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STUDIES ON THE PROTEIN MOIETY OF ENDOTOXIN FROM GRAM-NEGATIVE BACTERIA: CHARACTERIZATION OF THE PROTEIN MOIETY ISOLATED BY ACETIC ACID HYDROLYSIS OF ENDOTOXIN FROM *S. marcescens* 08

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Technical Report No. 30
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STUDIES ON THE PROTEIN MOIETY OF ENDOTOXIN FROM GRAM-NEGATIVE BACTERIA:

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Abstract

"Conjugated" protein isolated from the endotoxin complex of *Serratia marcescens* 08 by 1% acetic acid hydrolysis was characterized by analytical ultracentrifugation, electrophoretic and immunological properties and chemical analysis. The chemical composition of "conjugated" protein and other degraded fragments of endotoxin showed that all characteristic constituents of lipid A were present exclusively in "conjugated" protein. Successive treatment of "conjugated" protein with trypsin and pronase resulted in the isolation of corresponding tryptic and pronase cores which were characterized by an increased content of lipid A constituents. Lipid A was separated as an entity from the "conjugated" protein only after hydrolysis with 0.1 N hydrochloric acid. This result confirms our previous proposal that lipid A is covalently linked to the protein moiety in whole endotoxin of *S. marcescens* 08. "Conjugated" protein isolated by acetic acid hydrolysis and "simple" protein prepared by hot aqueous phenol treatment of whole endotoxin differ from one another not by the presence or

absence of lipid A but rather by the presence of the entire lipid A moiety in the "conjugated" protein and only a fragment of lipid A in the "simple" protein.

"Conjugated" protein and its tryptic and pronase cores were immunogenic and possessed a common antigenic determinant with "simple" protein and its corresponding cores. Toxicity studies revealed that both "conjugated" and "simple" proteins retained to different degrees the toxic properties of whole endotoxin. Sodium dodecyl sulfate had an enhancing rather than diminishing effect on the toxicity of endotoxin and its various fragments. The importance of lipid A as the toxic factor of endotoxins was established not only indirectly by determining the toxicity of each fragment containing lipid A, but also directly by demonstrating that lipid A dispersed in a Tris-SDS buffer displayed toxic properties similar to those of the lipopolysaccharide preparation.

Morgan and Partridge (1-3) demonstrated that hydrolysis of whole endotoxin of Shigella dysenteriae by 1% acetic acid resulted in the isolation of the so-called "conjugated" protein which may be converted into "simple" protein by treatment with either 90% phenol or 0.1 N sodium hydroxide. Because "conjugated" protein from Shigella paradysenteriae retained the typical toxic properties, Goebel and his coworkers (4, 5) have referred to protein preparations isolated by 1% acetic acid hydrolysis as "toxic" proteins. Since tryptic digestion of the intact endotoxin and of "toxic" protein did not destroy the toxic component, authors concluded that the toxin is either not a protein or that it represents a portion of the protein resistant to the action of proteolytic enzymes. The suggested existence of a toxic component other than protein and polysaccharide marked the beginning not only of an extensive search for but also of a lasting controversy about the chemical nature of the toxophore group in the endotoxin complex. Several investigators (6-13) have detected the presence

of a lipid moiety and identified fatty acids and glucosamine as its principal constituents. However, Westphal and his coworkers (14-16) were the first to suggest that the firmly bound lipid moiety (lipid A) was the most probable carrier of typical endotoxic effects and, therefore, identical with the active component of "toxic" or "conjugated" protein. Although the chemical composition and structure of "conjugated" protein is not known, it has been tacitly assumed (17) that the possible presence of lipid A in "conjugated" protein represents the only difference between this protein fraction and "simple" protein. To test this assumption, the physical, chemical and immunological properties of "conjugated" protein were compared with those of "simple" protein.

MATERIALS AND METHODS

Isolation of the "Conjugated" Protein (C.P.) from Endotoxin of S. marcescens 08

To remove free lipids, nucleic acid-free endotoxin isolated by trichloroacetic acid extraction of wet cells of *S. marcescens* 08

(18) was extracted by chloroform-methanol (2:1, v/v) in a Soxhlet extractor for 12 hours. About 500 mg of this endotoxin preparation (LPS-Ud-S) were hydrolyzed by stirring with 250 ml of preheated 1% acetic acid (Fig. 1) for 4 hours at 90° according to the method of Morgan and Partridge (1, 2). After cooling for one hour at 4°, a flocculating, slightly yellow-colored precipitate was separated from the clear supernate by centrifuging at 17,300 x g for 20 minutes at 10°. The protein preparation was dissolved in 0.02 M ammonium carbonate buffer, pH 8.6, and after three successive isoelectric precipitations at pH 3.5 was washed continuously with distilled water for 24 hours and lyophilized. The "conjugated" protein was tested for impurities according to the procedure described for "simple" protein (18). Chloroform extract of "conjugated" protein contained no free lipids.

After the "conjugated" protein had been separated, the clear supernate was extracted three times with chloroform to remove lipids. The lyophilized aqueous phase containing the "degraded"

polysaccharide moiety was dissolved in and dialyzed against distilled water for 7 days. The outer and inner dialysates were lyophilized.

Preparation of the Tryptic Core and Pronase Core, and Isolation of Lipid A from "Conjugated" Protein

The tryptic and pronase cores of "conjugated" protein and lipid A were obtained according to the procedures utilized for the preparation of cores and isolation of lipid A from the "simple" protein (19).

Peptide Mapping

The two-dimensional separation of peptides was carried out according to the procedure by Katz *et al.*, (20). Tryptic peptides (2-3 mg) of "simple" proteins or of "conjugated" proteins from *S. marcescens* 08 (PX-S) or *E. coli* O 1A1:K85(B) (PX-E) 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 one hour. The peptides were stained with 0.05% ninhydrin in ethanol and heated to 70° for 20 minutes.

Analytical Methods and Physical Measurements

Procedures for the quantitative analysis of amino acids, fatty acids, glucosamine, ash, phosphorus, nitrogen, carbon and hydrogen were the same as described in a previous paper (18). Analytical ultracentrifugation, agarose electrophoresis and infrared spectroscopy of "conjugated" protein and/or its fragments were carried out according to the procedures described previously (18).

