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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

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Two *Rickettsia prowazeki* seeds, an "early" seed in the logarithmic or exponential growth phase and a "late" seed in the stationary or possibly early decline phase, were prepared in chicken embryo (CE) cell cultures and compared with respect to morphology and infection cycle in CE cells in culture. Differences in size and ultrastructure of the organisms in the two seeds were similar to those seen in other gram-negative bacteria at comparable stages of growth. Vacuolar structures, rare in log-phase organisms, were common in stationary-phase organisms. Minute spherical forms reminiscent of minicells were seen in the stationary-phase preparations. In quantitative uptake experiments, organisms, typical in size and morphology of each preparation, had comparable capacity per plaque-forming unit to penetrate into CE cells in suspension when the seeds had been depleted of host cell membrane fragments and other debris. This suggests that host cell fragments, presumably of membrane origin, competitively inhibit rickettsial uptake by intact CE cells. Organisms of the log-phase seed, upon entry into a host cell, entered the logarithmic or exponential phase of intracellular growth without a measurable lag phase, whereas stationary-phase organisms displayed a lag phase of about 7.5 h, during which they enlarged and increased in intensity of staining, before entering the log phase of growth.

The similarity between the intracellular growth cycle of *Rickettsia prowazeki* in chicken embryo (CE) cells in culture and that of a bacterium in fluid medium was demonstrated in a recent report (19). One of the puzzling features of the rickettsial growth cycle described in that report was the apparent absence of a lag phase. However, subsequent studies with certain different rickettsial seed preparations indicated that some displayed a lag period of about 7 h. Since the seed preparations in all of these studies had been prepared from rickettsiae grown in the yolk sac of embryonated hen eggs, in which the phase of growth at the time of harvest could not be controlled or determined by then existing methods, the variation in properties among seeds might be a function of the phase or phases of the growth cycle represented in the different seed preparations. Accordingly, to test the influence of the phase of the growth cycle of a rickettsial inoculum on the growth properties of *R. prowazeki* in cell culture, two seeds were prepared from defined stages of the first infection cycle in CE cell cultures and were partially characterized according to principles outlined in other reports (19; C. L. Wisseman, Jr., and A. D. Waddell, in preparation): (i) rickettsiae in the exponential (log) phase of growth and (ii) rickettsiae estimated to be in the stationary phase or early phase of decline. The morphology of the organisms in these two seeds was studied by both light and electron microscopy and their behavior was compared at two points in the infection cycle (19): (i) the uptake or penetration phase (Wisseman and Waddell, in preparation) and (ii) the early portion of the intracellular growth cycle (19).

MATERIALS AND METHODS

General. Secondary X-irradiated and unirradiated CE cells from specific-pathogen-free eggs (SPAFAS, Norwich, Conn.) were prepared as previously described (19-21). The tissue culture (TC) medium was Dulbecco's modification of Eagle basal medium with Earle salts, which contained 0.1% glucose (GIBCO, Grand Island, N.Y.) (15) and 10% fetal calf serum (FCS) (GIBCO). Slide chamber cultures (Lab-Tek Division of Miles Laboratories, Inc., Naperville, Ill.; catalogue no. 4808), used to study the

intracellular growth cycle, were prepared, infected, incubated at 32 C in a humid atmosphere of 5% CO_2 in air, and stained and counted as previously described (19–21). Plaque counts (PFU) were performed in CE cell monolayers by a modification of the method of Wike et al. (17), using 60-mm plastic petri dishes (Falcon Plastics, Oxnard, Calif.) and Dulbecco medium containing 5% fetal calf serum. Absolute counts of rickettsia-like bodies (RLB) were performed by a modification of the method of Silberman and Fiset (13).

Dose-response-type rickettsial uptake kinetics were measured in the suspended cell system that was developed and characterized by Wisseman and Waddell (in preparation). Equal volumes of suspensions of unirradiated CE cells (2×10^7/ml) and rickettsiae (2× desired final concentration) in TC medium at 32 C were mixed in siliconized tubes and incubated for 30 min at 32 C with gentle mixing every 5 to 10 min. After dilution in cold TC medium, Cytofuge (Shandon-Elliot, Sewickley, Pa.) smears were prepared, stained with Giménez stain (6), and counted.

Rickettsial seed suspensions. The starting rickettsial preparation (Seed K-1, E155/TC3/E3) was the Breinl strain of *R. prowazeki*, which had been freed of extraneous agents in the laboratory of B. L. Elisberg (Bureau of Biologics, Food and Drug Administration, Washington, D.C.). The seed had been plaque purified by us by our routine procedure (M. R. Jones, A. D. Waddell, and C. L. Wisseman, Jr., in preparation) and subsequently had been propagated in the yolk sac of embryonated hen eggs (SPAFAS). A 20% suspension of infected yolk sacs in 3.7% brain heart infusion broth (BBL, Bioquest, Cockeysville, Md.) was distributed to ampoules in 0.4- to 1.5-ml amounts. The ampoules were flame-sealed, quick-frozen in a dry ice-alcohol mixture, and stored at −70 C.

