipid A preparation was 24%.

Comparison of the "Simple Proteins" PX-S and PX-E and "Conjugated Protein"

Table 15 compares the chemical composition of the "conjugated protein," PX-S, and PX-E.

From these results it is obvious that "conjugated protein" contained the highest, and PX-E the lowest amount of lipid A. Molar ratio of amino acids of "conjugated protein" was closer to that of PX-S than

TABLE 13

AMINO ACID COMPOSITION OF "CONJUGATED PROTEIN," TRYPTIC CORE AND PRONASE CORE

	"Conjugated Protein"		Tryptic Core		Pronase Core	
	μMoles/g	mg/100	μMoles/g	mg/100	μMoles/g	mg/100
Lysine	135	1.97	82	1.20	73	1.07
Histidine	31	0.48	35	0.53	15	0.23
Arginine	82	1.43	48	0.84	42	0.73
Aspartic acid	457	6.08	328	4.36	197	2.62
Threonine	157	1.87	108	1.29	53	0.63
Serine	212	2.23	139	1.46	108	1.13
Glutamic acid	327	4.81	212	3.12	120	1.77
Proline	85	0.98	59	0.69	55	0.63
Glycine	332	2.49	294	2.21	203	1.52
Alanine	315	2.81	227	2.02	140	1.25
Valine	202	2.37	153	1.79	83	0.97
Methionine	45	0.67	39	0.30	-	-
Isoleucine	95	1.25	68	0.90	42	0.55
Leucine	224	2.94	158	2.07	75	0.98
Tyrosin	156	2.83	140	2.47	51	0.92
Phenylalanine	134	2.21	99	1.63	54	0.89

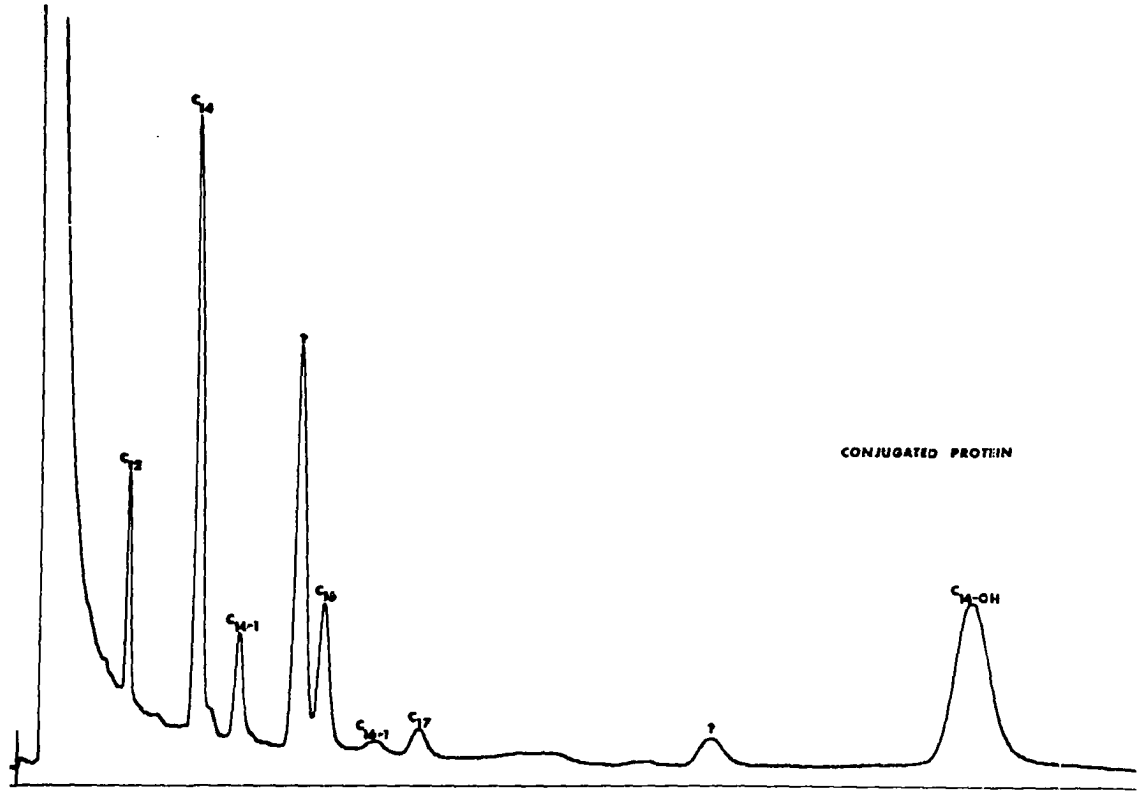


Figure 26. Gas-liquid chromatogram of fatty acid methyl esters of "conjugated protein."

TABLE 14

PERCENT FATTY ACID DISTRIBUTION OF "CONJUGATED PROTEIN," TRYPTIC CORE AND PRONASE CORE

	"Conjugated Protein"	Tryptic Core	Pronase Core
C_8	-	-	0.74
C_{10}	-	-	1.93
C_{12}	3.81	2.80	4.96
C_{14}	21.56	25.84	15.27
C_{14-1}	4.22	-	-
Unknown	20.90	23.88	13.36
C_{16}	9.65	12.36	17.18
C_{16-1}	1.45	1.86	3.41
C_{18}	3.28	-	3.22
C_{18-1}	-	-	11.82
Unknown	3.56	-	-
C_{14-OH}	31.58	33.26	28.10

TABLE 15

CHEMICAL COMPOSITION OF "CONJUGATED PROTEIN," PX-S AND PX-E

%	"Conjugated Protein"	PX-S	PX-E
Amino acids	35.26	72.89	84.42
Glucosamine	9.35	2.87	1.31
Fatty acids	46.08	10.10	5.98
PO_4^{3-}	4.98	2.01	1.59

it was to the molar ratio of PX-E (Table 16).

Mapping of the tryptic peptides shows a great resemblance, if not identity, between the peptides of "conjugated protein" (Figure 27) and PX-S (Figure 28). However, mapping of the peptides of E. coli (Figure 29) revealed differences in comparison with "conjugated protein" and PX-S.

Toxicity of the "Conjugated" and "Simple Protein"

Determination of the LD_{50} in mice disclosed the dependency of toxicity on solubilization of substances and strain of mice used (Table 17).

Both endotoxin complex and "conjugated protein" when tested in Tris-SDS buffer were highly toxic. Removal of the polysaccharide moiety decreased the toxicity of the endotoxin complex drastically, although the isolated polysaccharide moiety was non-toxic. Although the "conjugated protein" was more toxic than "simple protein," the latter should still be considered as moderately toxic. Preparations solubilized in 0.05 M

TABLE 16

COMPARISON OF MOLAR RATIOS* OF AMINO ACIDS OF "CONJUGATED PROTEIN," PX-S, AND PX-E

	"Conjugated Protein"	PX-S	PX-E
Lysine	4	3	3
Histidine	1	1	1
Arginine	3	2	2
Aspartic acid	14	11	9
Threonine	6	6	4
Serine	6	6	4
Glutamic acid	10	7	10
Proline	3	2	3
Glycine	10	9	8
Alanine	10	9	9
Valine	6	5	5
Methionine	1	1	2
Isoleucine	3	3	4
Leucine	7	6	7
Tyrosin	5	3	2
Phenylalanine	4	3	3

*Molar ratios based on histidine taken as unity.

