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Biochemical Characteristics of Typhus Group Rickettsiae with Special Attention to the *Rickettsia prowazekii* Strains Isolated from Flying Squirrels

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Six strains of *Rickettsia prowazekii*, two derived from human infections and four isolated from flying squirrels, two strains of *R. typhi*, and the single available strain of *R. canada*, were characterized by several biochemical procedures. The electrophoretic patterns on polyacrylamide gels of rickettsial proteins solubilized by sodium dodecyl sulfate revealed several species differences, but strains of the same species appeared to have identical patterns. Cytoplasmic fractions of the rickettsiae were examined for enzymatic activities and for polyacrylamide gel isoelectric focusing patterns. Some species differences were encountered in the activities or ratios of activities of glutamate-oxaloacetate transaminase, glutamate dehydrogenase, and malate dehydrogenase. When polyacrylamide gels were stained for malate dehydrogenase after electrophoresis, a single band became apparent with single extracts or mixtures of two strains of *R. prowazekii*, but two bands were seen with mixtures of a strain of *R. prowazekii* and one of *R. typhi*. The isoelectric focusing patterns of the soluble proteins revealed numerous species differences, especially between *R. canada* and the other two species, and a few differences among the strains of *R. prowazekii*. The patterns of the two human strains, Breinl and E^R, differed in at least one location, and both differed from the flying squirrel strains in the displacement of one band. One of the flying squirrel strains, GvF-16, contained a protein band not seen in the other five strains. Despite these minor differences, a striking similarity was revealed by all the biochemical tests performed between the *R. prowazekii* strains of human and flying squirrel origin.

Until very recently, it has been difficult to characterize species and strain variation within the typhus group of rickettsiae, which include *Rickettsia prowazekii*, *R. typhi*, and *R. canada*. The primary difficulty has been that these unstable, obligately intracellular bacteria are not amenable, as are many other bacteria, to comparison by the numerous biochemical tests that require growth on differential media. Typhus rickettsiae are classified on the basis of relatively small differences in antigenic specificity (18). The immunogenicity and antigenic properties of strains of each species of *R. typhi* or *R. prowazekii* appear to be completely homogeneous by all the tests that have been performed, but comparisons of strains by sensitive immunoelectrophoresis or crossed immunoelectrophoresis techniques have not been reported. Many biological properties such as plaque type and antibiotic sensitivity are inadequate to distinguish even the species (27). Although other biological properties, most notably virulence for chicken embryos, laboratory animals, or man, do differ substantially between strains (23), the stability of this trait and its relationship to passage level in a particular host has been subject to some controversy (2, 13, 14). The biochemical basis of the differences in virulence remains obscure. Studies on variation in growth characteristics of rickettsial strains in primary or continuous cell lines or macrophages (11, 26) and the relationship of these parameters to their virulence (11) show promise. However, the value of these approaches for extensive strain characterization in the typhus group of rickettsiae has not yet been demonstrated.

In the last decade, numerous biochemical methods have been developed for directly comparing species and strains of bacteria, particularly the nutritionally fastidious mycoplasma (7, 20). These principally include specific zymogram techniques for enzymes that have been separated by electrophoresis on starch or polyacrylamide gels (3, 21), the comparison of the protein patterns of whole cells or various envelope fractions obtained by sodium dodecyl sulfate (SDS)-

polyacrylamide gel electrophoresis (PAGE) (1, 7), or isoelectric focusing (20), and the comparison of relative activities of specific enzymes (12). Although several distinct differences have been demonstrated in the profiles of the proteins of highly purified *R. typhi*, *R. prowazekii*, and *R. canada* on neutral SDS-gels (9, 19), too few strains of typhus rickettsiae have been compared to determine the extent of such differences within each species.

The recent unexpected isolation of strains of *R. prowazekii* from flying squirrels, *Glaucomys volans volans*, in the Eastern United States (4, 5) provided the impetus for our present efforts to assess the extent of strain variation in typhus group rickettsiae. We recently reported (27) that the biological properties of these strains, virulence for chicken embryos, plaque type, erythromycin susceptibility, capacity to catabolize glutamate but not glucose, and capacity to hemolyze erythrocytes, clearly place these strains in the typhus group of rickettsiae. However, little evidence as to rickettsial species or strain type could be obtained with these parameters. We report here the application of a variety of biochemical methods for distinguishing the species of typhus group rickettsiae. By isoelectric focusing of soluble protein extracts of the rickettsiae in polyacrylamide gels, we have also demonstrated distinctive biochemical differences between strains of *R. prowazekii* of both human and flying squirrel origin.

MATERIALS AND METHODS

Rickettsial strains. The origin, passage histories, and biological characteristics of each strain have been described previously in detail (27). The Breinl and E^R strains of *R. prowazekii* were of human origin, while the strains obtained from flying squirrels, *G. volans*, were collected in Virginia (GvV-250, GvV-257) and Florida (GvF-12, GvF-16). The ATCC VR 738 strain of *R. typhi* was isolated from a rat, and the Wilmington strain was from a human case of murine typhus.

