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Vole Agent Identified as a Strain of the Trench Fever Rickettsia, *Rochalimaea quintana*

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The vole agent described by Baker in 1946 was studied as an example of a bacterium that has been mistakenly regarded a rickettsia. Unlike rickettsiae, the vole agent killed chicken embryos with great irregularity, multiplied primarily at the surface of avian or mammalian cells and not intracellularly, produced colonies rather than plaques on chicken embryo monolayers under agar, and developed small colonies after 4 to 7 days of cultivation on blood plates. It was most conveniently cultivated on monolayers of irradiated L cells and was purified by minor modifications of the Renografin gradient procedure used for rickettsiae. It actively catabolized glutamate, glutamine, succinate, and pyruvate, but not glucose or glucose-6-phosphate. Enzymatic activities of cell extracts were consistent with above findings. The base ratio (molar percent guanine plus cytosine) of its deoxyribonucleic acid was shown to be 39, which was identical to the base ratio of the deoxyribonucleic acid of *Rochalimaea quintana* tested simultaneously. Serological studies indicated no cross-reactivity with *Rickettsia tsutsugamushi*, but strong cross-reaction with *R. quintana* was observed when a hyperimmune rabbit serum and a convalescent human serum were tested. We conclude that the vole agent is a strain of the trench fever rickettsia, *R. quintana*.

In 1943 Baker (1) isolated a microorganism, which he designated a rickettsia, from voles (*Microtus pennsylvanicus*) captured in Grosse Isle, Province of Quebec, Canada. The reason for his studies was that this isle, used again during World War II, had a tragic history as a quarantine station during the 19th century. In 1847, during the height of the immigration from Ireland, thousands of immigrants died of typhus and were buried on this small island. The possibility was considered that the rickettsia might have persisted in the isle's fauna. However, to Baker's surprise, the microorganism that he isolated more closely resembled the Far Eastern scrub typhus rickettsia than the European rickettsia of epidemic typhus.

During the course of our investigations (9, 34) of strains of *Rickettsia prowazekii* isolated from southern flying squirrels (3, 5), we included the "vole rickettsia." It appeared clear, however, early in these studies that this microorganism was not an obligate intracellular parasite. As the microorganism was further investigated, it became obvious that it resembled the trench fever rickettsia, *Rochalimaea quintana*, and that it can be best regarded as a strain of this species.

MATERIALS AND METHODS

Vole agent's egg passage history. After its isolation from the spleen of a vole in yolk sacs of chicken embryos, Baker (1) passaged the agent serially in eggs 39 times (CE39). The agent was then sent to the Rocky Mountain Laboratory, where it was passaged an additional four times (CE43) by C. B. Philip, once by F. J. Bell, and twice from CE43 by F. M. Bozeman at the Walter Reed Army Institute of Research. Philip, Bell, and Bozeman provided to the American Type Culture Collection (ATCC), respectively, lots 4, 5, and 6 of VR152, vole rickettsia. Our seeds were derived from ATCC lots 4 and 6, and from a subsequent passage by Bozeman. Including our own chicken embryo passages, our seeds represented, respectively, CE44, CE46, and CE48 passages. No differences among them were encountered.

Preparations of seeds, purified suspensions, and cell extracts. The sources of chicken embryos and mouse LM_3 cells and procedures for their inoculation were described previously (32, 34).

Seeds were prepared from the yolk sacs of heavily infected surviving chick embryos. They were purified through the bovine plasma albumin step 2 (32) and stored at −70°C as 50% yolk sac suspensions in brain heart infusion (Difco).

For the preparation of purified suspensions of the vole agent, irradiated L-cell monolayers in 20 to 24 flasks (16-ounces, ca. 475 ml) were inoculated with a multiplicity of 0.05 to 0.5 viable microorganism/cell. The microorganisms were harvested, in what appeared to be late-log phase of growth, 4.5 to 5.5 days after inoculation of the flasks. The previously described Renografin purification procedure (32) was modified as follows. The culture fluids were removed from the flasks and centrifuged for 30 min at 8,000 rpm in a

Sorvall GSA rotor. The cell monolayers were removed by brief treatment with 0.25% Bactotrypsin (Difco) diluted in Dulbecco phosphate-buffered saline (PBS) and were combined with the pellets from the culture fluids. The cells from all flasks were digested for 30 min at 34°C in 100 ml of 0.25% trypsin and were centrifuged at 1,000 rpm for 10 min in a Damon/IEC PR-J clinical centrifuge. Cell debris and nuclei were discarded, and the supernatant was centrifuged at 12,000 rpm for 15 min in a Sorvall SS-34 fixed-angle rotor. The pelleted vole agent was layered over six 34-ml 20 to 45% Renografin gradients prepared in PBS. After centrifugation in an SW-27 rotor at 25,000 rpm for 1 h in an L2-65B Beckman ultracentrifuge at 4°C, the heavy band midway down the gradient was collected with an 18-gauge cannula, diluted in PBS, and centrifuged at 12,000 rpm for 15 min. The pellets were resuspended in 15 ml of PBS, and the vole agent again was banded in 25 to 45% Renografin gradients. The collected bands were washed twice and resuspended in PBS for experiments with intact cells or in 0.04 M potassium phosphate buffer, pH 7.2, when processed as described below.

