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Some Characteristics of Heavy and Light Bands of *Rickettsia prowazekii* on Renografin Gradients

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Suspensions of partially purified *Rickettsia prowazekii* yielded two bands of organisms when centrifuged to equilibrium in Renografin density gradients. Rickettsiae from the lower, heavy band were defective in their infective and metabolic activities, as compared to organisms from the light band. The greater density in Renografin of heavy-banding organisms was due to their lack of permeability barrier to it, as evidenced by the absence of plasmolysis in hypertonic Renografin. In contrast, light-banding rickettsiae were able to exclude Renografin, since they were plasmolyzed in it. The proportion of heavy-banding organisms in a rickettsial suspension was influenced by the growth phase they were in when harvested from infected yolk sacs, as well as by the conditions and media to which they subsequently were exposed. We have concluded that these defective forms arise from the degeneration of light-banding rickettsiae. This separation of two functional classes of rickettsiae in Renografin density gradients has been exploited (i) to increase the uniformity of the suspensions by removing many noninfectious particles and (ii) to determine rapidly the integrity of certain properties of the cytoplasmic membrane of organisms exposed to a variety of conditions.

Definitive studies of rickettsiae of diverse types often require the use of well-characterized, uniform, highly viable, and highly purified preparations of organisms. Unfortunately, the purification procedures commonly used, including rapid freezing for storage, sometimes seem to have a deleterious effect on rickettsiae in degrees ranging from increased permeability to inorganic ions (12), to reversible inactivation (1, 2), to outright killing. Thus, even though a rickettsial suspension is very pure, it likely contains inactive as well as active organisms.

The heterogeneity of partially purified rickettsial suspensions is evidenced by the fact that both phase I *Coxiella burnetii* (22) and *Rickettsia* species (20) produce at least two bands of rickettsiae when centrifuged to equilibrium in Renografin density gradients, which are commonly used for rickettsial purification (6, 20). In the case of *C. burnetii*, organisms in both bands were viable, but they could be distinguished morphologically, the lighter organisms being smaller and containing condensed rather than dispersed nucleoid material (22). In *Rickettsia* species, there has been little characterization of the organisms which sediment to different densities in Renografin.

Consequently, we have analyzed the phenomenon of double band formation by *Rickettsiae prowazekii* in Renografin, exploring its cause and significance. The distribution of heterogeneous rickettsial populations into separate bands in Renografin gradients has been exploited (i) to increase the uniformity of the suspensions by removing many noninfectious particles and (ii) to determine rapidly the integrity of certain properties of the cytoplasmic membrane of organisms exposed to a variety of conditions.

(A portion of this material was presented at the Rickettsiology Conference held in the University of Maryland Donaldson-Brown Center, Port Deposit, Md., in March 1979.)

MATERIALS AND METHODS

Rickettsiae. The Breinl and E strains of *R. prowazekii* initially were prepared as 20% infected yolk sac suspensions from plaque-purified seeds at the 157th and ca. 270th egg passage levels, respectively. Infectivity was determined by a standard plaque assay in chicken embryo cultures which did not employ centrifugation of organisms onto the cell monolayers (24, 26). Rickettsial particles (rickettsia-like bodies, or RLB) were counted directly by the method of Silverman et al. (15). The association of rickettsiae with chicken embryo cells was determined by incubating rickettsiae with suspended chick cells for 30 min at 32°C with frequent shaking and subsequently counting the RLB associated with the stained cells (26).

Purification. Before gradient centrifugation, the yolk sac suspensions were partially purified by a "batch sucrose" method, which was developed (Wisseman, unpublished data) to maximize recovery of infec-

tious rickettsiae. Rapidly thawed yolk sac suspensions were mixed with 1.67 volumes of 50% (wt/vol) sucrose in brain heart infusion broth (BHI; 3.7%; BBL Microbiology Systems, Cockeysville, Md.). After centrifugation at 16,300 × *g* (Sorvall GSA rotor), the rickettsial pellet was suspended in 50 to 100 ml of a 1/10 dilution of rabbit anti-normal yolk sac serum (10) in 6% bovine serum albumin (BSA; Armour fraction V, Reheis Chemical Co., Phoenix, Ariz.) (3) in 122 mM KCl + 23 mM NaCl (pH 7.0). The suspension was held in an ice bath for 60 to 90 min, and the flocculated host material was removed by centrifugation at 500 × *g* for 10 min. The supernatant rickettsial suspension was removed and held at 0°C. The pellet was resuspended in fresh BSA-antiserum and held on ice for 30 min. The flocculant was again removed by low-speed centrifugation, and the resulting supernatant fluid was added to the first supernatant rickettsial suspension. These batch sucrose, or partially purified, rickettsiae were either frozen as such or pelleted and resuspended in the desired diluent, usually SPG (218 mM sucrose, 10 mM potassium phosphate buffer [pH 7], 5 mM potassium glutamate; 4). The final step in the purification procedure was Renografin density gradient centrifugation. The combination of batch sucrose partial purification and Renografin gradient centrifugation (see below) has, on occasion, enabled us to obtain ratios of rickettsial particles to plaque-forming units approaching 1:1 (Wisseman, unpublished data). Ormsbee et al. (13) have recently published their modification of the batch sucrose method.