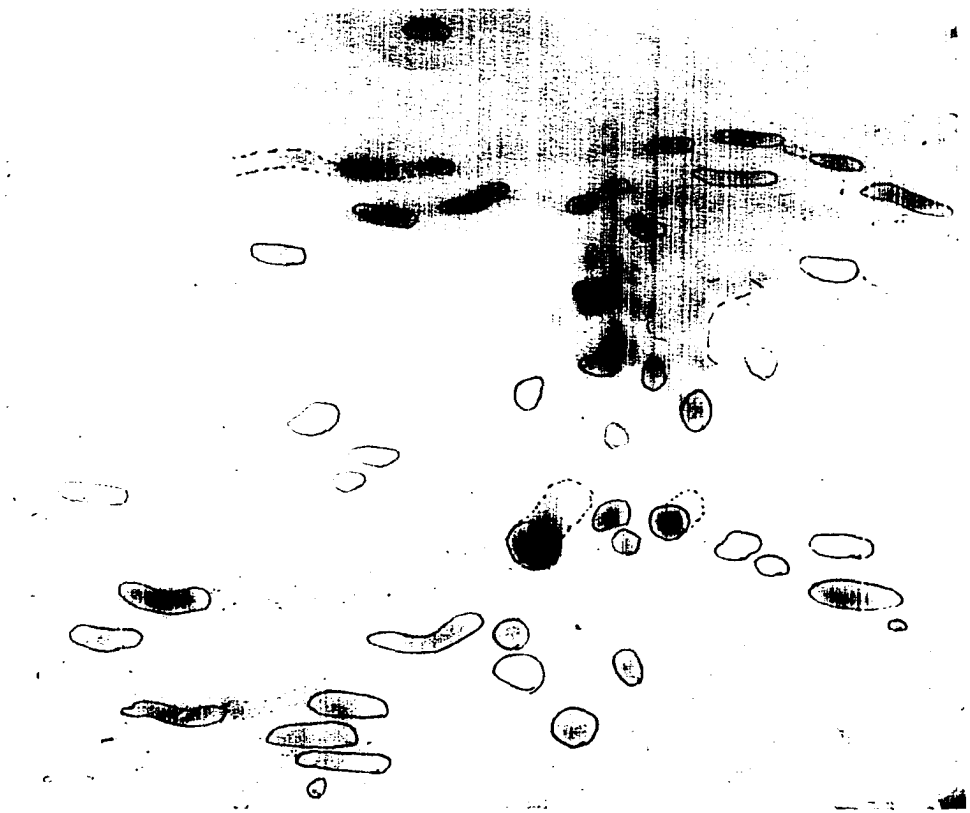


Figure 27. Peptide mapping of tryptic peptides of "conjugated protein."

First dimension: descending paper chromatography (n-butanol/acetic acid/water 4:1:5, v/v). Second dimension: high voltage electrophoresis (1 hour, 3000 V, pH 3.65). Stain: ninhydrin.

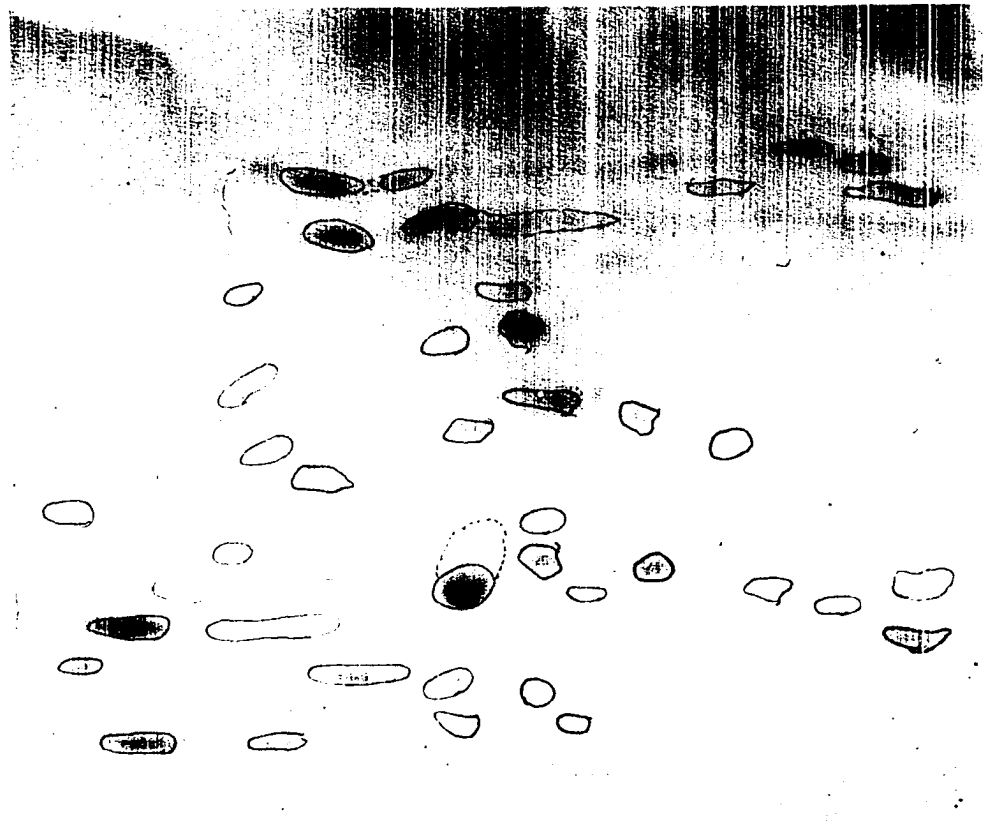


Figure 28. Peptide mapping of tryptic peptides of PX-S

Experimental conditions as in Figure 27.

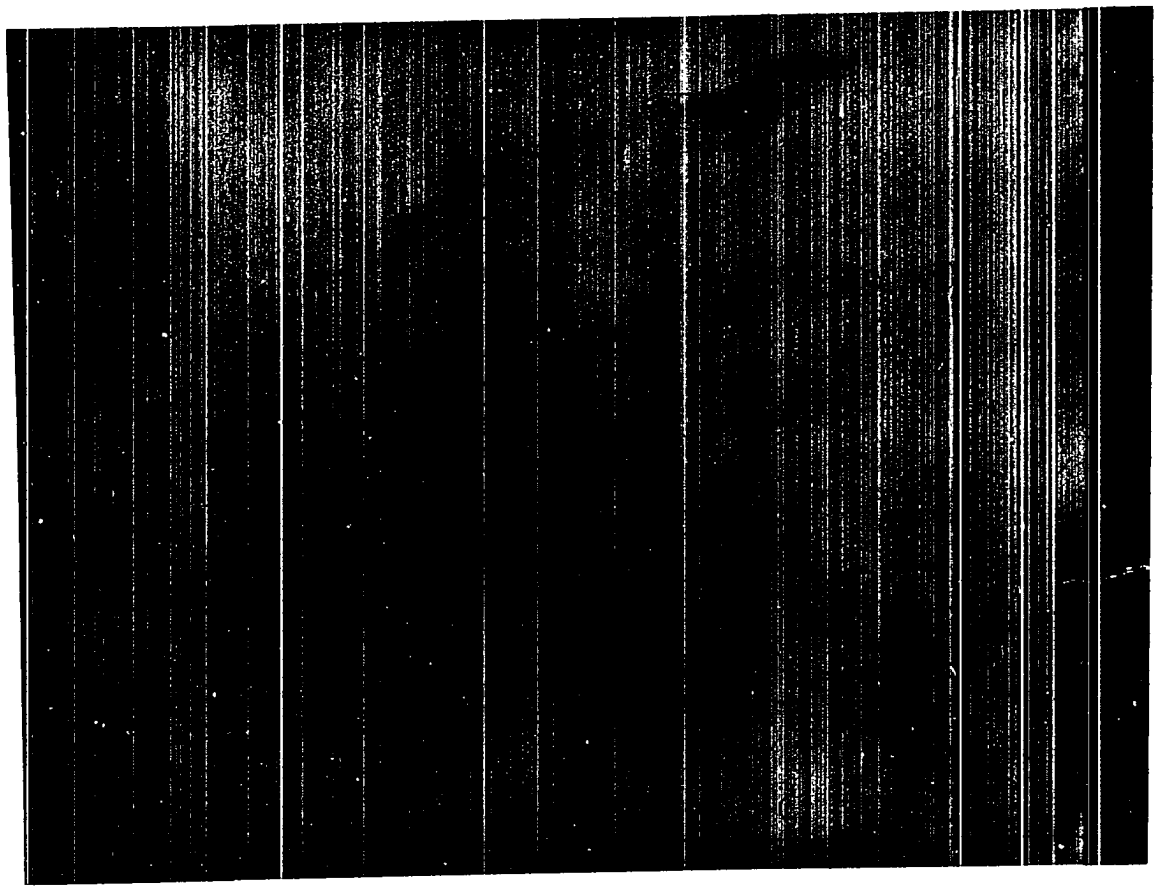


Figure 29. Peptide mapping of tryptic peptides of PX-E.

Experimental conditions as in Figure 27.

