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Partial Purification and Characterization of the Major Species-Specific Protein Antigens of *Rickettsia typhi* and *Rickettsia prowazekii* Identified by Rocket Immunoelectrophoresis

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Species-specific antigens from *Rickettsia typhi* and *Rickettsia prowazekii* were readily solubilized by French pressure cell extraction or sonication of Renografin density gradient-purified rickettsiae and were identified by rocket immunoelectrophoresis. As measured by quantitative rocket immunoelectrophoresis, the species-specific typhus rocket antigens (STRAs) appeared to be proteins; they were denatured by heating at 56°C for 30 min but not by 50°C treatment, and they were sensitive to pronase and trypsin but were not affected by periodate oxidation, glycosidases of various specificities, phospholipase A, or lipase. STRAs from both *R. typhi* and *R. prowazekii* were separated from common antigens by DE52 column chromatography of 100,000-×-*g* supernatant fractions of rickettsial extracts. The purified STRAs were characterized by crossed immunoelectrophoresis, by polyacrylamide gel electrophoresis on Davis and sodium dodecyl sulfate gels, and by an enzyme-linked immunosorbent assay. The two purified STRAs were proteins with similar native electrophoretic mobilities in agarose and polyacrylamide gels, and these proteins had similar polypeptide patterns on sodium dodecyl sulfate gels. Most of the STRA activity migrated as a single protein band on sodium dodecyl sulfate-polyacrylamide and Davis polyacrylamide gels, although minor protein bands with STRA activity were also detected. The major STRA proteins constituted 10 to 15% of the total cellular protein of *R. typhi* and *R. prowazekii*. According to sensitive enzyme-linked immunosorbent assay titrations, the STRA of *R. prowazekii* had substantial cross-reactivity with rabbit antiserum prepared against *R. typhi*, as shown also by rocket immunoelectrophoresis, whereas the STRA of *R. typhi* reacted only very weakly with antiserum prepared against *R. prowazekii* according to the enzyme-linked immunosorbent assay and not at all according to rocket immunoelectrophoresis.

Epidemic and murine typhus infections can be differentiated serologically by several methods, which employ quite different antigen preparations (13, 26). The complement fixation and rickettsial tube (33, 37) or micro-agglutination (14) procedures employ yolk sac-grown rickettsiae that have been ether extracted and washed extensively by centrifugation. The fluorescent antibody procedure employs unfractionated smears of infected yolk sacs (16). More recently, highly purified rickettsiae have been used in the complement fixation and microagglutination tests (10, 27), in the micro-immunofluorescent antibody procedure (27, 32), and in the enzyme-linked immunosorbent assay (ELISA) (18, 19). Finally, the toxin neutralization procedure employs live rickettsiae and is particularly useful for identifying new rickettsial isolates as *Rickettsia typhi* or *Rickettsia prowazekii* (20, 42).

Little is known about the chemistry and subcellular location of the rickettsial antigens involved in any of these species-specific serological tests, possibly because of the multiplicity of common antigens in the typhus rickettsiae and the large amounts of contaminating host cell materials which, until recently, were present in many rickettsial preparations. For example, although Reiss-Gutfreund et al. (34) confirmed that species-specific antigens could be identified in soluble extracts of *R. typhi* and *R. prowazekii*, four common antigen components were also detected. Presumably, one of these common antigens is the erythrocyte-sensitizing substance characterized by Osterman and Eisemann (29). The erythrocyte-sensitizing substance probably contains a carbohydrate moiety since it is sensitive to sodium metaperiodate but not trypsin.

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Another typhus group antigen, which is distinct from the erythrocyte-sensitizing substance antigen but also probably contains a carbohydrate, appears to be responsible for the production of antibodies which agglutinate *Proteus* OX-19 cells in the Weil-Felix reaction (3, 21, 25). Recently, Smith and Winkler (41) have provided evidence that *R. prowazekii* contains 2-keto-3-deoxyoctulosonic acid, a marker for lipopolysaccharide. Schramek et al. (35, 36) extracted a hydrophobic endotoxic lipopolysaccharide from *R. typhi* and *R. prowazekii* which was antigenic and group reactive in the complement fixation test, but these workers did not examine its relationship to the erythrocyte-sensitizing substance and OX-19-like antigens. In contrast to the heat-stable erythrocyte-sensitizing substance, lipopolysaccharide, and OX-19 antigens, species-specific antigens of both *R. typhi* and *R. prowazekii* are destroyed in 45 min at 56°C (8) or in 4 min at 60°C (38). Although heated typhus antigens elicit antibodies in rabbits and guinea pigs which soluble antigens fix complement with, flocculate heated soluble antigens, and agglutinate heated rickettsiae (8, 15, 38), these antisera are not species specific, are not effective in the mouse toxin neutralization assay (17, 38), and do not flocculate or agglutinate unheated antigens (15, 38). Heated antigens also do not protect guinea pigs against challenge with the homologous strain (17, 38). Consequently, the species-specific serological reactions of *R. typhi* and *R. prowazekii* seem to be due to similar heat-labile components, presumably proteins, which are also essential for full protection of vaccinated animals against challenge by these agents.

Quantitative immunoelectrophoretic techniques, most notably rocket immunoelectrophoresis and two-dimensional crossed immunoelectrophoresis (CrIE), have proven to be invaluable in the separation and identification of individual components in highly complex mixtures of antigens (2, 44). These techniques were useful in identifying a serologically important antigen found in *Chlamydia trachomatis* but not in *Chlamydia psittaci* (6, 7), in distinguishing strains of *Mycoplasma arginini* (1), and in assessing distant antigenic relationships between *Bordetella pertussis* and 28 other bacterial species (22). We report here the identification by rocket immunoelectrophoresis of the heat-labile species-specific protein antigens of *R. typhi* and *R. prowazekii*. The two specific antigens were partially purified by diethylaminoethyl-cellulose column chromatography and then characterized by polyacrylamide gel electrophoresis (PAGE), CrIE, and ELISA. The species-specific antigens of *R. typhi* and *R. prowazekii* were quite similar in their physical properties and constituted 10 to 15% of the total protein of each rickettsial species; these proteins are apparently responsible for the species-specific serological reactions of the typhus group rickettsial antigens which have been described by previous investigators. Other reports will deal in more detail with the molecular and immunological characteristics of these species-specific antigens and their use in serological tests for the detection of specific antibodies against *R. typhi* and *R. prowazekii* (Dasch et al., manuscripts in preparation).

MATERIALS AND METHODS

Rickettsial strains. The origin, passage history, and biological and biochemical characteristics of each rickettsial strain used have been described elsewhere in detail (9, 47).