Gradient centrifugation. Renografin 76 (66% diatrizoate meglumine and 10% diatrizoate sodium in 0.32% sodium citrate and 0.04% edetate disodium) was obtained from E. R. Squibb and Sons, Inc., Princeton, N.J., as a 76% solution. Renografin usually was diluted in phosphate-buffered saline (PBS; 5.2 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 116 mM NaCl; pH 7.0) or BD buffer (62.5 mM KCl, 62.5 mM NaCl, 10 mM potassium phosphate buffer [pH 7.2], 5 mM potassium glutamate). The particular buffers used in given experiments are noted below. Batch sucrose-purified rickettsiae were layered onto 28 to 43% preformed linear Renografin gradients or, for preparative work, onto discontinuous gradients containing 6 to 8 ml of 42%, 14 to 16 ml of 36%, and 10 to 12 ml of 30% Renografin. In the latter gradients, heavy-banding (H) rickettsiae (see Results) sedimented on the 42% cushion and light-banding (L) rickettsiae collected at the 36%–30% interface. The gradients were centrifuged to equilibrium at 50,000 × *g* for 60 min in a Beckman SW25.1 or SW27 rotor. Fractions were collected through punctures in the bottoms of the tubes. From preparative gradients, rickettsial bands were collected separately and diluted approximately 1:2 with the buffer contained in the gradient. The rickettsiae were pelleted by centrifugation at 20,200 × *g* for 30 min in a Sorvall SS34 rotor and suspended in the desired diluent. Unless otherwise noted, gradient-purified rickettsiae were stored frozen at −70°C in SPG until used. Neither rapid freezing and thawing in SPG or BHI nor storage at −70°C in the same diluents for at least 3 months caused a detectable diminution of rickettsial infectivity (unpublished data). In any given experiment, only H and L rickettsiae prepared in the same gradients were compared.

Glutamate oxidation. Glutamic acid oxidation assays were carried out essentially by the method of Weiss et al. (21). The glutamate oxidation buffer consisted of K36 buffer (50 mM potassium phosphate buffer, pH 7.4, and 15 mM NaCl) supplemented with 2.5 mM $MgCl_2$, 0.3 mM $MnCl_2$, and 3 mg of BSA per ml. Volumes of 2.5 ml of the suspensions were put into triplicate 30-ml flasks with glass center wells containing 0.2 ml of Hyamine hydroxide, 1 M in methanol (Amersham Searle, Des Plaines, Ill.). After a 30-min equilibration at 32°C, 0.5 to 1 μCi of L-[U-^{14}C]glutamic acid (276 mCi/mmol, Amersham Searle) was added to each flask. The sealed flasks were shaken at 32°C for 2 h, and the reaction was halted by the addition of 1 ml of 30% trichloroacetic acid. The flasks were shaken for 30 min longer to trap residual CO_2. Then the contents of the center wells plus two 0.2-ml methanol washes were transferred to scintillation vials for counting in 8 ml of OCS scintillation fluid (Amersham Searle). A preliminary experiment confirmed that the amount of glutamic acid oxidized per organism was not affected by the rickettsial concentration in the presence of 3 mg of BSA per ml (14). This validated the comparison of suspensions containing slightly different numbers of organisms.

Protein assay. Protein was assayed by the method of Lowry et al. (11), using BSA as a standard.

Electron microscopy. For electron microscopic examination, pelleted rickettsiae were suspended and fixed in acrolein-glutaraldehyde, postfixed in osmium tetroxide, and embedded in Epon 812 as previously described (17).

RESULTS

Gradient profiles of *R. prowazekii* in Renografin density gradients. A typical Renografin density gradient profile of partially purified *R. prowazekii* is shown in Fig. 1. The major band of rickettsiae occurred at a Renografin density of 1.20 g/ml, and the minor band was found at a density of 1.23 g/ml. Both bands were free of host cell material, as determined by electron microscopic examination. Occasionally, the relative proportion of rickettsiae in the heavy and light bands deviated from the usual pattern. Figure 1B shows the gradient profile of a suspension which contained a disproportionate number of H rickettsiae. This suspension had accidentially undergone a gradual partial thawing during a freezer breakdown; it was known to contain many inactivated rickettsiae. This profile suggested that damaged rickettsiae might sediment to a greater Renografin density than intact organisms. To determine whether the H rickettsiae were defective, we compared them with the L rickettsiae in several ways. In addition, yolk sac suspensions prepared on different days after egg inoculation were examined.