For the present study, rickettsial seeds in the early (log) and late (probable stationary) phases of the growth cycle, as defined in a previous publication (19), were prepared in CE cell cultures under controlled growth conditions. Thus, secondary unirradiated CE cells were infected in suspension, as described in our studies on rickettsial uptake (Wisseman and Waddell, in preparation), by mixing equal volumes of a trypsinized suspension of secondary CE cells (2×10^7 cells per ml in Dulbecco medium containing 10% fetal calf serum) and a 1:14.5 dilution of *R. prowazeki* seed K-1 in the same medium and incubating at 32 C with gentle mixing at 5- to 10-min intervals for 60 min, to give a suspension with 80 to 90% of the cells infected with an average of about 4 RLB per cell. Then, both eight-chambered slide cultures (0.3-ml suspension per chamber) and 75-cm^2 plastic tissue culture flasks (Falcon Plastics, catalogue no. 3024) (15 ml per flask) were simultaneously inoculated with the infected suspension. Both types of culture were incubated at 32 C in moist air containing 5% CO_2. Growth of the rickettsiae was monitored at intervals during the incubation period by microscopic examination of Giménez-stained slide cultures.

The early (log phase) seed was prepared by harvesting the cells from the flask cultures when examination of the slide chambers indicated that the infected cells were well into the log phase of growth (42 h in this instance with an average of 37 RLB per infected cell). The late (stationary phase) seed was prepared by harvesting the flask cultures when examination of the slide cultures indicated that the majority of infected cells were packed with minute rickettsial forms (92.5 h). Infected cells were removed from the flasks with trypsin-ethylenediaminetetraacetic acid, collected by centrifugation ($200 \times g$ for 5 min), and resuspended in a small volume of Dulbecco medium containing 10% fetal calf serum. Cells were disrupted by blending for two 90-s periods separated by a 90-s cooling period in an ice-chilled Sorvall Omnimixer. Phase contrast microscopy revealed that over 99% of the cells were disrupted by this treatment. After removal of intact cells and large particles by low-speed centrifugation ($200 \times g$, 5 min), the rickettsial suspensions were ampouled, frozen, and stored as described above.

Log- and stationary-phase seeds depleted in host cell membrane fragments were prepared by layering the crude tissue culture seeds on a column of 40% (wt/wt) sucrose, centrifuging the rickettsiae through the column, and resuspending the pellet to original volume in TC medium.

Calculations. Uptake characteristics were calculated as described by Wisseman and Waddell (in preparation) in a basic study of uptake models and kinetics. Thus, the uptake rate, m, was calculated as the average number of RLB taken up per cell per minute. The uptake efficiency, as defined for the suspended cell model, is the ratio of the number of chances of collision between a CE cell and the rickettsiae (either RLB or PFU) to the number of effective collisions, i.e., those actually resulting in the entry of an RLB into a CE cell. In the growth studies, the best-fit line was determined for the exponential growth phase by the method of least squares, using the equation

$$\log_2 N = \log_2 N_0 \text{ (calculated)} + Rt \quad (1)$$

as previously described (19), where N is the number of rickettsia-like bodies (RLB) per infected cell at time t, $\log_2 N_0$ (calculated) is the y intercept, and R is the exponential growth rate constant. The generation time (g) was calculated from the equation

$$g = \frac{1}{R} \quad (2)$$

The length of the lag phase was calculated by two methods: (i) graphically by the method of Monod (11); and (ii) mathematically, by solving equation (1) for t when the observed zero-time value (N_0 [observed]) is substituted for N:

$$t \text{ (lag)} = \frac{\log_2 N_0 \text{ (observed)} - \log_2 N_0 \text{ (calculated)}}{R} \quad (3)$$

This equation is in fact a modification of the one given by Lodge and Hinshelwood (9), simply making use of the information contained in the equation for

the regression line of the exponential growth phase. Neither graphic nor mathematical method takes into account the duration of the acceleration phase (11).

Electron and light microscopy. The two seeds of *R. prowazeki* were examined by negative staining and thin-sectioning techniques. For negative staining, 20 μl-drops of the seed suspension were placed on 300-mesh Formvar-coated copper grids, and the specimens were negatively stained with a 0.5% aqueous uranyl acetate solution.