Tris buffer containing 0.5% SDS displayed higher toxicity than those suspended in 0.9% NaCl. The buffer system itself was not toxic for mice.

TABLE 17

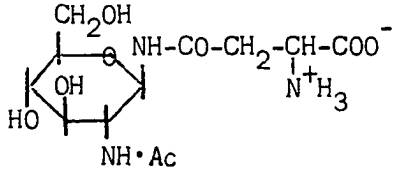
LD_{50} OF "CONJUGATED" AND "SIMPLE PROTEIN" (PX-S)

	Random bred Swiss-regular Mice		Inbred Balb-C Mice	
	In Tris-SDS	In Saline	In Tris-SDS	In Saline
	mg/kg		mg/kg	
Endotoxin complex (LPS-Ud)	7.5	50.0	1.3	13.4
"Conjugated Protein"	37.5	187.5	22.3	
"Simple Protein"	55.0			
Protein-free Endotoxin (LPS-A)			12.1	39.9
Lipid A			14.2	
Polysaccharide	non-toxic		non-toxic	

Studies on the Protein-Carbohydrate Linkage

Since lipid A was removed from the "conjugated" and "simple protein" by acid hydrolysis rather than by such mild procedures as chloroform extraction or separation by gel filtration or agarose electrophoresis, it was assumed that lipid A was attached to the protein moiety through a covalent linkage. The amino acid analysis of pronase cores revealed aspartic acid, glutamic acid and serine as amino acids most likely to be involved in a linkage with glucosamine. An attempt to release serine from an O-glycosidic linkage by β-elimination according to

the method of Carubelli (101) failed. Glutamic acid or glutamine has not yet been reported as being involved in a carbohydrate-protein linkage (102). However, it is well known that asparagine is linked to glucosamine in glycoproteins in form of 2-acetamido-1-β-(L-β-aspartamido)-1,2-dideoxy-D-glucose or β-aspartyl-acetylglucosaminylamine (BAGA) (102, 103):



The half-life of this compound upon hydrolysis with 2 N HCl at 100° is 45 minutes (102). Therefore, the pronase core of "conjugated protein" was hydrolyzed under these conditions for 20 minutes, and high voltage electrophoresis at pH 3.65, 3000 V according to Winzler et al. (103) revealed the presence of aspartic acid, glucosamine and a spot corresponding to an authentic sample of BAGA (Figure 30). Since this substance is characterized by the same Rf value as threonine (104), the latter amino acid was used for comparison. Fractionation of mild acid (2 N HCl, 100°, 20 minutes) hydrolysate on amino acid analyzer disclosed a peak (6.1 cm) eluted in front of aspartic acid (9.1 cm); this peak was absent after strong acid hydrolysis (5.7 N HCl, 105°, 24 hrs). A sample of authentic β-aspartyl-acetylglucosaminylamine appeared at the same position (6.1 cm) (Figure 31) and had the same retention (18.8%) with respect to norleucine (32.3 cm) as the unknown compound. Addition of the authentic compound to the hydrolysate revealed no additional peak. From these experiments it seems very likely that lipid A moiety is covalently linked to the pro-

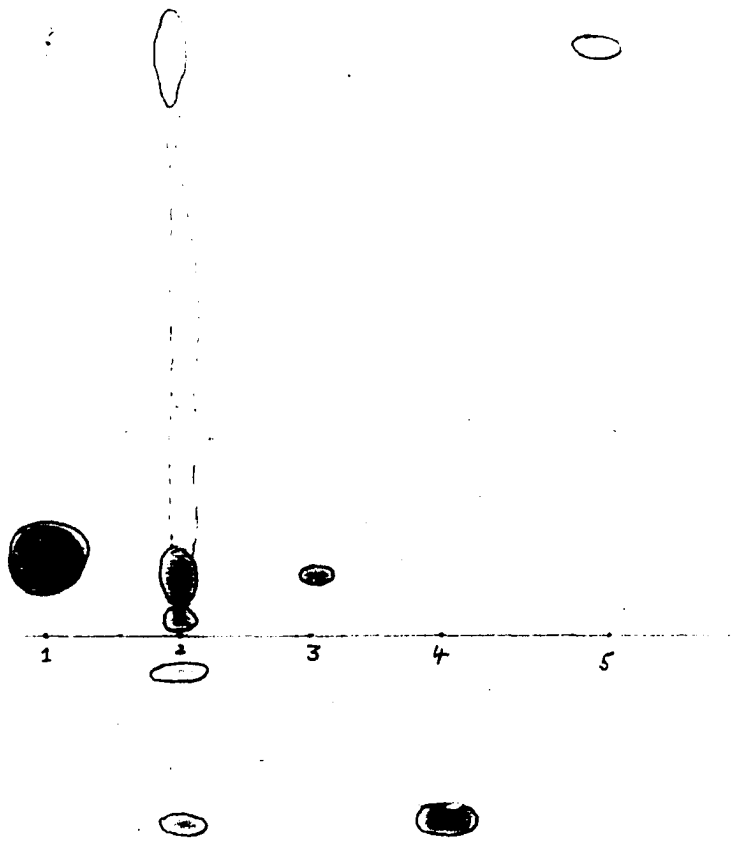


Figure 30. High voltage electrophoresis of hydrolysate of "conjugated protein" pronase core.

Hydrolysis with 2 N HCl, 30 minutes, 100°C. 1, threonine; 2, hydrolysate; 3, β-aspartyl-glucosaminylamine; 4, aspartic acid; 5, glucosamine.

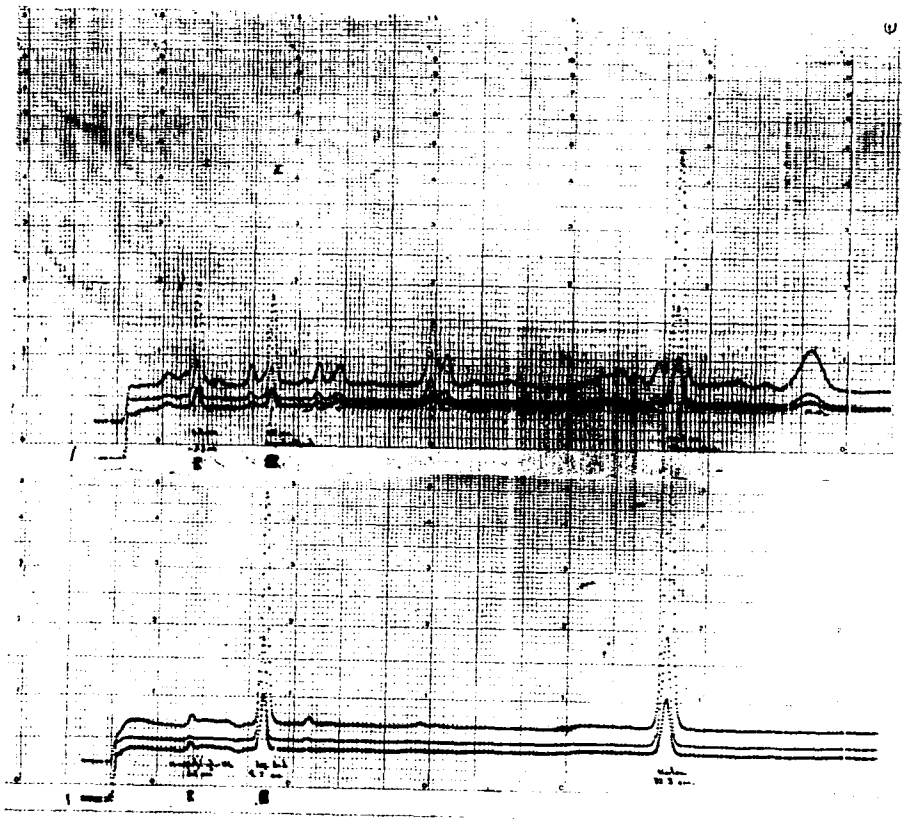


Figure 31. Ion exchange chromatography of hydrolysate of "conjugated protein" pronase core (top), and reference substances (bottom).