Immunological Methods

Preparation of antibodies to "conjugated" protein and tryptic and pronase cores, and double diffusion and immunoelectrophoresis in 1% agarose gel were performed according to the procedures previously described (18).

Antigens and the homologous antibodies are symbolized by

the same letters; capital letters designate antigens and small letters antibodies:

Antigens		Antibodies	
"Conjugated" protein	G	Anti-"conjugated" protein	g
C.P. tryptic core	H	Anti-tryptic core	h
C.P. pronase core	I	Anti-pronase core	i

Lethality for Mice (LD_{50})

Two different strains of white male mice (Swiss-register, random bred, Pel-Freeze, Bio-Animals, Inc., Rogers, Arkansas, and Balb-C inbred mice, Texas Inbred Mouse Co., Houston, Texas) weighing 17-21 g were used for toxicity studies. Each group consisted of six mice. The various preparations were tested at least two times each. The substances, suspended either in 0.9% NaCl or in 0.05 M Tris buffer containing 0.5% SDS, pH 7.6, were injected intraperitoneally. Control animals were injected with the same buffer system. Deaths occurring within 7 days were registered. The LD_{50}-values were calculated according to Cornfield and Mantel (21). Categories of toxicity were assigned

according to Loomis (22).

RESULTS

Isolation of "Conjugated" Protein and Corresponding Tryptic and Pronase Cores

The endotoxin complex (LPS-U-S) isolated by trichloroacetic acid extraction of *S. marcescens* 08 contained approximately 20% lipids extractable by chloroform-methanol. "Conjugated" protein isolated from the defatted endotoxin complex (LPS-Ud-S) by hydrolysis with 1% acetic acid represented 36% of the starting material (Fig. 1). The acetic acid hydrolysate was extracted with chloroform and the small amount of material present in the chloroform phase was submitted to hydrolysis with 0.1 N HCl; the hydrolysate contained only traces of fatty acids. 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, representing approximately

52% of the polysaccharide moiety, contained 41.8% C, 6.2% H, 3.3% N and less than 0.4% P. Amino acid analysis revealed only trace amounts of amino acids and 38.4% glucosamine. Results of quantitative gas-liquid chromatography (23) indicated that the inner dialysate contained glucose, galactose and glucosamine in a molar ratio 1:1:2. This fraction, representing most probably the O-specific "side chain" of the polysaccharide moiety, gave a single precipitin line with antibodies to LPS-Ud-S (18). The outer dialysate contained 2.2% P, 5.6% N and heptose as the most characteristic sugar component (23). Since heptose is the typical component of the polysaccharide "core" (17), the outer dialysate represents a partially degraded "core" portion of the polysaccharide moiety. Both fractions were soluble in distilled water.

The tryptic core represented 75% of "conjugated" protein and the pronase core 60% 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, pH 8.6, and precipitable by addition of acetic acid to pH 3.5-3.8. However, after repeated lyophilizations the protein preparations were soluble only in 0.05 M Tris buffer containing 0.2% SDS, pH 7.6.

The "conjugated" protein exhibited a single, symmetrical peak in the analytical ultracentrifugal with s_{obs} = 2.4S (Fig. 2).

In agarose electrophoresis, "conjugated" protein, both cores and the endotoxin complex (LPS-Ud) migrated as single bands (Fig. 3).

Infrared spectra of "conjugated" protein and its tryptic and pronase cores are shown in Fig. 4. Although spectra resemble those of the "simple" protein and its cores (18, 19), 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 tryptic and pronase cores than in the spectra of "simple" protein and its corresponding 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 1.

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

Like "simple" protein and its cores (18, 19), the "conjugated" protein and the 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) (Fig. 5, pattern 1). The tryptic core (H) and pronase core (I) gave also single precipitin lines with antibodies to "conjugated" protein (g) (Fig. 5, pattern 2), tryptic core (h) and pronase core (i) (Fig. 5, pattern 3). The precipitin lines

of these three protein preparations showed complete fusion with antibodies to "conjugated" protein as well as with antibodies to pronase core (Fig. 5, patterns 2 and 3). Immunoelectrophoretic patterns of the tryptic (H) and pronase (I) cores show clearly that both fragments of "conjugated" protein exhibited single precipitin arcs (Fig. 6).

The "conjugated" protein (G), "simple" protein (A) from S. marcescens 08 and "simple" protein (B) from E. coli O 141K85(B) gave lines of total coalescence with antibodies to "simple" protein (a) from S. marcescens 08 (Fig. 7, pattern 1). A similar line of total fusion was also obtained in the reaction of "conjugated" protein (G) with antibodies to "conjugated" protein (g), "simple" protein (a), C.P. tryptic core (h) and C.P. pronase core (i) (Fig. 7, pattern 2). However, the precipitin line of "conjugated" protein with antibodies to endotoxin complex, LPS-Ud-S, (k) showed only partial identity with lines obtained with antibodies to various protein fragments.

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

The "conjugated" protein and both cores contained fatty acids, glucosamine and phosphate, the characteristic constituents of lipid A, but not even traces of other sugars or lipids (Table 2). A comparison of the percent chemical composition of "conjugated" protein with that of "simple" protein (18) shows a greater content of fatty acids, glucosamine and phosphate and a smaller content of amino acids in the "conjugated" protein. Successive proteolytic degradation of the "conjugated" protein with trypsin and pronase resulted in a decreased content of amino acids and nitrogen and an increased content of carbon, hydrogen, phosphate, glucosamine and fatty acids. Pronase digestion resulted in more pronounced change in the chemical composition than trypsin hydrolysis. The molar ratio fatty acids/glucosamine/phosphate of the "conjugated" protein and its cores (3:1:1) was higher than that of the "simple" protein and its corresponding cores (2:1:1). Thus, the fatty

acid content of "conjugated" protein was higher than that of the "simple" protein.

The amino acid composition of "conjugated" protein and its tryptic and pronase cores is presented in Table 3. Aspartic acid was the major amino acid and half-cystine was absent. Neither glucosamine nor fatty acids were detected upon hydrolysis of the tryptic and pronase peptides.