Concentrated cell suspensions (2 to 3 mg of protein/ml) were disrupted twice at 20,000 lb/in^2 with a French pressure cell. The disrupted cells were centrifuged at 32,000 rpm for 2 h in a fixed-angle Beckman type 40 rotor. The supernatant fluid, stored at −70°C, served as the soluble extract in enzymatic assays.

Isolation and determination of the base composition of DNA. Deoxyribonucleic acid (DNA) was isolated from four preparations of vole agent by the procedure of Marmur (20) and was further purified by hydroxyapatite column chromatography (6, 23). The spectral properties and hyperchromicity of three preparations indicated that a high degree of purification of the DNA had been achieved, but all four preparations were satisfactory and yielded results that were identical to one another. The base composition of the DNA was determined by its thermal denaturation temperature, T_m (21), in a Beckman Acta III spectrophotometer equipped with an automatic sampling system. Control DNA preparations were obtained from *Escherichia coli* K-12 and *Cytophaga johnsonae* (ATCC 17061).

Miscellaneous procedures. Plaque assays were attempted as done previously (34) on monolayers of primary chicken embryo cells. Antibiotic susceptibility was tested by placing antibiotic sensitivity disks (Difco) atop the agar overlay with sterile forceps, as described by McDade (17).

Mice (NIH/Nmri CV) were approximately 3 weeks old at the time of first inoculation. They were inoculated with the vole agent and/or with the Karp strain of *R. tsutsugamushi* in a manner comparable to that used by Baker (1). The Karp strain was obtained from F. M. Bozeman and was used in its CE49 passage.

CO_2 formation from various ^{14}C-labeled substrates was determined as described by Weiss et al. (32). The diluent was PBS and the total volume was 2 ml. The final concentrations of the reagents were as follows: 0.1, 1, or 5 mM substrate with 0.1 μCi of ^{14}C-labeled substrate per flask, as indicated, 1 mM $MgCl_2$, and 0.35% bovine plasma albumin (Pentex, from Calbiochem). The protein content of the vole agent cells added to each flask was approximately 0.3 mg.

Enzyme activities were assayed as described previously (8), except that fructose diphosphate aldolase was measured with a Stat-Pack (Calbiochem) and hexokinase was measured as described by Joshi and Jagannathan (15) scaled to a 1-ml total assay volume. Control L cells, *E. coli,* and yolk sac extracts were prepared as described previously (8).

RESULTS

Growth in eggs, cell cultures, and cell-free media. The first suspicion that the vole agent might not be an obligately intracellular parasite originated from the irregular pattern of embryo deaths after inoculation of various dilutions of the vole agent into their yolk sacs (Table 1). The correlation between the dilution of the agent and embryo mortality was not good. With dilutions of 10^{-1} to 10^{-4}, some embryos died within 6 days postinoculation, while other embryos survived. Baker (1) likewise noted that most embryos died 5 to 7 days after inoculation, but a few lived to hatching. Smears of yolk sacs from the early deaths generally contained numerous microorganisms, whereas some of the smears from later deaths or survivors contained few if any microorganisms. This was true even when the inoculum was diluted 10^{-1} or 10^{-2}. This suggested that the embryos which survived the first onslaught of infection were able to contain it, a situation not expected to occur when the invading microorganism is an obligately intracellular parasite (30).

TABLE 1. *Virulence of the vole agent for chicken embryos*[a]

Seed	Dilution[b]	Embryo mortality[c]		
		Days 4–6	Days 7–12	Survivors[d]
CE44	1	10		5
	2	6	2	6
	3	7	5	3
	4	4	1	10
	5	2	2	9
	6		1	11
CE48	2	5	3	7
	3	4		9
	4	2	3	10
	5	1	1	11
	6	1		14

[a] Seven-day-old chicken embryos were inoculated via yolk sac with 0.4-ml volumes of vole agent suspensions and were incubated at 35°C.

[b] Inverse of the $\log_{10}$ dilution of yolk sac.

[c] Fifteen eggs were used for each dilution, and deaths occurring on days 4 to 6 and 7 to 12 are shown. Deaths occurring during the first 3 days were not attributed to infection and were disregarded.

[d] At 12 days postinoculation.