Purification of rickettsiae and preparation of extracts. Seeds and pools of the rickettsiae were prepared from infected yolk sacs of embryonated chicken eggs as described previously (24). Only rickettsial suspensions purified from frozen pools of yolk sacs by two cycles of Renografin density centrifugation and filtration through a glass filter (Millipore AP-20; Millipore Corp., Bedford, Mass.) were used in these studies (8). Samples (0.05-ml volume) of the purified rickettsiae (50 μg of protein per sample estimated from the optical density at 420 nm) in K36 buffer were frozen at −70°C until used in SDS-PAGE. Extracts were prepared in 0.04 M KPO_4 buffer, pH 7.2, as described previously (8) and stored at −70°C until used. Protein determinations were made by the procedure of Lowry et al. (16).

Enzyme assays. All assays used the methods of Coolbaugh et al. (6). Frozen soluble rickettsial extracts (0.3 to 2.0 mg of protein/ml) essentially free of cell membranes (supernatant fluid after centrifugation at 80,000 × *g* for 1 h) were used in all cases. Consequently, the activities reported previously with crude extracts containing cell membranes (6) are not necessarily comparable to those reported here.

PAGE. Thawed and undialyzed soluble rickettsial extracts were electrophoresed on 7.5% Davis disc gels and stained for malate dehydrogenase (MDH) activity as described previously (8). Thawed samples of whole cells (50 μg of protein) were solubilized in Laemmli's sample buffer (15) by boiling for 3 min, and the protein was electrophoresed on 10% neutral SDS-disc gels (22). SDS-solubilized whole-cell proteins were also separated on 3 to 30% polyacrylamide gradient pore disc gels according to Esposito and Obijeski (10). Densitometric scans of polyacrylamide disc gels were made at 550 nm with a Beckman Acta III spectrophotometer equipped with a gel scanner.

Isoelectric focusing. Slab gels (2 by 125 by 260 mm) were made using ampholines (pH 3.5 to 9.5 range, LKB Instruments) according to the direction of the LKB instruction manual, except that the final riboflavin concentration was increased to 3.7 μg/ml. Just before use in isoelectric focusing, the soluble rickettsial extracts were concentrated with B-15 Minicon concentrators (Amicon Corp., Lexington, Mass.) to approximately 5 to 10 mg of protein per ml. Samples (20 μl containing 100 to 200 μg of protein) were placed on squares (9 by 9 mm) of Whatman no. 4 qualitative filter paper and placed 0.5 cm from the anode and 1 mm apart. Voltage was held constant and increased at 10-min intervals in 100-V increments from an initial voltage of 100 V until 700 V had been maintained. The sample papers were then removed, and electrophoresis was continued for 10 min at 800 V and finally at 1,000 V for an additional 40 min. A circulating bath at 4°C was used to maintain the cooling plate of the LKB Multiphor at less than 10°C during the isoelectric focusing procedure. At the completion of focusing, 1-cm^2 sections of gel were cut along the length of the gel and eluted overnight in 1 ml of water in capped tubes, and the pH profile of the gel was determined at 25°C.

The gels were fixed in 25% trichloroacetic acid for 1 h with one change of acid at 30 min. The trichloroacetic acid was removed by a wash in destaining fluid (see below) for 30 min. The gels were stained for 4 to 12 h in 0.2% Coomassie brilliant blue in an ethanol-water-acetic acid mixture (9:9:1) and destained by diffusion in ethanol-acetic acid-water (3:1:8) until the background stain was removed. Precipitated stain on the gel surface could be readily removed by gentle scraping with the wood end of a cotton swab.

RESULTS

SDS-PAGE patterns of rickettsial proteins. Proteins solubilized from Renografin-purified whole cells of nine strains of typhus rickettsiae were compared by electrophoresis in neutral 10% SDS-polyacrylamide gels (Fig. 1). The electrophoretic patterns of the proteins were highly reproducible, whether the same extract