Gas-liquid chromatography of the fatty acids isolated from "conjugated" protein and both cores revealed again that β-OH-myristic acid was the major fatty acid (Table 4). Hydrogenation and acetylation of the fatty acid methyl esters confirmed the presence of unsaturated and hydroxy fatty acids.

Isolation of Lipid A from "Conjugated" Protein

Lipid A was isolated from "conjugated" protein by mild hydrolysis with 0.1 N HCl according to the procedure used for its isolation from "simple" protein (19). The infrared spectrum of lipid A from "conjugated" protein (Fig. 4) was identical with

the spectrum of lipid A isolated from the lipopolysaccharide fragment of endotoxin complex (24, 25). The ester/amide I ratio of lipid A (0.96) was higher than that of the pronase core (0.86) (Table 1). The glucosamine content of lipid A was 24%.

Comparison of the Chemical Composition of the "Simple" Proteins and "Conjugated" Protein

Results shown in Table 5 point out clearly some differences in the chemical composition of the "simple" proteins isolated from S. marcescens 08 (PX-S) and E. coli O 141K85(B) and the "conjugated" protein prepared from S. marcescens 08. The "conjugated" protein contains a higher relative content of glucosamine, fatty acids and phosphate and, therefore, a higher content of lipid A than the "simple" proteins. On the other hand, the "simple" protein PX-S seems to contain more lipid A than PX-E.

Molar ratios of amino acids of "conjugated" and "simple" proteins from S. marcescens were very similar, if not identical;

however, they differed to some extent from those of "simple" protein from E. coli. Similarly, while two-dimensional patterns of tryptic peptides of C.P. and PX-S were almost identical, they differed in certain regions from that of PX-E (Figs. 8 and 9)

Toxicity Studies

The LD_{50} values of the endotoxin complex and its fragments were influenced to a large extent by solubility of the substances in the two different solvent systems used and by the strain of mice tested (Table 6). Endotoxin complex, "conjugated" protein, lipopolysaccharide and lipid A were highly toxic in inbred mice when administered in Tris-SDS buffer. Removal of the polysaccharide or protein moiety from the endotoxin complex decreased but did not abolish the toxicity of the remaining fragments ("conjugated" protein and lipopolysaccharide, respectively). Although the "conjugated" protein exhibited a higher toxicity than the "simple" protein, the latter should still be considered as moderately toxic. However, removal of the "conjugated" protein from the

endotoxin resulted in the isolation of a non-toxic, partially degraded polysaccharide moiety. Preparations solubilized in Tris-SDS buffer displayed much higher toxicity than those suspended in 0.9% NaCl. The 0.05 M Tris buffer containing 0.5% SDS, pH 7.6, was not toxic for mice.

DISCUSSION

Chemical analysis of "conjugated" protein has confirmed the assumption of Westphal and Lüderitz (15) that this fragment of endotoxin complex consists of lipid A and protein. "Conjugated" protein contained no other compounds except amino acids and typical constituents of lipid A. "Simple" and "conjugated" protein preparations differed in quantitative rather than qualitative chemical composition. The relative content of lipid A constituents of "conjugated" protein was greater than that of "simple" protein. Like "simple" protein (19), lipid A was firmly bound to the protein moiety of "conjugated" protein and was extracted by chloroform only after hydrolysis of the protein

preparation by 0.1 N HCl. Similarity of the molar ratios of amino acids, distribution of tryptic peptides and immunological properties demonstrated clearly the identity of the protein moieties of "simple" and "conjugated" proteins. It has been suggested recently (17) that the presence of lipid A in the "conjugated" protein represents the only difference between these two protein fragments. However, results of our studies show that "conjugated" and "simple" proteins are not distinct 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. Accordingly, the "conjugated" protein consists of the protein moiety linked to the entire lipid A, whereas in "simple" protein 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 differing effects of aqueous phenol and trichloroacetic acid on

the structure of endotoxin in general and of lipid A in particular. The structure of lipid A is not yet fully understood. Westphal and Lüdertiz (15) have considered lipid A as a single molecular entity constituting an integral part of the endotoxin complex. However, the fractionation of lipid A isolated from lipopolysaccharides by mild acid hydrolysis disclosed a variety of compounds such as free fatty acids and glucosamine on one hand, and unaltered phosphorylated acylpolyglucosamines of different chain length on the other (25-28). Although the breakdown products resulting from acid hydrolysis may be visualized as parts of a single structural unit of lipid A, it has also been suggested that lipid A may represent a mixture of related but not identical structures derived from different subunits of the complex endotoxin structure (29). Our conclusion supporting the concept of lipid A as a single structural entity is based on following experimental facts: 1) after 1% acetic acid hydrolysis of the endotoxin complex, the lipid A constituents were detected only in the

"conjugated" protein, 2) lipid A constituents could not be extracted by organic solvents from electrophoretically- and immunochemically-homogeneous "conjugated" protein, 3) "conjugated" protein contained no sugars other than glucosamine; and 4) lipid A constituents were absent in the mixtures of free amino acids and/or peptides released by proteolytic degradation of "conjugated" protein by trypsin and pronase. 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, lipid A possesses a branched structure consisting of several polyglycosamine chains of different length linked together through phosphodiester linkages. Although the predominant bond between O,N-acylated glucosamine phosphate units seems to be of glycosidic nature (17, 24, 25, 28, 30), a phosphodiester linkage even within a single polyglucosamine should not be excluded (25, 31). In contrast to the stable glucosamine glycosides, the phosphodiester linkages are

very labile to mild acid hydrolysis (32-34). As a result of mild acid hydrolysis of phosphodiester linkages between or within polyglucosamine chains, the phosphate groups should remain linked to glucosamine especially as very stable monoesters in positions C_4 and C_6 (30, 35). Mild acid hydrolysis used for the isolation of lipid A may cleave the phosphodiester linkages and result in breakdown products of different sizes.