The position of the vole agent with respect to its host cell was resolved by placing infected L cells on slide cultures, incubating them for various time intervals, and staining them with Giemsa (Fig. 1). Although it is not possible to exclude the possibility that some of the microorganisms were intracellular, the great majority appeared to be simply attached to the surface of the L cells. When an attempt was made to plaque the vole agent on chicken embryo monolayers by the technique commonly used for rickettsiae (18), white colonies were seen on top of the cells and below the agar (Fig. 2). They first appeared on day 5 or 6 and achieved a diameter of about 0.5 mm which did not increase during subsequent days. When neutral red was applied, the colonies were surrounded by narrow unstained zones which had the appearance of plaques. In fact, when first seen after neutral red staining they were mistaken for plaques. The numbers of colonies were proportional to the concentration. The CE48 seed was shown to have a titer of 2×10^6 colony-forming units (CFU)/ml, whereas CE43 and CE45 seeds, as received in our laboratory from the ATCC prior to passage, had 10^4 and 10^5 CFU/ml, respectively.

The method of growing the vole agent under an agar overlayer was utilized to test its antibiotic susceptibility. With an inoculum of approximately 2×10^3 CFU/25-cm^2 tissue culture flask, the effect of disks impregnated with 30 μg of antibiotic (10 U in the case of penicillin G) was as follows: erythromycin and tetracycline, wide zone of inhibition; carbenicillin and penicillin, narrow zone of inhibition; novobiocin and sulfadiazine, no inhibition.

A systematic study of the growth of the vole agent in cell-free media has not been made, but the following observations were made on cultures used to test for the presence of contaminating bacteria. The vole agent did not grow in various combinations of tissue culture media with or without agarose in the absence of avian or mammalian cells. No growth was obtained on Mueller-Hinton agar or thioglycollate broth. However, when heavy suspensions were placed on Mueller-Hinton blood agar and incubated in a candle jar, small translucent colonies appeared and were clearly visible with the naked eye 4 to 7 days after inoculation. Some of the colonies achieved a diameter of 0.5 mm, but most of them remained considerably smaller. Efficiency of plating appeared to be low. A heavy suspension