Preparation of the seeds for thin sectioning was done as follows. The specimens were prefixed for 3 h at room temperature in a solution containing 5% acrolein and 0.25% glutaraldehyde (Polysciences, Inc., Warrington, Pa.) prepared in 0.05 M sodium cacodylate buffer, pH 7.4 (4). The cells were washed three times in the same buffer, postfixed in 1% osmium tetroxide in cacodylate buffer for 1 h at room temperature, and embedded in Epon 812 according to the method of Luft (10). Ultrathin sections (60 to 90 nm) were cut on a Porter-Blum MT-2 ultramicrotome with a Dupont diamond knife, picked up on Formvar-coated copper grids, and double stained, first with an aqueous solution of uranyl acetate (0.5%) and then with lead citrate (12). The specimens were examined in a Siemens IA electron microscope operating at 80 kV and equipped with a 400-μm condenser and a 50-μm objective aperture.

Photomicrographs were made of Giménez-stained (6) preparations (using Kodak Panatomic X film in a Zeiss photomicroscope as previously described [19]).

RESULTS

Morphology of the rickettsiae in the log- and stationary-phase seeds. The morphology of the early (log phase) and the late (stationary phase) seeds of *R. prowazeki* differed significantly from one another in several respects.

Strongly staining, large bacillary and bipolar (dividing) forms typical of the exponential growth phase (19) were apparent in photomicrographs of infected CE cells at time of harvest (Fig. 1a) and of smears of the final log-phase seed preparation (Fig. 1b).

Seed material was also examined by electron microscopy using negative staining and thin-sectioning techniques. Both techniques were used to measure cell size (Table 1). Although some lateral shrinkage was apparent in the uranyl acetate-stained preparations, the technique proved amenable to determining the relative sizes of the seed materials. Negatively stained rickettsial cells (Fig. 2) were rod shaped and had a highly convoluted outer envelope. These surface convolutions, however, may have been due to shrinkage during the staining procedure. Cell division constrictions characteristic of cells in logarithmic growth are seen in this electron micrograph. Extraneous host cell debris remained associated with the bacterial cells. The size of the rickettsiae in uranyl acetate-stained preparations was about 1.4 by 0.32 μm (Table 1). When viewed by thin-sectioning techniques (Fig. 3), the structure of cells in the log phase appeared very similar to that of typical gram-negative bacteria. Internally there were many ribosomes, and within the electron-lucent nuclear region were numerous fibrous strands, which presumably represented part of the bacterial chromosome. Cells observed in the log phase of growth did not contain mesosomes or other visible internal cellular structures. The high membrane content of associated host cell debris is evident.

In contrast, the organisms in the late (probably late stationary phase) seed were predominantly minute coccobacillary to coccoid in shape in the CE cells at the time of harvest (Fig. 1e), in the smear of the final seed (Fig. 1f), and in the electron micrographs (Fig. 4–7). Because this seed was prepared from cultures well beyond the end of the first infection cycle (19), the seed consisted of a few larger rickettsiae from the second infection cycle (Fig. 1f). However, by electron microscopy of negatively stained preparations, most of the rickettsiae were found to be distinctly smaller (about 0.7 by 0.27 μm) than those organisms in the log-phase seed (Table 1).

Ultrastructural studies of stationary-phase rickettsiae showed numerous differences in cell size, external morphology, and internal organization when compared with those of the log-phase cells. Figure 4 shows cells typical of those harvested from the stationary phase as they appeared when stained with uranyl acetate. Most of the cells were about one-half the size of the log-phase cells. In addition, stationary-phase cells appeared to have fewer surface convolutions than those from the logarithmic stage, a characteristic that might reflect surface changes in the outer envelope. Another prominent structural feature of cells in the stationary phase was the appearance of small spherical bodies that seemed to bud from the poles of the parent cell (Fig. 5). These small bodies may represent aberrant division forms similar to those described in cells of *Escherichia coli* that lacked deoxyribonucleic acid and were termed minicells (1, 5). Whether these small rickettsial bodies contain deoxyribonucleic acid has not been determined.

In thin section (Fig. 6), cells from the stationary phase appeared significantly different in their internal organization from logarithmic-phase cells. Perhaps the most dramatic feature was the presence of large inclusion bodies in about 70% of the stationary-phase cells. These inclusions, whose chemical nature is unknown, ranged in size from 50 to 125 nm and, in some