Purification of rickettsiae and preparation of extracts. Frozen seeds and pools of rickettsiae were prepared from infected yolk sacs of embryonated chicken eggs as described previously (10, 46). The rickettsiae were purified from pools of 60 to 100 yolk sacs as described previously (9, 46), except that 12 gradients were used in the second cycle of Renografin density gradient centrifugation. Total rickettsial extracts were prepared by French pressure cell disruption at 22,000 lb/in^2 of cells suspended in 0.01 M sodium phosphate (pH 7.0) or 0.04 M potassium phosphate (pH 7.2), followed by centrifugation at 17,400 $\times$ *g* for 15 min to remove intact cells. Some extracts were prepared by sonication in a model W-375 sonicator-cell disruptor (Heat Systems-Ultrasonics, Inc.). Rickettsiae were sonicated in 10 ml of 0.01 M tris(hydroxymethyl)aminomethane (Tris) hydrochloride buffer (pH 7.6) in a 0°C, ethylene glycol-cooled, 8- to 15-ml, sealed-atmosphere treatment chamber for 10 min at 375 W, using the automatic pulsed mode for 30% of each second. Intact cells and membranes were collected by centrifugation at 17,400 $\times$ *g* for 15 min, suspended in 10 ml of Tris buffer, and sonicated again. The remaining crude debris was removed by centrifugation at 7,700 $\times$ *g* for 5 min. Total pressure cell or sonic extracts were generally rendered noninfectious by adding Formalin to a final concentration of 0.1% (vol/vol) and storing at 4°C for at least 2 days. In some cases the extracts were first centrifuged at 100,000 $\times$ *g* for 60 min to separate soluble and membrane fractions. The soluble fraction was then filtered through a 0.22-μm membrane filter to remove any residual viable cells.

Preparation of rabbit anti-typhus rickettsia hyperimmune sera. The rabbit antisera against *R. typhi* and *R. prowazekii* used in the direct ELISA (see below) were identical to those characterized previously (10, 19). To obtain the larger amounts of antisera needed for rocket immunoelectrophoresis and CrIE, rabbits were given an additional booster immunization with live rickettsiae as described previously, and the antisera were collected by exsanguination 2 weeks later. The specificity of these hyperimmune antisera was similar to the specificity of the sera characterized previously, except that the antibody titers measured by either microagglutination or the ELISA were two-

to fourfold higher.

Rocket immunoelectrophoresis. Rocket immunoelectrophoresis was performed overnight at 5 V/cm by using an LKB Multiphor apparatus with the cooling plate at 4°C. Rocket plates contained 11 ml of 1% (wt/vol) Litex HSA agarose (Accurate Chemical and Scientific Corp., Hicksville, N.Y.) in either 0.02 M sodium barbital-hydrochloride buffer (pH 8.6) or Tris-Tricine buffer (0.08 M Tris, 0.024 M Tricine, 0.3 mM calcium acetate, 0.02% [wt/vol] sodium azide, pH 8.6) on lantern slides (100 by 75 by 1 mm) that had been coated previously with 1% (wt/vol) agarose in water and dried. Hyperimmune rabbit antiserum prepared against *R. typhi* (50 μl) or *R. prowazekii* (100 μl) was included in a 7-ml anodic antibody strip (100 by 55 mm), and antigens (5 μl) were applied in a 4-ml cathodic, antibody-free agarose strip.

PAGE. Electrophoresis on 7.5% (wt/vol) Davis gels (without incorporated Triton X-100) or on 10% (wt/vol) neutral sodium dodecyl sulfate (SDS)–polyacrylamide gels was as described previously (10). Discontinuous SDS-PAGE on 9% (wt/vol) disc gels was by the method of Laemmli (23).

CrIE. First-dimension separations of antigen were performed in 1% Litex agarose strips (in Tris-Tricine or barbital buffer as described above for rocket immunoelectrophoresis) for 90 min at 5 V/cm. The first-dimension agarose strips were then embedded directly in the cathodal antibody agarose strip of a rocket plate prepared as described previously, which was then electrophoresed at 5 V/cm overnight; this second electrophoresis was perpendicular to the first-dimension separation into antibody agarose.

Alternatively, antigens were separated in Davis or discontinuous SDS-PAGE disc gels (diameter, 6 mm), and then the gels were sliced into four strips with a Longitudinal Gel Slicer II (Miles Research Products). The Davis gel strips were then sliced in half with a 15-cm razor blade (Bio-Rad Laboratories), soaked in water for 60 min, and laid on a standard rocket immunoelectrophoresis plate for the second-dimension electrophoresis (12). The SDS-PAGE strips were soaked for 60 min in 1.5% Triton X-100 in water to remove SDS and renature the antigen and then electrophoresed on a rocket plate which contained 1.5% Triton X-100 in the cathodal agarose strip.

DE52 column chromatography. Diethylaminoethyl cellulose (Whatman DE52) was suspended in 100 mM Tris, and the pH was adjusted to 7.6. The DE52 was allowed to settle, was suspended twice in 10 mM Tris-hydrochloride–1 M NaCl–0.01% sodium azide (pH 7.6), and then was packed in Lab-Crest columns (2.5 by 25 cm; Fisher and Porter Co.) at 150 to 200 ml/h at room temperature. The column was then equilibrated to 10 mM Tris-hydrochloride–0.01% sodium azide (pH 7.6). Antigen samples (10 to 40 ml) were dialyzed against 10 mM Tris, applied to the column, and followed by starting buffer to a total of 100 ml. Elution was with a linear 600-ml 0.0 to 0.3 M NaCl gradient generated with a Sorvall GF-2 gradient marker, followed by 100 ml of 0.3 M NaCl and 300 ml of 1 M NaCl (all in Tris buffer). Fractions (9 ml) were collected at a flow rate of 135 ml/h by using a Buchler multistaltic pump and an LKB UltroRac 7000 fraction collector.

ELISA for antigen. In a direct ELISA for antigen, column fractions (10 μl) or Davis gel fraction eluates (50 μl) were placed into duplicate wells of micro-ELISA plates (Microbiological Associates), diluted to 100 μl with coating buffer (0.1 M Na_2CO_3, pH 9.8), and incubated overnight at 37°C. Washing of the plates, antibody and conjugate additions, and substrate incubations were as described previously (18, 19), except that the substrate buffer was diethanolamine (45) instead of carbonate.

An indirect double antibody sandwich ELISA was used to detect antigen when detergents (SDS, Triton X-100) were present that inhibited direct binding of the antigen or when the presence of antigens which bound poorly was suspected. A gamma globulin fraction (three times precipitated at 35% saturation with ammonium sulfate) of hyperimmune rabbit antiserum against either *R. typhi* or *R. prowazekii* was adsorbed to the plate in coating buffer for 8 h. Antigen diluted in working buffer (10 μl of column fractions or 50 μl of gel eluates, diluted to 100 μl) was added to each well and incubated overnight. The bound antigen was detected with convalescent human antityphus antisera or immune guinea pig antisera, followed by alkaline phosphatase-conjugated rabbit anti-human immunoglobulin G or rabbit anti-guinea pig gamma globulin; each antibody incubation was for 2 h at 37°C. All antibody and antigen incubations were followed by washing the ELISA plates five times with working buffer. The alkaline phosphatase reaction was assayed as described above.