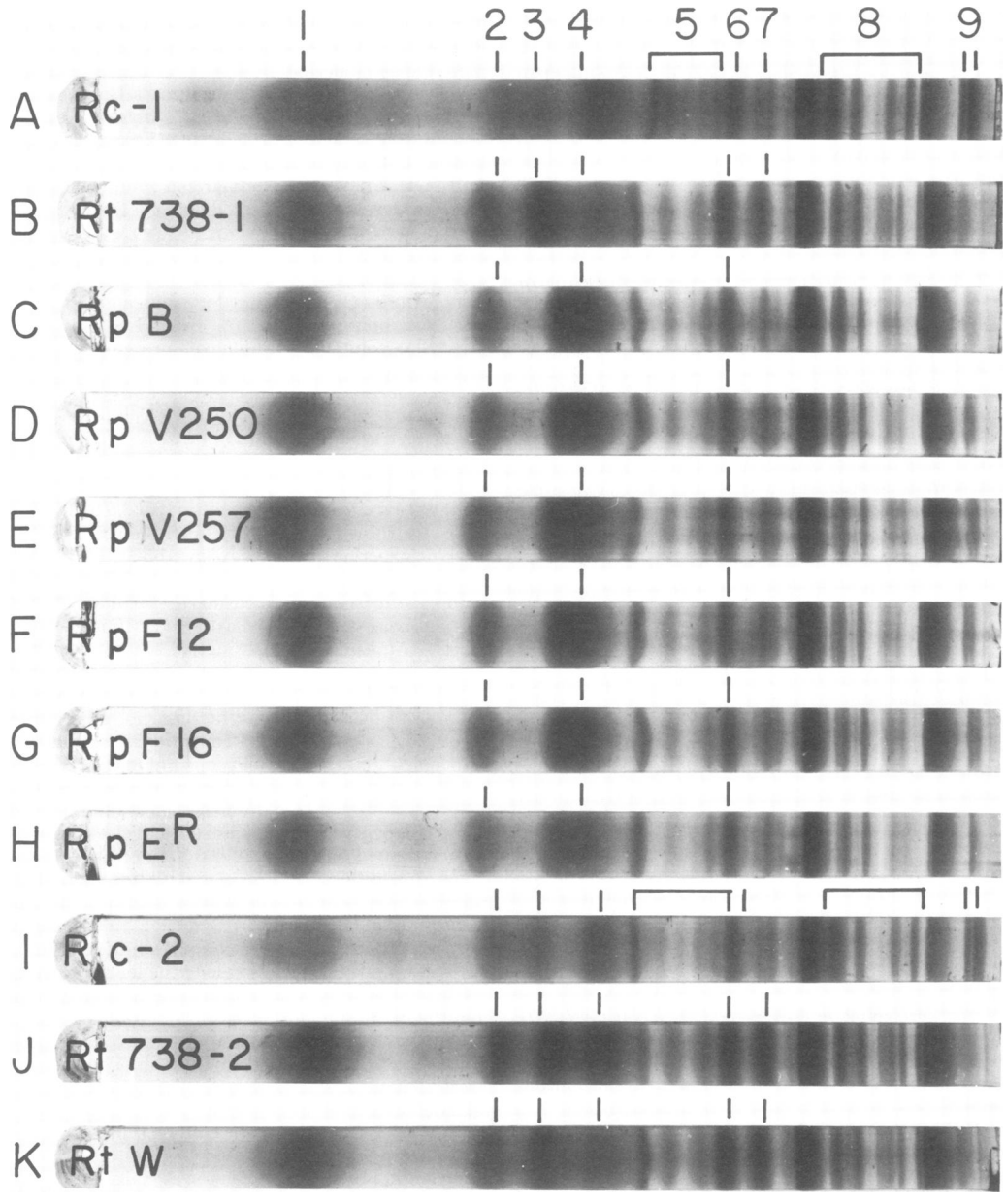


FIG. 1. *SDS-PAGE patterns of solubilized proteins from whole cells of rickettsial strains. Electrophoresis was from right (cathode) to left (anode). Abbreviations: Rc, R. canada; Rt, R. typhi; Rp, R. prowazekii; B, Breinl; W, Wilmington. Numbers 1 to 9 refer to areas in the gels where species differences in protein migration patterns have been observed.*

was tested at different times or different extracts of the same strain were compared (Fig. 1, gels A and I or gels B and J). The protein profiles shown in Fig. 1 are similar to those reported previously for *R. canada*, the Breinl strain of *R. prowazekii*, and the Wilmington strain of *R. typhi* (9, 19) despite differences in growth conditions and/or purification procedure. In the previous studies, the rickettsiae were grown in L-929 cells and purified by sucrose gradient centrifugation (9) or grown in yolk sacs, pretreated with Formalin and Celite, and purified by glycerol-tartrate viscosity equilibrium gradient centrifugation (19). We demonstrated consistent dif-

ferences among the protein patterns of *R. canada*, *R. prowazekii*, and *R. typhi*. These differences are labeled 1 through 9 in Fig. 1, but no attempt was made to identify each separable protein or to use the numbering system of previous authors (9, 19). In sharp contrast, the protein patterns of the same species were identical. This was true of the *R. typhi* strains of human and rat origin or the two human strains and the four flying squirrel isolates of *R. prowazekii*.

The protein profiles of the rickettsiae were evaluated by quantitative densitometric scans of the stained SDS-gels. The scans of the first four gels are illustrated in Fig. 2. By this method, minor differences were encountered between the extracts prepared from the same strain, but these were of the same magnitude as the variation encountered between strains of the same species. The differences among the three species of typhus rickettsiae are again readily apparent (Fig. 2, traces A–C), while the protein profiles of two strains of the same species are almost superimposable (Fig. 2, traces C, D).

Attempts were also made to detect strain differences by electrophoresis of the whole-cell proteins in SDS-3 to 30% polyacrylamide gradient gels. The patterns (not shown) were very similar to those obtained in 10% neutral SDS-gels, and little improvement in protein resolution was achieved.

Enzymatic activities of rickettsial extracts. All the strains of typhus rickettsiae examined so far metabolize glutamate with formation of CO_2, but do not catabolize glucose (27). We determined, therefore, the specific activities of three enzymes, previously shown to be present in *R. typhi* (6), in nine strains of the typhus group (Table 1). Although all of the strains contained these three enzymes, as expected, significant species differences in these activities could be clearly demonstrated. *R. canada* differed most markedly from the rest. It had significantly lower levels of glutamate-oxaloacetate transaminase (GOT) than all other strains ($P < 0.05$, Student's t test) and higher levels of nicotinamide adenine dinucleotide-dependent glutamate dehydrogenase (GDH) than the other species ($P < 0.05$). Although the MDH levels of *R. canada* were similar to those found in *R. prowazekii*, they were lower than those of *R. typhi* ($P < 0.05$). Differences in enzyme levels were even more clearly seen when the ratios of the specific enzymatic activities were calculated (Table 1). The high GDH/GOT ratio of *R. canada* was most useful in distinguishing it from *R. typhi* and *R. prowazekii* strains ($P < 0.01$), while the MDH/GOT ratio separated it from all *R. prowazekii* strains ($P < 0.01$) except Breinl ($P = 0.10$), and the MDH/GDH ratio distinguished it most markedly from the *R. typhi* strains ($P < 0.001$).

