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Surface Proteins of Typhus and Spotted Fever Group Rickettsiae

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Six proteins, previously established as major constituents of intact organisms, were identified in cell envelopes obtained from intrinsically radiolabeled *Rickettsia prowazekii*. Extrinsic radioiodination of intact organisms conducted at 0.5 μM iodide indicated that protein 4 was the most peripheral, although protein 1 also had reactive groups exposed on the surface of the organisms. A 10-fold increase in iodide concentration resulted in labeling of protein 2, and at 50 μM iodide, all six major proteins were radiolabeled. Similar selective labeling was not achieved with *R. conorii*. Analysis of both typhus and spotted fever group organisms radiolabeled with galactose suggested that carbohydrate was associated with proteins 1, 3, and 4. Typhus soluble antigen included all major proteins except protein 2, which remained attached to particulate rickettsiae after ether extraction. Protein 4 appeared to be prominent in the surface topography of *R. prowazekii*, was a component of soluble antigen and may have an important role in rickettsiae-host interactions.

The proteins of intact rickettsiae have been analyzed by electrophoretic techniques, and the established profiles have been used for identification and comparison of different species of rickettsiae (1, 3, 12, 13). The importance of these proteins to the disease process, including the establishment of infection and the immune response of the animal host, is likely to be dependent upon their morphological location in the organism. Surface proteins, especially those on the periphery of the rickettsial cell envelope, are most accessible to the immune system of the host, and probably constitute the major antigenic stimulus for a protective humoral or cellular response. The cell envelope of both typhus group and spotted fever group rickettsiae has been shown to be the source of soluble antigen (6, 21, 23), a peripheral, group-specific antigen important in rickettsial serology (19, 20). Polyacrylamide gel electrophoresis (PAGE) of soluble antigen obtained from *Rickettsia rickettsii* indicated the antigen was complex and contained at least nine proteins (21). Further studies on peripheral proteins are lacking, and without reliable identification of rickettsial cell envelope proteins and analysis of their topographical relationship, it is difficult to hypothesize their role in rickettsiae-host cell interactions. The purpose of this study was to identify peripheral proteins by two different approaches: (i) isolation of cell envelopes and analysis of intrinsically radiolabeled proteins, and (ii) selective peripheral labeling of intact organisms by extrinsic radioiodination.

MATERIALS AND METHODS

Reagents. Carrier-free ^{125}I, [^{3}H]adenine, and [^{3}H]tyrosine were purchased from New England Nuclear Corp., Boston, Mass. Other radioisotopes, including ^{14}C-labeled L-amino acid mixture, ^{3}H-labeled L-amino acid mixture, and [^{3}H]galactose were obtained from Schwarz/Mann, Orangeburg, N.Y. Ribonuclease and deoxyribonuclease were purchased from Sigma Chemical Co., St. Louis, Mo. Lactoperoxidase was obtained from Calbiochem, Los Angeles, Calif. Chloramine-T was obtained from Eastman Kodak Co., Rochester, N.Y., and sodium metabisulfite was obtained from Fisher Scientific Co., Fairlawn, N.J. Reagent grade diethyl ether was purchased from Mallinckrodt, Inc., St. Louis, Mo. TEN buffer was prepared as 0.05 M tris(hydroxymethyl)aminomethane (pH 7.5), 0.001 M ethylenediaminetetraacetic acid, and 0.1 M NaCl. Phosphate buffer for radioiodination was prepared as 0.25 M phosphate (pH 7.4) from stock solutions of 0.25 M Na_2HPO_4 and 0.25 M KH_2PO_4. Dulbecco phosphate-buffered saline was purchased from Grand Island Biological Co., Grand Island, N.Y.

Growth and radiolabeling of rickettsiae. *R. prowazekii* Breinl and *R. conorii* Casablanca were grown in Spinner cultures of gamma-irradiated L-929 cells and radiolabeled with [^{3}H]tyrosine (3 μCi/ml), ^{14}C-labeled amino acids (1 μCi/ml), [^{3}H]adenine (3 μCi/ml), or [^{3}H]galactose (5 μCi/ml), as previously described (3).