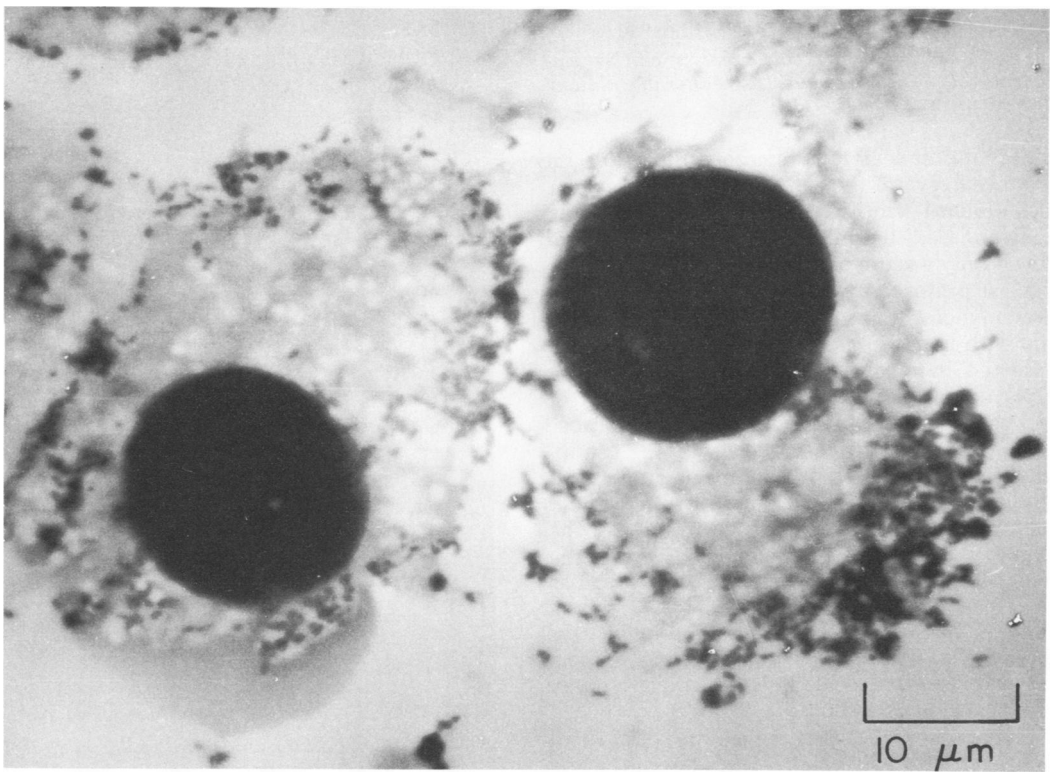

FIG. 1. *L cells 48 h after infection with the vole agent. Giemsa staining.*

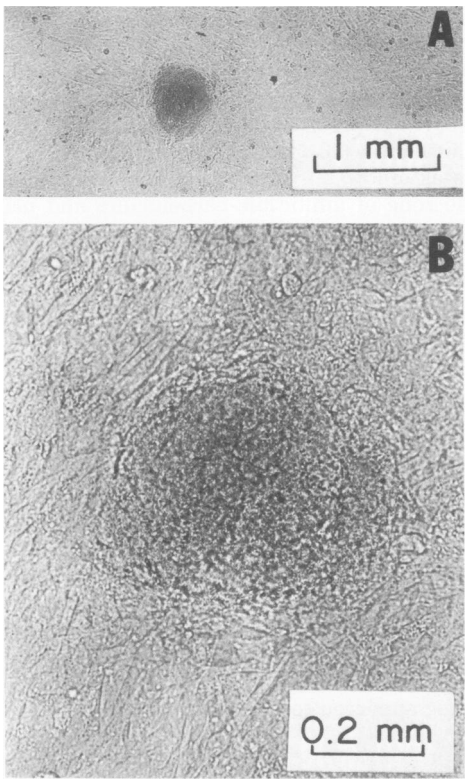

FIG. 2. *Colonies of the vole agent on chicken embryo monolayers under agar 6 days after inoculation. Unstained.*

containing at least 10^8 microorganisms/0.1 ml produced several thousands of colonies but not a confluent lawn (Fig. 3). Seed CE44 containing an estimated 10^5 microorganisms/0.1 ml produced only a score of colonies.

Most profuse growth was obtained, however, over monolayers of chicken embryo cells overlaid with liquid tissue culture medium. Growth, reflected in moderately high turbidity of the supernatant fluid, was obtained after 48 h of incubation at 32°C. Particularly useful, because of the simplicity of the procedure, was the inoculation of L cells which had been irradiated 7 days previously. Although the supernatant fluid acquired considerable turbidity by day 5, the majority of organisms remained attached to the cells and had to be released with trypsin as described in Materials and Methods. Yields of purified vole agent per 16-ounce flask with the three seeds were 1.9 to 2.0 mg of protein, or more than four times the yield of *R. typhi* grown under similar conditions (32).

Figure 4 illustrates the appearance of purified vole agent, which matches the description by Baker (1). It is gram-negative and stains well by the Gimenez method (12), a modification of the Macchiavello stain (16) used by Baker. Most satisfactory was Giemsa staining.

Biochemical activities. Table 2 illustrates some of the catabolic activities of cells of the vole agent. Glutamate, glutamine, succinate, and pyruvate were utilized, with the production of moderate amounts of CO_2. With the first three substrates considerably larger amounts of CO_2 were produced with 5 mM than with 1 mM concentrations, but it is not known whether 5 mM was adequate for optimal utilization. Of the microorganisms studied in this laboratory under comparable conditions, *R. typhi* utilized glutamate most rapidly, and *Rochalimaea quintana* utilized succinate most rapidly (13). The vole agent appeared to metabolize glutamate at a much faster rate than *R. typhi* (32, 34), and it appeared to metabolize glutamine and succinate at about the same rates as *R. quintana* (13). Although the investigation of pyruvate metabolism was limited to CO_2 formation from carbon position 1, there is evidence that there were subsequent steps. In a single experiment done under comparable conditions, it was shown that

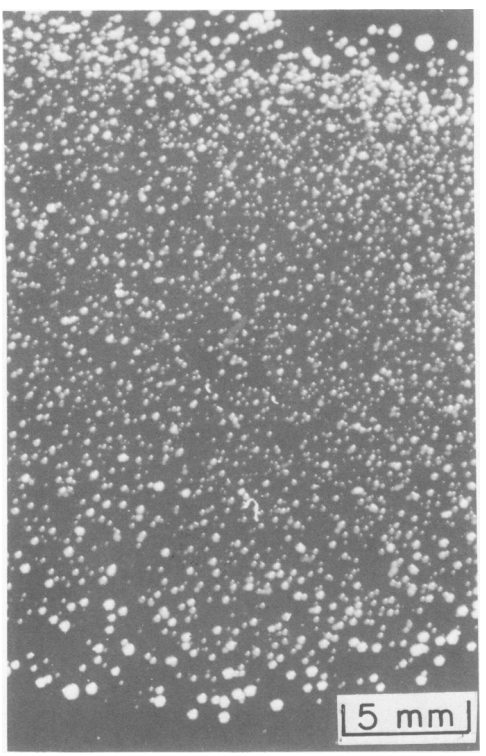

FIG. 3. *Colonies of the vole agent on blood agar 7 days after inoculation with a heavy suspension of purified microorganisms.*