Electron microscopic comparison of H

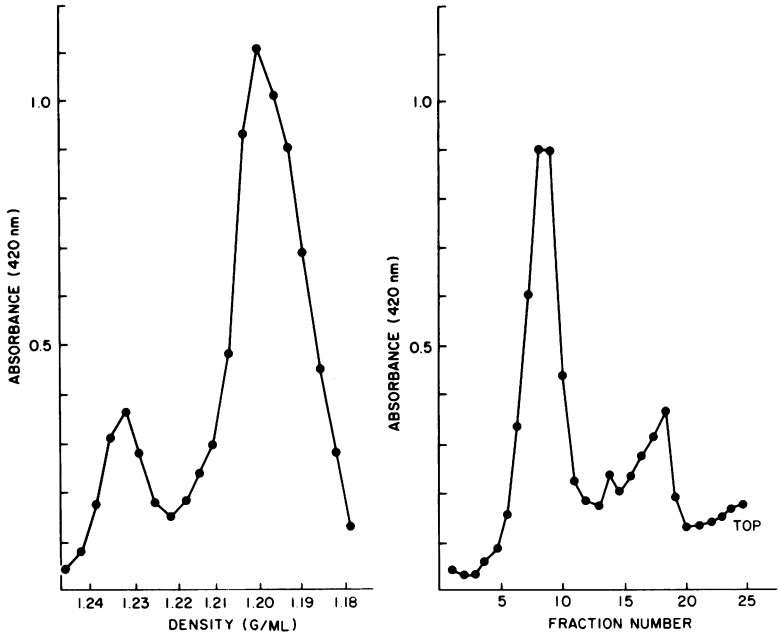

FIG. 1. *Profiles of partially purified R. prowazekii (Breinl) in continuous Renografin (28 to 43%, wt/vol, in BD buffer) gradients. (A) Profile typical of most partially purified yolk sac suspensions prepared 8 days after egg inoculation. (B) profile of partially degraded yolk sac suspension.*

and L rickettsiae. Electron micrographs of rickettsiae taken from the light and heavy regions of the same gradients revealed some consistent differences (Fig. 2). The majority of the rickettsiae sedimenting in the light band were somewhat irregular in shape, with highly convoluted outer envelopes. The outer envelopes were closely apposed to the cytoplasmic membranes, which bounded a cytosol so electron dense that visualization of individual ribosomes and strands of deoxyribonucleic acid was very difficult. On the other hand, organisms from the heavy band appeared to be swollen and tended to have less highly convoluted outer membranes. Also, in contrast to L rickettsiae, their outer envelopes and cytoplasmic membranes were not closely apposed. The cytoplasm in H rickettsiae was less dense than in L rickettsiae, and ribosomes and deoxyribonucleic acid fibers were easily discerned. Some visibly damaged organisms were usually present in these preparations. The same general morphological picture held whether rickettsial bands were prepared from yolk sac suspensions obtained 7, 8, or 9 days after egg inoculation (not shown). Electron-lucent inclusion bodies described in *R. prowazekii* at the stationary phase of its growth cycle in chicken embryo cell cultures (16, 26) were rarely observed in either H or L organisms from the Renografin-purified yolk sac preparations.

Viability of H and L rickettsiae. An analysis of the plaque-forming capacities of organisms collected from each band in the same gradient showed that the H rickettsiae had a strikingly lower infectivity than did L organisms, whether seen as RLB per plaque-forming unit or plaque-forming units per milligram of protein (Table 1). These experiments left no doubt that the heavy Renografin band contained damaged or defective rickettsiae, although some infectious organisms seemed to remain in these populations as well.

To determine whether H rickettsiae did not form plaques because they could not enter host cells, H and L organisms were exposed to chick cells in suspension in our standard uptake assay. During the 30-min incubation, uptake of H rickettsiae was low, whereas substantial uptake was achieved by the same numbers of L rickettsiae (Fig. 3). To determine whether the H organisms were still metabolically active, their capacity to oxidize glutamic acid was compared with that of L rickettsiae taken from the same gradients. Table 1 shows a marked reduction in the ability of H rickettsiae to form CO_2 from glutamic acid as compared to L rickettsiae, regardless of the day on which the yolk sacs were harvested. Thus, the H rickettsial population has reduced metabolic as well as infective activity.

Effect of diluents on gradient profiles.

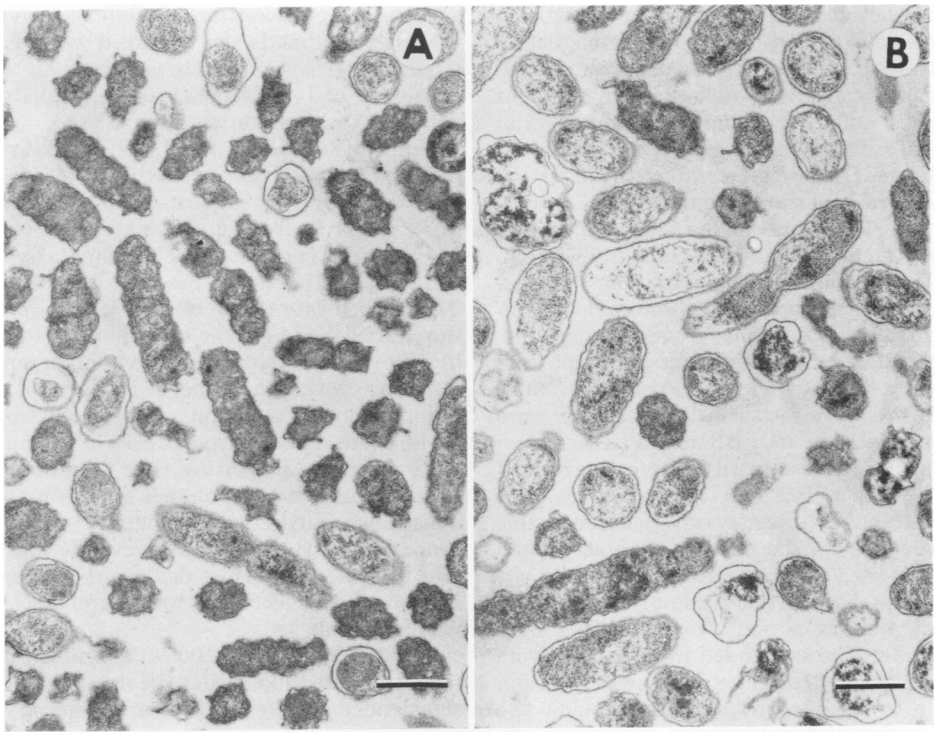

FIG. 2. *Ultrathin sections of R. prowazekii (Breinl) removed from Renografin (30 to 45%, wt/vol, in PBS) gradients, pelleted, and suspended in fixative. (A) Rickettsiae from light (1.20 g/ml) bands; (B) rickettsiae from heavy (1.23 g/ml) bands. Note relative electron densities of organisms in (A) and (B). Bar = 0.5 μm.*