Preparation of cell envelopes. Sucrose gradient-purified *R. prowazekii* (3), dually labeled with [^{3}H]-adenine and ^{14}C-labeled amino acids, were suspended

in TEN and irradiated with 300 krads in a Gammacell 220 irradiator (Atomic Energy of Canada, Lt., Commercial Products, Ottawa, Canada), in order to inactivate the rickettsiae (4). Cell envelopes were prepared by passing the organisms twice through a French pressure cell (American Instrument Co., Silver Spring, Md.) at 18,000 lb/in^2, then centrifuging at 6,500 × *g* for 30 min at 4°C to pellet any unbroken organisms. The supernatant was treated with ribonuclease (100 μg/ml), deoxyribonuclease, (100 μg/ml), and $MgCl_2$ (30 mM) for 1 h at 37°C, then diluted with TEN, and centrifuged at 59,000 × *g* for 3 h at 4°C, using an SW41 Ti rotor (2). Cell envelopes were resuspended in a small volume of TEN and were analyzed without further washing.

Gradient centrifugation. Intact rickettsiae and cell envelopes prepared from dually labeled [^{3}H]adenine and ^{14}C-labeled amino acid-labeled organisms were layered onto 10-ml potassium tartrate density gradients, 0 to 35% in TEN, and centrifuged at 148,000 × *g* for 18 h at 4°C. Fractions (0.2 ml) were collected from the bottom of the tube, and 0.1-ml aliquots were counted, after they had been dried on glass fiber filters (Whatman, GF/C, Maidstone, England) in a Packard Tri-Carb scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

PAGE analysis. Sodium dodecyl sulfate-PAGE was performed as previously described (3). Gels were 5 mm in diameter by 75 mm in length. Samples of cell envelopes and whole organism controls were adjusted to contain 1.0% agarose; all other samples were subjected to electrophoresis as aqueous preparations.

Radioiodination. Rickettsiae were radioiodinated by both the chloramine-T (8) and lactoperoxidase (14) methods. For radioiodination using chloramine-T, carrier-free ^{125}I was diluted to an iodide concentration of 1.2 μg/ml with 0.25 M phosphate buffer. Sucrose gradient-purified organisms (0.2 to 0.3 mg of protein), 0.03 ml, were mixed with 0.005 ml of phosphate buffer, 0.005 ml ^{125}I, and 0.07 ml of chloramine-T, 3.5 mg/ml, in distilled water. The mixture was agitated in an ice bath for 30 s, then the reaction was terminated by the addition of 0.1 ml of sodium metabisulfite, 4.8 mg/ml, in distilled water.

For lactoperoxidase-catalyzed radioiodination, carrier-free ^{125}I was diluted with phosphate buffer to an iodide concentration of 0.6 μg/ml. Sucrose gradient-purified organisms (0.03 ml) were mixed for 30 min at 26°C with 0.01 ml of lactoperoxidase (1 mg/ml in Dulbecco phosphate-buffered saline), 0.005 ml of ^{125}I, 0.005 ml of phosphate buffer, and 0.005 ml of hydrogen peroxide (0.03% in distilled water).

For both iodination methods, the final radioiodide concentration was 0.5 μM. Labeling at 5.0 and 50.0 μM iodide concentrations was achieved by incorporating non-radioactive potassium iodide into the phosphate buffer of the reaction mixture. At the completion of the reactions, the iodination mixtures were layered onto 26-ml linear sucrose gradients, 5 to 30% (wt/wt) in TEN, and centrifuged at 2,600 × *g* for 35 min at 4°C in a Beckman L2-65B ultracentrifuge using an SW25.1 rotor (Beckman Instruments, Inc., Palo Alto, Calif.). Fractions (0.8 ml) were collected from the bottom of the tube, and 0.1-ml aliquots were counted in an automatic gamma counting system (Nuclear-Chicago Corp., Des Plaines, Ill.). Peak radioactive fractions were diluted with TEN and centrifuged at 57,700 × *g* for 1 h at 4°C. Pelleted organisms were resuspended in a small volume of TEN.