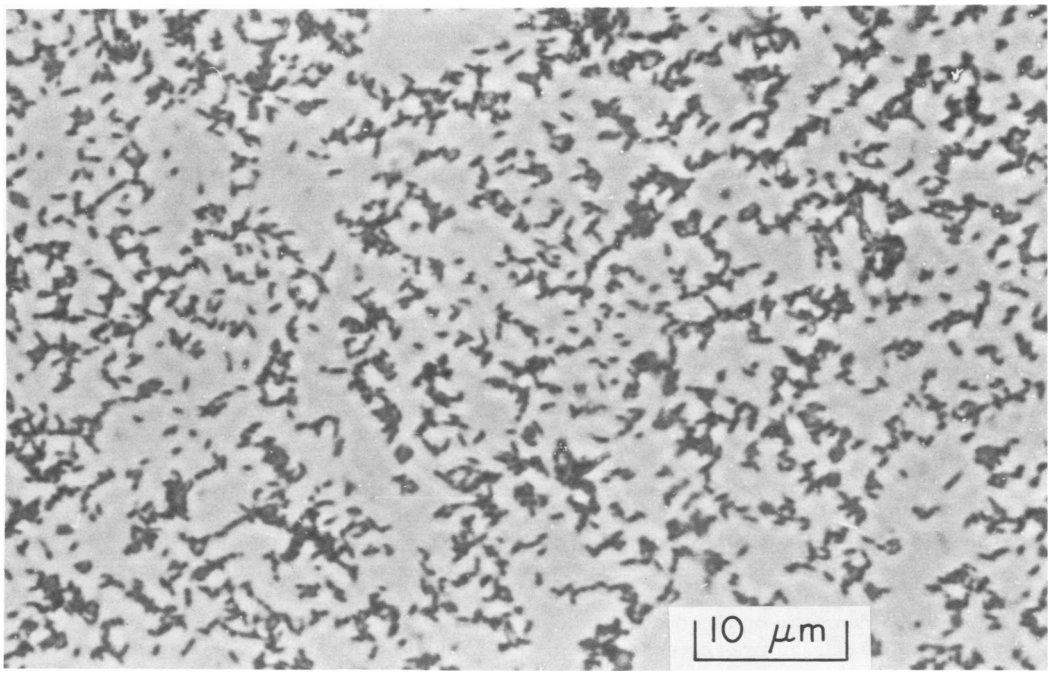

FIG. 4. *Smear of purified vole agent. Giemsa staining.*

TABLE 2. *CO_2 formation from various substrates[a] by vole agent cells*

Substrate	Carbon positions labeled	Mean ± SD[b] with substrate concn of	
		1 mM	5 mM
Glutamate	1-5	2.74 ± 0.76	6.44 ± 1.70
Glutamine	1-5	1.05 ± 0.11	2.47 ± 0.42
Succinate	1,4	3.70 ± 0.58	6.27 ± 0.27
Pyruvate	1	2.71 ± 0.74	2.51 ± 0.55

[a] Micromoles per milligram of vole agent protein after 2 h of incubation at 34°C. Each flask contained approximately 0.3 mg of bacterial protein in a total volume of 2 ml.

[b] Mean ± standard deviation (SD) from three experiments with CE44, CE46, and CE48 seeds.

0.66 μmol of CO_2 per mg of protein per 2 h of incubation was formed from C_1 of acetate.

The vole agent shares with the rickettsiae (30) the inability to catabolize glucose or glucose-6-phosphate. These two compounds were tested at 1 and 0.1 mM concentrations, but in neither case was the radioactivity in the trapped CO_2 significantly above background. With the smaller concentration, an amount of CO_2 as small as 1 nmol would have been measurable.

Table 3 illustrates the enzymatic activities of cell extracts of the vole agent. The results confirm and extend the data obtained with whole

TABLE 3. *Enzymatic activities[a] of cell-free extracts derived from the vole agent and from L cells*

Enzyme	Vole agent[b]	L cells
Glutamate dehydrogenase[c]		
NAD-dependent	91 ± 20	45
NADP-dependent	<13	8
Transaminase		
Glutamate-oxalacetate	244 ± 110	554
Glutamate-pyruvate	34 ± 12	36
Malate dehydrogenase	1,690 ± 330	2,340
Citrate synthase	95 ± 12	376
Hexokinase	<13	58
Dehydrogenase		
Glucose-6-phosphate	<21	55
6-Phosphogluconate	<13	4
Phosphoglucose isomerase	<13	447
Fructose −1,6-diphosphate aldolase	14 ± 1	49
Pyruvate kinase	<13	447

[a] Nanomoles of substrate utilized per milligram of protein per minute. Mean of triplicate assays of each of three preparations of vole agent ± standard deviation (when measurable) and results of assay of a single preparation of L cells. Positive control preparations also included extracts derived from yolk sacs and *E. coli* (not shown).

[b] From same preparations listed in Table 2.

