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Chemical Composition and Biological Activity of the *Yersinia pestis* Envelope Substance

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Purification of the envelope antigen of *Yersinia pestis* EV with passive hemagglutination activity is described. The purification procedure consisted of pancreatin digestion, chromatography on human erythrocyte stroma set on Celite, and rechromatography on Sephadex G-200. Chemical, physical, and biological properties of this antigen were investigated. The results show the lipid-polysaccharide structure of the isolated antigen. The carbohydrate moiety of the galactolipid antigen consists of galactose and fucose. The lipid fraction contained phosphatidylethanolamine and phosphatidylserine. The preparation showed high specificity in the hemagglutination reaction and in *Y. pestis* phage receptor activity. In two-dimensional immunoelectrophoresis, the isolated pancreatic envelope digest antigen appeared as a single line. Two-dimensional immunoelectrophoresis was modified for tandem separation and was employed to electrophoretically identify the pancreatic envelope digest, trypsin envelope digest preparation, and F1 envelope antigen of *Y. pestis*. Related or identical antigens showed confluence of peaks with reactions of identity.

Plague bacteria cultivated on bacteriological media at 37°C produce a capsular substance (18). Chen and Elberg (9) showed that the capsular antigen viewed by scanning electron microscopy forms a granular layer on the surface of cells, overflowing onto the surrounding medium. According to Amies (3) this macromolecular substance is easily soluble in water (10). It contains fractions which are able to sensitize erythrocytes for passive hemagglutination (3, 8, 11).

Baker et al. (5) isolated the capsular antigen F1 by extracting the dry mass of *Yersinia pestis* bacteria with a physiological solution of salt and fractionated the extract by precipitation with ammonium sulfate. They obtained the IA fraction, a protein-carbohydrate complex, and the IB fraction, a simple protein. This antigen was unable to sensitize normal erythrocytes (8).

By means of electrophoresis in acrylamide gel, Habig et al. (13) showed that the F1 antigen contains heterogeneous protein populations. They suggested that these protein populations were a series of macromolecular aggregates. With reference to these studies, Bennett and Tornabene (6) later reported that the soluble F1 fraction of the *Y. pestis* envelope antigen occurs in the buffered solution as aggregates with molecular weights larger than 300,000. Each of the aggregated molecules can be separated, in a solution of sodium dodecyl sulfate, into simple antigen subunits containing proteins and glycoproteins with molecular weights in the range of 15,000 to 17,000. Detailed examination of the carbohydrate of the glycoproteins showed only the presence of galactose. These workers did not find any amino acids or hexuronic acids described earlier by Bakhrakh et al. (reviewed by Brubaker, reference 7) in the F1 antigen.

This paper contains a description of the isolation and purification procedures and the chemical composition of those fractions of the *Y. pestis* envelope antigen which exhibit hemagglutination and phage receptor activity. Immunoelectrophoresis properties and comparison of the pancreatic envelope digest (PED) preparation, trypsin envelope digest (TED) preparation, and envelope antigen F1 are also described.

MATERIALS AND METHODS

Organisms. *Y. pestis* EV and *Y. pestis* bacteriophage were obtained from the Pasteur Institute, Paris, France.

Cultivation. Bacteria were grown for 48 h at 37°C on a medium consisting of 3% agar (Difco Laboratories, Detroit, Mich.), 0.1% glucose, 0.3% beef extract (Difco), 0.25% yeast extract (Difco), 0.025% Na_2SO_3, 0.06% $Na_2S_2O_3 \cdot 5H_2O$, 0.2% cysteine hydrochloride, and 0.5% NaCl. They were collected in water and quickly mixed with acetone (1:3, vol/vol). After centrifugation at 2,200 × *g*, the sediment was suspended in acetone and left overnight at 37°C. During the next 3 days acetone was changed daily, and then the bacteria were air dried.

Preparation of antisera. The immunization procedure for preparing rabbit antisera to complete organisms was as follows: *Y. pestis* was suspended in 0.85% NaCl (10^8 organisms per ml), and a formolized suspension of *Y. pseudotuberculosis* and *Y. enterocolitica* was diluted in 0.85% NaCl to the same concen-

tration as mentioned above. The immunogens were administered by five intravenous injections at 5-day intervals using volumes of 0.5, 0.1, 1.5, 2.0, and 2.5 ml, respectively. Sera were obtained within 7 days of the last immunization.