Hydrolysis: 2 N HCl, 30 minutes, 100°C. I, β-aspartyl glucosaminylamine; II, aspartic acid. Peak to the right: norleucine.

tein most probably through β-aspartyl-acetylglucosaminylamine. However, the possible involvement of glutamine in a carbohydrate-protein linkage has not yet been excluded by these experiments, since the electrophoretic and chromatographic behavior of a theoretical glutaminyl-acetyl-glucosaminylamine is not known.

CHAPTER V

DISCUSSION

Treatment of both trichloroacetic acid extracted Boivin antigen from the chromogenic strain of S. marcescens 08 and whole cells of E. coli by aqueous phenol (17) led to the isolation of the protein moieties of endotoxins from corresponding phenol phases. Both proteins were found to contain the characteristic components of lipid A, namely, fatty acids, especially β-hydroxy myristic acid, phosphorus, and glucosamine. The proteins PX-S and PX-E, and the oxidation products PXOD-S and PXOD-E were found to be homogeneous on the basis of ultracentrifugal analysis, agarose electrophoresis, immunodiffusion, immunoelectrophoresis, and, in the case of oxidized proteins, gel chromatography. Could the components of lipid A be regarded as contaminations? Protein moiety could be contaminated either by lipopolysaccharides or lipid A, or by degradation products of both compounds. Lipopolysaccharides can be excluded, since hydrolysis of the protein preparations revealed presence of no other sugars except glucosamine; furthermore, the phenol phases had been washed with water until no immunochemically-detectable lipopolysaccharides could be identified in two successive washings. That leaves chloroform soluble lipid A or its degradation products as possible impurities. However, exhaustive extraction of protein preparations by chloroform or chloroform/methanol

(2:1) did not remove lipid A, but free lipids such as fatty acids, glycerides, and phospholipids. Only after hydrolysis of PX-S with 0.1 N HCl could lipid A be isolated by chloroform extraction. Similarly, repeated isoelectrical precipitations of tryptic and pronase cores did not result in the removal of lipid A. As a matter of fact, the content of non-extractable lipid A was higher in cores than in the starting protein preparations. Thus, the lipid A moiety should be considered as an entity covalently bound to the protein moiety. Consequently, the protein moieties of endotoxins from S. marcescens and E. coli, isolated by aqueous phenol, cannot be classified as "simple proteins."

This conclusion is contrary to those of some earlier investigators who claimed the isolation of "simple proteins" by similar methods (17, 19, 21, 45, 56, 55). Morgan (19, 45, 55) and Goebel (21, 46), indeed, realized the existence in endotoxins of a component other than protein and polysaccharide, but it remained for Westphal and Lüderitz (16) to initiate intensive investigation into the nature of the lipid moiety, to recognize the principle components of lipid A, and to assume that Goebel's toxic "factor T" and Morgan's "prosthetic group" were principally identical with lipid A (16, 105). Thus, early investigators, not knowing the composition of lipid A, used almost solely the absence of or a low content of phosphorus as a chemical criterion for "simple proteins," or as a means to differentiate between "simple" and "conjugated" proteins. Goebel's failure (21) to detect glucosamine in "simple" and "conjugated (toxic) protein" of Shigella paradysenteriae might have been the reason why later investigators were not searching at all for glucosamine in endotoxin proteins. The β-hydroxy myristic acid was not recognized as a

relatively specific marker for lipid A until 1953 (36). These facts and, possibly, the lack of sensitive analytical methods may explain why lipid A was not recognized as an integral part of "simple proteins."

The analysis of "conjugated protein" of S. marcescens, derived from the endotoxin complex by mild acid hydrolysis, confirmed the assumption of Westphal and Lüderitz (16) that it consists of lipid A and protein. No other compounds but those characteristic for lipid A and amino acids were detected upon hydrolysis. "Simple" and "conjugated" proteins of S. marcescens 08 differed in quantitative rather than qualitative chemical composition. Fatty acids (46.08%), glucosamine (8.73%), phosphate (4.98%), and, thus, lipid A were found in higher concentrations in "conjugated" than in "simple" protein (FA 1.1%, glucosamine 2.87%, phosphate 2.01%). Similar to PX-S, lipid A was firmly bound to the protein moiety of "conjugated protein" and could only be extracted by chloroform after hydrolysis with 0.1 N HCl. The close similarity of the molar ratios of amino acids and the distribution of tryptic peptides indicated clearly the identity of both proteins. Thus the assumption (15) about the identity of the two protein moieties was found to be correct. However, "conjugated" and "simple" proteins are not distinct, as proposed (15) by the presence or absence of lipid A, but rather by the presence of a larger or smaller amount of lipid A linked to the protein moiety. Hence, the "conjugated protein" consists of the protein moiety linked to the entire lipid A, whereas in "simple protein" (PX-S) the identical protein moiety is bound only to a fragment of lipid A.

These unexpected results have raised intriguing questions not only about the structure of lipid A, but also about the difference in

the action of aqueous phenol and trichloroacetic acid on endotoxins in general and on lipid A in particular. As already mentioned, the structure of lipid A is not yet fully understood. Westphal and Lüderitz (16, 35, 105) have considered lipid A as a single molecular entity integrated into the endotoxin complex. However, fractionation of lipid A obtained from lipopolysaccharides by mild acid hydrolysis disclosed a variety of compounds, such as free fatty acids and glucosamine on one hand, and complex, unaltered phosphorylated acylpolyglucosamines of different chain length (34, 106) on the other. One can easily visualize that these breakdown products of hydrolysis represent parts of a single structural unit, lipid A, as proposed by Westphal and Lüderitz (16, 105). Nowotny (106), on the other hand, prefers the term "lipid moiety" implicating that the constituents of lipid A derive either from the different subunits of complex endotoxin structure in form of chemically identical repeating units, or from different lipid-rich zones of related but not identical structures. The results of this investigation support strongly the former proposal describing lipid A moiety as a single structural entity. Nevertheless, it is difficult to visualize lipid A as a simple linear structure consisting of a phosphorylated polyglucosamine chain with ester- and amide-bound fatty acids. More likely, it is a branched or even three-dimensional network structure consistent with previous results concerning the structure of lipid A (34, 35). For example, several polyglucosamine chains of different length could be linked together by phosphodiester linkages. Although the predominant bond between O,N-acylated glucosamine phosphate units seems to be of glycosidic nature (34, 35), a phosphodiester linkage within a single polyglucosamine chain, first proposed by

Nowotny (37), should not be excluded (34, 35). In contrast to the stability of glucosamine glycosides, phosphodiester linkages are very labile to mild acid hydrolysis (107, 108). Simple phosphodiesters such as dimethylphosphate are hydrolyzed below pH 4 (109). Adjacent hydroxy groups have an enhancing effect on the rate of phosphodiester hydrolysis. Thus, as a result of mild acid hydrolysis of phosphodiester linkages either between or within polyglucosamine chains the phosphate groups should remain linked to glucosamine as monoesters in C_4 or C_6 position, since these monophosphates are very acid stable compared with the phosphate at position C_1 (35, 110). Mild acid hydrolysis used for the liberation of lipid A, could thus cause the cleavage of phosphodiester linkages and result in breakdown products of different sizes.

Aqueous phenol extraction has been widely used for the isolation of lipopolysaccharides since this method was introduced by Westphal et al. (17) in 1952. It has been generally assumed that, except for the dissociation of endotoxins into the protein moiety and lipopolysaccharide, this procedure causes little, if any, cleavage of covalent linkages. Proteins, indeed, do not seem to be altered or denaturated by phenol treatment as shown for physical-chemical parameters and enzyme activity of ribonuclease (111). Tsang (34) studied intensively the effects of phenol treatment on the chemical composition of lipopolysaccharides from S. marcescens 08 and reported a markedly decreased fatty acid and glucosamine content of a multiply phenol-treated lipopolysaccharide preparation. However, even after multiple phenol treatment, a small amount of amino acids, mainly aspartic acid and histidine, was found in the lipopolysaccharide preparation. Thus, phenol treatment caused cleavage of

ester and amide bound fatty acids, a structural degradation of the lipopolysaccharide, and dissociation of protein.