Preparation of soluble antigen. An aliquot of *R. prowazekii*, radioiodinated by the chloramine-T method at a concentration of 50 μM iodide, was mixed with an equal volume of diethyl ether, and the suspension was shaken vigorously for 5 min at 26°C. Aqueous and ether phases were allowed to separate for 1 h at 26°C. Soluble antigen was recovered in the supernatant after centrifuging the aqueous phase at 12,000 × *g* for 1 h at 4°C.

RESULTS

Preparation and analysis of cell envelopes. Intact rickettsiae intrinsically radiolabeled with adenine and amino acids banded uniformly in a potassium tartrate density gradient (Fig. 1A). Cell envelopes, prepared by disruption in a French pressure cell, were less dense than intact organisms and were devoid of adenine (Fig. 1B). Physical differences between intact rickettsiae and cell envelope preparations were also evident in sucrose velocity gradients. Intact organisms banded uniformly, as previously reported (3), but cell envelopes were unable to enter a similar gradient and remained at the meniscus. Thus, the cell envelope fraction of *R. prowazekii* was easily distinguished from intact organisms by gradient centrifugation procedures, contained no detectable contaminating whole cells, and was used as an enriched source of peripheral proteins.

Protein composition of cell envelopes. PAGE of intrinsically radiolabeled cell envelopes showed a migration pattern very similar to intact rickettsiae (Fig. 2). The six major proteins of *R. prowazekii*, previously identified and characterized as components of intact organisms (3), were also prominent in the gel pattern of cell envelopes. These data established the morphological location of the major proteins, but the intrinsic labeling technique could not discriminate their relative location in the cell envelope.

A selective peripheral labeling technique was required to identify the outermost proteins of the rickettsial cell envelope. Since radioiodination techniques often rely on substitution reactions involving tyrosine, we examined the six major proteins for the presence of this amino acid before initiating extrinsic labeling studies. The PAGE pattern obtained after intrinsic radiolabeling of *R. prowazekii* with tyrosine is shown in Fig. 3 and compared to the protein labeling obtained with the mixture of radioactive amino acids. Although there is a relative dimunition in tyrosine labeling of proteins 1 and 2, an a relative increase in tyrosine labeling of proteins 3 and 4, it was evident that none of the major