TABLE 1. *Infectivity and glutamate oxidation of H and L rickettsiae*

Gradient band	RLB/PFU[a]	PFU/mg of protein[b]	cpm of $^{14}CO_2$ released per 10^{10} RLB[c]
L	6.3 (±0.2)[d] × 10^1	6.3 (±0.1) × 10^8	1,406 ± 52
H	2.0 (±0.5) × 10^4	4.0 (±0.4) × 10^5	58 ± 9
L/H	0.003	1,575	24

[a] Geometric mean, three rickettsial suspensions. PFU, Plaque-forming units.

[b] Geometric mean, five rickettsial suspensions, including one of the E strain of *R. prowazekii*.

[c] Arithmetic mean, two rickettsial suspensions.

[d] Value ± 1 standard error of the mean; the rickettsiae were prepared from viable eggs 7, 8, or 9 days after infection. Neither the day of harvest nor the strain of *R. prowazekii* affected the values for fractionated suspensions reported in this table.

Because the relative infectivity of a rickettsial suspension can be estimated by the proportions of light and heavy band material in Renografin gradients, we could monitor the effects on rickettsial integrity of certain manipulations by analyzing the gradient profiles of rickettsiae. Any manipulation which increased the proportion of H rickettsiae would be considered deleterious.

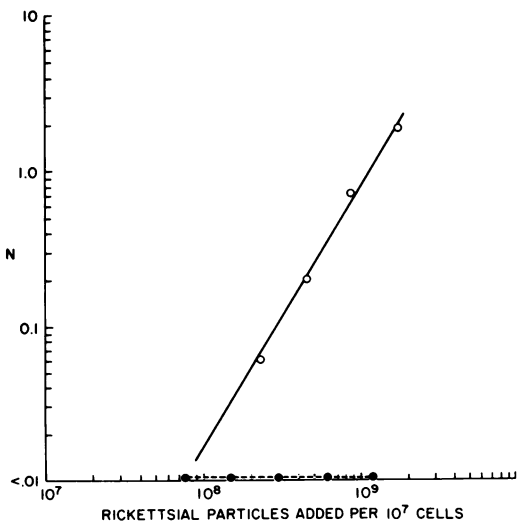

FIG. 3. *Association of L and H R. prowazekii (Breinl) with chicken embryo cells. N, Number of RLB per cell.*

One parameter we investigated was the effect of the diluent on the rickettsial profiles. In each experiment, samples of the same batch sucrose-

purified rickettsial suspension were pelleted, suspended in different diluents, and immediately layered onto Renografin gradients. After centrifugation of the gradient, rickettsiae in the light bands were collected, pelleted, resuspended in their respective diluents, and run in a second set of gradients. Rickettsial banding in all of the first gradients was similar to the typical pattern seen in Fig. 1A. However, the choice of diluent did affect the profiles of the second gradients. In each of two experiments, maintaining the rickettsiae in BHI throughout caused no degeneration of organisms into heavy-banding material, but suspension in all other selected diluents did: faint heavy bands were generated consistently from unfrozen L rickettsiae when the latter were processed entirely in PBS or SPG, or when organisms suspended in SPG were centrifuged in Renografin in BD (not shown). Since BHI best preserved the integrity of L rickettsiae, this would seem to be the diluent of choice, when compatible with the experiment.

The suspension of rickettsiae in either of two hypotonic buffers caused a major shift in density. L rickettsiae suspended in 19% PBS (vol/vol in water) just before gradient centrifugation formed a broad band in Renografin below the usual L region and including an increase in the H band (Fig. 4). The breadth of the "intermediate" peak indicates substantial heterogeneity in the rickettsial population after exposure to low salt concentrations. It should be emphasized that the effect seen was due to changes which occurred in the rickettsiae before they entered the gradient, since the gradients themselves were identical for the hypotonic and isotonic suspensions of rickettsiae. A shift to heavy and intermediate bands also occurred when L rickettsiae were suspended in a hypotonic buffer consisting of 10 mM potassium phosphate (pH 7.0) and 5 mM glutamate (not shown).

Changes in the H and L rickettsial populations during the growth cycle in yolk sacs. Infected yolk sac suspensions prepared on different days after egg inoculation were examined for their H and L particle contents by direct counting of RLB in the gradient bands (Fig. 5). The growth rate of the total rickettsial population diminished between days 8 and 9, reflected by a slowdown in the production of L organisms. However, during this time, as the general population reached the stationary phase of its growth cycle, there was a sharp increase in the number of H particles, suggesting that they may arise from the degeneration of once active organisms. This view is further substantiated by the higher proportion of H particles in nonviable eggs from day 9, where extensive degeneration was evidenced by the decline in total number of organisms per yolk sac.