Whereas "simple proteins" were obtained by phenol treatment of the complete Boivin antigen (PX-S) or by direct phenol extraction of whole bacterial cells (PX-E), the "conjugated protein" was isolated by mild acetic acid hydrolysis of trichloroacetic acid extracted endotoxin complex. Although phenol ($pK_a^{25°} = 10.0$) is a weaker acid than acetic acid ($pK_a^{25°} = 4.76$) or trichloroacetic acid ($pK_a^{25°} = 0.08$), it has to be kept in mind that trichloroacetic acid extraction was performed at 4°C, whereas phenol treatment was carried out at 70°C. Jenkin and Rowley (112) demonstrated the effect of temperature and time on the toxicity of Boivin antigen from Vibrio cholerae. Toxicity decreased markedly with an increased time of heating at 100° indicating an alteration of the chemical structure. This effect may be specific for the endotoxin of Vibrio cholerae since endotoxin complexes from other Gram-negative bacteria are heat stable. On the other hand, cold phenol extraction did not dissociate the protein moiety from the endotoxin complex (17). It can also be visualized that phenol, an excellent solvent for proteins (111), may cause unfolding of the entire endotoxin structure and thus maximal exposure of susceptible linkages to the action of phenol. Electronmicroscopic pictures of phenol extracted lipopolysaccharides revealed, indeed, filamentous structures (13).

Under these conditions, the difference between the approximate molar ratios of fatty acids/phosphate/glucosamine of "conjugated protein" and corresponding tryptic and pronase cores (3:1:1) and PX-S and its tryptic and pronase cores (2:1:1) may be explained by the enhanced cleav-

age of ester bound fatty acids by phenol treatment. It has been assumed until recently (17) that phenol causes a complete dissociation or removal of the protein moiety from the lipopolysaccharide portion of endotoxin complex. On the basis of experimental data presented in this dissertation it is now suggested that the cleavage by hot phenol actually takes place within the lipid A structure leaving a part of lipid A still bound to the protein moiety and constituting the "simple protein." Weak covalent phosphodiester linkages may be attacked by phenol more readily than the β-aspartyl-N-acetylglucosamine, the suggested linking compound between protein moiety and lipid A. The N-glycosidic linkage between asparagine and N-acetyl glucosamine is stronger than O-glycosidic linkages and the half-life of this compound upon hydrolysis with 2 N HCl at 100°C was found to be 45 minutes (101).

There are no complete data available concerning the chemical composition of properly defined "conjugated" or "simple" proteins from other Gram-negative bacterial species. Homma (64) isolated the endotoxin protein from Pseudomonas aeruginosa and found 13.1% N, 1.6% P, and 2.3% galactosamine. Alkaline ethanol treatment resulted in the isolation of a protein moiety with 15.4% N. Glutamic acid was present in the highest concentration (12 mol. %). Fatty acids were not determined. Jenkin and Rowley (112) isolated toxic proteins from Vibrio cholerae and Water Vibrios by dissolving the bacteria in 2.5 M urea and by subsequent fractional precipitation with ammonium sulfate. The toxic protein accounted for the major portion of the toxicity of whole organism. Immunological and chemical data suggested that the toxic protein was identical with the protein moiety of the Boivin antigen. Nitrogen accounted for 13.8%, and

phosphorus for 1.5% in the toxic protein from Vibrio cholerae, Ogawa strain 5596. No values for carbohydrate or fatty acids were presented. In other strains of Vibrio cholerae no values for nitrogen or phosphorus were reported, but the carbohydrate content was 1.2-3.0% of the protein content (100%).

The successive digestion of "simple protein" (PX-S and PX-E) and "conjugated protein" resulted in an incomplete hydrolysis of the protein moieties. Morgan (45) reported an almost complete removal of the protein moiety from "conjugated protein" of Shigella dysenteriae, but only a partial hydrolysis of that of Salmonella typhosa by the action of trypsin (55). Although the endotoxin complex and the "conjugated protein" from Shigella paradysenteriae were readily attacked by trypsin, the hydrolysis of the protein moiety was incomplete (21, 46). Whether a partial tryptic hydrolysis of the protein moieties of endotoxins is due to partial amino acid sequences or to the inhibitory effect of protein bound lipid A on the enzyme, is not known. Even after pronase digestion considerable amounts of amino acids were found in the pronase cores of "conjugated protein" (8.84%) and of PX-S (11.44%). Recently, Rosselet et al. (113) isolated an "endotoxin-free biologically active component" from E. coli. This proteinaceous fraction, called protodyn, was isolated by a modified phenol method and found to contain 87.7% protein, 1.2% carbohydrate, 0.5% amino sugars, and 0.9% lipid. Upon pronase digestion, an insoluble residue was obtained containing 62.9% protein, 1.2% carbohydrate, 0.3% amino sugars, and 8.6% lipid. The parent substance and the residue were submitted to acid hydrolysis in 0.1 N H_2SO_4 at 100°C and, since no 2-keto-3-deoxy-octonate (KDO) was discovered, the authors concluded that their

preparations contained no lipopolysaccharides and differed from these cell wall constituents. This may have been a false conclusion, because KDO, linking lipid A with the polysaccharide moiety, is present in the water soluble lipopolysaccharide fraction and not in the phenol soluble protein moiety. It seems, therefore, that protodyn and the pronase residue are similar to PX-S and its pronase core. However, also in this case, pronase digestion was not complete and the "pronase core" still contained 63% protein.

Although β-aspartyl-N-acetylglucosaminylamine has still to be isolated and identified, there is already some evidence based on the high voltage electrophoresis and ion exchange chromatography of protein hydrolysates, that this compound is involved in the lipid A-protein linkage of PX-S and "conjugated protein." The existence of a linkage of this type would explain the frequent finding of various amounts of amino acids, especially aspartic acid, in lipopolysaccharide and lipid A preparations (16, 33, 34). The usually mild hydrolytic procedures utilized for the liberation of either lipopolysaccharide or lipid A would seem to be too weak to cleave the N-glycosidic linkage of β-aspartyl-N-acetylglucosaminylamine. However, it must be pointed out that in addition to the characteristic protein moiety, the existence of small peptides linking endotoxin complex to the murein layer cannot be excluded (13) from various endotoxin preparations; the amino acids detected in lipopolysaccharide or lipid A preparations may actually stem from these peptides. A strong evidence for the existence of a covalent linkage between lipid A and protein moiety is the fact that lipid A could be isolated from PX-S or "conjugated protein" by chloroform extraction only after mild acid

hydrolysis. A residual "conjugated protein" liberated from lipid A by hydrolysis with 0.1 N HCl still contained 3.44% glucosamine and 3.8% fatty acids. The glucosamine content was very similar to that of "simple protein" PX-S (2.87%). Thus this result supports the existence of a covalent N-glycosidic linkage, not cleaved under mild hydrolytic conditions; on the other hand, it suggests also a cleavage within the lipid A moiety similar to that postulated for the action of phenol.

The molar ratio glucosamine/aspartic acid of the pronase core (5:1) and carboxypeptidase core (10:1) of PX-S and the molar ratio of major amino acids of these cores suggest a single point of attachment of lipid A and protein, though the amino acid content of the pronase cores of PX-S (11.44%) and "conjugated protein" (8.84%) is still relatively high. This would be a rather unusual arrangement, since carbohydrate chains seem to be randomly attached along the polypeptide backbones of several structurally known glycoproteins (114). Nevertheless, it should be recalled that tryptic and pronase peptides of PX-S and "conjugated protein" were entirely free of glucosamine and fatty acids.

In contrast to the presently proposed covalent linkage between the protein moiety and lipopolysaccharide, Morgan (45) postulated a non-covalent attachment of the protein moiety. Results of Webster *et al.* (48) demonstrating dissociation of nitrogeneous material from a protein-poor endotoxin of *Salmonella typhosae* by extremely high salt concentrations in ethanol, and those of Westphal *et al.* (17) describing isolation of a phosphorus-free protein preparation by phenol treatment of endotoxin, would support the latter proposal.