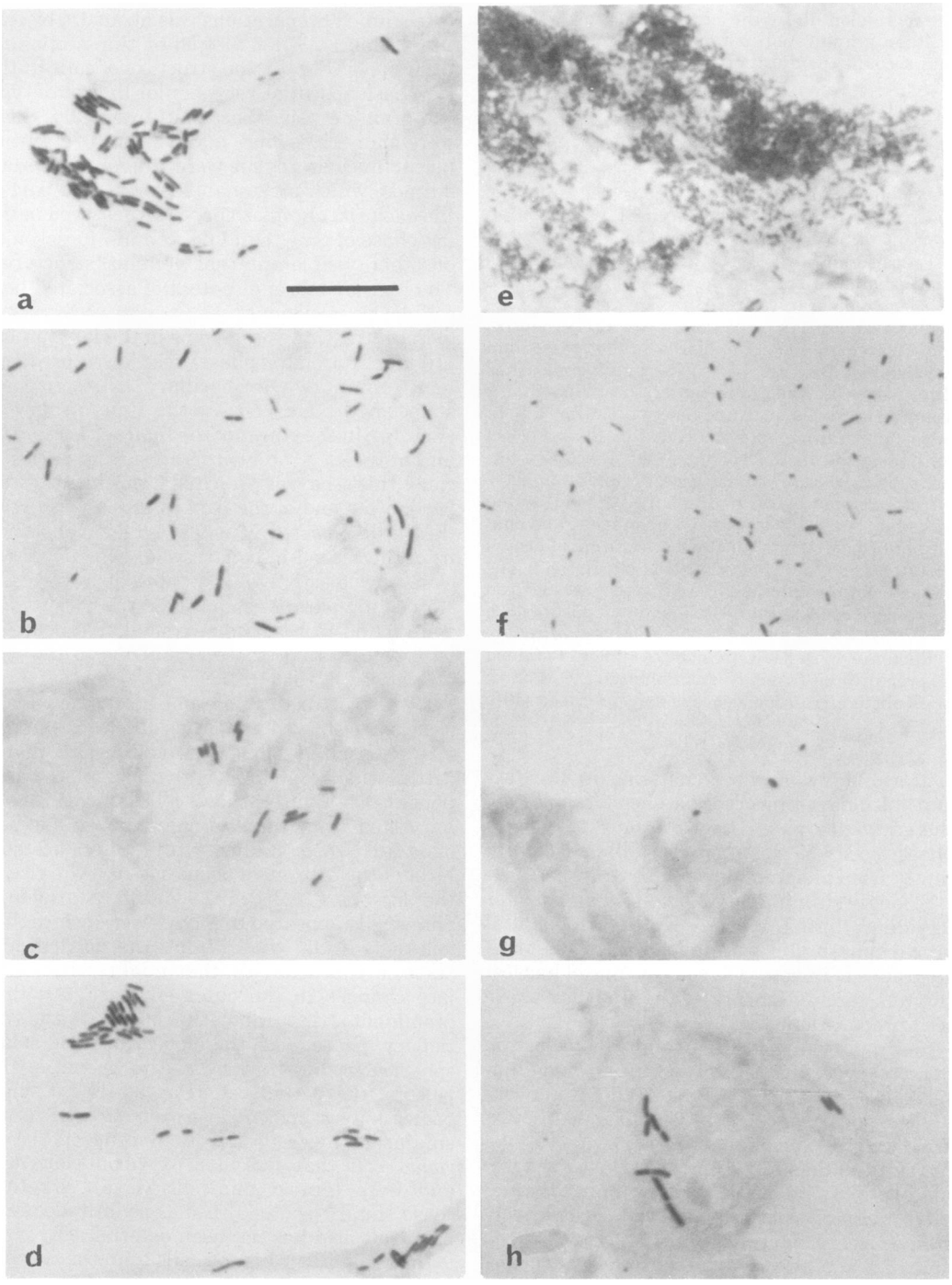

FIG. 1. *Photomicrographs of various Giménez-stained preparations of log- and stationary-phase seeds of R. prowazeki. (a–d) Log-phase seed. (a) Appearance of intracellular rickettsiae at time of harvest for seed preparation. (b) Smear of final seed preparation. (c) Intracellular rickettsiae at zero time in growth experiment. (d) Intracellular rickettsiae after 12 h of incubation in growth experiment. (e–h) Stationary-phase seed. (e) Appearance of intracellular rickettsiae at time of harvest for seed preparation. (f) Smear of final seed preparation. (g) Intracellular organisms at zero time in growth experiment. (h) Intracellular rickettsiae after 12 h of incubation in growth experiment. Bar, 10 μm.*

TABLE 1. *Some morphological characteristics of early and late R. prowazeki seeds*

Seed	Dominant morphology by light microscopy[a]	EM measurements[b]		Vacuoles[c]
		Length (μm)	Diameter (μm)	
Early (log phase)	Strongly staining bacillary forms	1.4[d] (1.0–1.7)[e]	0.32 (0.30–0.35)	Rare
Late (stationary phase)	Lightly staining coccobacillary forms	0.7 (0.48–1.0)	0.27 (0.2–0.35)	Numerous

[a] Giménez-stained preparations.
[b] EM, Electron microscope. Cell length measurements made on thin-sectioned material were similar to those made on the uranyl acetate preparations. However, the average cell width measurements made on thin-sectioned cells were significantly greater (0.5 μm for early and 0.45 μm for late) than those made on the uranyl acetate-stained preparations. Mean/range.
[c] Observations on ultrathin sections.
[d] Mean.
[e] Range.