Isolation and purification of the capsular envelope antigen: preparation of crude extract. The 2% suspension of dry bacteria in a 0.9% NaCl solution was shaken with a few glass beads at 37°C for 1 h and then centrifuged for 30 min at 14,000 × *g*. The clear, yellow supernatant was used for another preparation.

The PED preparation was made by our modification of the method of Taylor (20). The crude extract was precipitated with 1:5 (vol/vol) acetone, centrifuged at 2,200 × *g*, and dried. The acetone sediment was suspended in 0.01 M acetic acid (pH 5) and reprecipitated with 1:5 (vol/vol) acetone. After centrifugation at 2,200 × *g*, the sediment was suspended in phosphate-buffered saline (PBS) containing (in 100 ml) 0.80 g of NaCl, 0.22 g of KCl, 0.29 g of $Na_2HPO_4 \cdot 12H_2O$, 0.2 g of KH_2PO_4, 0.01 g of $CaCl_2$, and 0.01 g of $MgCl_2 \cdot 6H_2O$ and was digested with pancreatin (K & K Laboratories Inc., Plainview, N.Y., lot 18896F). Digestion took place for 4 h at 39°C in a Visking (Serva, Feinbiochemica, Heidelberg, Germany) tube against PBS. One gram of acetone sediment suspended in 40 ml of PBS was mixed with 32.2 mg of pancreatin suspended in 10 ml of 0.85% NaCl. After dialysis in water, the PED was adjusted to pH 5 with acetic acid and precipitated with 1:5 (vol/vol) ethanol. The sediment dissolved in water was further purified by column chromatography on erythrocyte membranes mixed with Celite. Erythrocyte membranes were prepared from 1,000 ml of human blood (Blood Donor Centre, Gdańsk, Poland) by the method of Taylor (20). The mixture of 40 g of Celite 535 with the membrane suspension, containing 20 ml of membrane sediment, was poured into a column (2.5 by 20 cm). The column was washed successively with the following: 500 ml of 2.0% formaldehyde, 200 ml of 0.5 M NaCl, 200 ml of water, 200 ml of 0.5 M NaCl, 200 ml of water, 200 ml of 0.1 M NaCl, 200 ml of water, and finally 200 ml of 0.1 M NaCl. A column thus prepared may be used only once. Forty milliliters of the specimen was put into the column and left overnight at room temperature to adsorb the antigen on the erythrocyte membranes. After 18 h, the column was washed with 0.1 M NaCl to remove unadsorbed material. Fractions active in hemagglutination were eluted with water, concentrated, and subjected to rechromatography.

Fractions of the condensed preparation (3 ml) were applied to a column (2.5 by 30 cm) filled with Sephadex G-200. Elution was performed with 0.1 M phosphate buffer containing 0.5 M of NaCl (pH 7.2) at room temperature.

The TED was prepared by our own method. A 28-mg amount of trypsin (Sigma Chemical Co., St. Louis, Mo., type III, lot 93012118) was added to 280 ml of the *Y. pestis* crude extract and kept at 37°C for 1 h. The mixture was transferred to a Visking tube and dialyzed against water (1.5 h at room temperature and then overnight at 4°C). This preparation was then adjusted to pH 5 with 0.1 M acetic acid and precipitated with 1:5 (vol/vol) ethanol. The sediment was dissolved in water, and 5-ml portions were put into the column (5 by 18 cm) filled with Sephadex G-200. Elution was accomplished in 0.1 M phosphate buffer containing 0.5 M of NaCl (pH 7.2) at room temperature. Six-milliliter fractions were collected.

Chemical analysis. Samples of PED and TED preparations were assayed colorimetrically for total carbohydrates, hexosamines, uronic acids, and proteins by the methods of Dubois et al. and Lowry et al., respectively, as described by Herbert et al. (15). Phosphorus was determined by the method of Ames (2). Lipids were extracted from PED and TED preparations with chloroform and methanol by the method of Kates et al. (16).