RESULTS

Identification and characterization of species-specific antigens of *R. typhi* and *R. prowazekii* by rocket immunoelectrophoresis. Complex polyspecific antisera raised conventionally by prolonged immunization schedules with disrupted or killed bacterial cells emulsified with Freund adjuvants (1, 2, 6, 22) are usually used to compare fine antigenic differences in closely related microorganisms by CrIE. However, our objective was limited to the identification of the heat-labile antigens(s) responsible for the species-specific serological reactions observed after infection with typhus group rickettsiae (8, 15, 17, 38). Consequently, we used only antisera resulting from infection of rabbits with these rickettsiae and boosted with viable cells. Previously, these antisera were found to react nearly identically with both typhus species in group-reactive complement fixation and ELISA tests; however, some species specificity was demonstrable in complement fixation tests with ether-extracted particulate antigens or in micro-agglutination tests with whole cells (10, 19). We obtained species-specific reactivity by electrophoresis of subcellular fractions of the rickettsiae into agarose containing rabbit antityphus sera (rocket immunoelectrophoresis) (Fig. 1). The rocket immunoprecipitate patterns obtained with these sera were remarkably sim-

ple; a single strong and distinct rocket was obtained when the *R. typhi* or *R. prowazekii* fractions were subjected to electrophoresis into the homologous antiserum but not when the fractions were subjected to electrophoresis into the heterologous antiserum. Additional diffuse rockets were apparent with each antigen when both homologous and heterologous antisera were used. The weak rockets could have been due to a common typhus antigen(s) that reacted poorly with both antisera, to weak heterologous cross-reactivity of the specific antigens, or to both reactions. For simplicity, we refer to the antigens that reacted to produce the strong homologous rocket immunoprecipitates as the species-specific typhus rocket antigens (STRAs).

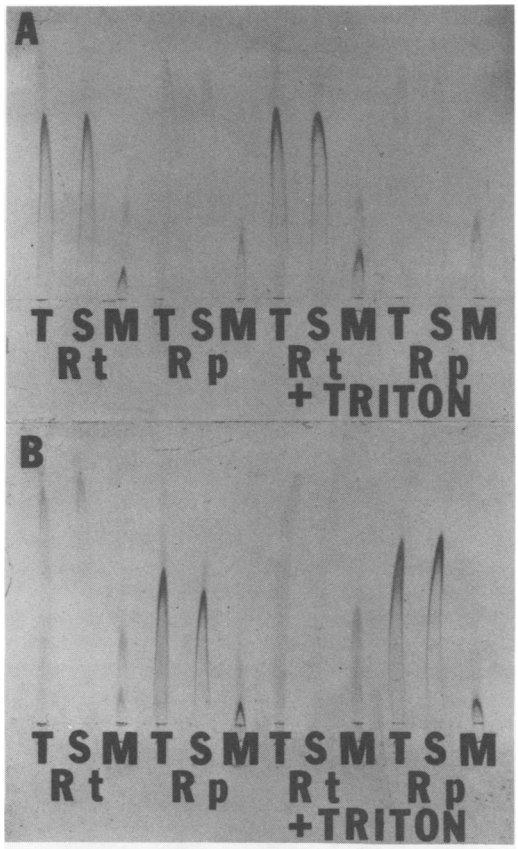

FIG. 1. *Rocket immunoelectrophoresis of fractions of Formalin-inactivated French pressure cell extracts of Renografin density gradient-purified R. typhi Wilmington (Rt) and R. prowazekii Breinl (Rp). Total extracts (lanes T), with or without treatment with 0.1% Triton X-100 for 60 min at 37°C, were separated into soluble (lanes S) and membrane (lanes M) fractions by centrifugation at 100,000 × g for 60 min, and each membrane pellet was resuspended in the original volume of sample. Electrophoresis was in barbital buffer for 4 h. (A) Antiserum 7 prepared against R. typhi Wilmington. (B) Antiserum 11 prepared against R. prowazekii E.*

Detergents have been used widely to solubilize membranes before quantitative immunoelectrophoresis in order to increase the number of antigenic components that can be identified by this technique (1, 4, 6). Although the peak heights of the typhus membrane fraction rockets were slightly increased by Triton X-100 treatment, no additional components could be detected (Fig. 1). Antigens treated more extensively or with higher concentrations of Triton X-100 or with several Tween and Brij detergents elicited similar simple rocket patterns with these sera. Whether Triton X-100 was added or not, the *R. typhi* and *R. prowazekii* STRAs were recovered nearly quantitatively in the 100,000-×-*g* supernatant fractions of the French pressure cell extracts since the rocket peak heights of the total and supernatant fractions were nearly identical in each case (Fig. 1, lanes T and S). In other experiments (data not shown), we found that membrane fractions obtained immediately after sonication contained greater amounts of the STRAs, but that the STRAs were gradually released into the soluble fraction when the extracts were stored at 4°C. Bound STRA was released only gradually by simple washing of the membranes, but it was solubilized readily by additional sonication or Triton X-100 treatment. Most of the STRA released from the membrane fraction was not sedimented even by centrifugation at 200,000 × *g* for 2 h. These results suggested that the STRA was initially associated with the membrane fraction but was readily detached by the mechanical forces of French pressure cell disruption and sonication.

Since the species-specific antigens studied in the classical analyses of typhus antigens were heat labile, thus suggesting a protein nature (8, 15, 38), the physical and chemical properties of the STRAs were next examined with this in mind. Samples of the supernatant fractions were treated with various proteases, phospholipase A_2, lipase, sodium metaperiodate, and glycosidases of various specificities before rocket immunoelectrophoresis against their homologous antisera (Fig. 2). Trypsin and pronase had the most pronounced effects on the peak heights of both *R. typhi* and *R. prowazekii* STRAs. Collagenase had a slight effect on the *R. prowazekii* STRA, but none of the other treatments significantly affected the rocket immunoprecipitate pattern. These results suggest that the antigenicity of the STRAs required an intact protein component but not a sugar or lipid moiety. Studies of the heat lability of the STRAs supported