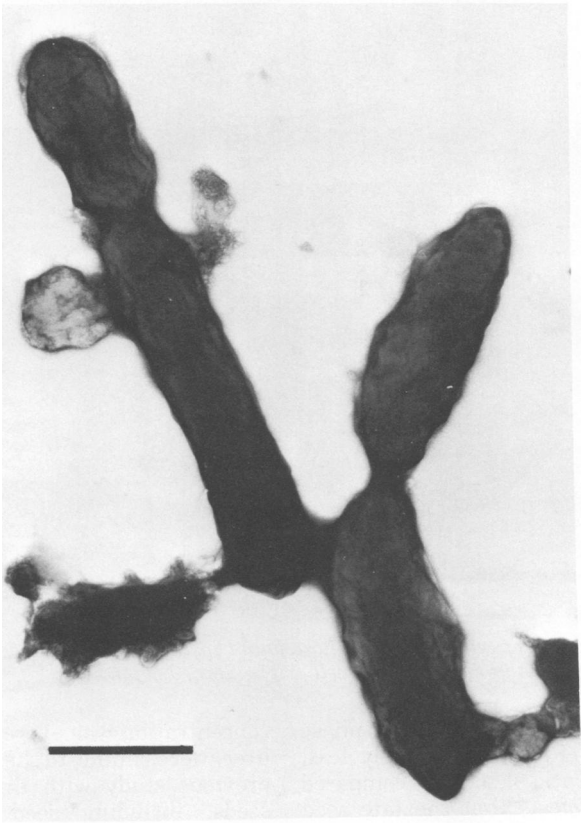

FIG. 2. *Uranyl acetate-stained preparation of the log-phase seed of R. prowazeki. Bar, 0.5 μm.*

cells such as that shown in Fig. 7, they appeared to make up as much as 20 to 30% of the sectional area of the cell. They have been found only rarely in cells harvested from the log phase of growth. In addition, some cells in the stationary phase appeared to have fewer ribosomes than those cells from the log phase. However, a quantitative study was not carried out and, moreover, no way exists to distinguish between viable and dead cells (see below). Abundant host cell debris, including membrane fragments, was also visible in this preparation (Fig. 6).

Quantitation of rickettsial content of seeds. Counts of RLB and PFU (Table 2) revealed that, although the stationary-phase seed con-

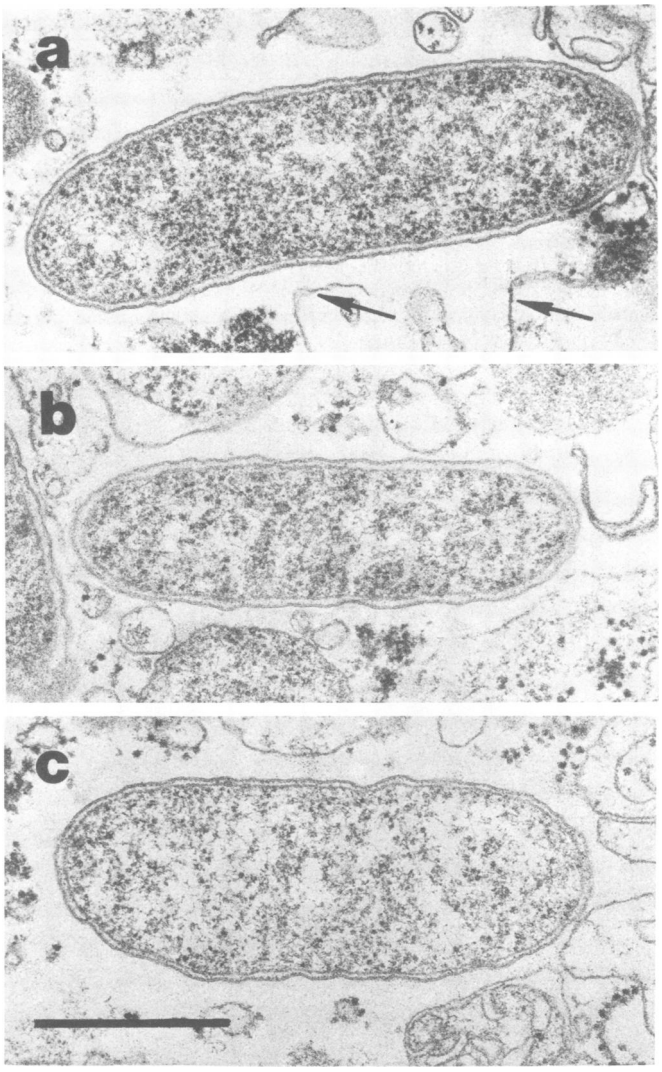

FIG. 3. *Ultrathin sections of cells typical of those found in the log-phase seed of R. prowazeki. Arrows indicate membrane fragments probably of host cell origin. Bar, 0.5 μm.*

tained many more RLB than did the log-phase seed, the number of PFU was actually less, giving an RLB/PFU ratio of 266:1 as compared with 18.4:1. This suggests that the late seed may have contained a high proportion of dead or noninfectious organisms and may actually have been in a stage of accelerating organismal death rather than a true stationary phase.