Paper and thin-layer chromatography. Hydrolysis was performed in 0.1 N and 6 N HCl at 105°C for 18 h and in 23.5 M formic acid at 105°C for 2 h. Chromatography of hydrolyzed PED and TED products was performed on Whatman no. 1 paper and on MN-300 cellulose by using the following mixtures: (a) *n*-butanol-acetic acid-water (4:1:5); and (b) *n*-amyl alcohol-*izo*-butanol-*n*-propanol-pyridine-water (5:5:5:15:15). Compounds were detected with $AgNO_3$ and *p*-anisidine-hydrochloride. Total and hydrolyzed lipids were studied by thin-layer chromatography on glass plates covered with Silica Gel G by solvent mixture (c) chloroform-acetone-methanol-acetic acid-water (50:20:10:10:5). Spots were visualized by exposure to I_2 vapors.

Hemagglutinin test. The hemagglutinin test was carried out by preparing 0.25 ml of the twofold serial dilutions of eluted fractions with 0.1-ml portions of a 2% suspension of formolized sheep erythrocytes. After 1 h of incubation at 37°C, 0.25 ml of the *Y. pestis* antiserum diluted 1:100 was added, and the mixture was stored at room temperature.

Electrophoresis. Two-dimensional immunoelectrophoresis (4) was performed on glass plates (9.5 by 10 cm) covered with 1% agarose gel (International Enzymes Ltd., Windsor, Berkshire, England) in Veronal buffer (pH 8.6, ionic strength of 0.05). Thirty-microliter amounts (150 μg) of antigen solubilized in 10 M urea solution were added to a well 3 mm in diameter cut in the corner of the slide on the cathode side.

First-phase electrophoresis was carried out for 1.5 h at 80 V per plate. Agar, except for a 1.5-cm strip in the antigen migration path, was removed. A total of 1.2 ml of antiserum-containing agarose mixture was then poured over the cleared area, and the second phase was run at right angles to the electrophoresis direction of the first phase for 18 h at 20 V per plate at +10°C. Precipitin lines were allowed to further intensify in a moist chamber at room temperature for 24 h. The gels were finally pressed, washed twice in cold 0.1 M NaCl, air dried, and stained with Coomassie brilliant blue, Oil red O, or with Schiff-periodic acid reagent (19).

The two-dimensional immunoelectrophoresis procedure was modified for tandem separation of two samples as follows. Two sample wells with a 1.5-cm distance between centers were punched at the origin. The reference sample (F1 antigen or TED preparation) was placed in the outer well. Either the TED preparation or PED antigen was placed in the inner well. The other details for these runs were as described above.

Immunoelectrophoresis was performed on microscope slides (about 7.5 by 2.5 cm) covered with 3 ml of the agarose gel. A 10-μl amount (5 mg of antigen solubilized in 0.1% Triton X-100) of antigen was added to the 3-mm-diameter well and was electrophoresed for 1 h at 5 V/cm in Veronal buffer (pH 8.9) which also contained 0.5% Triton X-100. *Y. pestis* antiserum (0.1 ml) was added to the 2-mm-wide troughs, and precipitin arcs were allowed to develop in a moist chamber at 4°C. Gels were stained with Oil red O or Schiff-periodic acid reagent (19).

Passive hemagglutination reaction. Erythrocytes were sensitized for 1 h in a water bath at 37°C by the addition of 1 volume of 2% erythrocytes to 1 volume of antigen solution concentration, 100 μg/ml.

The diagnostic sera, anti-*Y. pestis*, anti-*Y. pseudotuberculosis*, anti-*Y. enterocolitica*, and anti-*Salmonella* BO (National Salmonella Centre, Gdańsk, Poland) were diluted again from 1:10 to 1:5,000 on a Plexiglas plate in a volume of 0.25 ml, and 0.1 ml of the 2% sensitized erythrocytes was added. The mixture was stored at room temperature for 18 h. Formolized erythrocytes were prepared by the method of Weinbach (22).