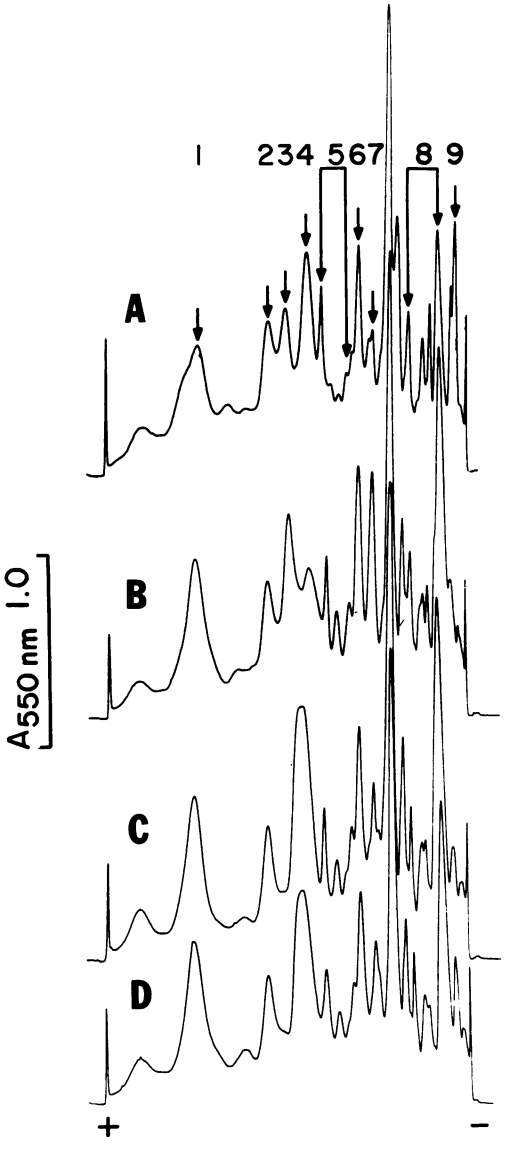


FIG. 2. *Spectrophotometric scans at 550 nm of the first four gels shown in Fig. 1.*

The Wilmington and VR 738 strains of *R. typhi* were remarkably similar to each other, differing, possibly, only in their levels of MDH. The specific activities were very similar to those of the *R. prowazekii* strains, with somewhat higher MDH levels and somewhat lower GDH levels, resulting in a significantly different MDH/GDH ratio ($P < 0.01$) compared with all strains of *R. prowazekii* with the possible excep-

TABLE 1. *Comparative enzymatic activities of strains of typhus rickettsiae*

Species and strain	No. of extracts	Sp act of enzymes[a]			Ratios of sp act[b]		
		GOT	GDH	MDH	GDH/GOT	MDH/GOT	MDH/GHD
R. prowazekii							
GvF-12	3	240 ± 66	161 ± 29	266 ± 84	0.74 ± 0.11	1.10 ± 0.08	1.56 ± 0.27
GvF-16	3	160 ± 40	132 ± 50	177 ± 63	0.78 ± 0.10	1.06 ± 0.13	1.39 ± 0.20
GvV-250	3	144 ± 37	130 ± 31	207 ± 71	0.92 ± 0.17	1.41 ± 0.44	1.48 ± 0.25
GvV-257	3	115 ± 10	101 ± 30	188 ± 31	0.94 ± 0.31	1.62 ± 0.19	2.70 ± 1.46
Breinl	4	85 ± 16	108 ± 12	228 ± 52	1.50 ± 0.38	3.30 ± 0.76	2.07 ± 0.31
E^R	5	113 ± 27	67 ± 9	177 ± 24	0.78 ± 0.20	1.79 ± 0.29	2.78 ± 0.60
R. typhi							
Wilmington	4	139 ± 30	53 ± 7	462 ± 89	0.41 ± 0.07	3.37 ± 0.26	8.78 ± 1.25
ATCC VR 738	3	110 ± 10	53 ± 6	334 ± 31	0.48 ± 0.06	3.10 ± 0.46	6.41 ± 0.36
R. canada							
2678	4	42 ± 6	338 ± 44	228 ± 22	8.32 ± 1.74	5.48 ± 0.83	0.70 ± 0.07

[a] Expressed as nanomoles of substrate per milligram of protein per minute; mean ± standard error.

[b] Ratios determined from activities of same extract. They were not derived from the means of specific activities of enzymes listed on this table. Mean ± standard error.