Uptake kinetics. The capacity of the log- and stationary-phase rickettsial seeds to infect CE cells was compared in dose-response-type uptake experiments, using the suspended cell system (Wisseman and Waddell, in preparation). The regression lines obtained in a single experiment with the log-phase seed, when rickettsial concentration was expressed as PFU per milliliter, were similar to the means obtained in the previous study with three separate yolk sac seeds, including seed K-1, differing only slightly in slope (Fig. 8). In contrast, the regression lines obtained from two replicate experiments with the stationary-phase seed were displaced upward substantially and significantly from those of both the yolk sac seeds and the log-phase tissue culture seed, which suggested a higher uptake efficiency.

Substantial differences were also found when the efficiency of uptake (number of chances of collision between CE cell and rickettsia expressed as PFU per effective collision) was cal-

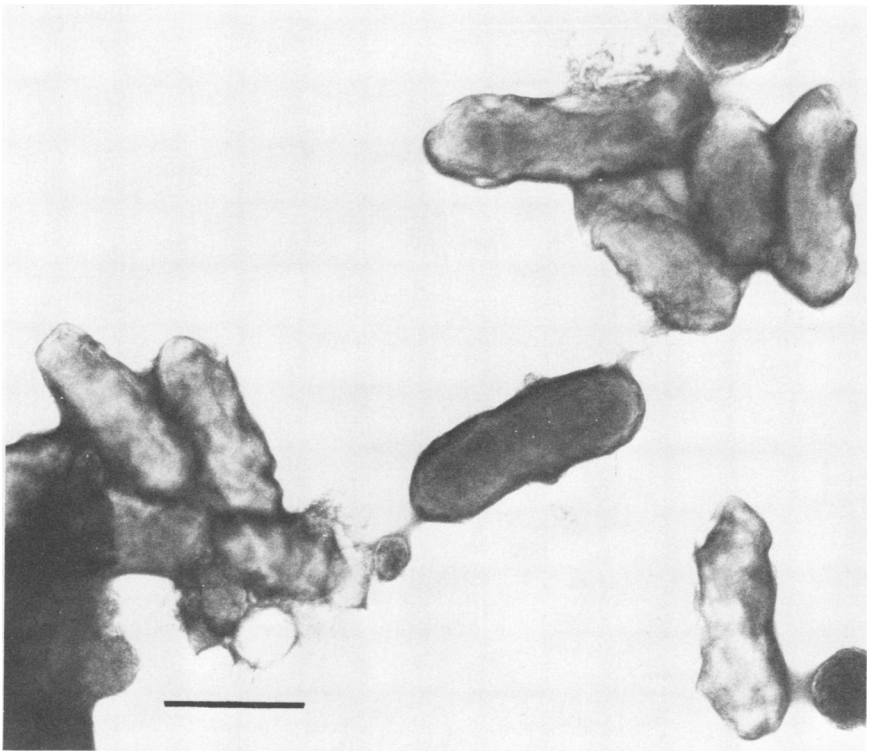

FIG. 4. *Uranyl acetate-stained preparation of a portion of the stationary-phase seed of R. prowazeki. Bar, 0.5 μm.*