Since its introduction by Westphal et al. (36), aqueous phenol extraction has been one of the most frequently used methods for the isolation of lipopolysaccharides. It has been assumed that this procedure causes primarily a dissociation of endotoxin into the protein and lipopolysaccharide fragments with little, if any, cleavage of covalent linkages. Proteins, indeed, do not seem to be altered by phenol treatment as shown by the unchanged physical-chemical properties and enzymatic activity of ribonuclease (37). Tsang (25) studied the effect of aqueous phenol treatment on the chemical composition of

lipopolysaccharides from S. marcescens 08 and reported a markedly decreased fatty acid and glucosamine content of a lipopolysaccharide preparation submitted to repeated extraction by aqueous phenol. He concluded from these results that phenol treatment caused cleavage of ester- and amide-bound fatty acids and at least a partial degradation of lipid A.

One can summarize the various effects of phenol (pKa = 10.0), acetic acid (pKa = 4.76) and trichloroacetic acid (pKa = 0.08) on whole endotoxin in the following manner. Although phenol and acetic acid ware weaker acids than trichloroacetic acid, at high temperatures (70° and 90°, respectively) they cause a selective partial cleavage of endotoxins. Phenol cleaves some ester- and amide-bound fatty acids and dissociates the protein moiety by splitting a particular (phosphodiester?) linkage within the lipid A. Acetic acid, on the other hand, seems to break specifically the linkages between the lipid A and the polysaccharide moiety as well as the linkages between the "side chain" and the "core" portions of the polysaccharide. The isolation

of a relatively intact endotoxin complex by trichloroacetic acid is due most probably to the fact that the extraction of bacterial cells is carried out at low temperature (4°). It has been shown, however, in a preceding paper of this series (18) that even at that temperature the endotoxin complex contains degradation fragments such as "simple" protein, side chain and free fatty acids. The importance of the reaction temperature is best illustrated by the finding that cold phenol extraction does not dissociate the protein moiety from the endotoxin complex (36). The effectiveness of phenol may be due also to its property as an excellent solvent for proteins (37); by unfolding the entire endotoxin structure as a solvent it may cause the maximal exposure of susceptible linkages to its action as a reagent.

The difference between the approximate molar ratios of fatty acids/phosphate/glucosamine of "conjugated" protein and its tryptic and pronase cores (3:1:1) and "simple" protein and its corresponding cores (2:1:1) may be explained now by

the enhanced cleavage of ester bound fatty acids by phenol treatment. Although it has been assumed (36) that phenol causes a complete separation of the protein moiety from the lipopolysaccharide fragment of the endotoxin complex, results of our studies indicate that hot phenol treatment cleaves the endotoxin within the lipid A portion; the resulting "simple" protein consists then of the protein moiety still linked to a fragment of lipid A.

There are very few reports describing the chemical composition of well defined "simple" and "conjugated" proteins from other Gram-negative bacteria. Homma (38) isolated the protein moiety of endotoxin from Pseudomonas aeruginosa and found that it contained 13.1% N, 1.6% P and 2.3% galactosamine. Alkaline ethanol treatment resulted in the isolation of a protein with 15.4% N. Fatty acids were not determined. Since the protein moiety contained 11% glucose, it is very difficult to classify it either as a "simple" or as a "conjugated" protein. Jenkin and Rowley (39) isolated

toxic proteins from Vibrio cholerae and Water vibrios. Toxic protein accounted for the major portion of the toxicity of the whole organism. Immunological and chemical data suggested that the toxic protein was identical with the protein moiety of the Boivin antigen. Toxic protein from Vibrio cholerae contained 13.8% N and 1.5% P; no values for carbohydrate or fatty acids were reported. Previous studies on the isolation of "conjugated" protein from Brucella melitensis (40), Shigella dysenteriae (1, 41) and Shigella paradysenteriae (Flexner) (5) showed that these preparations had nitrogen values (9-11%) higher than that of the "conjugated" protein but still lower than that of the "simple" protein from S. marcescens 08. It remains for future studies to establish whether this difference in nitrogen content reflects a lower content or an incomplete removal of lipid A from endotoxins of other species of Gram-negative bacteria.

Antibodies prepared against "conjugated" protein and its tryptic and pronase cores showed the presence of a common antigenic

determinant in all three protein preparations. Several investigators (3-5, 41) have already shown that "conjugated" proteins treated with trypsin retained their immunogenic properties. It appears that the antigenic site is located most probably on the protein moiety close to the lipid A.

Since toxicity represents 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 (29). Goebel and his coworkers (4, 5) suggested that a toxic "factor T" present in the so-called "toxic" protein preparation (a preparation equivalent to "conjugated" protein) was neither a protein nor a polysaccharide. Westphal and Lüderitz (15) proposed later that the toxic "factor T" was identical with lipid A, and detoxification studies (29) by partial alkaline hydrolysis showed that the presence of ester-bound fatty acids in endotoxins was essential

for eliciting toxic reactions. The polysaccharide moiety was found not to be essential for the toxicity, because endotoxin preparations from R-mutants of various Salmonella strains lacking the entire polysaccharide moiety still retained potent toxic properties (42-44). On the other hand, Ribi and his coworkers (45) isolated from S. enteritidis a highly toxic endotoxin characterized by a low fatty acid content and suggested (46) that a macromolecular complex of critical size is a major requirement for toxicity and other biological properties of endotoxins.

Results of the present investigation show clearly that endotoxin and its fragments dissolved in Tris-SDS buffer exhibit much higher toxicity than the same fractions administered as saline suspensions. It appears that SDS, by decreasing the particle size of the endotoxin fractions, had an enhancing rather than a diminishing effect on their toxic properties; similarly, McIntire et al. (46) have shown that disaggregation of a lipopolysaccharide preparation from E. coli K-235 by SDS did not

decrease pyrogenicity in the rabbit. The whole endotoxin complex, LPS-Ud-S, was the most toxic preparation. The removal of the polysaccharide moiety affected the toxicity of the remaining fragment more than a corresponding cleavage of the protein moiety; however, both the "conjugated" protein (LD_{50} = 22 mg/kg) and the lipopolysaccharide (LD_{50} = 12 mg/kg) should be considered as highly toxic fragments. Although "simple" protein, containing only a small part of lipid A, was less toxic than "conjugated" protein, it still displayed a moderate toxicity. It was shown for the first time that lipid A, due to an increased solubility in SDS, is a highly toxic fraction (LD_{50} = 14 mg/kg). These results demonstrate clearly the importance of the lipid A for the toxic properties of endotoxins and support strongly the proposal by Westphal and Lüderitz (5, 42) that lipid A is the toxic factor of endotoxins.