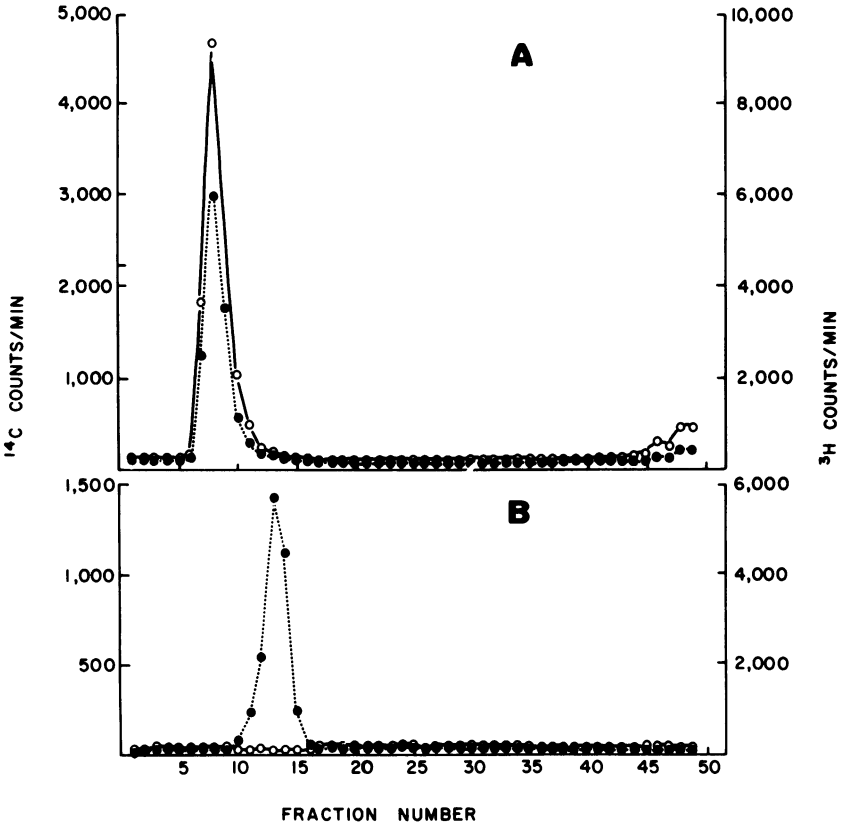

FIG. 1. *Sedimentation of intact rickettsiae and cell envelopes in potassium tartrate density gradients. (A) Intact R. prowazekii labeled with [^{3}H]adenine (○) and ^{14}C-labeled amino acids (●); (B) cell envelopes prepared from dually labeled organisms.*

proteins was either devoid of tyrosine or heavily enriched in this amino acid.

Radioiodination of intact rickettsiae. Rickettsiae were radioiodinated by either the chloramine-T or lactoperoxidase technique, sedimented in sucrose velocity gradients to remove free iodine, and then examined by the PAGE procedure. Figure 4A indicates the gel pattern observed after labeling *R. prowazekii* by both techniques with 0.5 μM radioiodine. Proteins 1 and 4 were selectively labeled, although the latter component exhibited substantially greater incorporation of isotope regardless of which technique was used to couple iodine to cell envelope proteins. Reduction in the concentration of radioactive iodine diminished the amount of isotope incorporation, but did not change the labeling pattern.

If the concentration of radioactive iodine was held constant, but the total iodide concentration was increased 10-fold, the labeling of protein 1 was more pronounced, and protein 2 was also accessible for radioiodination (Fig. 4B). Further increase in total iodide concentration to 50 μM, with the radioactive iodine held constant, produced the PAGE pattern shown in Fig. 4C. At this concentration of iodide, all six major cell envelope proteins were labeled regardless of the iodination method employed. Preferential labeling of protein 4 was abolished, and the quantitative labeling pattern of proteins 1, 2, 4, 5, and 6 resembled that observed with intrinsic radiolabeling. Protein 3 was poorly labeled by either radioiodination technique when compared to intrinsic labeling patterns (Fig. 2 and 3).

Intact spotted fever group organisms were also extrinsically labeled with radioiodine, but unlike the typhus rickettsiae, selective labeling was not achieved at the lower iodide concentration. Gel patterns of *R. conorii* obtained with organisms radioiodinated at both 0.5 μM and 50 μM iodide were similar to those previously obtained (3) with rickettsiae intrinsically radiolabeled with amino acids.

Intrinsic labeling of carbohydrates. The cell envelope fraction of typhus group rickettsiae contains carbohydrates comprised of several sugars, including galactose (16, 22), and it was of