Effect of Triton X-100 on rickettsial density. It seemed likely that the defective rickettsiae banded at a greater Renografin density than viable L organisms because they were unable to exclude Renografin from their intracellular spaces, becoming heavier due to the high density of Renografin. To determine whether rickettsiae laden with Renografin indeed band at 1.23 g/ml (the density of H organisms), L rickettsiae were exposed to Triton X-100 (final concentration,

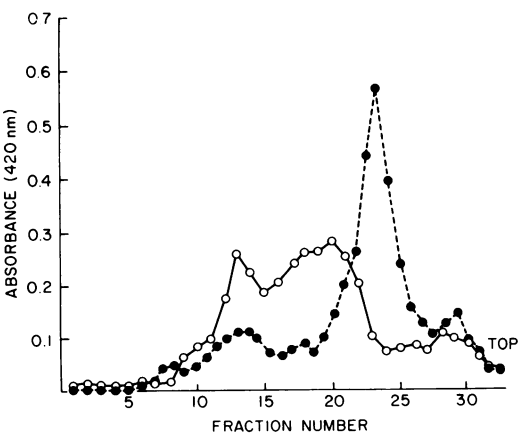

FIG. 4. *Profiles in Renografin-purified R. prowazekii (Breinl) L bands suspended in 19% PBS (vol/vol in water)* (○) *or in PBS* (●) *and centrifuged in 30 to 45% Renografin (wt/vol in PBS) gradients.*

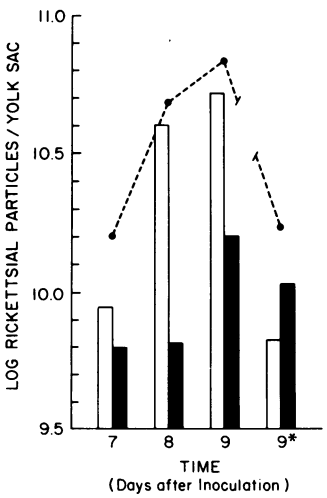

FIG. 5. *Yield of R. prowazekii (Breinl) obtained from purified infected yolk sacs collected 7, 8, or 9 days after inoculation. Open bars, L rickettsiae; closed bars, H rickettsiae;* ●, *total rickettsiae;* *, *yield from nonviable eggs.*

0.1%) in PBS for 20 min at 0°C before layering onto Renografin-PBS gradients. (This treatment renders the cytoplasmic membrane of *R. prowazekii* completely permeable to sucrose [25] and, presumably, to Renografin). A sample of the same rickettsial suspension was similarly treated, except that detergent was omitted. In contrast to the control L rickettsiae which banded at 1.20 g/ml, L rickettsiae treated with Triton X-100 did sediment at 1.23 g/ml (Fig. 6), implying that the H rickettsiae band at this density of Renografin by virtue of their lack of a permeability barrier to it.

Renografin-induced plasmolysis of rickettsiae. The above idea was tested directly by attempting to plasmolyze the purified organisms in Renografin, which is very hypertonic at gradient concentrations. Only those cells which were impermeable to Renografin could be plasmolyzed by it (12); thus, L rickettsiae were expected to be plasmolyzed in Renografin whereas H rickettsiae were not.

The extent of rickettsial plasmolysis was determined by direct visualization of the organisms in Renografin and in isoosmotic buffers. First, small drops of H and L rickettsiae suspended in different diluents were observed by dark-phase microscopy (12). L rickettsiae in 38% Renografin were distinguishable from those in SPG by the typical signs of plasmolysis, i.e., retraction of their protoplasm within the outer envelope. L rickettsiae in hypertonic sucrose (32.5%) were similar in appearance to those in the Renografin. On the other hand, the morphology of H rickettsiae in 38% Renografin or 32.5% sucrose was identical to that of H rickettsiae in SPG. Thus, in contrast to the obvious plasmolysis of L rickettsia in the hypertonic Renografin or sucrose, there was no apparent plasmolysis of H rickettsiae.

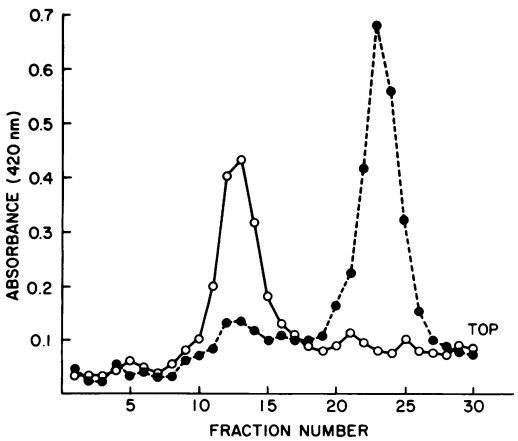

FIG. 6. *Profiles of Renografin-purified R. prowazekii (Breinl) L bands suspended in PBS (●) or in PBS + 0.1% Triton X-100 (○) and centrifuged in 30 to 45% Renografin (wt/vol in PBS) gradients.*