REFERENCES

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

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

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

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

6. Miles, A. A., and Pirie, N. W., Brit. J. Exptl. Med. 20 (1939) 278.

7. Freeman, G. G., and Anderson, T. H., Biochem. J. 35 (1941) 564.

8. Shear, M. J., and Turner, F. C., J. Nat. Cancer Inst. 4 (1943) 81.

9. Hartwell, J. L., and Shear, M. J., J. Nat. Cancer Inst. 4 (1943) 107.

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

11. Ikawa, M., Koepfli, J. B., Mudd, S. G., and Niemann, C., J. Am. Chem. Soc. 75 (1953) 1035.

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

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

14. Westphal, O., Lüderitz, O., Eichenberger, E., and Keiderling, W., Z. Naturforsch. 7b (1952) 536.

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

16. Westphal, O., and Nowotny, A., Lüderitz, O., Hurin, H., Eichenberg, E., and Schönholzer, G., Pharm. Acta Helv. 33 (1958) 401.

17. Lüderitz, O., Jann, K., and Wheat, R. In Comprehensive Biochemistry, Vol. 26A (edited by M. Florkin and E. H. Stotz). Elsevier Publishing Company, Amsterdam, 1968, p. 105.

18. Wober, W., and Alaupovic, P., Eur. J. Biochem.

19. Wober, W., and Alaupovic, P., Eur. J. Biochem.

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

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

22. Loomis, T. A., Essentials of Toxicology. Lea & Febiger, Philadelphia, Penn. 1968, p. 18.

23. Wang, C. S., and Alaupovic, P. Unpublished results.

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

25. Tsang, J. C., Dissertation, University of Oklahoma, Oklahoma City, 1968.

26. Nowotny, A., J. Bacteriol. 85 (1963) 427.

27. Kasai, N., and Nowotny, A., J. Bacteriol. 94 (1967) 1824.

28. Adams, G. A., and Singh, P. P., Biochim. Biophys. Acta 202 (1970) 553.

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

30. Gmeiner, J., Lüderitz, O., and Westphal, O., Eur. J. Biochem. 7 (1969) 370.

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

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

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

34. Archibald, A. R., and Baddiley, J., Advan. Carbohydrate Chem. 21 (1966) 328.

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

36. Westphal, O., Lüderitz, O., and Bister, F., Z. Naturforsch. 7b (1952) 148.

37. Kickhöfen, B., and Burger, M., Biochim. Biophys. Acta 65 (1962) 190.

38. Homma, J. Y., Z. Allg. Mikrobiol. 8 (1968) 227.

39. Jenkin, C. R., and Rowley, D., J. Gen. Microbiol. 21 (1959) 191.

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

41. Tal, C., and Olitzki, L., J. Immunol. 58 (1948) 337.

42. Lüderitz, O., and Westphal, O., Angew. Chem. Intern. Ed. Engl. 5 (1966) 198.

43. Tripodi, D., and Nowotny, A., Ann. N. Y. Acad. Sci. 133 (1966) 604.

44. Kun, Y. B., and Watson, D. W., J. Bacteriol. 94 (1967) 1320.

45. Ribi, E., Haskins, W. T., Landy, M., and Milner, K. C., Bacteriol. Rev. 25 (1961) 427.

46. McIntire, F. C., Sievert, H. W., Barlow, G. H., Finley, R. A., and Lee, A. Y., Biochemistry 6 (1967) 2363.

Table 1

Ester/Amide I Ratios of "Conjugated Protein", Tryptic and Pronase 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

Table 2

Elementary Analysis and Chemical Composition of "Conjugated" Protein, Tryptic Core and Pronase Core

	C %	H %	N %	PO_4 %	Glucosamine %	Fatty Acids %	Amino Acids %	Recovery[a] %	Ash %
"Conjugated" Protein	54.74	8.26	6.30	4.98	9.4	46.0	35.3	95.8	0.46
CP tryptic core	54.86	8.37	5.26	5.52	11.7	49.6	30.7	97.5	None or Trace
CP pronase core	57.39	9.23	3.45	6.48	18.1	53.2	12.9	90.7	None or Trace

[a]Recovery is calculated from phosphate, glucosamine, fatty acids and amino acids.

Table 3

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
1/2 Cystine	-	-	-	-	-	-
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

Table 4

Percent Fatty Acid Composition of "Conjugated" Protein and

Its Tryptic and Pronase Cores

	"Conjugated" Protein	Tryptic Core	Pronase Core
C_{12}	4.0	2.1	5.1
C_{14}	17.0	17.2	21.8
C_{14-1}	-	-	2.1
Unknown	11.6	11.6	7.5
C_{16}	13.0	13.6	12.0
C_{16-1}	5.4	6.2	4.1
Unknown	9.6	5.2	4.6
C_{14}-β-OH	39.5	41.4	41.8

Table 5

Chemical Composition of "Conjugated" Protein, PX-S and PX-E

Constituents	"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

Table 6

LD_{50} of Intact Endotoxin and Its Various

Fragments from S. marcescens 08

	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-S)	7.5	50.0	1.3	13.4
"Conjugated" Protein	37.5	197.5	22.3	> 75.0
"Simple" Protein (PX-S)	55.0	n.d.[a]	27.5	>100.0
Protein-free Endotoxin (LPS-A)	n.d.	62.5	12.1	39.9
Lipid A	n.d.	n.d.	14.2	n.d.
Polysaccharide side chain	non-toxic		non-toxic	

[a]Not determined.