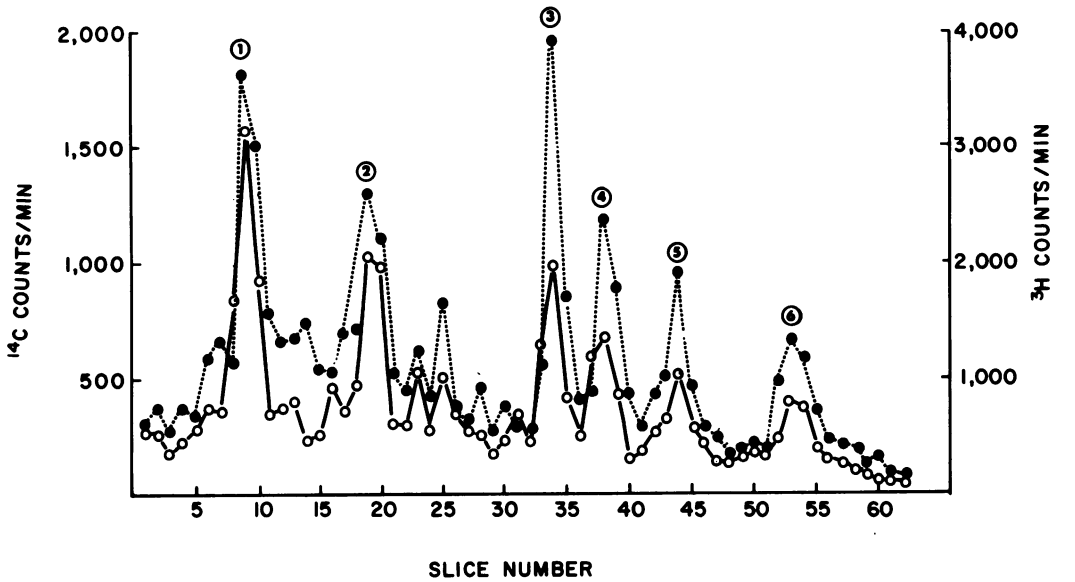

FIG. 2. *Co-electrophoresis of proteins from cell envelopes and intact organisms of R. prowazekii. Symbols:* ○, *^{3}H-labeled amino acid-labeled rickettsiae;* ●, *^{14}C-labeled amino acid-labeled cell envelopes.*

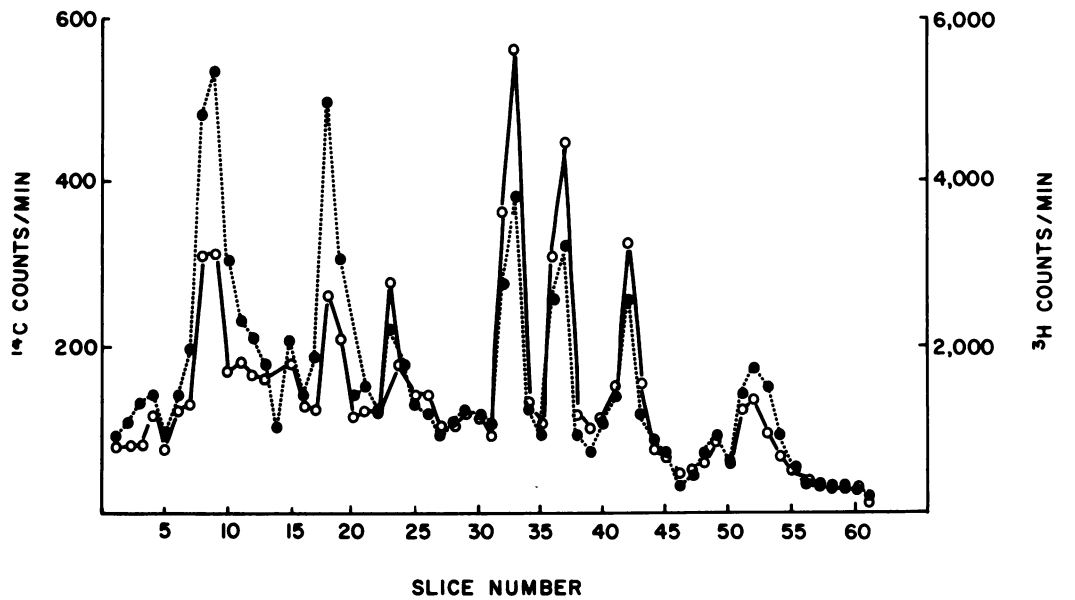

FIG. 3. *Co-electrophoresis of R. prowazekii proteins labeled with tyrosine or amino acids. Symbols:* ○, *[^{3}H]tyrosine;* ●, *^{14}C-labeled amino acids.*

interest to determine which of the six major cell envelope proteins were associated with carbohydrates. *R. prowazekii* was intrinsically labeled with [^{3}H]galactose and subjected to electrophoresis together with rickettsiae radiolabeled with ^{14}C-labeled amino acids. The gel pattern shown in Fig. 5A indicates that galactose incorporation was selective and that the three labeled macromolecules migrated at the same rate as proteins 1, 3, and 4.