Assay of receptor activity. Phage stock (0.1 ml) containing 10^{11} plaque-forming units (PFU)/ml was mixed with 3 ml of 10% formolized sheep erythrocytes sensitized with the PED and TED preparations studied (concentration, 1 mg/ml of 10% erythrocytes). The mixture was incubated for 5 min at 0°C. After incubation the mixture was quickly centrifuged at 1,500 × *g* for 5 min. The receptor activity of the preparations examined was measured by the quantity of unadsorbed *Y. pestis* phages, determined by the method of Adams (1).

RESULTS

The crude extract of the *Y. pestis* capsular envelope contained 32% protein, 2% sugar, and a significant amount of nucleic acids. It showed very high hemagglutination and *Y. pestis* phage receptor activity. The level of protein was reduced up to 8% as a result of pancreatin digestion.

A 40-ml sample of pancreatic-digested and ethanol-precipitated preparation was then introduced into a column with erythrocyte membranes and eluted with 0.1 M NaCl and afterwards with redistilled water. The elution profile is illustrated in Fig. 1. Fractions 1 to 9 had low hemagglutination activity, but ultraviolet spectrophotometric measurement showed a high level of protein, e.g. the optical density of fraction 5 at 280 nm was 1.5 (Fig. 1). The highest positive hemagglutination reactions occurred in fractions 16 and 17; fractions 13 to 25 (Fig. 1) were nevertheless concentrated.

A 2-ml sample of the pancreatic-digested, ethanol-precipitated and chromatography-purified preparation was then introduced into a Sephadex G-200 column and eluted with 0.1 M phosphate buffer. The elution profile is illustrated in Fig. 2. The preparation with the highest hemagglutination activity was eluted as a single peak in fractions 9 to 13. When these fractions were examined by immunodiffusion by using antisera prepared against a dry mass of *Y. pestis*, one band was discernible. Fractions 7 to 15 were dialyzed, condensed, and lyophilized. The lyophilized PED preparation (10 mg) was dissolved in 1 ml of phosphate buffer and then subjected to molecular sieve chromatography on Sephadex G-200. Compounds comprising PED eluted from

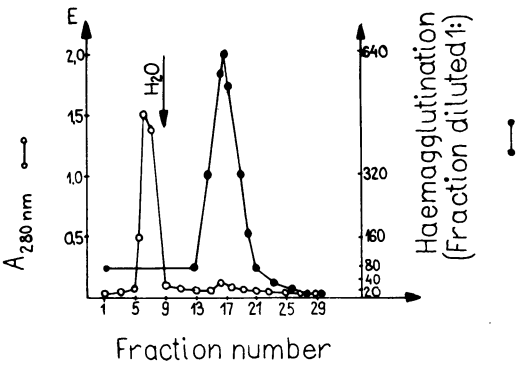

FIG. 1. *Separation of PED preparation on erythrocyte stroma-Celite 535. Column, 2.5 by 20 cm; eluent, 0.1 M NaCl and redistilled water; flow rate, 100 ml/h; fraction size, 20 ml. The absorbance of protein was measured at 280 nm ($A_{280\ nm}$). Hemagglutination tests were performed with fractions diluted from 1:10 to 1:1,280 as described in the text.*

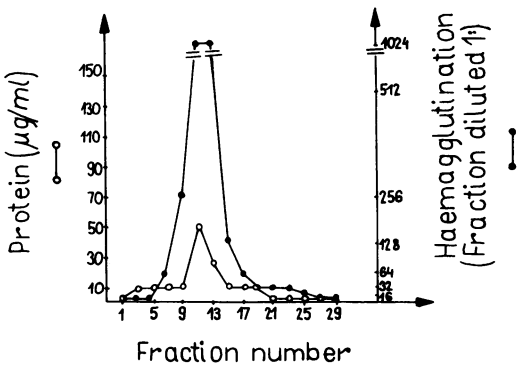

FIG. 2. *Rechromatography of PED preparation on Sephadex G-200. Column, 2.5 by 30 cm; eluent, phosphate buffer solution (pH 7.2) containing NaCl (0.5 M); flow rate, 30 ml/h; fraction size, 6 ml. Protein was determined by the method of Lowry (described by Herbert et al. [15]). Samples were read against a blank at 720 nm in an SP 1750 ultraviolet spectrophotometer by using 20-mm cuvettes, and crystalline albumin bovine solution from Fluka A.G. Busch S.G., Switzerland, 401206 was used as a standard. Hemagglutination tests were performed by fractions diluted from 1:4 to 1:1,024, as described in the text.*

the column in a regular peak at the void volume (as determined with blue dextran 2000).