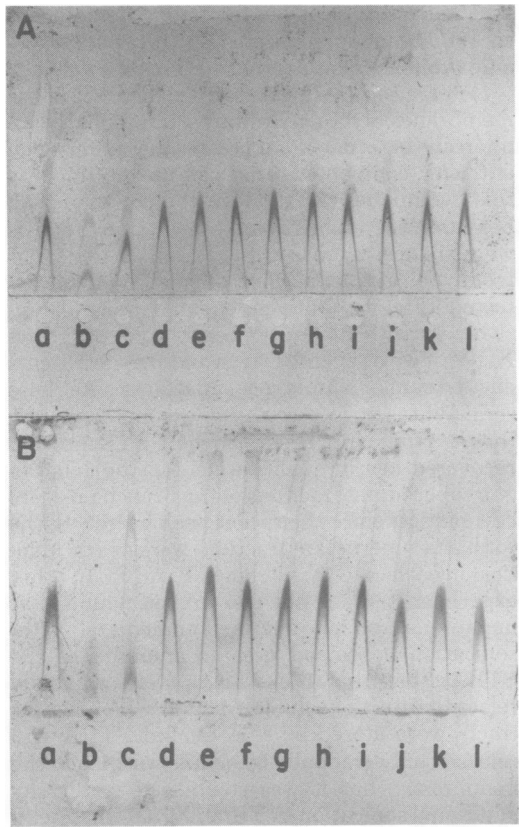

FIG. 2. *Rocket immunoelectrophoresis of enzyme- and chemical-treated soluble fractions of Formalin-inactivated French pressure cell extracts of Renografin-purified R. typhi Wilmington against antiserum 5 prepared against R. typhi Wilmington (A) and R. prowazekii Breinl against antiserum 10 prepared against R. prowazekii E (B). The antigen extracts were diluted at least 1:5 to 200 μg/ml in each of the following in 0.04 M KPO_4 buffer (pH 7.3) and then incubated for 60 min at 37°C. Samples (5 μl [1 μg]) were then subjected to electrophoresis overnight in barbital buffer. Lane a, Buffer control; lane b, 0.6% trypsin (Difco Laboratories); lane C, 100 μg of pronase per ml; lane d, 0.05 M sodium metaperiodate; lane e, 0.01 M sodium metaperiodate; lane f, 10 μg of bee venom phospholipase A_2 per ml; lane g, 100 μg of Candida lipase per ml; lane h, 100 μg of mixed glycosidases per ml; lane i, 50 U of Vibrio cholerae neuraminidase per ml; lane j, 100 μg of collagenase per ml; lane k, 100 μg of Macerase per ml; lane l, 100 μg of Cellulase per ml.*

this conclusion (Fig. 3). The STRAs from both *R. typhi* and *R. prowazekii* were almost completely stable to heating at 50°C for 30 min. Some of each antigen was lost at 52°C, 50% or more was lost at 54°C, and both were completely destroyed at 56°C.

None of the faint rocket precipitates (Fig. 1) obtained with *R. typhi* extract against both homologous and heterologous antisera was affected by heat (Fig. 3). This suggested that the faint *R. typhi* rockets were due to a stable typhus group antigen. Similarly, a heat-stable faint rocket pattern due to a group antigen was observed with the *R. prowazekii* extract. However, the faint rocket reaction with the heterologous serum declined in intensity as the temperature was raised in parallel with the disappearance of the STRA, although the heat-stable reaction remained (Fig. 3A and B). These results suggested that the *R. prowazekii* STRA cross-reacted weakly with the *R. typhi* antiserum, whereas there was no cross-reactivity between the *R. typhi* STRA and the *R. prowazekii* antiserum.

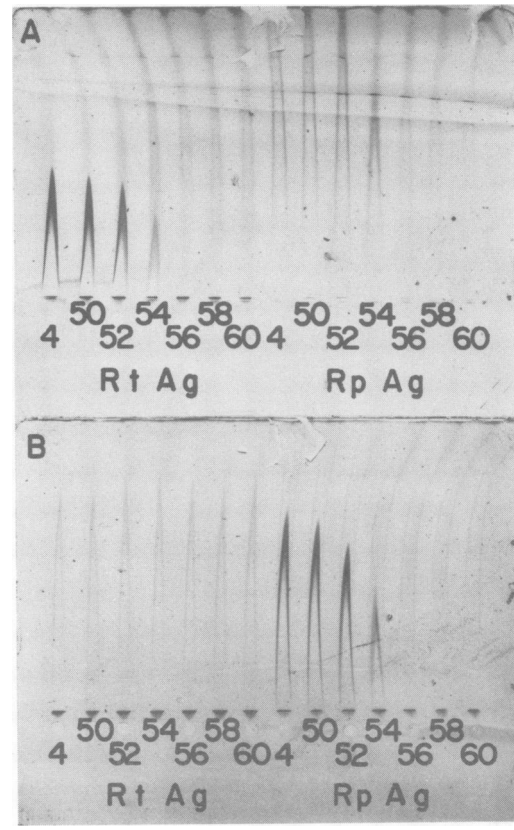

FIG. 3. *Rocket immunoelectrophoresis of heat-treated soluble fractions of Formalin-inactivated French pressure cell extracts of purified typhus rickettsiae. R. typhi Wilmington extract (360 μg/ml) (Rt Ag) or R. prowazekii Breinl extract (475 μg/ml) (Rp Ag) was held at various temperatures (in degrees centigrade) for 30 min in Tris-Tricine buffer, and duplicate samples were subjected to electrophoresis into antiserum 5 prepared against R. typhi Wilmington (A) or antiserum 11 prepared against R. prowazekii E (B).*

Using line-rocket immunoelectrophoresis, we have also shown (Dasch et al., manuscript in preparation) that the STRAs demonstrated here are indeed species specific and are not peculiar to the Wilmington strain of *R. typhi* or the Breinl strain of *R. prowazekii*. Four strains of *R. typhi* had immunologically identical STRAs, which were distinct from the identical STRAs found in 11 strains of *R. prowazekii*; *Rickettsia canada* lacked both antigens.

Partial purification of the species-specific rocket antigens of *R. typhi* and *R. prowazekii* by DE52 column chromatography. Approximately 70 to 80% of the protein of Renografin-purified rickettsiae was not pelleted by centrifugation at 17,400 × *g* for 15 min after sonication or French pressure cell extraction. Of this protein, 40% was pelleted into the membrane fraction by centrifugation at 100,000 × *g* for 60 min. The remaining supernatant could be filter sterilized (pore size, 0.22 μm) without measurable loss of protein, rocket, or ELISA antigen activity, and the protein in this supernatant represented about 45% protein recovery from the purified whole cells. The filtered soluble extract contained no detectable viable rickettsiae, as measured by infectivity in yolk sacs or irradiated mouse LM_3 cells.

DE52 chromatography of these filter-sterilized soluble extracts was a simple and highly reproducible procedure for the separation of the STRAs from the Renografin, nucleic acids, and unwanted group antigens and proteins in the extracts (Fig. 4). Extracts from *R. prowazekii* and *R. typhi* were similar in their column behavior (Fig. 4). Renografin (detected by absorbance at 240 nm) was only partly removed from the extracts by extensive dialysis and seemed to bind tenaciously to both nucleic acids and proteins. Both the major portion of the Renografin and a nucleic acid fraction (detected by optical density at 260 nm) eluted (0.02 to 0.14 M NaCl [Fig. 4A and D]) before rickettsial antigens detectable by rocket immunoelectrophoresis, direct ELISA (Fig. 4B and E), or sandwich ELISA (data not shown). The rocket antigen in the crude extracts eluted in a narrow salt range (0.125 to 0.17 M NaCl). These STRA fractions were the only fractions showing a marked specificity in ELISA reactivity with rabbit antisera against *R. typhi* and *R. prowazekii* (Fig. 4B and E, pool I). Fractions eluting at NaCl concentrations of more than 0.17 M reacted equally with both antisera and thus contained common typhus antigen(s) (Fig. 4B and E). The *R. prowazekii* STRA fractions showed much greater reactivity with the heterologous rabbit antiserum in the ELISA than did the *R. typhi* STRA fractions. When the direct ELISA for antigen was used, highly species-specific human and guinea pig antisera from infections with *R. typhi* reacted predominantly against the *R. typhi* STRA fractions and only weakly with subsequent fractions containing common antigen detectable with the rabbit antisera (data not shown). With some extracts, a diffuse rocket antigen with low ELISA activity eluted before the STRA fraction. Fused rocket antigen patterns indicated that this antigen was in part identical to the STRA. Since this diffuse STRA-like rocket activity was most apparent in several formalinized rickettsial extracts that had been stored at 4°C for 1 to 2 years, it may have represented a partially degraded STRA.