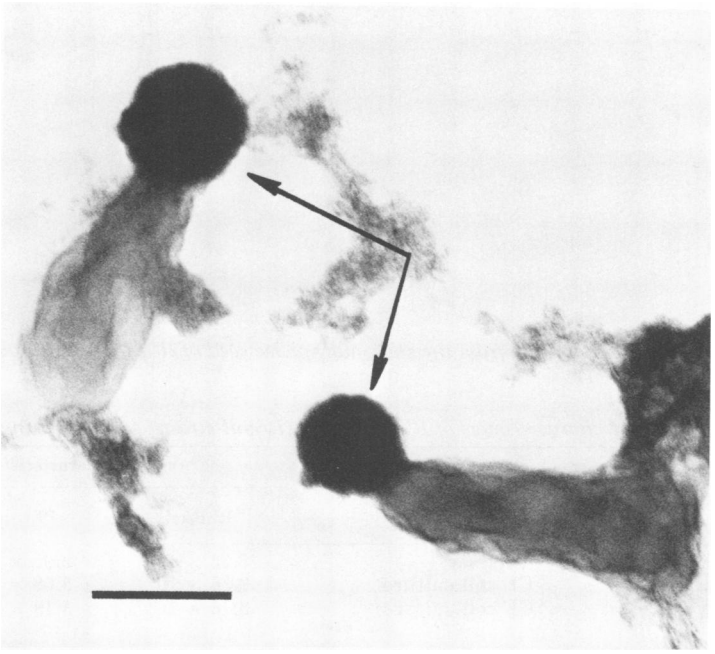

FIG. 5. *Uranyl acetate-stained preparation of a portion of the stationary-phase seed of R. prowazeki showing small spherical bodies at the poles of the parent cells (arrows). Bar, 0.5 μm.*

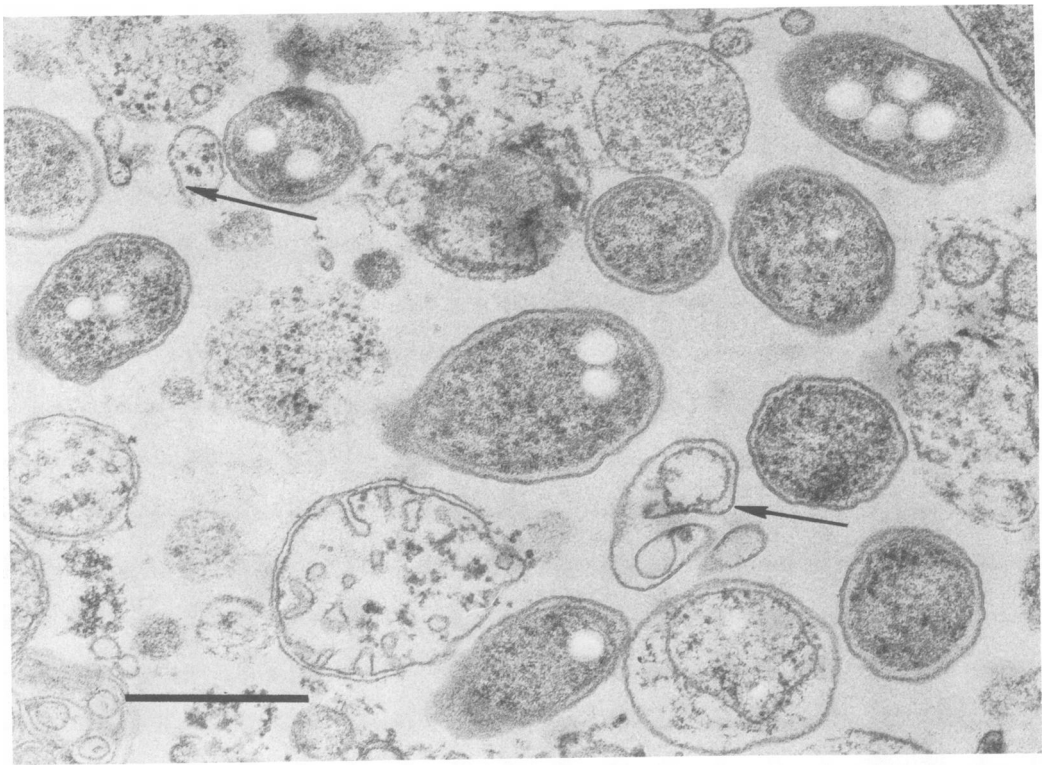

FIG. 6. *Ultrathin section of cells typical of those found in the stationary-phase seed of R. prowazeki. Arrows indicate membrane fragments probably of host cell origin. Bar, 0.5 μm.*

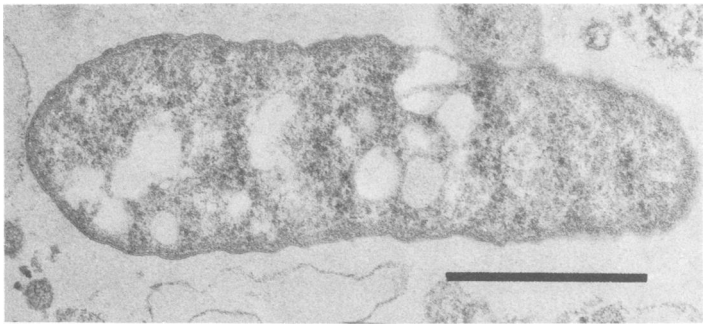

FIG. 7. *Ultrathin section of a cell from the stationary-phase seed of R. prowazeki showing many inclusion bodies. Bar, 0.5 μm.*

TABLE 2. *Some characteristics of R. prowazeki (Breinl strain) seeds used in this study*