These observations were confirmed when H and L rickettsiae freshly removed from gradients were pelleted and resuspended in acrolein-glutaraldehyde fixative prepared either in the usual phosphate buffer or in Renografin (double-strength fixative mixed 1:1 with 76% Renografin). The control organisms, fixed in the usual way, looked identical to those shown in Fig. 2, and thus micrographs of them have been omitted. However, in the hypertonic Renografin fixative, the L rickettsiae were substantially plasmolyzed, as evidenced by large spaces between the outer and inner membranes (Fig. 7A). In contrast, the H rickettsiae fixed in Renografin showed no such signs of plasmolysis (Fig. 7B) and, in fact, appeared to be more "normal" than the H controls. Despite their "healthy" appearance when maintained in Renografin, these H organisms nevertheless still were deficient in their ability to metabolize glutamate (Table 2), ruling out the possibility that the decreased metabolic activity observed previously was due to damage occurring during the removal of Renografin and return to isoosmotic conditions.

The differential plasmolysis of H and L rickettsiae in sucrose and Renografin, therefore, indicates that H rickettsiae have lost their permeability barrier to these molecules. In all probability, the penetration of the very dense Renografin into the H rickettsiae then caused them to sediment at a greater density in gradients of Renografin than did organisms which remained impermeable to it.

DISCUSSION

These experiments demonstrated that typhus rickettsiae are separable on Renografin density gradients according to the functional integrity of their cytoplasmic membranes. Organisms which were freely permeable to Renografin sedimented to a density of 1.23 g/ml. In contrast, rickettsiae which maintained their permeability barrier to small organic molecules such as Renografin, and thus excluded them, sedimented to a density of 1.20 g/ml in Renografin.

The heavy rickettsiae were largely inactive in terms of metabolic and infectious activity. An intriguing possibility is that these organisms, which allow small organic molecules to diffuse inside, are related to the "leaky" rickettsiae described by Bovarnick and Allen (1, 2) which, under certain conditions, lost critical amounts of nicotinamide adenine dinucleotide and some other factors to the surrounding medium, while at the same time becoming biologically inactive.

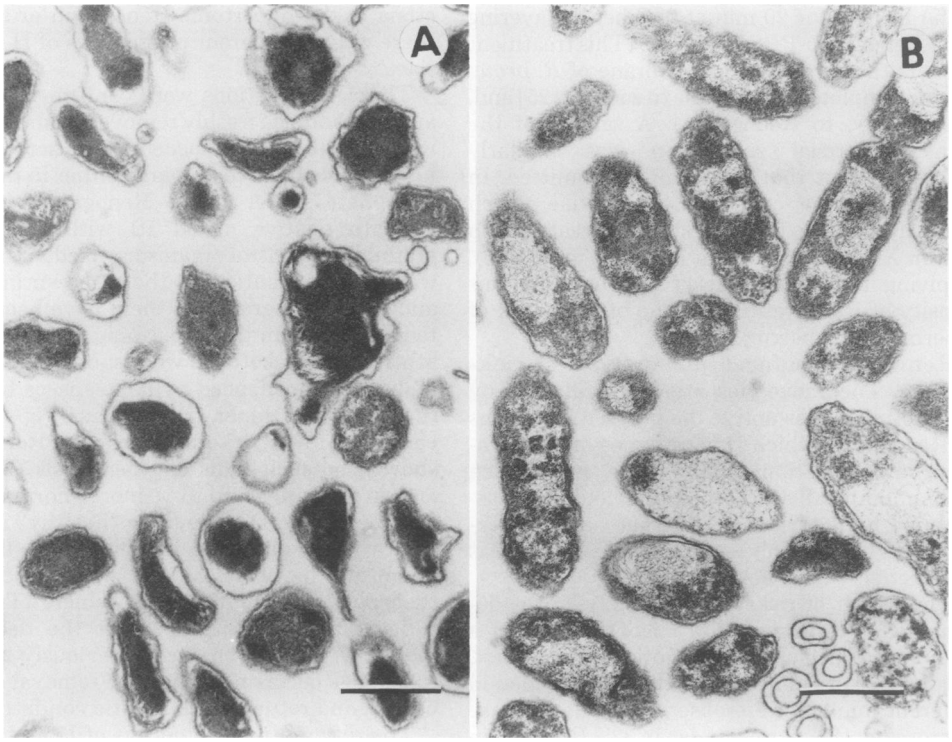

FIG. 7. *Ultrathin sections of R. prowazekii (Breinl) removed from Renografin (28 to 43%, wt/vol, in BD buffer) gradients, pelleted, and resuspended in fixative containing 38% Renografin (final concentration). (A) Rickettsiae from light bands. (B) Rickettsiae from heavy bands. Note electron density of rickettsial cytoplasm, distortion of typical bacillary morphology, and degree of plasmolysis in (A) compared to (B). Bar = 0.5 μm.*

TABLE 2. *Glutamate oxidation by H and L rickettsiae in the presence of Renografin*

Medium	Band	cpm of $^{14}CO_2$ released per 10^{10} RLB
GO buffer	L	17,700
	H	855
	H/L	0.05
GO buffer + Renografin[a]	L	14,468
	H	231
	H/L	0.02

[a] Glutamate oxidation (GO) buffer mixed 1:1 with 76% Renografin and containing 3 mg of BSA per ml, final concentration.