STRA recovery was determined by quantitative rocket immunoelectrophoresis, using a standard curve obtained with crude soluble antigen (Table 1). Recovery of STRA varied from 30 to 60%, with a three- to sixfold increase in specific activity. Upon rechromatography of the pool I STRA fractions, additional common typhus antigen (fractions eluting at NaCl concentrations higher than 0.17 M NaCl) and Renografin (data not shown; eluting as in Fig. 4A and D) could be separated from the STRAs (Fig. 4C and F). Since there was an additional 50% loss of STRA, little improvement in specific activity was obtained (Table 1). However, this rechromatography clearly demonstrated that the ELISA species-specific reactivity coincided with the STRA activity. The *R. prowazekii* STRA fractions clearly reacted more strongly in the ELISA with heterologous antiserum than did the *R. typhi* STRA, but even the latter was not devoid of cross-reactivity in the sensitive ELISA (Fig. 4C and F). The STRA fractions were pooled (Fig. 4C and F, pool II) for further characterization (see below).

In other experiments (data not shown), STRAs were partially purified by DE52 column chromatography from extracts of crude yolk sac suspensions of rickettsiae disrupted by French pressure cell treatment or sonication. The STRAs present in ether-extracted soluble antigen or pressure cell-disrupted particulate antigens, which were obtained from either crude suspensions or Renografin-purified rickettsiae (10, 19), were also readily separated from common rickettsial antigens by DE52 chromatography. However, the yields of STRA from ether-treated fractions (soluble or disrupted particulate) were lower than the yields from untreated rickettsiae. Formalin-inactivated preparations had the same chromatographic profiles of rickettsial antigen as filter-sterilized preparations. Preparative isoelectric focusing in granulated gels or sucrose gradient stabilized columns was

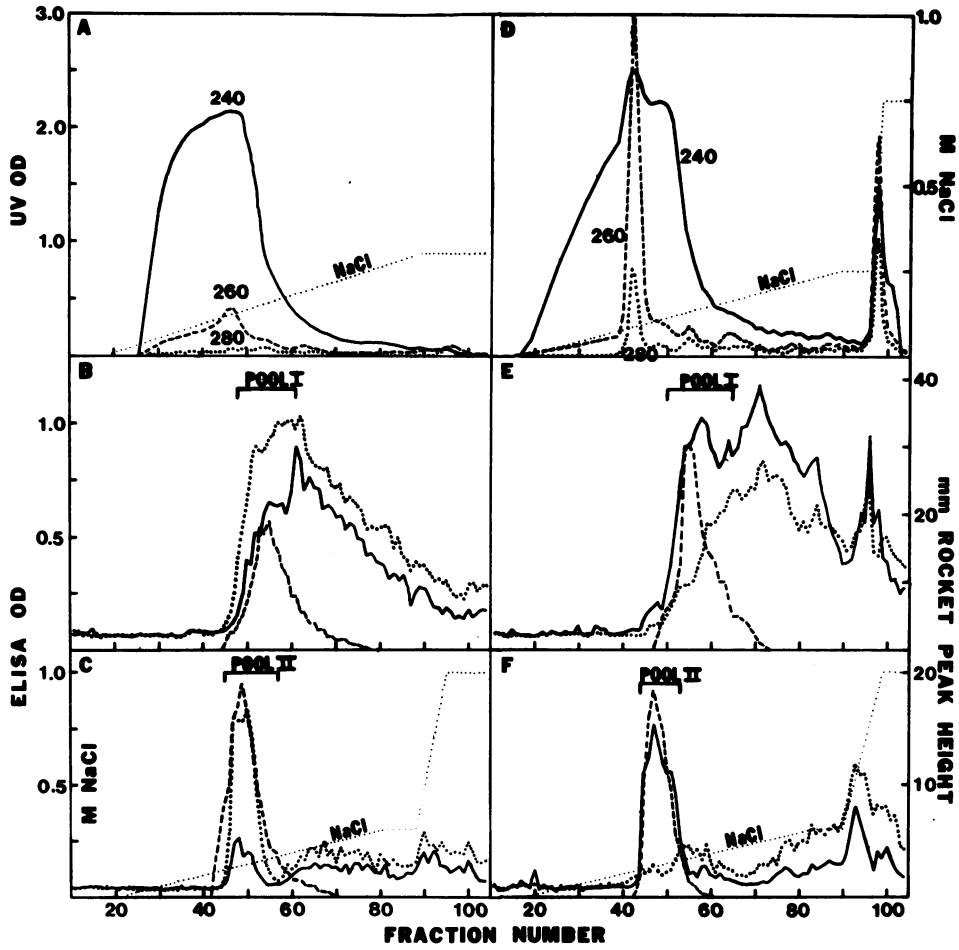

FIG. 4. *DE52 column chromatography of the soluble fractions of extracts of Renografin-purified typhus rickettsiae. (A and B) Chromatography of filter-sterilized 100,000-×-g supernatant of sonicated R. prowazekii Breinl. (C) Rechromatography of STRA pool I of R. prowazekii. (D and E) Chromatography of 100,000-×-g supernatant of a filter-sterilized French pressure cell extract of R. typhi Wilmington. (F) Rechromatography of STRA pool I of R. typhi. (A and D) Solid lines, dashed lines, and heavy dotted lines show optical densities (OD) at 240, 260, and 280 nm, respectively. UV, Ultraviolet. (B, C, E, and F) Solid lines show direct ELISA activity with antiserum 7 prepared against R. typhi Wilmington, heavy dotted lines show direct ELISA activity with antiserum 10 prepared against R. prowazekii E, and dashed lines show rocket peak heights with the homologous antisera. Pool II fractions were collected for further characterization.*

also used to purify the STRA from soluble pressure cell extracts of purified *R. typhi*. Although an antigen having a purity similar to that obtained by two DE52 column fractionations was obtained, less than 10% of the DE52 yield was obtained. This result suggested that the *R. typhi* STRA might be unstable at its rather acidic isoelectric point (pI 4.1 to 4.3).

Partial characterization of DE52-purified STRAs. STRAs purified by DE52 column chromatography did not produce the diffuse rockets previously obtained when the crude STRA extracts were electrophoresed against homologous antiserum (data not shown; Fig. 1 through 3). However, according to rocket immunoelectrophoresis, the purified *R. prowazekii* STRA still reacted weakly with the *R. typhi* antiserum, whereas no reaction was observed with *R. typhi* STRA and *R. prowazekii* antiserum. A single highly symmetrical rocket immunoprecipitate was obtained with either purified STRA in CrIE with agarose in the first dimension (data not shown). Purified STRAs obtained from filter-sterilized extracts had heat labilities and protease sensitivities identical to those observed with STRAs from formaldehyde-treated ex-

tracts (data not shown; Fig. 2 and 3), thus suggesting that the formaldehyde treatment had no effect on these properties of the antigens.