One may suggest that the PED preparation is a homogeneous macromolecular species. It contains about 90% lipids, 4% protein, and 2.8% sugar. Colorimetric tests did not show any hexosamines, hexuronic acids, or phosphorus.

The trypsin-digested and ethanol-precipitated preparation was divided into 5-ml portions, put into the Sephadex G-200 column, and eluted with 0.1 M phosphate buffer. Figure 3 shows the elution profile. A positive hemagglutination reaction took place in a 1,000-fold dilution of fractions 3 and 4. However, the extinction of these fractions at 280 nm amounted to 1.5, fractions 1 to 9 were dialyzed, condensed, and lyophilized. The TED preparation contained about 70% lipids, 11% protein, 2.2% saccharides, 0.2% hexosamines, and 0.6% phosphorus. The colorimetric studies provided no evidence for the presence of uronic acids.

The ultraviolet spectrum shows the difference between the PED and TED preparations (Fig. 4). The high peak at 256 nm with optical density of 1.2 indicates the presence of nucleotide contamination in TED. The PED preparation was devoid of this contamination, and its optical density at 256 nm was 0.1.

The infrared spectrum (Fig. 5) of the PED preparation shows the absorption band at 3,400 to 3,600 cm^{-1}, characteristic of carbohydrates, and another one at 2,800 to 3,000 cm^{-1}, characteristic of lipids.

Chromatographic separation of formic acid hydrolysates on thin-layer plates with MN-300 cellulose in solvent system (a) is shown in Fig. 6.

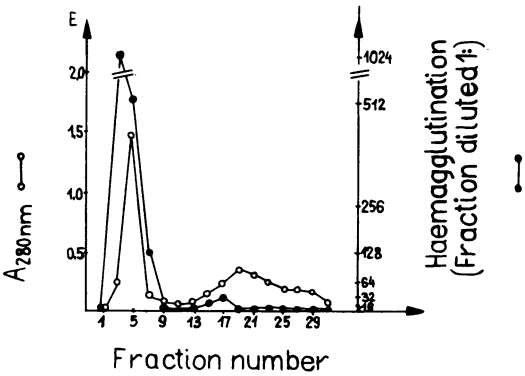

FIG. 3. *Chromatography of TED preparations on Sephadex G-200. Column, 5.0 by 18 cm; eluent, phosphate buffer solution (pH 7.2) containing NaCl (0.5 M); flow rate, 30 ml/h; fraction size, 6 ml. The absorbance of protein was measured at 280 nm ($A_{280\ nm}$). Hemagglutination tests were performed by fractions diluted from 1:4 to 1:1,024, as described in the text.*

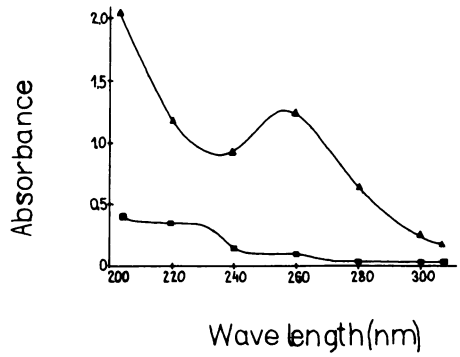

FIG. 4. *Ultraviolet spectra of the purified PED and TED preparations. Measurements were taken with an SP 1750 ultraviolet spectrophotometer by using 0.5-cm cuvettes. ■, 0.1% water solution of PED; ▲, 0.1% water solution of TED.*