It has been repeatedly reported (2, 6, 8, 10, 13) on the basis

of electronmicroscopic evidence that the outermost layer of Gram-negative cell walls consists of a lipoprotein. Weidel et al. (8) first postulated the existence of lipoprotein layer in 1954 when working on the isolation of receptor substances for phages T_3, T_4, T_7 and T_5 by phenol extraction of cell walls. The evidence for a lipoprotein layer was based on the finding that the extraction of protein-containing phenol phase resulted in the isolation of a mixture of lipids (115). However, until very recently (65), there has been no chemical evidence for the occurrence of a separate lipoprotein entity in cell walls. One possible reason for failure to isolate an intact lipoprotein may be due to the frequent use of phenol as a solvent for cell wall extraction and its known dissociating action on non-covalently protein-bound lipids which are then isolated and characterized as "free" rather than "bound" lipids. Recently, De Petris (13) found no electronmicroscopic evidence for the existence of two separate lipoprotein and lipopolysaccharide layers, and proposed, as an alternative, protein-lipid (to indicate that defined lipoprotein complexes have not yet been isolated) and lipopolysaccharide components arranged in a single interconnected mosaic pattern. De Petris' scheme assumes that there is a single lipid-containing stratum consisting of "free lipids" and the lipid moiety of lipopolysaccharides associated with polysaccharide and possibly one major protein fraction distributed in a mosaic-like fashion on the cell surface. Another large protein fraction is situated between the lipid-containing middle layer and the murein sacculus (innermost layer). Clarke et al. (14, 65, 68) investigated isolated cell walls of Pseudomonas aeruginosa and concluded that the cell wall consisted of 30% protein, 55-65% lipids and lipopolysaccharides, and

5-15% of murein. Aqueous phenol extraction yielded 36-37% cell wall material in the aqueous phase, 42-43% in the phenol phase, and about 13% unextractable residue consisting of about 50% murein and 50% protein. This latter protein fraction could possibly correspond to De Petris' protein fraction attached to the murein sacculus. A protein fraction obtained by methanol precipitation from the phenol phase and characterized by high content of dicarboxylic amino acids comprised 24.5% of cell wall; in many respects, this protein preparation was comparable to PX-S, PX-E and "conjugated protein." The residual, soluble material in the phenol phase (18.4% of the cell wall) consisted largely of free lipids (69%) and protein (13%). This protein had a different amino acid composition than the precipitated protein fraction and, recalculated, accounted only for about 2.4% of the cell wall. It was mentioned in the same publication that a lipoprotein with a composition similar to that of small protein fraction had been isolated after extraction of the cell wall with organic solvents. No further data were reported. Thus, the possible occurrence of a small lipoprotein fraction cannot yet be entirely excluded.

Results of present investigation gave no evidence for a separate lipoprotein fraction. The phenol phase was entirely free of any other protein-containing material after precipitation of PX-S with ethanol. Therefore, it is suggested that, in accordance with De Petris (13) modified scheme, basically only two protein fractions occur in the cell walls of Gram-negative bacteria; one not extractable by phenol is attached to the murein sacculus and the other one extracted by aqueous phenol is linked to the lipopolysaccharide moiety. The similarity of endotoxin proteins of various Gram-negative bacteria has been demonstrated in the

case of S. marcescens and E. coli; these two proteins had not only similar amino acid composition and peptide maps, but showed also the presence of a common antigenic determinant.

Antibodies prepared against "simple" and "conjugated" proteins and their corresponding tryptic and pronase cores as well as those obtained against the oxidized form of "simple protein" from E. coli revealed the presence of a common antigenic determinant (116) in all these protein preparations. Oxidation of PX-S and PX-E by performic acid did not destroy the antigenic sites, since PXOD-S and PXOD-E were still immunogenic. The above mentioned antigens also gave a complete fusion of precipitin lines when tested against anti-LPS-U-S (endotoxin) serum. However, when "conjugated protein" was tested simultaneously with antibodies to whole endotoxin complex (LPS-U-S) and to protein moieties (PX-S or "conjugated protein") the two precipitin lines showed a reaction of only partial identity. The endotoxin complex showed with antibodies to LPS-U-S three immunoprecipitin arcs. Absorption of this antiserum with PX-S eliminated one of the bands which was thus identified as "simple protein;" traces of free protein moiety were obviously present as contamination in the LPS-U-S preparation used for immunization. A second precipitin arc was due to the presence of liberated polysaccharide side chain, and the third one was that of intact endotoxin.

Morgan (19) recognized the "simple" and "conjugated protein" of Salmonella typhosa and Shigella dysenteriae as weak antigens. The "simple" and the "conjugated protein" of Shigella paradysenteriae (21) produced antibodies which reacted with the intact O-antigenic complex as well as with the homologous antigen. The "simple protein" was a weaker

antigen than the "conjugated protein," an observation also made semi-quantitatively for the "simple" and "conjugated protein" of S. marcescens. It was postulated that lipopolysaccharides of Enterobacteriaciae possess two types of antigenic determinants, the specific O-antigen, located in the side chain of the polysaccharide moiety, and the other one common to all members of this family of bacteriae and other unrelated species; lipopolysaccharides of S. marcescens were excluded, since the strains tested did not possess common antigen (117). Whether this antigenic site is really located on lipopolysaccharides is questionable (15). However, interbacterial cross reactions unrelated to the O-antigen are known. Whether common antigen is responsible for the identical cross reaction between PX-E, PX-S and "conjugated protein" is not known. Since both the tryptic and the pronase core were immunogenic, the antigenic site could be expected either on the residual protein fragment close to lipid A or on the lipid A moiety itself. To test this alternative, a lipopolysaccharide preparation, LPS-A, containing less than 2% amino acids and "simple protein" were reacted with antibodies to LPS-A and/or antibodies to "simple protein." Since LPS-A contains the polysaccharide and the lipid A moiety, but not the protein moiety, whereas PX-S contains the protein moiety and a portion of lipid A, but not the polysaccharide moiety, a reaction of partial identity would indicate the location of a second antigenic determinant within lipid A and that of non-identity in the protein moiety. The observed non-identity reaction indicated clearly the presence of the second antigenic site in the protein moiety.

There have been many attempts to correlate the chemical structure and biological functions of endotoxins (106). Since toxicity rep-

resents one of the most characteristic biological properties of endotoxins, the search for a toxic principle or site in the complex has been based on two alternative hypotheses proposing either the presence of a toxic constituent in or a toxic "conformation" of endotoxin structure (106). Goebel et al. (21, 46) concluded from their experiments that a toxic "factor T" existed which was neither identical with the protein nor with the polysaccharide moiety of endotoxins. Westphal and Lüderitz suggested later (16, 105) that the toxic "factor T" was identical with lipid A, and detoxification studies by partial hydrolysis and by other means showed that the presence of fatty acids in endotoxins was essential for the exhibition of toxic reactions (118). The polysaccharide part of endotoxins was found not to be essential for toxicity, since Lüderitz et al. (40) could demonstrate potent endotoxic properties of R-mutants of Salmonella which lacked either a part or the entire polysaccharide moiety. On the other hand, Ribi et al. (119, 120) isolated a highly toxic endotoxin from S. enteritidis which was found to be very low in fatty acid content. Then, in a series of papers, Ribi and his co-workers supported Robertson and Cromartie (121) who claimed that a certain particle size of endotoxin was necessary for maximal toxicity. This proposal led, consequently, to the hypothesis that the endotoxin macromolecule did not contain toxic groups, but had nontoxic functional groups arranged in such a way that the entire molecule exhibited a toxic conformation (106).

Results of present investigation showed clearly that endotoxin fractions solubilized in SDS-Tris buffer exhibited much higher toxicity than the same fractions administered as saline suspensions. It may be concluded that dissociating agents such as SDS by decreasing the particle

size of endotoxin fractions had an enhancing rather than diminishing effect on the toxic properties of endotoxins from S. marcescens. The whole endotoxin complex, LPS-U, was the most toxic preparation (LD_{50} = 1 3 mg/kg). The removal of the polysaccharide moiety, not toxic by itself, decreased the toxicity of the "conjugated protein" (LD_{50} = 22 mg/kg). "Simple protein," PX-S, containing only a part of the lipid A moiety was less toxic than "conjugated protein," but still retained a moderate toxicity. Protein-free lipopolysaccharide, LPS-A, (LD_{50} = 12 mg/kg) and lipid A (LD_{50} = 14 mg/kg) could be shown for the first time to be highly toxic fractions, because of the increased solubility in SDS-Tris buffer. These data demonstrate clearly the importance of the lipid A moiety for the toxicity of endotoxins and support Westphal and Lüderitz' (16, 105) hypothesis of lipid A as the toxic factor of endotoxins.