The crude fractions and purified STRAs were also examined by PAGE, using both native protein conditions on Davis disc gels and denaturing conditions on SDS gels (Fig. 5). On Davis gels most of the protein in the total formalinized French pressure cell extracts of *R. typhi* and *R. prowazekii* was retained by the stacking gel (data not shown) or at the top of the running gel (Fig. 5, lanes A and C). In addition, a major intense band was usually observed, which had a migration relative to that of the tracking dye (R_f) of about 0.25. In contrast, the purified STRAs consisted almost completely of a single band, which was slightly anodal with respect to the major crude band and had an R_f of about 0.3 (Fig. 5, lanes B and D). The bands at R_f 0.25 and R_f 0.3 contained most of the *R. prowazekii* STRA activity that could be detected by CrIE of the longitudinally sliced Davis gels (Fig. 6C and F). Although this same *R. prowazekii* STRA was homogeneous by standard agarose CrIE (data not shown), a pair of minor rockets anodal to the main peak were obtained by disc gel CrIE (Fig. 6E). Similarly, an additional CrIE rocket was sometimes found in soluble extracts (Fig. 6B), but it was clearly minor compared with the more cathodic major STRA when more antigen was applied (data not shown). These results were confirmed by ELISA analyses on eluates of the remainder of the disc gels used for CrIE (Fig. 6A and D). In each case, ELISA antigen activity coinciding with both the major and the minor STRA activities was obtained, but additional antigen activity was also detected at the top of the soluble extract gel (Fig. 6A). In contrast, all of the ELISA antigen activity of the purified STRA of *R. prowazekii* coincided with the STRA rockets (Fig. 6D). Other preparations of purified STRAs from *R. prowazekii* and *R. typhi* produced only single intense protein bands on Davis gels which had all of the STRA activity according to CrIE (data not shown). These STRA preparations completely lacked the minor STRA bands found in the DE52-purified *R. prowazekii* STRA of Fig. 5, lane D. Further confirmation that the prominent band at R_f 0.3 was the STRA protein was provided by heat lability studies; this band was no longer observed

TABLE 1. *Recovery of the species-specific rocket antigens of R. typhi and R. prowazekii after DE52 chromatography*[a]

DE52 fraction	Amt of protein (mg)	No. of RU[b]	No. of RU/μg of protein	% RU recovery[c]
R. typhi Wilmington[d]				
Sample I	22.80	40,660	1.8	100
STRA pool I	2.21	22,730	10.3	56
Sample II	2.10	21,600	10.3	56
STRA pool II	0.92	8,250	9.0	21
R. prowazekii Breinl[e]				
Sample I	33.30	74,600	2.2	100
STRA pool I	2.19	25,170	11.5	34
Sample II	2.10	24,100	11.5	34
STRA pool II	1.05	12,200	11.6	17

[a] See Fig. 4.

[b] One rocket unit (RU) is the amount of STRA in a 5-μl sample which produced a 10-mm rocket height with Tris-Tricine as the buffer after electrophoresis overnight at 5 V/cm, using antiserum 5 prepared against *R. typhi* or antiserum 10 prepared against *R. prowazekii*. Several dilutions were averaged for each sample, based on a standard curve run simultaneously.

[c] Corrected for the amount of sample removed before DE52 column II.

[d] Sample I was the filter-sterilized 100,000-×-*g* supernatant of French pressure cell-extracted rickettsiae; pool fractions are shown on Fig. 4E and F.

[e] Sample I was the filter-sterilized, 100,000-×-*g* supernatant of sonicated rickettsiae; pool fractions are shown on Fig. 4B and C.

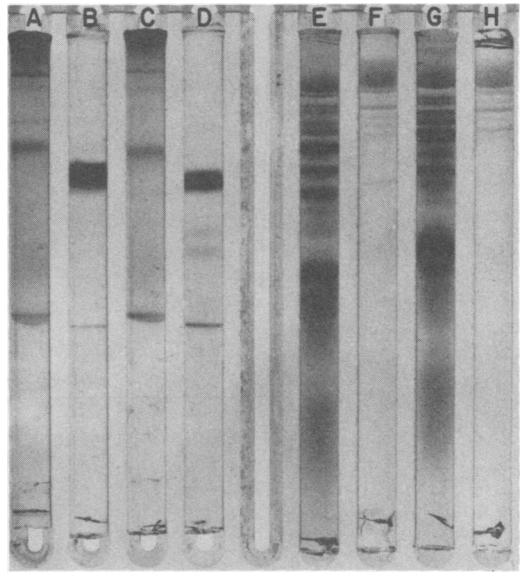

FIG. 5. *PAGE polypeptide patterns of fractions and DE52-purified STRAs of R. typhi and R. prowazekii. Lanes A through D, Native proteins on 7.5% Davis disc gels; lanes E through H, denatured polypeptides on neutral phosphate 10% SDS gels. A 50-μg amount of protein was applied to each gel except those in lanes E and G, which contained 100 μg. Gels were stained with Coomassie brilliant blue R250, and the anodic tracking dye front was marked with India ink (bottom). Lanes A, B, E, and F, R. typhi fractions; lanes C, D, G, and H, R. prowazekii fractions. Lanes A and C, Total formalinized French pressure cell extracts; lanes B, D, F, and H, DE52-purified STRAs; lanes E and G, whole rickettsial cells.*

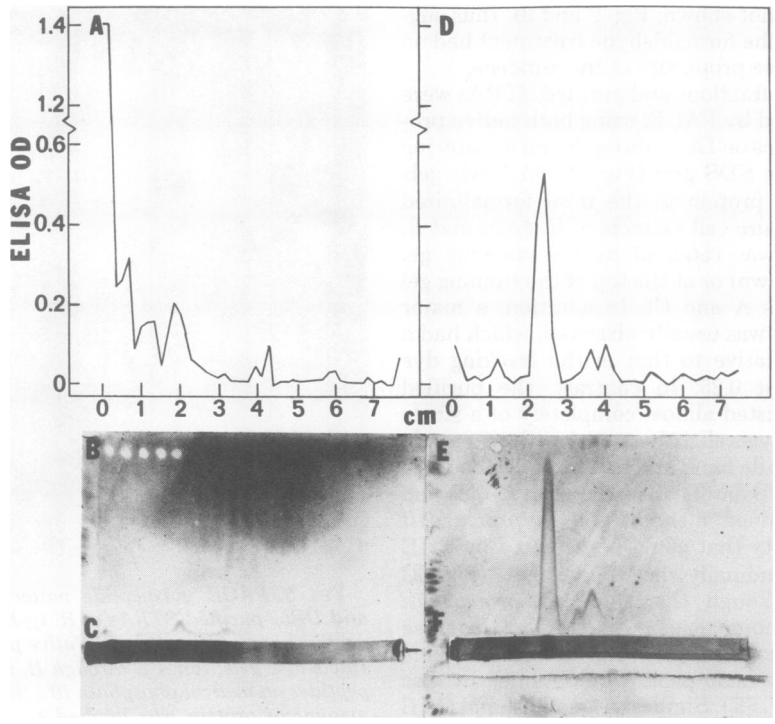

FIG. 6. *CrIE and ELISA localization of rickettsial antigens electrophoresed on Davis polyacrylamide gels. (A through C) Filter-sterilized soluble fractions of sonicate of R. prowazekii Breinl. (D through F) DE52-purified STRA of R. prowazekii Breinl. (A and D) ELISA antigen activities of 1.8-mm lateral slices of ¾ of Davis gels eluted in 400 μl of ELISA coating buffer for 24 h. Mean of duplicate assays of 50 μl of eluate with antiserum 10 prepared against R. prowazekii. (B and E) CrIE patterns of longitudinal ⅛ slices of disc gels with antiserum 10. (C and F) Duplicates of the Davis gels used for CrIE and ELISA, which were stained for protein with Coomassie brilliant blue R250. The anodic tracking dye front was marked with injected India ink (right).*