[c] NAD, Nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate.

cells. They indicate that the vole agent catabolizes glutamate via a nicotinamide adenine dinucleotide-dependent dehydrogenase and glutamate-oxalacetate and glutamate-pyruvate transaminases and that it has representative enzymes of the citric acid cycle. Enzymes of the glycolytic and pentose pathway are present in very small amounts or are absent. The results are qualitatively similar to those obtained with *R. typhi* by Coolbaugh et al. (8). Quantitative comparisons of the more significant enzymatic activities are more difficult to make because, after French pressure cell disruption, the preparations were centrifuged at a much higher speed than in experiments by Coolbaugh et al. (8). Nevertheless, preparations of extract from the vole agent appeared to be more active enzymatically than those derived from *R. typhi*.

Protection against *R. tsutsugamushi*. As shown by Baker (1), the virulence of the vole agent for mice was very low. Only an occasional mouse inoculated with 50 or 10% yolk sac suspension of the vole agent died, but the vole agent was not isolated from the organs of these mice by inoculation of cell cultures or eggs. An attempt was made to repeat the experiment by Baker in which he showed that the median lethal dose (LD_{50}) of the Karp strain of *R. tsutsugamushi* was about 10-fold higher for mice previously infected with the vole agent than for control mice. The mice had been infected by Baker with an intraperitoneal injection of 1 ml of 10% yolk sac suspension of the vole agent 13, 30, or 33 days prior to challenge with the Karp strain. Our results as well as those of Baker are shown in Table 4. In our experiments a small difference in mortality was obtained which is of doubtful significance. It can be estimated that the LD_{50} for the vole-infected mice was about fourfold higher than for the control mice. Thus, our results neither confirm the results of Baker nor exclude the possibility that his results can be confirmed by the use of a somewhat larger number of mice.

TABLE 4. *Protection against the Karp strain of R. tsutsugamushi of mice previously inoculated with the vole agent*

Dilution of Karp strain[b]	Dead/total inoculated			
	Our results		Baker's results (1)	
	Inoculated[a]	Control	Inoculated[a]	Control
2	5/5	10/10	15/15	15/15
3	5/5	8/10	13/15	15/15
4	3/5	10/10	0/15	15/15
5	2/5	6/10	0/15	0/15
6	2/5	5/10		
7	0/5	0/10		

[a] In our experiments mice were inoculated intraperitoneally with 1 ml of a 10% infected yolk suspension of vole agent 14 days prior to challenge. Baker similarly inoculated groups of five mice 13, 30, or 33 days prior to challenge. In both cases the challenge consisted of 0.5 ml of diluted yolk sac suspension of the Karp strain.

[b] Inverse of the $\log_{10}$ dilution of yolk sac.

DNA base composition. The T_m values of four preparations of vole agent DNA were determined in parallel with *E. coli*, *C. johnsonae*, or *R. quintana* DNA. The results were corrected on the basis of a T_m of *E. coli* equal to 90.5°C and a molar percent guanine plus cytosine of 51. The T_m of *C. johnsonae* was, as expected (10), 83.4°C. The mean T_m values of triplicate determinations of the four vole agent DNA preparations were 85.4, 85.6, 85.6, and 85.8°C. The mean T_m value of triplicate determinations of a sample of *R. quintana* DNA, prepared by F. J. Tyeryar, Jr. (27), was identical to that of the vole agent, 85.6°C. The calculated molar percent guanine plus cytosine (10) of either the vole agent or *R. quintana* is therefore 39.

Immunofluorescence. The antigenic relationship of the vole agent to scrub typhus rickettsiae and to *R. quintana* was tested by immunofluorescence as described by Bozeman and Elisberg (2, 3, 11). The vole agent did not react with hyperimmune rabbit sera prepared against the Karp, Gilliam, and Kato strains of *R. tsutsugamushi*. However, the vole agent reacted strongly with *R. quintana* hyperimmune rabbit serum (prepared by J. W. Vinson) and a human serum from a patient who had recovered from trench fever infection. The titer of the rabbit serum used in a series of fourfold dilutions was 1:2,560 in the homologous reaction and 1:640 (1:160 in a repeat test) with the vole agent. The human serum titered 1:160 with both *R. quintana* and the vole agent. These tests were kindly performed by F. M. Bozeman, Bureau of Biologics, Food and Drug Administration.

DISCUSSION

When this investigation was started, the vole agent was studied simply as an example of a bacterium which had been mistakenly regarded as a rickettsia. It has been evident for sometime that members of the genus *Rickettsia* have clearly defined properties (30, 31, 33), but *Bergey's Manual* (7) lists numerous organisms, described decades ago or more recently, which have improperly been designated rickettsiae. The differentiation between rickettsiae and organisms which bear only superficial resemblance to them has become particularly important since the discovery about 14 years ago of a new rick-

ettsial species, *R. canada* (19), and the more recent discovery of strains of *R. prowazekii* in American wildlife (3, 5).