CHAPTER VI

SUMMARY

A nucleic acid-free endotoxin preparation isolated from whole cells of S. marcescens 08 by trichloroacetic acid was submitted either to the hot aqueous phenol extraction or to mild acid hydrolysis. The resulting products, "simple" and "conjugated protein," were compared with "simple protein" from E. coli isolated by phenol extraction of whole cells. Protein preparations were characterized by elementary analysis, by determination of amino acids, glucosamine and fatty acids, by infrared absorption spectra, agarose and polyacrylamide electrophoresis, analytical ultracentrifugation and by immunological properties.

Chemical analysis of electrophoretically- and immunochemically-homogeneous "simple proteins" from S. marcescens 08 and E. coli revealed that these endotoxin proteins are not "simple" proteins, but contain a firmly attached portion of lipid A. Thus, the so-called "simple proteins" are quantitatively, but not qualitatively, different from "conjugated proteins" which consist of the entire lipid A moiety attached to the endotoxin protein. This study indicates that phenol treatment of endotoxin complex results in cleavage of a relatively weak covalent linkage within the lipid A moiety rather than in a sharp dissociation of the protein moiety from lipopolysaccharide part of endotoxin complex. It is pro-

posed that lipid A is linked to the protein moiety very likely through β-aspartyl-N-acetyl-glucosaminylamine. "Simple" and "conjugated protein" and the corresponding tryptic and pronase cores induced the formation of antibodies in rabbits and exhibited a common antigenic determinant. The antigenic site is located in the protein moiety.

Although endotoxin proteins of S. marcescens 08 and E. coli differed in molar ratios of amino acids and peptide maps, they were characterized by the presence of a common antigenic determinant.

Toxicity studies demonstrated lipid A as the toxic factor of endotoxin complex, and the "conjugated" and "simple protein" as highly or moderately toxic fragments.

BIBLIOGRAPHY

1. Salton, M. R. I. The Bacterial Cell Wall, Elsevier, Amsterdam, 1964, p. 293.

2. Murray, G. E., Steed, P., and Elson, H. E. Can. J. Microbiol., 11, 574 (1965).

3. Bladen, H. A., and Mergenhagen, S. E. J. Bacteriol., 88, 1482 (1964).

4. Mergenhagen, S. E., Bladen, H. A., and Hsu, K. C. Ann. N. Y. Acad Sci., 133, 279 (1966).

5. Martin, H. H., and Frank, H. Z. Naturforsch., 17b, 190 (1962).

6. Frank, H., and Dekegel, D. Zbl. Bakt., 198, 81 (1965).

7. Weidel, W., and Pelzer, H. Advan. Enzymol., 26, 193 (1964).

8. Weidel, W., Koch, G., and Bobosch, K. Z. Naturforschg., 9b, 573 (1954).

9. Nermut, M. V., and Murray, G. E. J. Bacteriol., 93, 1949 (1967).

10. Bayer, E., and Anderson, T. F. Proc. Natl. Acad. Sci. (U.S.), 54, 1592 (1965).

11. Shands, J. W. J. Bacteriol., 90, 266 (1965).

12. Shands, J. W. Ann. N. Y. Acad. Sci., 133, 292 (1966).

13. De Petris, S. J. Ultrastruct. Res., 19, 45 (1967).

14. Clarke, K., Gray, G. W., and Reaveley, D. A. Biochem. J., 105, 749 (1967).

15. Lüderitz, O., Jann, K., and Wheat, R. in Comprehensive Biochemistry, M. Florkin and E. H. Stotz, ed., Elsevier, Amsterdam, 1968, Vol. 26A, p. 106.

16. Westphal, O., and Lüderitz, O. Angew. Chem., 66, 407 (1954).

17. Westphal, O., Lüderitz, O., and Bister, F. Z. Naturforschg. 7b, 148 (1952).

18. Wardlaw, A. C. Can. J. Microbiol., 9, 41 (1963).

19. Morgan, W. T. J., and Partridge, S. M. Biochem. J., 35, 1140 (1941).

20. Davies, D. A. L., and Morgan, W. T. J. 6th Internatl. Congr. Microbiol., Rome, 1953, Abstr. No. 317.

21. Binkley, F., Goebel, W. F., and Perlman, E. J. Exptl. Med., 81, 331 (1945).

22. Osborne, M. J. Proc. Natl. Acad. Sci., U.S., 50, 499 (1963).

23. Edstrom, R. D., and Heath, E. C. Biochem. Biophys. Res. Commun., 16, 576 (1964).

24. Osborne, M. J. Ann. N. Y. Acad. Sci., 133, 375 (1966).

25. Heath, E. C., Mayer, R. M., Edstrom, R. D., and Beaudreau, C. A. Ann. N. Y. Acad. Sci., 133, 315 (1966).

26. Miles, A. A., and Pirie, N. W. Brit. J. Exptl. Pathol., 20, 83, 278 (1939).

27. Shear, M. J., and Turner, F. C. J. Natl. Cancer Inst., 4, 81, 107, 123 (1943).

28. Goebel, W. F., and Tal, C. J. Exptl. Med., 92, 25 (1950).

29. Goebel, W. F., and Jesaitis, M. A. J. Exptl. Med., 96, 409 (1952).

30. Burton, A. J., and Carter, H. E. Biochemistry 3, 411 (1964).

31. Kasai, N., and Yamano, A. Japan J. Exptl. Med., 34, 329 (1964).

32 Taylor, A., Knox, K. W., and Work, E., Biochem. J., 99, 53 (1966).

33 Alaupovic, P., Olson, A. C., and Tsang, J. Ann. N. Y. Acad. Sci., 133, 546 (1966).

34. Tsang, J. Ph.D. dissertation, University of Oklahoma, Oklahoma City, Oklahoma, 1968.

35. Gmeiner, J., Lüderitz, O., and Westphal, O. European J. Biochem., 7, 370 (1969)

36 Ikawa, M., Koepfli, J. B., Mudd, S. C., and Niemann, C. J. Am. Chem. Soc., 75, 3439 (1953).

37. Nowotny, A. J. Am. Chem. Soc., 83, 501 (1961).

38. Ikawa, M. Ann. N. Y. Acad. Sci., 133, 476 (1966).

39. Ribi, E., Anacker, R. L., Fukushi, K., Haskins, W. T., Landy, M., and Milner, K. C. in Bacterial Endotoxins, M. Landy and W. Braun (eds.), Rutgers University Press, New Brunswick, N. J., 1964, p. 16.

40. Lüderitz, O., Galanos, L., Risse, H. J., Ruschmann, E., Schlecht, S., Schmidt, G., Schulte-Holthausen, H., Wheat, R., Westphal, O., and Schlosshardt, Jr. Ann. N. Y. Acad. Sci., 133, 349 (1966).