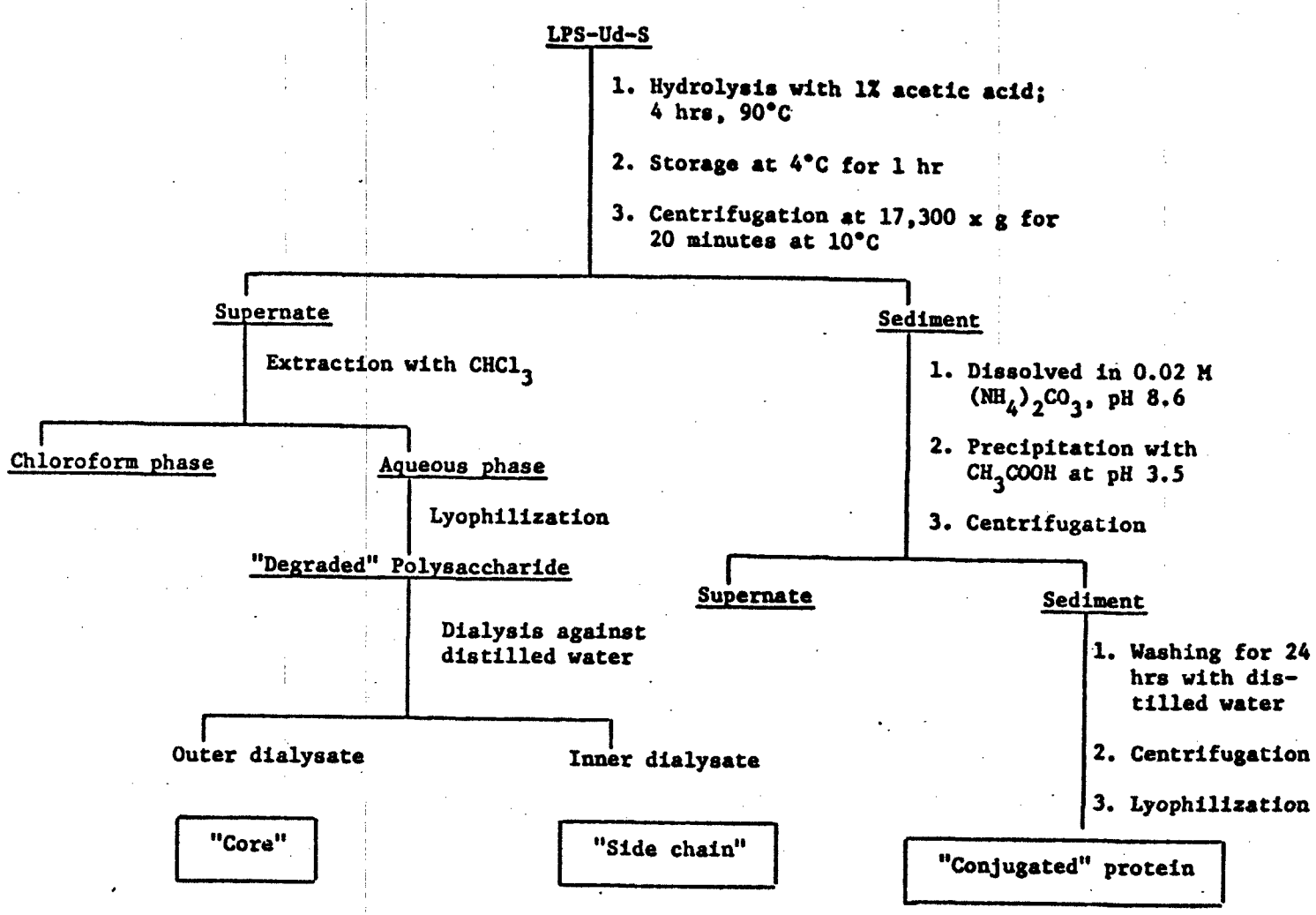

Figure 1. Isolation of "conjugated" protein from LPS-Ud-S.

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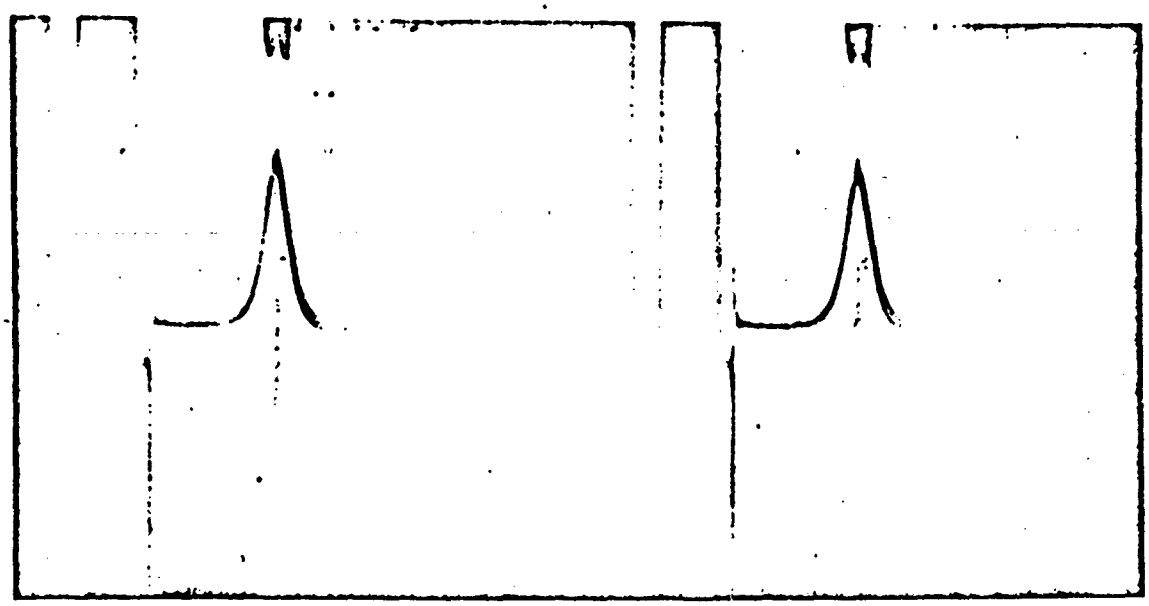

Figure 2. Sedimentation velocity pattern of "conjugated" protein. "Conjugated" protein (6 mg/ml) dissolved in 0.05 M Tris-Cl buffer, pH 7.6, containing 0.2% SDS, solvent density 1.004 g/ml, was centrifuged in a synthetic boundary cell at 52,640 rpm at 25 . Exposures were taken from left to right at 4 minute intervals for a total run of 64 minutes. Photographs shown were obtained at 12 and 32 minutes after reaching speed.