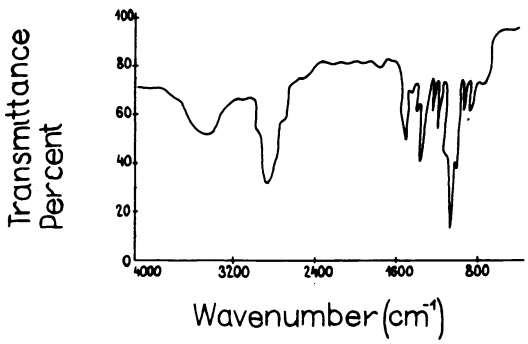

FIG. 5. *Infrared spectrum of the PED preparation.*

Two components were observed in PED and TED hydrolysates. These components were identified by their relative R_f values to known standards, which correspond to those of galactose and fucose. The same components (with R_f values similar to those of the authentic galactose and fucose) were observed in PED and TED hydrolysates on paper chromatograms developed in solvents (a) and (b).

Chromatographic separation of total lipids extracted from PED and TED preparations on thin-layer plates in solvent (c) is shown in Fig. 7. Two ninhydrin-positive components were observed in both preparations, their R_f values being similar to those of authentic phosphatidylethanolamine and phosphatidylserine. The fatty acids present in the lipid fraction of isolated preparations were not identified.

Investigation of PED in two-dimensional immunoelectrophoresis showed the presence of a single precipitation arc with *Y. pestis* antiserum (Fig. 8A). The precipitate was also colored with Oil red O, but did not color with Schiff-periodic acid reagent. No precipitation bands of PED

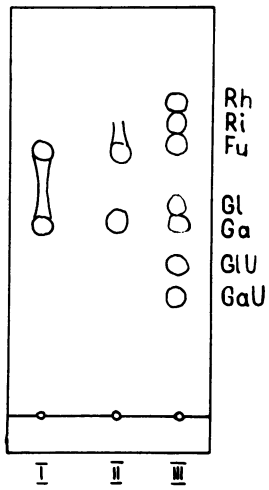

FIG. 6. *Chromatogram of the PED and TED products obtained by formic acid hydrolysis after separation over MN-300 cellulose thin-layer plates. The chromatogram was developed in solvent system (a). Spots were visualized by p-anisidine spray. I, PED hydrolysate; II, TED hydrolysate; III, standards: Rh, rhamnose; Ri, ribose; Fu, fucose; Ga, galactose; Gl, glucose; GaU, galacturonic acid; GlU, glucuronic acid.*

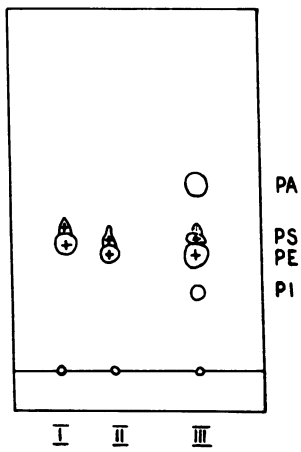

FIG. 7. *Chromatogram of total lipids extracted from PED and TED preparations after separation over Silica Gel G-coated thin-layer plates. Chromatogram was developed in solvent system (c). Spots were visualized by exposure to iodine vapors. +, Components that were ninhydrin positive; I, PED extract; II, TED extract; III, standards; PA, phosphatidic acid; PS, phosphatidylserine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.*

preparation with *Y. pseudotuberculosis* and *Y. enterocolitica* antisera were observed.

Immunoelectrophoretic separation of PED antigen dissolved in Triton X-100 showed the presence of a single precipitation band colored with Oil red O, but did not color with Schiff-periodic acid reagent.

The TED preparation and F1 antigen showed in two-dimensional immunoelectrophoresis the presence of four precipitin bands with anti-plague serum (Fig. 8B and C, respectively). The presence of common precipitation bands with anti-*Y. pseudotuberculosis* serum showed only the F1 antigen.