In their studies, addition of cofactors, especially nicotinamide adenine dinucleotide, to the medium restored rickettsial functions, sometimes fully. Although we have seen no spontaneous reversion of H rickettsiae to L organisms which would signal membrane repair, we have made no attempts to "reactivate" them in any way by adding cofactors to the suspending medium. H organisms, which are inactive and which are known to be leaky at least insofar as influx of substances is concerned, should be useful in further analyzing the question of inactivation and reactivation of rickettsiae.

Conditions which were unfavorable to rickettsial viability, such as exposure to Triton X-100 (25), changed the density of L rickettsiae from 1.20 to 1.23 g/ml. It seems likely that H organisms also arise naturally from the degeneration of viable L rickettsiae. This was indicated by the increasing numbers of H organisms as the total rickettsial population reached the stationary phase of its growth cycle in infected yolk sacs and particularly by the increased proportion of H organisms when rickettsial degeneration had occurred in nonviable eggs. Increased membrane permeability often is an early manifestation of cell injury, and our experiments suggest that this may be true for typhus rickettsiae as well.

Renografin itself did not cause degeneration of L rickettsiae into Renografin-permeable forms since no H organisms were formed from L rickettsiae freshly purified in Renografin mixed with BHI. The possibility that H rickettsiae were inactivated by the shock of being transferred from very hypertonic Renografin into an

isoosmotic buffer was also discounted because even before transfer out of Renografin, the H organisms still were unable to oxidize glutamic acid as well as control L rickettsiae in Renografin. The morphological dissimilarities between H and L rickettsiae reported here probably have no relation to those observed between log-phase and stationary-phase tissue culture preparations of *R. prowazekii*, which were not Renografin purified (16, 26). In contrast to the stationary-phase organisms described in the published studies, our yolk sac preparations did not reveal a high incidence of rickettsiae containing electron-lucent inclusion bodies.

It was found that the choice of Renografin diluents and suspending media was critical to the maintenance of the rickettsial membrane integrity. Of particular interest was the finding that SPG, a very commonly used rickettsial diluent, did not allow preservation of the permeability barrier to Renografin in all L organisms. The superiority in this respect of BHI to the other solutions tested extends the demonstration by Wike et al. (23, 24) that suspension of highly diluted rickettsiae in BHI permitted greater infectivity than did the use of alternative suspending diluents, including SPG.

At this point, the structural alterations which cause changes in rickettsial density in Renografin remain unknown, beyond the supposition that some type of cytoplasmic membrane damage is involved. The broad intermediate band formation after brief exposure of L rickettsiae to hypotonic buffers could be caused by a transient increase in cytoplasmic membrane permeability due to cellular swelling. This could allow the entry of varying amounts of Renografin into the organisms before the permeability barrier is restored, as the rickettsiae pass from the hypotonic buffer into the hypertonic gradient.

Other variations observed in L rickettsial density have been more subtle. The Renografin density at which the light bands of rickettsiae form has been found to vary between 1.204 and 1.197, often with a trailing shoulder extending upward (unpublished data). Although the shoulder is more likely to occur in some diluents (e.g., SPG) than others, neither the cause nor the significance of this apparent heterogeneity is understood. Weiss et al. (20) reported that they occasionally found two barely separable L bands after centrifugation of *Rickettsia typhi* in Renografin.

Other bacteria have shown heterogeneous banding in Renografin gradients. Chylamydiae formed two bands in Renografin (7, 9); the major band (density 1.20 g/ml) contained both the infectious elementary and the noninfectious reticulate bodies characteristic of chlamydial suspensions. The minor band (density 1.14 g/ml) was not further characterized. Likewise, *Bacillus subtilis* sedimented to two densities in Renografin (5, 8). This banding pattern was of great significance, since the minor, less dense band was greatly enriched with cells capable of being transformed. In neither of these instances was there any indication of the reason for the occurrence of organisms with two densities, and consequently there can be no assessment of their relevance to the present study.

The experiments reported here do pertain to reports of heterogeneous rickettsial banding in CsCl gradients, however. *C. burnetii* in phase I gave rise in two bands in CsCl (22), and inactivation with Formalin (18, 19, 22) or ultraviolet light (18, 19) caused a shift of organisms from the light to the heavy band. (It also has been reported that lengthy centrifugation of *C. burnetii* at lowered pH in sucrose caused the appearance of a heavy band, but that ultraviolet light or Formalin treatment, which caused heavy band formation in CsCl, did not have that effect in sucrose [18]). Noninactivated phase I *C. burnetii* which naturally formed two bands in CsCl also were easily distinguished morphologically, the rickettsiae in the upper band being smaller with more condensed nucleoids than those in the lower band (22). *C. burnetii* organisms which sedimented in heavy and light bands in CsCl also formed heavy and light bands, respectively, in Renografin and in sucrose. A major difference between *C. burnetii* and *R. prowazekii*, however, is the fact that *Coxiella* organisms in heavy and light bands were nearly equally infectious (22).

The results of this study have demonstrated that, in addition to providing a system of monitoring rickettsial cytoplasmic membrane integrity, Renografin gradients offer another advantage for the purification of *Rickettsia* species. This lies in the capacity of these gradients selectively and efficiently to remove large proportions of inactive organisms from the original suspension, leaving a preparation enriched with infectious rickettsiae. These findings add to the previously described (6, 20) benefits of purification in Renografin gradients when highly infectious and uniform rickettsial suspensions are required.