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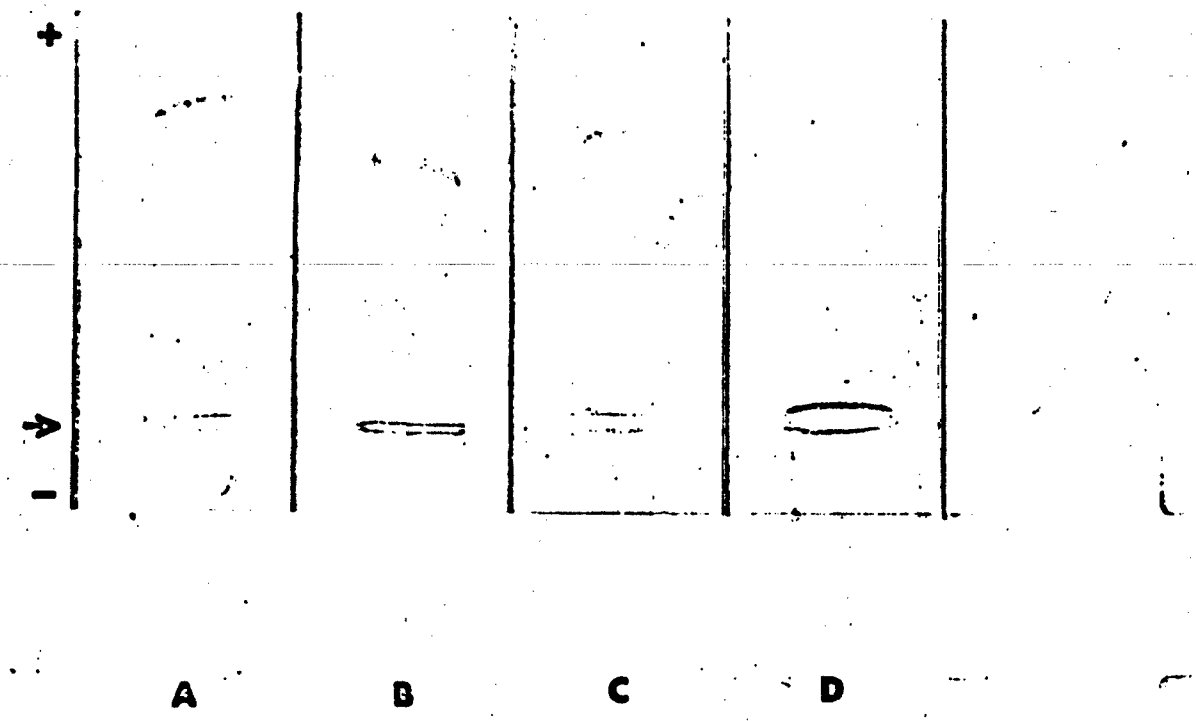

Figure 3. Agarose electrophoresis of LPS-U-S (A), "conjugated" protein (B), tryptic core (C) and pronase core (D). Protein preparations (20 mg/ml) were dissolved in 0.05 M Tris-Cl buffer, pH 7.6, containing 0.5% SDS and diluted to a final concentration of 10 mg/ml with 1% agarose in Veronal buffer. Electrophoresis was performed in 1% agarose gel using Veronal buffer, pH 8.6, ionic strength 0.05 for 40 minutes.

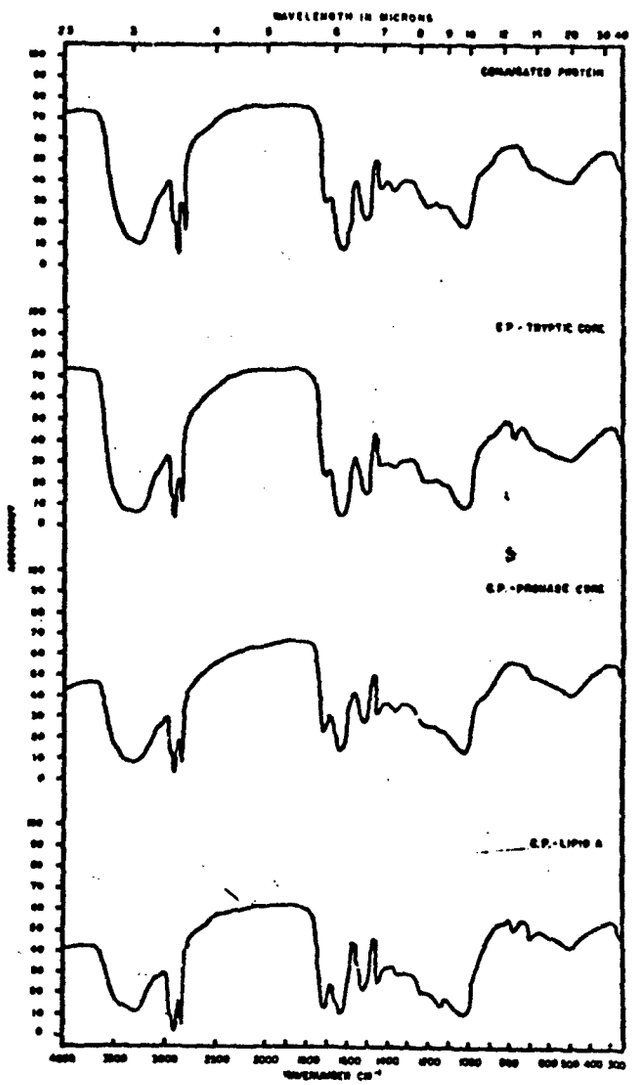

Figure 4. Solid infrared spectra of "conjugated" protein, tryptic and pronase cores and lipid A.