41. Westphal, O. Ann. Inst. Pasteur, 98, 789 (1960).

42. Clarke, K., Gray, G. W., and Reaveley, P. A. Nature, 208, 586 (1965).

43. Kasai, N., Aoki, Y., Watenaba, T., Odaka, T., and Yamano, T. Japan J. Microbiol., 5, 347 (1961).

44. Michich, E., Westphal, O., Lüderitz, O., and Neter, E. Proc. Soc. Exptl. Biol. Med., 107, 816 (1961).

45. Morgan, W. T. J., and Partridge, S. M. Biochem. J., 34, 169 (1940).

46. Goebel, W. F., Binkley, F., Perlman, E. J. Exptl. Med., 81, 315 (1945).

47. Ribi, E., Hoyer, B. H., Milner, K. C., Perrine, T. D., Larson, C. L., and Goode, G. J. Immunol., 84, 32 (1960).

48. Webster, M. E., Sagen, J. F., Landy, M., and Johnson, A. G. J. Immunol., 74, 455 (1955).

49. Ikawa, M., Koepfli, J. B., Mudd, S. G., and Niemann, C. J. Natl. Cancer Inst., 13, 157 (1952).

50. Hartwell, J. L., Shear, M. J., Adams, J. R., and Perrault, A. J. Natl. Cancer Inst., 4, 107 (1943).

51. Ikawa, M., Koepfli, J. B., Mudd, S. G., and Niemann, C. J. Natl. Cancer Inst., 14, 961 (1954).

52. Boivin, A., and Mesrobeanu, L. Rev. d'Immunol., 1, 553 (1935), 2, 113 (1936), 3, 319 (1937).

53. Mesrobeanu, L. Les antigenes glycido-lipidiques des bacteries, Masson et Cie, Paris, 1936.

54. Boivin, A., and Mesrobeanu, L. Compt. Rend. Soc. Biol., Paris, 112, 76 (1933).

55. Morgan, W. T. J., and Partridge, S. M. Brit. J. Exptl. Pathol., 23, 11 (1942).

56. Morgan, W. T. J. Biochem. J., 31, 2003 (1937).

57. Nowotny, A. M., Thomas, S., Duron, O. S., and Nowotny, A. J. Bacteriol., 85, 418 (1963).

58. Adams, G. A. Can. J. Biochem., 45, 522 (1967).

59. Boivin, A., Mesrobeanu, J., and Mesrobeanu, L. Compt. Rend. Soc. Biol., 113, 490 (1933).

60. Rathgeb, P., and Sylven, B. J. Natl. Cancer Inst., 14, 1099 (1954).

61. Bishop, D. G., and Work, E. Biochem. J., 96, 567 (1965).

62. Homma, J. Y., and Suzuki, N. J. Bacteriol., 87, 630 (1964).

63. Homma, J. Y., and Suzuki, N. Ann. N. Y. Acad. Sci., 133, 508 (1966).

64. Homma, J. Y. Zeitschr. Allg. Mikrobiol., 8, 227 (1968).

65. Clarke, K., Gray, G. W., and Reaveley, A. Biochem. J., 105, 755 (1967).

66. Clarke, K., Gray, G. W., and Reaveley, D. A. Biochem. J., 105, 759 (1967).

67. Okuda, S., and Weinbaum, G. Biochemistry, 7, 2819 (1968).

68. Reeves, P. Bacteriol. Rev., 29, 24 (1965).

69. Goebel, W. F., and Baray, G. T. J. Exptl. Med., 107, 185 (1958).

70. Amano, T., Goebel, W. F., and Smidth, E. M. J. Exptl. Med., 108, 731 (1958).

71. Rude, E., and Goebel, W. F. J. Exptl. Med., 116, 73 (1962).

72. Hinsdill, R. D., and Goebel, W. F. Ann. Inst. Pasteur, 107, 54 (1964).

73. Mesrobeanu, L., Mesrobeanu, I., and Mitrica, N. Ann. N. Y. Acad. Sci., 133, 685 (1966).

74. Shear, M. J., and Turner, F. C. J. Natl. Cancer Inst., 4, 31 (1943).

75. Creech, H. J., Breuninger, E., and Adams, G. A. Can. J. Biochem., 42, 593 (1964).

76. Srivastava, H. C., Breuninger, E., Creech, H. J., and Adams, G. A. Can. J. Biochem. Physiol., 40, 905 (1962).

77. Mueller, J. M., Pierce, J. G., Davoll, H., Du Vigneaud, V. J. Biol. Chem., 191, 309 (1951).

78. Shachman, K. H. in Methods in Enzymology, S. P. Colowick and N. O. Kaplan (eds.), Acad. Press Inc., N. Y., 4, 32.

79. Brown, H. B., Sivasankaran, K., and Brown, C. J. Org. Chem., 28, 214 (1963).

80. Wober, W., Thiele, O. W., and Urbaschek, B. Biochim. Biophys. Acta, 84, 376 (1963).

81. Rondle, C. J. M., and Morgan, W. T. J. Biochem. J., 61, 586 (1955).

82. Koehler, L. H. Anal. Chem., 24, 1576 (1952).

83. Gerlach, E., and Deuticke, B. Biochem. Z., 337, 477 (1963).

84. Colombo, P., Corbetta, D., Pirotta, A., Ruffini, G., and Sartori, A. J. Chromatog., 3, 343 (1960).

85. Mangold, H. K., Schmid, H. O., and Stahl, E. in Methods of Biochemical Analysis, D. Glick (ed.), Interscience Publishing, N. Y., 1964, Vol. 12, p. 343.

86. Thiele, O. W., and Wober, W. Zeitschr. Analyt. Chem., 205, 442 (1964).

87. Jatzkewitz, H., and Mehl, E. Z. physiol. Chem., 320, 251 (1960).

88. Dittmer, C. J., and Lester, R. L. J. Lipid Res., 5, 126 (1964).

89. Grabar, P., and Williams, C. A. Biochim. Biophys. Acta, 17, 67 (1955).

90. Davis, B. J. Ann. N. Y. Acad. Sci., 121, 404 (1964).

91. Ouchterlony, Ö. Acta Pathol. Microbiol., 32, 23 (1953).

92. Spies, J. R., and Chambers, D. C. Anal. Chem., 21, 1249 (1949).

93. Katz, A. M., Dreyer, W. J., and Anfinsen, C. B. J. Biol. Chem., 234, 2897 (1959).

94. Cornfield, J., Mantel, N. J. Am. Statist. Assoc., 45, 181 (1960).

95. Loomis, T. A. Essentials of Toxicology, Lea & Feliger, Philadelphia 1968, p. 18.

96. Bergey's Manual of Determinative Bacteriology, Williams & Wilkens Co., Baltimore, 1957, p. 360.

97. Cruz-Camarillo, R., and Sanchez-Zuniga. Nature, 218, 567 (1968).

98. Purkayasta, M., and Williams, R. P. Nature, 187, 349 (1960).

99. Kuhn, R., Rudy, H., and Wagner-Jauregg. Ber. 66, 1950 (1933).

100. Tamura, E., Nichihara, A., Jsobe, S., and Matsuno, N. Ann. Rep. Natl. Inst. Nutr., Tokyo, (1958), p. 46.

101. Carubelli, R., Bhavanandan, V. P., and Gottschalk, A. Biochim. Biophys. Acta, 101, 67 (1965).

102. Neuberger, A., and Marshall, R. D. in Glycoproteins, A. Gottschalk, (ed.), Elsevier Publishing Co., Amsterdam, 1969, p. 275, 276.

103. Wagh, P. V., Bornstein, I., and Winzler, R. J. Biol. Chem., 214, 658, 1969.

104. Wagh, P. V. State University of New York, Buffalo, Personal communication, 1969.

105. Westphal, O., Nowotny, A., Lüderitz, O., Hurni, H., Eichenberger, E., and Schönholzer, G. Pharm. Acta Helv., 33, 401 (1958).

106. Nowotny, A. Bacteriol. Rev., 33, 72 (1969).

107. Moggridge, R. C. G., and Neuberger, A. J. Chem. Soc., 745 (1938).

108. Foster, A. B., Horton, D., and Stacey, M. J. Chem. Soc., 81 (1957).

109. Archibald, A. R., and Baddiley, J. Adv. Carbohydrate Chem., 21, 328 (1966).

110. Maley, F., and Landy, H. A. J. Am. Chem. Soc., 78, 1393 (1956).

111. Kickhöfen, B., and Bürger, M. Biochim. Biophys. Acta, 65, 190 (1962).

112. Jenkin, C. R., and Rowley, D. J. gen. Microbiol., 21, 191 (1959).

113. Rosselet, J. P., Murphy, S. G., Adamski, R. J., Fletscher, M. J., and Ludwig, B. J. J. Bacteriol., 98, 434, 1969.

114. Gottschalk, A. Biochemistry Seminar, OMRF, Oklahoma City, 1969.

115. Weidel, W., Koch, G., and Lohss, F. Z. Naturforsch., 9b, 398 (1954).

116. Ouchterlony, Ö. Handbook of Immunodiffusion and Immunoelectrophoresis. Ann Arbor Science Publishers, Inc., 1968, p. 37.

117. Kunin, C. M. J. Exptl. Med., 118, 565 (1963).

118. Nowotny, A. in M. Landy and W. Braun (ed.) Bacterial Endotoxins, Rutgers University Press, New Brunswick, N. J., 1964, p. 29.

119. Ribi, E., Haskins, W. T., Landy, M., and Milner, K. C. J. Exptl. Med., 114, 647 (1961).

120. Ribi, E., Milner, K. C., and Perrine, T. D. J. Immunol., 82, 75 (1959).

121. Robertson, B. S., and Cromartie, W. J. J. Bacteriol., 84, 882 (1962).