R. conorii was also intrinsically radiolabeled with [^{3}H]galactose and analyzed by PAGE (Fig. 5B). The results were essentially identical to those observed with typhus rickettsiae; sugar

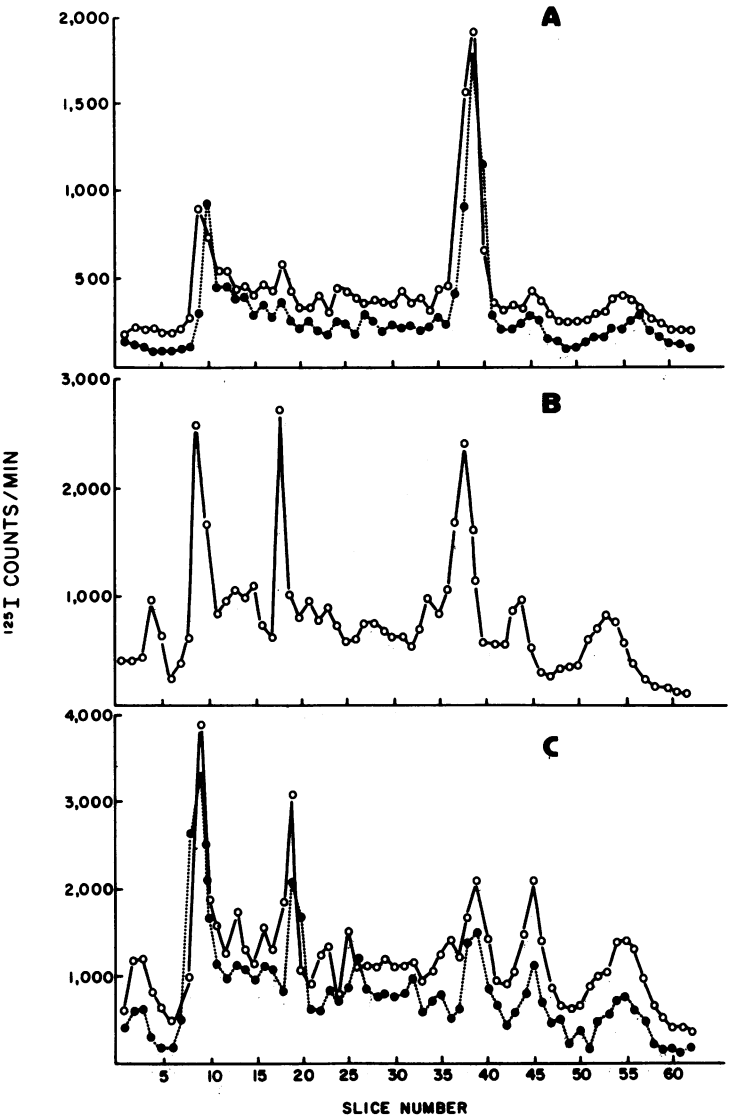

FIG. 4. *Radioiodination of intact R. prowazekii. (A) 0.5 μM radioiodine; (B) 0.5 μM radioiodine plus 4.5 μM iodide; (C) 0.5 μM radioiodine plus 49.5 μM iodide. Symbols: ○, Chloramine-T technique; ●, lactoperoxidase technique.*

labeling was restricted to three macromolecules which comigrated with *R. conorii* proteins 1, 3, and 4.

Soluble antigen. Intact typhus rickettsiae, labeled with 0.5 μM radioiodine at a final iodide concentration of 50 μM, were extracted with diethyl ether, and the soluble antigen was examined by PAGE. Figure 6 indicates that the soluble antigen contained five of the six major cell envelope proteins. Protein 2 was not detected in soluble antigen, although gels of the residual corpuscular antigen remaining after ether extraction revealed this protein had been effectively labeled.