Had Baker isolated the agent from a patient with a febrile illness with a history of louse infestation, its identification as trench fever rickettsia would not have been difficult. Since it was isolated from a vole, its true identity was not suspected until the DNA work was completed. The serological reaction with two sera from entirely different sources, plus all the other data, clearly establishes the vole agent as a strain of *R. quintana.*

Few of the experiments of this study were entirely comparable to those performed with *R. quintana,* but it is obvious that, although the Fuller strain of *R. quintana* and the vole agent are similar, they are not identical. The only detailed report of the growth of the Fuller strain of *R. quintana* in yolk sac of chicken embryos and cell cultures is that of Vinson and Fuller (29). In the yolk sac growth was scant, but after 16 serial passages a 2% suspension killed 42% of the embryos, not unlike our experience with the vole agent. Their description of the growth in HEp-2 cells, and especially their photomicrograph, is reminiscent of our results, but the vole agent apparently grew more profusely with the cells and media that we used. Comparisons of growth in bacteriological media are more difficult to make. Although a considerable amount of information has accumulated on the nutritional requirements of the Fuller strain (22, 24, 25, 27-29), the vole agent was cultivated on commercial sheep blood agar, which does not appear to be particularly well suited for *R. quintana.* A single attempt to grow the vole agent in a modified Evans medium (27) yielded equivocal results. It is quite possible that the two strains have somewhat different nutritional requirements, but resemble each other in the production of small smooth colonies which appear several days after the inoculation of the blood plates. The similarity between the two agents in biochemical activities has already been noted. The only difference, which is not necessarily a major one, is the ratio of CO_2 production from glutamate to that from glutamine: it is approximately 1:2.5 in the Fuller strain (13) and 2.5:1 in the vole agent (Table 2). A more detailed comparison of the two strains must await the performance of experiments in which both strains are used in identical fashion.

If future studies confirm our impression that there is only a limited number of genetic differences between the two strains, these results may aid us in understanding the natural history of louse-borne human disease. A hypothesis that trench fever originated in field mice would be difficult to sustain. Voles and other small rodents have been surveyed extensively, but there is no other record of vole agent isolation. For example, Bozeman et al. (4) isolated two strains of *Rickettsia rickettsii* from 185 voles captured in Virginia and Maryland and Jackson et al. (14) isolated *R. akari* from the Korean vole, but in none of these studies was the vole agent recovered. On the other hand, Baker (1) reported five successful isolations from 10 voles captured on Grosse Isle. A more appealing hypothesis is the one advanced by Baker (1) that on Grosse Isle he would find a vestige of the epidemics that occurred in 1847. Although trench fever was not recognized until 1915 (26), it is possible that it existed as one of the agents carried by the louse during previous louse-borne epidemics. Baker did not isolate the rickettsia of epidemic typhus, as he expected, but he isolated the agent of trench fever.

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LITERATURE CITED

1. **Baker, J. A.** 1946. A rickettsial infection in Canadian voles. J. Exp. Med. **84**:37-51.
2. **Bozeman, F. M., and B. L. Elisberg.** 1963. Serological diagnosis of scrub typhus by indirect immunofluorescence. Proc. Soc. Exp. Biol. Med. **112**:568-573.
3. **Bozeman, F. M., S. A. Masiello, M. S. Williams, and B. L. Elisberg.** 1975. Epidemic typhus rickettsiae isolated from flying squirrels. Nature (London) **255**:545-547.
4. **Bozeman, F. M., A. Shirai, J. W. Humphries, and H. S. Fuller.** 1967. Ecology of Rocky Mountain spotted fever. II. Natural infection of wild mammals and birds in Virginia and Maryland. Am. J. Trop. Med. **16**:48-59.
5. **Bozeman, F. M., M. S. Williams, N. I. Stocks, D. P. Chadwick, B. L. Elisberg, D. E. Sonenshine, and D. M. Lauer.** 1977. Ecologic studies on epidemic typhus infection in the southern flying squirrel. *In* J. Kazar (ed.), Proceedings of the Second International Symposium on Rickettsiae and Rickettsial Diseases. Slovak Academy of Sciences, Bratislava.
6. **Britten, R. J., M. Pavich, and J. Smith.** 1970. A new method for DNA purification. Carnegie Inst. Washington Yearb. **68**:400-402.
7. **Buchanan, R. E., and N. E. Gibbons (ed.).** 1974. Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
8. **Coolbaugh, J. C., J. J. Progar, and E. Weiss.** 1976. Enzymatic activities of cell-free extracts of *Rickettsia typhi.* Infect. Immun. **14**:298-305.