The results of two-dimensional immunoelectrophoresis in tandem separation are shown in Fig. 9. Identical lines between four precipitation arcs of envelope F1 antigen and TED preparation (Fig. 9A), as well as between the first peak

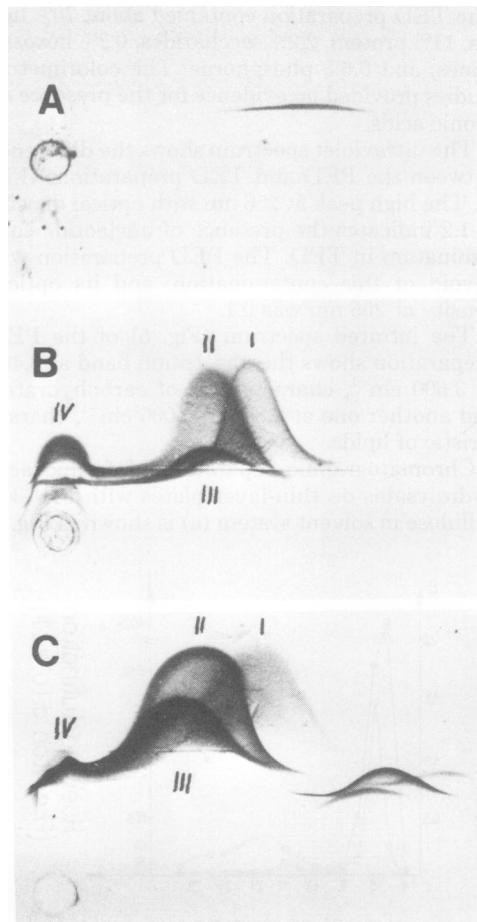

FIG. 8. *Two-dimensional immunoelectrophoresis profile of PED (A), TED (B), and F1 antigen (C). A 150-μg portion of each antigen was subjected to electrophoresis in the first phase anode on the left of the figure and developed against 1.2 ml of antiserum to Y. pestis EV dry mass in the second phase anode at the top of figure. The dominant precipitation arcs (B and C) are marked, I, II, III, and IV.*

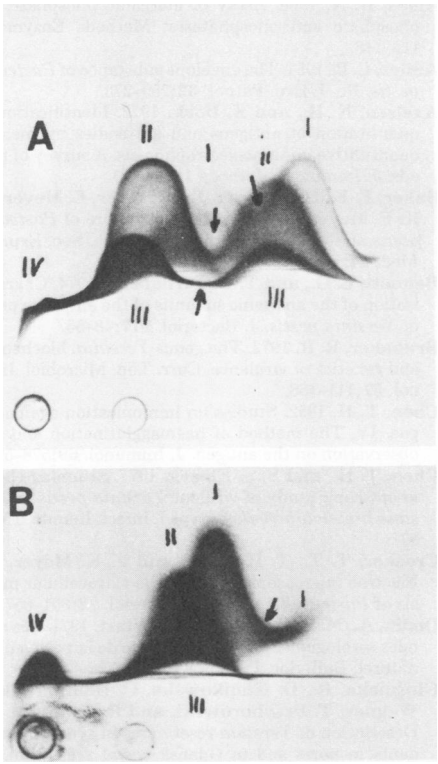

FIG. 9. *Serological comparison of Y. pestis envelope antigens by two-dimensional immunoelectrophoresis in tandem separation. (A) F1 antigen in the outer well; TED preparation in the inner well. (B) TED preparation in the outer well; PED antigen in the inner well. Other details for these runs are as described for Fig. 8. The precipitation arcs are marked I to IV. Arrows show the identity lines.*

of TED preparation and PED antigen, were apparent (Fig. 9B).

The PED and TED preparations showed a positive hemagglutination reaction with anti-plague serum in dilutions of 1:2,560 and 1:1,280, respectively. The hemagglutination reactions of PED antigen with *Y. pseudotuberculosis*, *Y. enterocolitica*, and *Salmonella* BO antisera were negative, but the hemagglutinin reactions of TED preparations were positive with anti-*Y. pseudotuberculosis* (serotypes II and IV) sera diluted 1:20 and also with anti-*Y. enterocolitica* (serotypes O:3 and O:9) and anti-*Salmonella* BO serum diluted 1:10.

Investigation of *Y. pestis* phage receptor activity of the isolated PED and TED preparations showed that the concentration of *Y. pestis* phage after incubation with erythrocytes sensitized with PED preparation was 10^7 PFU/ml and with TED antigen was 10^8 PFU/ml. However, both preparations showed very high receptor activity in comparison to that of stock solution bacteriophages at a concentration of 10^{11} PFU/ml. In addition, the adsorption of *Y. pestis* phage to unsensitized erythrocytes was observed; the concentration of *Y. pestis* phage after incubation with unsensitized erythrocytes was 10^9 PFU/ml.