DISCUSSION

This study clearly demonstrated that the six major proteins identified after PAGE of intact *R. prowazekii* (3) are located within the cell envelope fraction of the organism. Other workers have demonstrated that rickettsial cell envelopes constitute about 20% of the dry weight of intact organisms (22), and we anticipated that the principal components of intact organisms

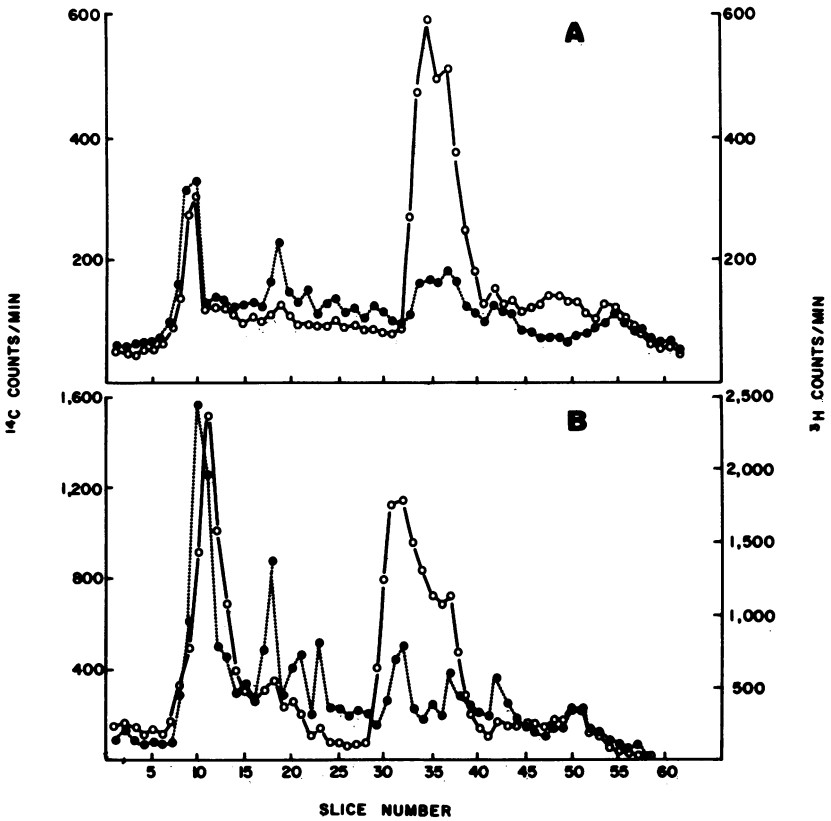

FIG. 5. *Co-electrophoresis of intact rickettsiae labeled with galactose or amino acids. (A) R. prowazekii; (B) R. conorii. Symbols: ○, [^{3}H]galactose; ●, ^{14}C-labeled amino acids.*

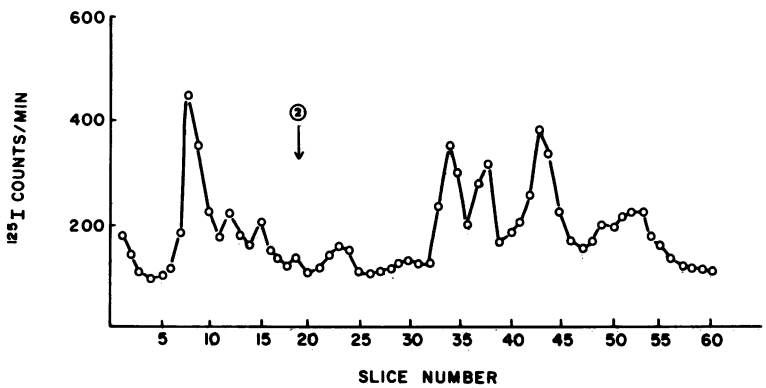

FIG. 6. *Electrophoresis of ^{125}I-radiolabeled soluble antigen obtained from R. prowazekii.*

would also be prominent in the gel pattern of cell envelopes.