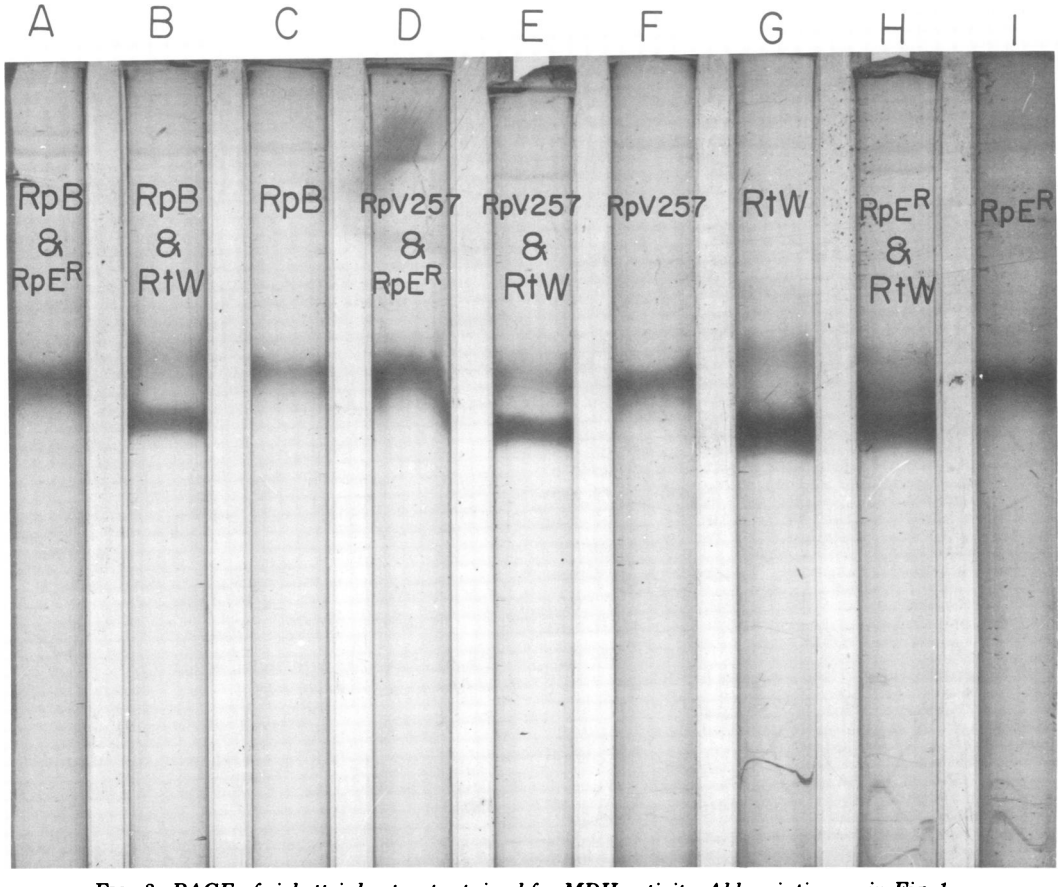


FIG. 3. *PAGE of rickettsial extracts stained for MDH activity. Abbreviation as in Fig. 1.*

tion of GvV-257 ($P = 0.07$ in comparison with VR 738).

Although some variation was apparent among the strains of *R. prowazekii* in individual enzymatic activities and, to a lesser extent, in computed ratios, no pattern was detected by which one strain could be differentiated from the rest.

Electrophoretic mobilities of MDHs in polyacrylamide gels. It was shown previously (8) that the MDH of the E^R strain migrates at

a different rate in neutral 7.5% polyacrylamide gel than the enzyme from the Wilmington strain of *R. typhi*. To determine whether this is a consistent species difference, similar determinations were made on soluble extracts of each of the nine strains (Fig. 3). Only single bands appeared on the gels with individual extracts (Fig. 3, gels C, F, I). Occasionally, upon prolonged storage, the extract of the Wilmington strain of *R. typhi* produced a faint band migrating more slowly than the primary band (Fig. 3, gel G), but the formation of the second band could be prevented by lower loading of protein or shorter incubation during enzyme staining. When extracts derived from any two strains of *R. prowazekii* (either E^R and Breinl with each other or with any of the flying squirrel strains) were mixed, only a single MDH band appeared in the gel (Fig. 3, gels A, D). When *R. prowazekii* extracts were mixed with either strain of *R. typhi*, two MDH bands appeared in the gels (Fig. 3, gels B, E). Occasionally, mixed extracts produced more complex MDH patterns (Fig. 3, gel H), suggesting the possibility of hybrid formation with an intermediate electrophoretic mobility. In general, however, the MDHs of the six *R. prowazekii* strains studied here appeared to be similar in electrophoretic mobility and distinct from the more rapidly migrating MDHs of the two *R. typhi* strains.

Isoelectric focusing of soluble rickettsial extracts. The same soluble protein extracts previously used for enzyme studies (Table 1 and Fig. 3) were subjected to isoelectric focusing in slab gels. Excellent resolution of a large number of proteins was obtained on gels ranging in pH from 3.5 to 9.5 (Fig. 4). The protein patterns obtained with different extracts of the same strain (compare a and b patterns of any of the strains in Fig. 4) or mixture of two extracts (compare Fig. 4b, N, O, T) were identical, as were the patterns obtained with the same ampholine on different gels (not shown). The main difficulty encountered was uniform protein loading (Fig. 4a, E, F; 4b, N, O) because of the errors inherent in concentrating some dilute extracts and in applying the samples.

The proteins in extracts of *R. canada*, *R. prowazekii*, and *R. typhi* had clearly different patterns of isoelectric points (Fig. 4a). The three species did have some proteins with identical isoelectric points, particularly in the acidic range (pH 4.0 to 5.5), but there were important differences in both the number and relative concentrations of the various proteins. With adequate protein loading, *R. canada* (Fig. 4a, F) appeared to have the largest number of proteins in the acidic range (pH 4.2 to 5.8) and the alkaline range (pH 7.0 to 7.8 and above 8.3). *R. typhi* had several proteins in the pH 7 to 8.5 range that were absent in the *R. prowazekii* strains, while the latter had many prominent bands in the pH 5.8 to 6.5 range that were absent from *R. typhi*.