after the DE52-purified STRAs from *R. typhi* and *R. prowazekii* were heated at 60°C for 30 min before electrophoresis (data not shown). This treatment apparently denatured the protein since considerable protein was now detected at the top of the running gel. Similarly, in additional experiments both the purified STRA Davis gel band and the STRA activity found in crude extracts were stable to treatment with Triton X-100 and sulfhydryl compounds, such as dithiothreitol (data not shown).

The apparent high degree of homogeneity of DE52-purified STRA was examined further by SDS-PAGE. The STRA polypeptide patterns (Fig. 5, lanes F and H) found on neutral phosphate SDS-PAGE gels were compared with those of whole cells (Fig. 5, lanes E and G). The banding pattern of each purified STRA was relatively simple compared with the respective whole-cell banding pattern. It consisted chiefly of one of the most prominent bands in the whole-cell profile; in addition, a number of more anodal minor bands of slightly lower apparent molecular weight were always present. Although the major SDS-PAGE bands of the *R. typhi* and *R. prowazekii* STRAs had nearly identical molecular weights, the minor banding patterns showed slight but consistent differences. Identical SDS-PAGE results were obtained with eluates of the most prominent STRA bands cut out of Davis PAGE gels (Fig. 5 and 6) or by STRA analysis in the high-resolution discontinuous system (data not shown).

Direct evidence was obtained by CrIE and sandwich ELISA that both the major band and at least some of the minor bands found by SDS-PAGE analysis of the DE52-purified STRAs represented the specific antigen. The STRAs of both *R. typhi* (Fig. 7A) and *R. prowazekii* (Fig. 7B) had fused multiple-peak CrIE patterns. The major CrIE peak originated from the prominent SDS-PAGE band, whereas additional peaks originated from the minor bands found on SDS-PAGE gels. The absence of spurs suggested the immunological identity of the multiple peaks. Because the CrIE patterns were consistent with

a given STRA preparation and varied little between preparations, it is highly unlikely that they were artifactual. Comparable results were also obtained by using double antibody sandwich ELISA on eluates of slices of the SDS-PAGE gels (data not shown, similar to Fig. 6A and D). Surprisingly, the *R. typhi* STRAs uniformly had CrIE patterns in which some of the peaks did not correspond to bands detectable by Coomassie brilliant blue staining (cf. Fig. 5, lane F, with Fig. 7A). The absence of similar peaks in the *R. prowazekii* STRA CrIE pattern probably only reflected the lower potency of that antiserum. However, the *R. typhi* CrIE pattern did suggest that very small amounts of the STRAs with very low apparent molecular weights were present and that they could regain their antigenicity despite the 100°C SDS-β-mercaptoethanol treatment. The possibility that the minor SDS-PAGE STRA bands were derived from the major band by proteolytic degradation during growth of the rickettsiae in the yolk sacs or in vitro during purification of the antigen is under investigation.

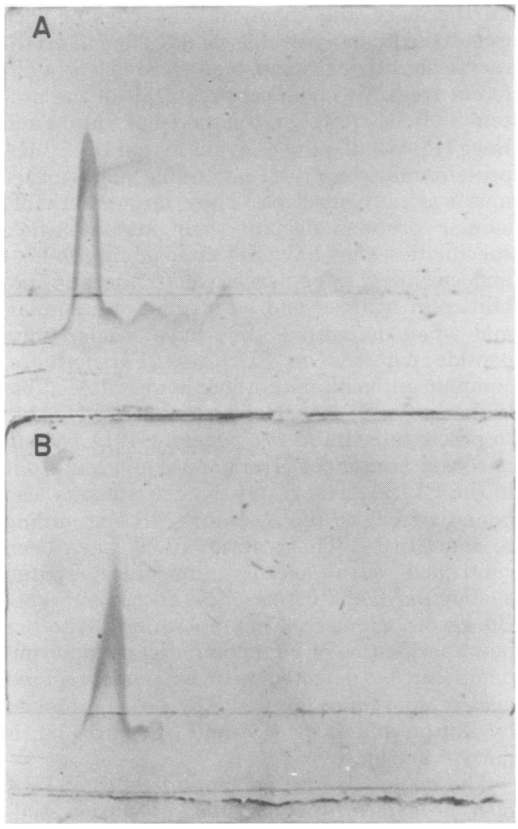

FIG. 7. *CrIE of DE52-purified STRAs electrophoresed on 9% discontinuous SDS-polyacrylamide gels. (A) Triton X-100 CrIE of a longitudinal ¼ slice of a SDS disc gel containing 25 μg of DE52-purified R. typhi STRA against antiserum 7 prepared against R. typhi (100 μl/plate). (B) Triton X-100 CrIE of a ¼ longitudinal slice of a SDS disc gel containing 50 μg of DE52-purified R. prowazekii STRA against antiserum 10 prepared against R. prowazekii (200 μl/plate).*

Assuming that all of the protein found on the SDS-PAGE patterns (Fig. 5, lanes F and H) was STRA, approximately 10 to 15% of the whole rickettsial protein was STRA. A similar but lower estimate (11% of the total protein) was made by multiplying the percent recovery of STRA after DE52 purification (21 and 17% [Table 1]) by the estimated 60% total protein recovered from the soluble fraction (without adding a correction for the STRA actually recovered in the membrane fraction).

The DE52-purified STRAs were compared with the total French pressure cell extracts in quantitative ELISA antigen titrations against both *R. typhi* and *R. prowazekii* antisera. The total French pressure cell antigen is currently used in our ELISAs for antibodies against typhus rickettsiae (18, 19). It is primarily sensitive to typhus group antibodies with rabbit (Fig. 8) and human sera, although it reacts more specifically with mouse and guinea pig sera. In contrast, the DE52-purified STRA antigen of *R. typhi* was highly reactive with rabbit antibodies against *R. typhi* but not *R. prowazekii* (Fig. 8A), whereas the *R. prowazekii* STRA showed somewhat less specificity (Fig. 8B). The binding characteristics of the highly acidic STRAs were decidedly inferior to those of the crude extracts under these conditions. The specificity of the ELISA reactivity of each STRA corresponded to the specificity previously obtained by rocket immunoelectrophoresis with these sera since the *R. prowazekii* STRA, but not the *R. typhi* STRA, had a weak heterologous reaction.