DISCUSSION

The methods described allowed extraction of a highly purified antigen by a very mild procedure. The analysis showed that PED is a macromolecular lipopolysaccharide (to distinguish it from the lipopolysaccharide of *Y. pestis*, we propose the name galactolipid).

One may suppose that the *Y. pestis* envelope antigen is a protein-lipid-polysaccharide complex. The purification of the antigen by precipitation of ammonium sulfate allowed isolate of the protein-carbohydrate complex (5), but the precipitation may cause depolymerization of the antigen. Depolymerization occurs with loss of the hemagglutination activity of plague extract purified by that compound (3, 8, 17).

Digestion with pancreatin caused elimination of the protein component of the envelope antigen, as well as nucleotide contamination, without any loss of hemagglutination activity. It allowed us to make a preliminary analysis of the other components of the *Y. pestis* envelope.

Chromatographic investigations showed that a carbohydrate moiety of galactolipid consists of galactose and fucose. That result is a partial confirmation of the work of Bennett and Tornabene (6). They found galactose in the F1 antigen, but did not find fucose.

About 90% of the PED antigen mass is lipids. The presence of phosphatidylserine, phosphatidylethanolamine, and undefined fatty acid in the above-mentioned lipids did not differ from that of lipid fractions of compounds isolated by Tornabene (21) from *Y. pestis* and *Y. pseudotuberculosis* whole cells. No glycolipids were found in *Y. pestis* strains (21).

Unlike the results of Hartley et al. (14), our own investigation of *Y. pestis* lipopolysaccharide refuted the association of the PED preparation with the lipopolysaccharide part of the cell wall because no glucose and no hexosamines were found in the PED. On the other hand, only traces of ethanolamine in the *Y. pestis* lipopolysaccharide were found (14).

Immunoelectrophoresis and two-dimensional immunoelectrophoresis investigations showed the PED preparation as a homogeneous antigen. Color reaction of PED with Oil red O confirmed the presence of the lipid component. The negative periodic acid-Schiff reaction may indicate lower carbohydrate moiety in PED than sensivity of this reagent.

Based on the two-dimensional immunoelectrophoresis in tandem separation, one may suppose that PED and TED antigens are connected with the *Y. pestis* envelope.

The TED preparation contained the same compounds of carbohydrate and lipids as did the PED antigen. However, the TED preparation contained contaminations of other compounds such as hexosamines and nucleic acids. Therefore by two-dimensional immunoelectrophoresis, TED showed the presence of four precipitation arcs, and an unspecific positive hemagglutination reaction with *Y. pseudotuberculosis*, *Y. enterocolitica*, and *Salmonella* BO antisera was ascertained.

Important, however, is the fact that the F1 fraction contains electrophoretically common antigenic components of both *Y. pestis* and *Y. pseudotuberculosis* organisms as well.

Both PED and TED preparations showed a positive hemagglutination reaction with a high dilution of antiplague serum.

The ability of *Y. pestis* bacteria to produce a substance with hemagglutination activity was described earlier by Amies (3), Chen (8), and Dodin et al. (11). These authors suggested that this is a polysaccharide substance. Investigations described in this paper showed that hemagglutination activity is rather bound with the galactolipid antigen isolated from the *Y. pestis* envelope. The results do not suggest which part of the galactolipid complex is responsible for the antigen specificity in the hemagglutination reaction. Probably it is not a lipid fraction, because the same lipid components were found in *Y. pseudotuberculosis* cells (21).

The application of PED antigen in epidemiological investigation in search of *Yersinia* reservoirs in ports revealed its serological specificity in comparison with that of the F1 antigen (12).

The other very important property of the described galactolipid antigen is its *Y. pestis* bacteriophage receptor activity. This property is the object of further examinations in our laboratory.

The presented results show that the PED antigen could be a diagnostic preparation in *Y. pestis* epidemiological investigations with the simple hemagglutination test. The useability of this antigen in diagnosis of plague can be determined after conducting an appropriate examination in plague endemic foci.

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