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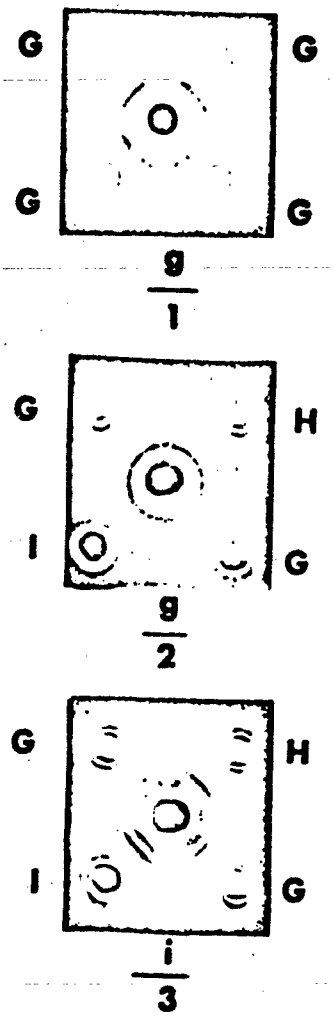

Figure 5. Immunodiffusion patterns of "conjugated" protein (G), tryptic core (H) and pronase core (I). Central wells contain antibodies to "conjugated" protein (g) and to pronase core of "conjugated" protein (i).

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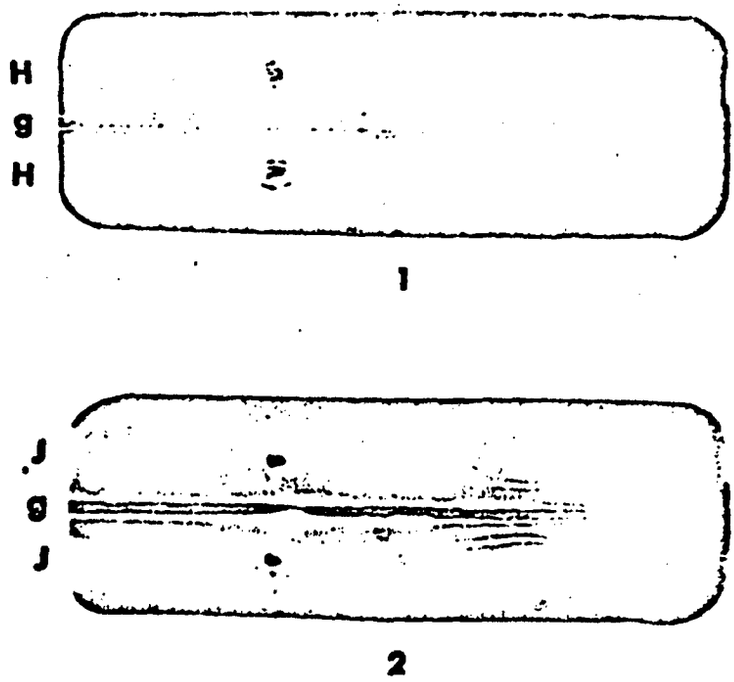

Figure 6. Immunoelectrophoresis patterns of tryptic core (H) and pronase core (I). The central troughs in both patterns contain antibodies to "conjugated" protein.

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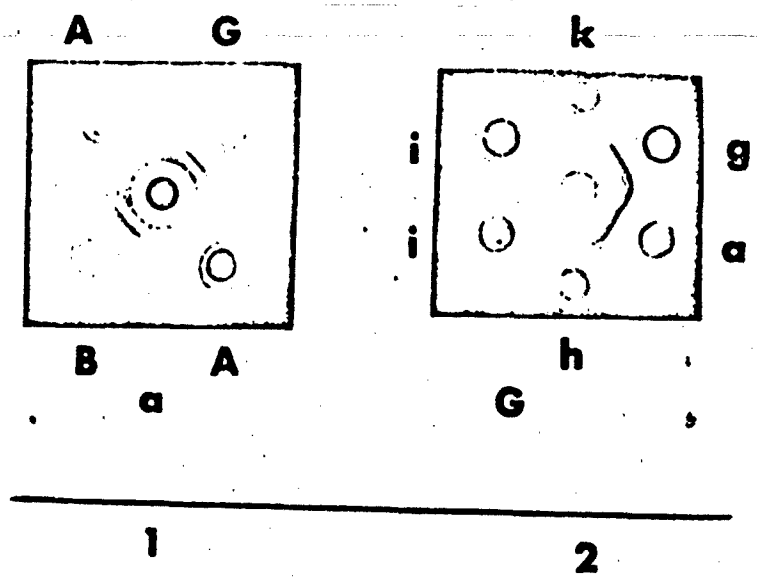

Figure 7. Immunodiffusion patterns of PX-S (A), PX-E (B) and "conjugated" protein (G). The central well in pattern 1 contains antibodies to PX-S (a). The outer wells in patters 2 contain antibodies to PX-S (a), LPS-U-S (k), "conjugated" protein (g), tryptic core of "conjugated" protein (h), and pronase core of "conjugated" protein (i).

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Figure 8. Peptide mapping of tryptic peptides of "conjugated" protein (upper pattern) and PX-S (lower pattern). Experimental conditions are described in the Materials and Methods.

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13. ABSTRACT

"Conjugated" protein isolated from the endotoxin complex of Serratia marcescens 08 by 1% acetic acid hydrolysis was characterized by analytical ultracentrifugation, electrophoretic and immunological properties and chemical analysis. The chemical composition of "conjugated" protein and other degraded fragments of endotoxin showed that all characteristic constituents of lipid A were present exclusively in "conjugated" protein. Successive treatment of "conjugated" protein with trypsin and pronase resulted in the isolation of corresponding tryptic and pronase resulted in the isolation of corresponding tryptic and pronase cores which were characterized by an increased content of lipid A constituents. Lipid A was separated as an entity from the "conjugated" protein only after hydrolysis with 0.1 N hydrochloric acid. This result confirms our previous proposal that lipid A is covalently linked to the protein moiety in whole endotoxin of S. marcescens 08. "Conjugated" protein isolated by acetic acid hydrolysis and "simple" protein prepared by hot aqueous phenol treatment of whole endotoxin differ from one another not by the presence or absence of lipid A but rather by the presence of the entire lipid A moiety in the "conjugated" protein and only a fragment of lipid A in the "simple" protein. "Conjugated" protein and its tryptic and pronase cores were immunogenic and possessed a common antigenic determinant with "simple" protein and its corresponding cores. Toxicity studies revelaed that both "conjugated" and "simple" proteins retained to different degrees the toxic properties of whole endotoxin. Sodium dodecyl sulfate had an enhancing rather than diminishing effect on the toxicity of endotoxin and its various fragments.

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