Although differences in protein patterns among the three rickettsial species were readily apparent, strains differences were infrequent, although in some cases they were clearly detectable. The Wilmington and VR 738 strains of *R. typhi* had indistinguishable patterns, except for very minor quantitative differences (Fig. 4a, A–D). Fig. 4b illustrates the patterns of the six strains of *R. prowazekii* with human and flying squirrel strains interspersed. No differences were noted among three of the flying squirrel strains (GvF-12, GvV-250, and GvV-257; Fig. 4b, K, L, N, O, Q, R, T). There were, however, some differences among the other strains, more clearly seen at higher magnification (Fig. 4c). The GvF-16 strain (Fig. 4c, H, I) had a band at about pH 5.2 that was not present in any of the strains of *R. prowazekii*. However, a less intense band of similar isoelectric point could be detected in *R. canada* and *R. typhi* (Fig. 4a). A major difference between the Breinl and all the other strains of *R. prowazekii*, including E^R, was apparent at pH 5.1, just to the acid side of the GvF-16 band. A distinct band was present in all strains except Breinl (Fig. 4c). On the other hand, just to the acid side of pH 5.0 there was a band that was more distinct in the Breinl strain (Fig. 4c, G) than in the other strains. Finally, a major difference occurred in the location of a heavy band (pH 6.4), which distinguished the human from the flying squirrel strains. In the latter, this band was displaced toward the acid side in relation to the corresponding band of the human strains. Some other differences could be seen, but they were either very minor or not reproducible. In another experiment, a pH range of 4 to 8 was chosen for focusing the extracts. Although the separation of the proteins of *R. prowazekii* strains was increased by expanding this portion of the gel, no new differences were discerned among the protein patterns.

In summary, the following differences were encountered when the cytoplasmic fractions of nine strains of rickettsiae were subjected to isoelectric focusing. Differences among the three species were quite numerous. Among the six strains of *R. prowazekii* they were as follows: the two human strains, Breinl and E^R, one and possibly two bands; human strains in comparison with flying squirrel strains, displacement of one major band; flying squirrel strains compared with each other, a band in GvF-16 not encountered in the other strains.

DISCUSSION

Our previous investigation (27) demonstrated that a number of biological parameters can be

used to define the typhus group biotype and that the four flying squirrel strains examined clearly belong to this group. However, the primary methods for identifying new isolates of typhus group rickettsiae, *R. prowazekii*, *R. typhi*, and *R. canada*, continue to be various serological tests, supplemented with toxin neutralization tests (4). As mentioned previously (27), the discovery by Bozeman et al. (4, 5) of *R. prowazekii* strains in American wildlife was so surprising that an independent method of identifying some of these strains was sought. A secondary objective of this study was to detect strain differences that might be helpful in epidemiological investigations and in speculations concerning evolutionary relationships.

This paper describes the successful application of some biochemical parameters to rickettsial species and strain differentiation. This work was facilitated by the availability of a procedure of separation of the rickettsiae from yolk sac components (24) that was satisfactory for all strains used (27). It was previously shown (6, 25) that some of the enzymatic activities may be affected by the time of harvest of the rickettsiae. In these studies the rickettsiae were harvested