9. **Dasch, G. A., J. R. Samms, and E. Weiss.** 1978. Biochemical characteristics of typhus group rickettsiae with special attention to the *Rickettsia prowazekii* strains isolated from flying squirrels. Infect. Immun. **19**:676–685.
10. **De Ley, J.** 1970. Reexamination of the association between melting point, buoyant density, and chemical base composition of deoxyribonucleic acid. J. Bacteriol. **101**:738–754.
11. **Elisberg, B. L., and F. M. Bozeman.** 1966. Serological diagnosis of rickettsial diseases by indirect immunofluorescence. Arch. Inst. Pasteur Tunis **43**:193–204.
12. **Gimenez, D. F.** 1964. Staining rickettsiae in yolk sac cultures. Stain Technol. **39**:135–140.
13. **Huang, K.-Y.** 1967. Metabolic activity of the trench fever rickettsia, *Rickettsia quintana*. J. Bacteriol. **93**:853–859.
14. **Jackson, E. B., J. X. Danauskas, M. C. Coale, and J. E. Smadel.** 1957. Recovery of *Rickettsia akari* from the Korean vole *Microtus fortis pelliceus*. Am. J. Hyg. **66**:301–308.
15. **Joshi, M. D., and V. Jagannathan.** 1966. Hexokinase. Methods Enzymol. **9**:371–375.
16. **Macchiavello, A.** 1937. Estudios sobre tifus exantematico. III. Un nuevo metodo para tenir *Rickettsia*. Rev. Chil. Hig. Med. Prev. **1**:101–106.
17. **McDade, J. E.** 1969. Determination of antibiotic susceptibility of *Rickettsia* by the plaque assay technique. Appl. Microbiol. **18**:133–135.
18. **McDade, J. E., J. R. Stakebake, and P. J. Gerone.** 1969. Plaque assay system for several species of *Rickettsia*. J. Bacteriol. **99**:910–912.
19. **McKiel, J. A., E. J. Bell, and D. B. Lackman.** 1967. *Rickettsia canada:* a new member of the typhus group of rickettsiae isolated from *Haemaphysalis leporispalustris* ticks in Canada. Can. J. Microbiol. **13**:503–510.
20. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. **3**:208–218.
21. **Marmur, J., and P. Doty.** 1962. Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature. J. Mol. Biol. **5**:109–118.
22. **Mason, R. A.** 1970. Propagation and growth cycle of *Rickettsia quintana* in a new liquid medium. J. Bacteriol. **103**:184–190.
23. **Meinke, W., D. A. Goldstein, and M. R. Hall.** 1974. Rapid isolation of mouse DNA from cells in tissue culture. Anal. Biochem. **58**:82–84.
24. **Myers, W. F., L. D. Cutler, and C. L. Wisseman, Jr.** 1969. Role of erythrocytes and serum in the nutrition of *Rickettsia quintana*. J. Bacteriol. **97**:663–666.
25. **Myers, W. F., J. V. Osterman, and C. L. Wisseman, Jr.** 1972. Nutritional studies of *Rickettsia quintana:* nature of the hematin requirement. J. Bacteriol. **109**:89–95.
26. **Strong, R. P. (ed.).** 1918. Trench fever. Report of Commission, Medical Research Committee, American Red. Cross. Oxford University Press, London.
27. **Tyeryar, F. J., Jr., E. Weiss, D. B. Millar, F. M. Bozeman, and R. A. Ormsbee.** 1973. DNA base composition of rickettsiae. Science **180**:415–417.
28. **Vinson, J. W.** 1966. *In vitro* cultivation of the rickettsial agent of trench fever. Bull. WHO **35**:155–164.
29. **Vinson, J. W., and H. S. Fuller.** 1961. Studies on trench fever. I. Propagation of rickettsia-like microorganisms from a patient's blood. Pathol. Microbiol. Suppl. **24**:152–166.
30. **Weiss, E.** 1973. Growth and physiology of rickettsiae. Bacteriol. Rev. **37**:259–283.
31. **Weiss, E.** 1977. Biological properties of rickettsiae. *In* J. Kazar (ed.), Proceedings of the Second International Symposium on Rickettsiae and Rickettsial Diseases. Slovak Academy of Sciences, Bratislava.
32. **Weiss, E., J. C. Coolbaugh, and J. C. Williams.** 1975. Separation of viable *Rickettsia typhi* from yolk sac and L cell host components by Renografin density gradient centrifugation. Appl. Microbiol. **30**:456–463.
33. **Weiss, E., and J. W. Moulder.** 1974. *Rickettsia, Coxiella, Rochalimaea,* p. 883–893. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
34. **Woodman, D. R., E. Weiss, G. A. Dasch, and F. M. Bozeman.** 1977. Biological properties of *Rickettsia prowazekii* strains isolated from flying squirrels. Infect. Immun. **16**:853–860.