DISCUSSION

The pioneering studies of Craigie et al. (8), Shepard (38), and Fulton and Begg (15) clearly demonstrated that heat so altered the in vitro and in vivo immunological properties of both *R. typhi* and *R. prowazekii* that these two species were no longer distinguishable by serological tests. The similarity in the heat sensitivities of the species-specific properties of these rickettsiae and the remarkable identity of their heat-stable properties suggested a number of complex models which related these two characteristics. The simplest model was that of Craigie et al. (8), who suggested that these specificities were due to two different surface components and that heat destroyed the species-specific components

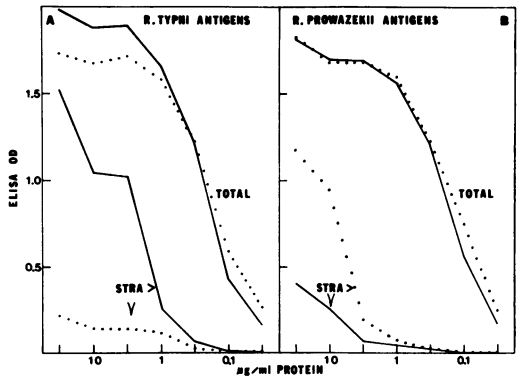

FIG. 8. *ELISA antigen titration of total French pressure cell extracts and DE52-purified STRAs of R. typhi and R. prowazekii. Duplicate 100-μl dilutions of extract or STRA were bound to microtiter plates overnight in coating buffer and detected by using the ELISA with rabbit antiserum 10 prepared against R. prowazekii (dotted lines) or antiserum 5 prepared against R. typhi (solid lines), both at a dilution of 1: 1,000. The goat anti-rabbit immunoglobulin G-alkaline phosphatase conjugate was used at a 1:2,000 dilution. The alkaline phosphatase assay was stopped at 60 min, the microtiter well contents were diluted 1:10, and the optical density (OD) at 400 nm was read against a control without antigen. (A) R. typhi extract and STRA. (B) R. prowazekii extract and STRA.*

and exposed the remaining stable common antigen. However, both Shepard (38) and Fulton and Begg (15) noted that heat denaturation of a protein may change, but not destroy, its antigenic specificity, and that this type of degradation might occur in vivo as well. Either in vitro or in vivo degradation of the rickettsial heat-labile antigens might stimulate the production of cross-reactive antibodies. Fulton and Begg (15) incorporated both ideas; they hypothesized that the rickettsiae have essentially identical surface structures and that the native heat-labile component has both specific and common antigenic determinants, as well as common determinants readily exposed by denaturation and other stable determinants, such as the OX-19-like antigen. However, Fulton and Begg (15) strongly opposed the model of Craigie et al. (8), who thought that different determinants were due to separable molecular entities. Instead, they suggested that the two species of *Rickettsia* contained mosaics of closely related, but not identical, labile surface antigenic structures which elicited in vivo a complex mixture of antibodies with various specificities.

These models are remarkably pertinent to the present study. First, the species-specific properties of *R. typhi* and *R. prowazekii* can now be ascribed to specific protein components, the STRAs, which have the expected heat lability properties since they are completely denatured after 30 min at 56°C. Craigie et al. (8) were correct when they proposed that the heat-labile antigens are distinct from other antigens common to *R. typhi* and *R. prowazekii*, since we have shown that the STRAs are separable from an antigen(s) common to the typhus rickettsiae by DE52 column chromatography (Fig. 4) and that an additional antigen stable at 56°C could be detected by rocket immunoelectrophoresis (Fig. 3). The large amount of common antigenic activity found with DE52 column separation cannot be due to antibody produced in vivo against degraded STRA, since the purified STRAs, when heat denatured, had no reactivity against these same sera by either rocket immunoelectrophoresis or ELISA (data not shown). Furthermore, the chemical properties of both rickettsial lipopolysaccharide (35, 36) and erythrocyte-sensitizing substance (29) are clearly different from the chemical properties of the protein STRAs. However, the model of Fulton and Begg (15) is still quite relevant in that the STRA proteins may comprise comparable labile surface mosaics in themselves. They are remarkably similar proteins despite their immunological specificities; they have the same lability to heat and enzymes, their native electrophoretic mobilities in agarose and acrylamide are similar, and when denatured, they have similar polypeptide patterns on SDS gels. Furthermore, common antigenic determinants were detectable in the native STRAs; *R. typhi* antisera were able to precipitate the *R. prowazekii* STRA weakly in rocket immunoelectrophoresis and bind to it in the ELISA. The *R. prowazekii* antisera also bound weakly to the *R. typhi* STRA according to the ELISA. These observations have been confirmed with specific antisera prepared against purified STRAs (G. A. Dasch and A. L. Bourgeois, manuscript in preparation). Whether new specificities or common antigenic determinants can be detected with antisera prepared against denatured purified STRAs, as proposed by Fulton and Begg (15) and Shepard (38), is not yet known.

The major SDS gel polypeptide of the STRA of *R. prowazekii* (polypeptide 1) has been studied by Smith and Winkler (41) and Osterman and Eisemann (11, 28). Smith and Winkler (41) found that it was present in purified outer membranes, although some of it was readily solubilized. Osterman and Eisemann (28) also identified this polypeptide in the cell envelope fraction. We have confirmed these results with both *R. typhi* and *R. prowazekii* by using rocket immunoelectrophoresis (Fig. 1). However, the STRA association with the membrane would be

unusual for an intrinsic membrane protein since it can be readily solubilized by mechanical forces, as shown here, or selectively by procedures which do not lyse the rickettsiae (Dasch, manuscript in preparation). These results suggest that the STRAs are actually surface proteins, similar to those described for other gram-negative and gram-positive bacteria (5, 40), rather than intrinsic membrane proteins. Osterman and Eisemann (28) found that polypeptide 1 was iodinated preferentially in intact rickettsiae under mild conditions, even though it had a relatively low content of tyrosine compared with other envelope proteins, thus suggesting its surface location. They also identified it in ether-extracted soluble antigen, which is believed to originate from the rickettsial cell envelope. Golinevitch and Voronova (17) have noted that granules (diameter, 6 to 8 nm) are associated with soluble antigen, which was also shown to contain the heat-labile protective antigen. Other investigators have described T-shaped structures in thin sections of rickettsiae (39), as well as repetitive granular surface structures which were visible with negative staining (30, 31). These structures are similar to structures described for other better-studied bacteria which have surface proteins with properties like the properties of rickettsial STRAs (5, 40). Whether the rickettsial granular structures are indeed the STRAs is presently under investigation.

In conclusion, rocket immunoelectrophoresis of soluble rickettsial extracts permits the rapid and direct identification of the major species-specific protein antigens that distinguish *R. typhi* and *R. prowazekii*. The simplicity of this procedure is particularly rewarding since it avoids the difficulties in identifying these species posed for other procedures by the presence of multiple common rickettsial antigens (13). The usefulness of the purified STRAs as reagents for identifying specific antibodies against *R. typhi* or *R. prowazekii* in clinical rickettsial infections or as experimental vaccines remains to be determined.

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