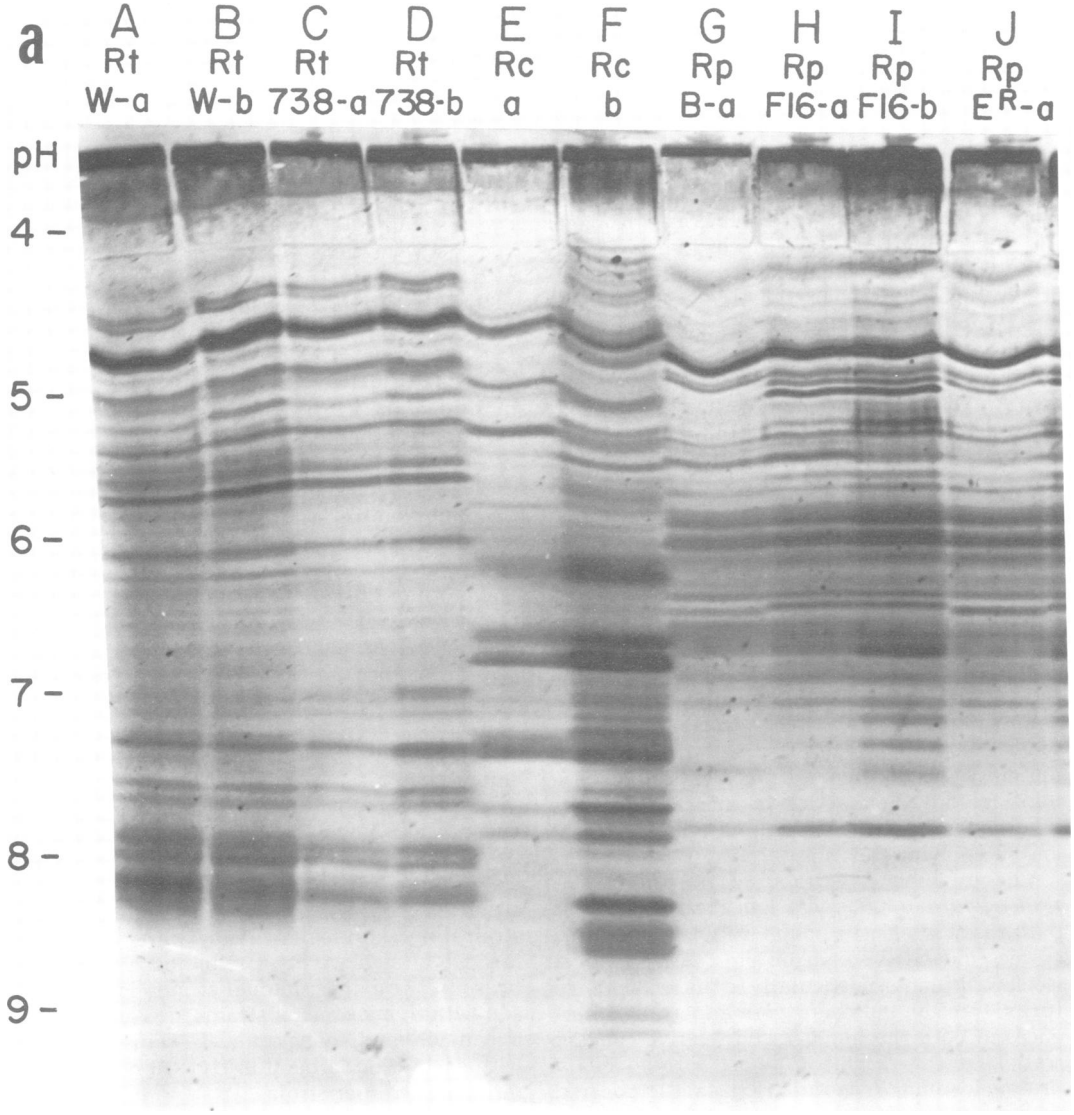
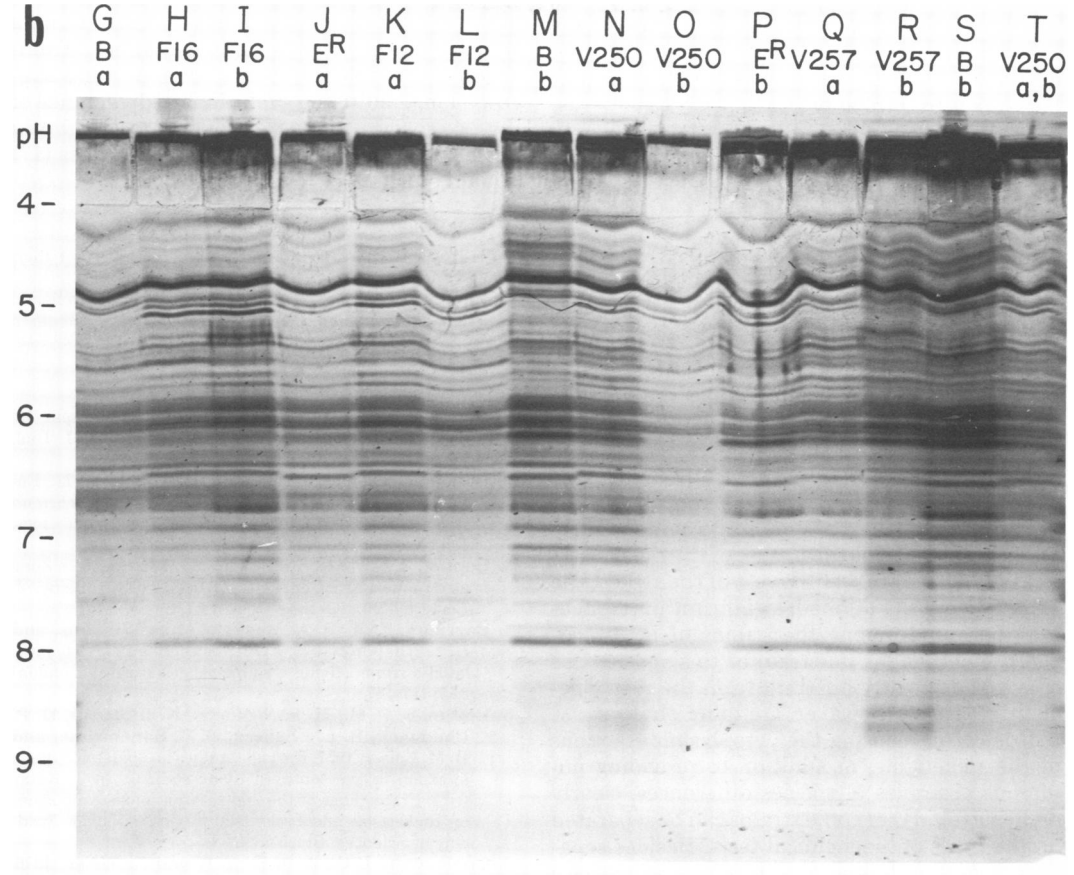