The tyrosine content of each of these proteins was similar and allowed the use of extrinsic radioiodination both to increase sensitivity of gel analyses and to explore the topographical relationship of cell envelope proteins. Radioiodination, performed by either the lactoperoxidase or chloramine-T procedures, yielded similar results at 0.5 μM iodide and established that protein 4 was fully accessible for labeling and was therefore likely to be the outermost protein in the cell envelope of *R. prowazekii*. Minor labeling of protein 1 also occurred, indicating that some

reactive sites were exposed on the surface of the rickettsiae. Incremental increases in iodide concentration resulted in other cell envelope proteins becoming reactive with radioiodine. More complete labeling of protein 1 and labeling of protein 2 was observed with a 10-fold increase in iodide concentration, while proteins 3, 5, and 6 were radiolabeled after a 100-fold increase in iodide concentration. We are not certain of the effect of iodide on the protein mosaic of the rickettsial cell envelope, but similar selective, concentration-dependent labeling has been achieved with Rous-associated virus (10) and influenza virus (18). The influence of iodide concentration on the extent and specificity of radioiodine labeling by the chloramine-T method has been attributed to kinetic phenomena (7) and the formation of concentration-dependent hydrophobic or hydrophilic iodinating species (10). The effect of iodide concentration on lactoperoxidase labeling has received less attention, although it is clear that low iodide concentrations promoted peripheral labeling of enveloped viruses (11, 17). In this study, as well as in a study of influenza virus (18), it was apparent that iodide concentrations equal to, or exceeding 50 μM affected the accessibility of proteins to the surface catalyzed enzyme labeling reaction mediated by lactoperoxidase (9). Regardless of whether changes in rickettsial labeling were due to the increased hydrophilic nature of the reactive iodine complex or due to perturbation of cell envelope morphology with increased accessibility of lactoperoxidase, we feel that the concentration-dependent iodide labeling technique provides a sensitive probe for determining the topographical orientation of cell envelope proteins. However, the specificity of labeling observed with *R. prowazekii* was not seen with the spotted fever group organism studied. Over 10 separate experiments were conducted to verify the uniform labeling of *R. conorii* at both 0.5 and 50 μM iodide. We feel that there is a fundamental difference in the array of proteins in the cell envelope of typhus and spotted fever group organisms.

On the other hand, galactose labeling of rickettsiae produced uniform gel patterns with both typhus and spotted fever group organisms. Proteins 1, 3, and 4 appeared to be specifically associated with carbohydrates, although similar migration in gels is only suggestive evidence that these macromolecules are glycoproteins. It was significant that these same proteins were major components of soluble antigen since Frygin (5) has demonstrated the presence of a glycoprotein in soluble antigen obtained from *R. prowazekii*. Other studies (J. V. Osterman and C. S. Eisemann, submitted for publication) concerned with a serologically reactive fraction derived from soluble antigen also indicated a constant association of protein and carbohydrate after various separative procedures, again suggesting the presence of glycoproteins.

The protein composition of typhus soluble antigen included proteins 1, 3, 4, 5, and 6. Thus, soluble antigen was comprised of all major cell envelope proteins except protein 2. We did not examine soluble antigen from *R. conorii* since a spotted fever antigen had been carefully examined by Tzianabos et al. (21). It is important to note, however, that *R. rickettsii* spotted fever soluble antigen analyzed by them on polyacrylamide gels was also devoid of a major protein (protein 2 in the nomenclature of this laboratory). It is possible that protein 2 may contribute to the species-specific complement-fixation antigen that is prepared by extensive washing of rickettsiae to remove soluble antigen (15).

The identification of protein 4 as a peripheral protein in the cell envelope of *R. prowazekii*, its preliminary characterization as a glycoprotein, and its contribution to typhus soluble antigen suggest that further study is warranted to determine its role in rickettsiae-host interactions.

ACKNOWLEDGMENTS

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