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LITERATURE CITED

1. **Bovarnick, M. R., and E. G. Allen.** 1957. Reversible inactivation of typhus rickettsiae at 0°C. J. Bacteriol.

73:56–62.
2. **Bovarnick, M. R., and E. G. Allen.** 1954. Reversible inactivation of typhus rickettsiae. I. Inactivation by freezing. J. Gen. Physiol. **38**:169–179.
3. **Bovarnick, M. R., and J. C. Miller.** 1950. Oxidation and transamination of glutamate by typhus rickettsiae. J. Biol. Chem. **184**:661–676.
4. **Bovarnick, M. R., J. C. Miller, and J. C. Snyder.** 1950. The influence of certain salts, amino acids, sugars, and proteins on the stability of typhus rickettsiae. J. Bacteriol. **59**:509–522.
5. **Cahn, F. H., and M. S. Fox.** 1968. Fractionation of transformable bacteria from competent cultures of *Bacillus subtilis* on Renografin gradients. J. Bacteriol. **95**: 867–875.
6. **Dasch, G. A., and E. Weiss.** 1977. Characterization of the Madrid E strain of *Rickettsia prowazekii* purified by Renografin density gradient centrifugation. Infect. Immun. **15**:280–286.
7. **Friis, R. R.** 1972. Interaction of L cells and *Chlamydia psittaci*: entry of the parasite and host responses to its development. J. Bacteriol. **110**:706–721.
8. **Hadden, C., and E. W. Nester.** 1968. Purification of competent cells in the *Bacillus subtilis* transformation system. J. Bacteriol. **95**:876–885.
9. **Howard, L., N. S. Orenstein, and N. W. King.** 1974. Purification on Renografin density gradients of *Chlamydia trachomatis* grown in the yolk sac of eggs. Appl. Microbiol. **27**:102–106.
10. **Karp, A.** 1954. An immunological purification of typhus rickettsiae. J. Bacteriol. **67**:450–455.
11. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
12. **Myers, W. F., P. J. Provost, and C. L. Wisseman, Jr.** 1967. Permeability properties of *Rickettsia mooseri*. J. Bacteriol. **93**:950–960.
13. **Ormsbee, R., M. Peacock, R. Philip, E. Caspar, J. Plorde, T. Gabre-Kidan and L. Wright.** 1977. Serologic diagnosis of epidemic typhus fever. Am. J. Epidemiol. **105**:261–271.
14. **Rees, H. B., Jr., and E. Weiss.** 1968. Glutamate catabolism of *Rickettsia rickettsii* and factors affecting retention of metabolic activity. J. Bacteriol. **95**:389–396.
15. **Silverman, D. J., P. Fiset, and C. L. Wisseman, Jr.** 1979. Simple differential staining technique for enumerating rickettsiae in yolk sac, tissue culture extracts, or purified suspensions. J. Clin. Microbiol. **9**:437–440.
16. **Silverman, D. J., C. L. Wisseman, Jr., and A. Waddell.** 1980. In vitro studies of rickettsia-host cell interactions: ultrastructural study of *Rickettsia prowazekii*-infected chicken embryo fibroblasts. Infect. Immun. **29**: 778–790.
17. **Silverman, D. J., C. L. Wisseman, Jr., A. D. Waddell, and M. Jones.** 1978. External layers of *Rickettsia prowazekii* and *Rickettsia rickettsii*: occurrence of a slime layer. Infect. Immun. **22**:233–246.
18. **Wachter, R. F., G. P. Briggs, J. D. Gangemi, and C. E. Pedersen, Jr.** 1975. Changes in buoyant density relationships of two cell types of *Coxiella burneti* phase I. Infect. Immun. **12**:433–436.
19. **Wachter, R. F., G. P. Briggs, and C. E. Pedersen, Jr.** 1977. Differences in buoyant-density properties of *Coxiella burnetii* and *Rickettsia rickettsii*. Infect. Immun. **15**:668–669.
20. **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.
21. **Weiss, E., H. B. Rees, Jr., and J. R. Hayes.** 1967. Metabolic activity of purified suspensions of *Rickettsia rickettsii*. Nature (London) **213**:1020–1022.
22. **Wiebe, M. E., P. R. Burton, and D. M. Shankel.** 1972. Isolation and characterization of two cell types of *Coxiella burneti* phase I. J. Bacteriol. **110**:368–377.
23. **Wike, D. A., R. A. Ormsbee, G. Tallent, and M. J. Peacock.** 1972. Effects of various suspending media on plaque formation by rickettsiae in tissue culture. Infect. Immun. **6**:550–556.
24. **Wike, D. A., G. Tallent, M. G. Peacock, and R. A. Ormsbee.** 1972. Studies of the rickettsial plaque assay technique. Infect. Immun. **5**:715–722.
25. **Winkler, H. H.** 1976. Rickettsial cell water and membrane permeability determined by a micro space technique. Appl. Environ. Microbiol. **31**:146–149.
26. **Wisseman, C. L., Jr., A. D. Waddell, and D. J. Silverman.** 1976. In vitro studies on rickettsia-host cell interactions: lag phase in intracellular growth cycle as a function of stage of growth of infecting *Rickettsia prowazeki*, with preliminary observations on inhibition of rickettsial uptake by host cell fragments. Infect. Immun. **13**:1749–1760.