FIG. 4. *Polyacrylamide gel isoelectric focusing of extracts of rickettsial strains. Abbreviations as in Fig. 1. (a) Six strains representing the three species of rickettsiae; (b) six strains of R. prowazekii; (c) detail of (b) with arrows indicating differences in isoelectric focusing patterns between strains.*


b
G B a
H F16 a
I F16 b
J E^R a
K F12 a
L F12 b
M B b
N V250 a
O V250 b
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Q V257 a
R V257 b
S B b
T V250 a,b
pH
4-
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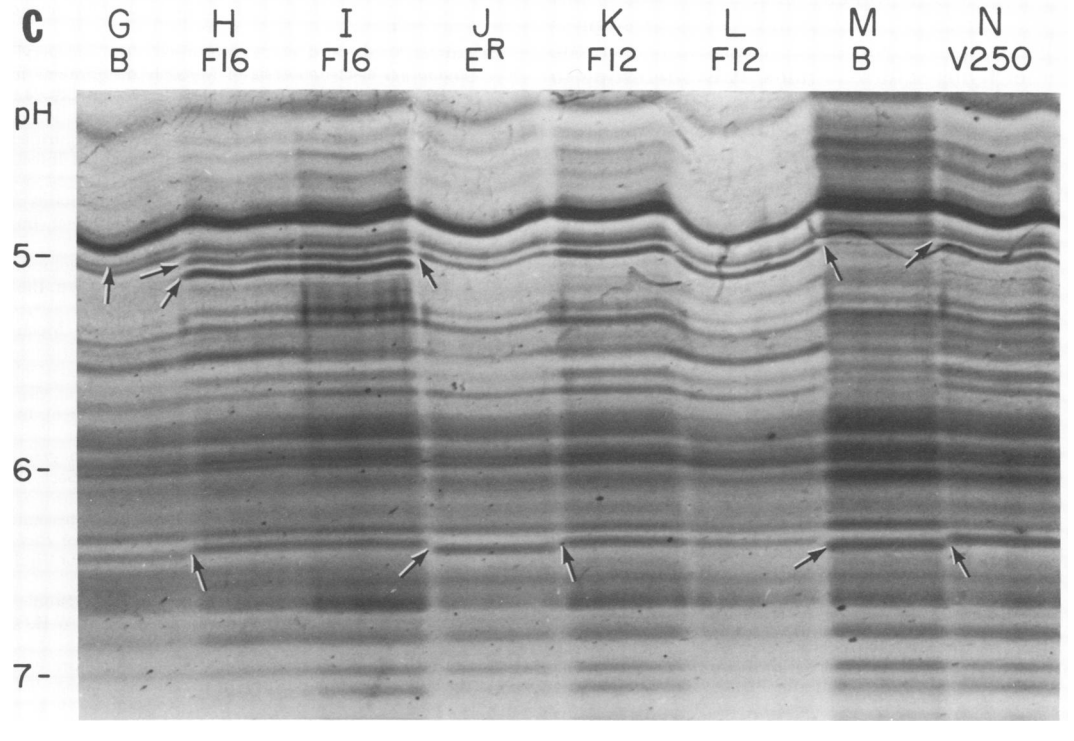
c
G B
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I F16
J E^R
K F12
L F12
M B
N V250
pH
5-
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according to a rigid schedule, from surviving embryos of a group in which some had died, and differences in phase of growth of the various harvests were probably small.

Of the methods used, SDS-PAGE is the only one that was previously used on rickettsiae in other laboratories. Close comparisons of our results with those of Eisemann and Osterman (9) and of Obijeski et al. (19) reveal remarkable similarities. Since differences were not detected among strains of the same species, SDS-PAGE might serve as a useful adjunct to serology for the species identification of new strains from unusual sources. The differential migration of MDH (Fig. 3) may have a similar application. Other methods are required, however, to detect strain differences.

The tests presented in Table 1 are subject to the greatest variation and are the ones most likely to have been influenced by minor differences in the physiological state of the rickettsiae. Possibly, if the time of harvest of the rickettsiae is more carefully defined, significant differences may be found among the strains of *R. prowazekii*, but thus far no strain of this species has emerged as clearly different from the rest. Species differences, however, are quite obvious.

Isoelectric focusing the cytoplasmic fractions of the rickettsiae, in addition to providing numerous examples of species differences, clearly demonstrated that some strains can be separated on the basis of biochemical tests. Besides separating the two human strains of *R. prowazekii* from each other and from the flying squirrel strains, it set aside the GvF-16 strain from the other flying squirrel strains. It is interesting to note that in our previous study (27), the GvF-16 strain was also shown to differ somewhat from the other strains, since it was the least virulent for chicken embryos. Most importantly, however, isoelectric focusing together with the other methods discussed here has brought to light an impressive similarity between the wildlife and human strains of *R. prowazekii*. The few differences that we detected can be explained readily on the basis of a limited number of evolutionary steps.

On the other hand, by the four biochemical criteria used in this work, *R. prowazekii*, *R. typhi*, and *R. canada* are indeed quite distinct species. The strong cross-reaction between *R. typhi* and *R. prowazekii* in serological tests (18) as well as the similarity in SDS-PAGE patterns suggest that they are much more closely related to each other than either of them is to *R. canada*. However, the many differences in soluble proteins between *R. typhi* and *R. prowazekii* exclude the possibility that these species differ only by relatively minor changes in surface proteins or that they could have arisen from a common ancestor by a limited number of mutations. The differences in soluble proteins suggest a long, multistep evolutionary divergence of the two species. Their relationship to *R. canada* as well as the natural habitat of this species (17) remain elusive.

ACKNOWLEDGMENTS

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