Prepn	Grown in:	Characteristics		
		RLB/ml	PFU/ml	RLB/PFU ratio
Seed K-1[a]	Yolk sac	5.35×10^9	2.05×10^8	26.1:1
Early (log-phase) seed[b]	CE cell culture	9.36×10^8	5.08×10^7	18.4:1
Late (stationary-phase) seed[b]	CE cell culture	3.16×10^9	1.19×10^7	266:1

[a] Plaque-purified *R. prowazeki* (Breinl).
[b] Inoculum: seed K-1.

culated (Table 3) as described in the basic uptake paper (Wisseman and Waddell, in preparation). Again, the log-phase seed gave a figure that was very similar to those calculated for yolk sac seeds in the previous study. The efficiency of uptake of the stationary-phase seed appeared to be considerably greater than that of both the log-phase tissue culture and yolk sac seeds.

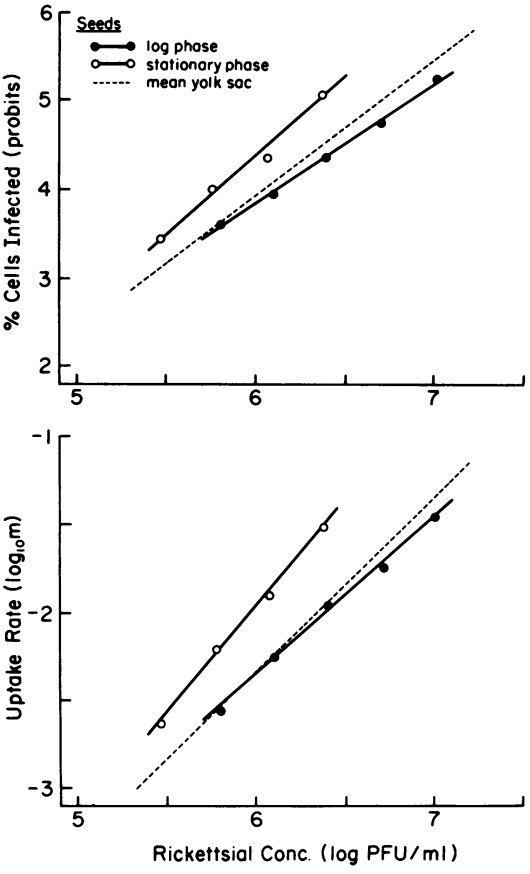

FIG. 8. *Dose-response uptake relationships in suspended CE cell system of crude tissue culture log- and stationary-phase R. prowazeki seeds compared with those of crude yolk sac grown seeds in previous study (Wisseman and Waddell, in preparation).*

The seed preparations were crude homogenates of concentrated infected CE cells that contained large quantities of host cell membranes (see Fig. 3 and 6). Since the uptake appears to require attachment between rickettsia and host cell membrane through postulated membrane receptor sites (Wisseman and Waddell, in preparation), it is possible that the differences observed between the two seeds could have resulted from competition between receptors on membrane fragments in the crude seed and on intact host cells for the rickettsiae, much as has been demonstrated in the case of rickettsial hemolysis (18; L. Warfel et al., in preparation). Accordingly, rickettsial suspensions, depleted of membrane fragments by centrifugation through a 40% sucrose column, were prepared from the small amounts of crude seed remaining and were tested again in a suspended cell dose-response experiment. Because of the small amounts of original seeds remaining, only rickettsial suspensions of reduced titer were possible (log-phase seed, 6.83×10^6 PFU/ml/ stationary seed, 3.21×10^6 PFU/ml). Hence, only three and two usable points were possible with the log-phase and stationary-phase seed, respectively. Microscopic examination revealed that the typical large rickettsiae from the log-phase seed and minute forms from the stationary-phase seed were actually taken up by the CE cells, which indicated that the purification process did not select for one form (Fig. 9). The displacement observed in the crude seeds disappeared and the five points from the two partially purified seeds appeared to fall around single regression lines with correlation coefficients of 0.93 and 0.95 with the probit-log PFU and log uptake rate per cell-log PFU plot, respectively, whose slopes were substantially steeper than those with any of the crude preparations (Fig. 8). Moreover, the uptake efficiencies of both membrane-depleted

TABLE 3. *Uptake efficiency of crude and membrane-depleted early and late R. prowazeki seeds by CE cells in dose-response suspended-cell experiments*

Seed	Rickettsial concn		Uptake efficiency[a]	
	RLB/ml (log_{10})	PFU/ml (log_{10})	RLB	PFU
Crude				
Early (log phase)	7.07–8.27	5.80–7.01	93 ± 15	5.04 ± 0.88
Late (stationary phase)	7.90–8.80	5.17–6.37	567 ± 165	2.11 ± 0.62
Membrane-depleted				
Early (log phase)		5.54–6.14		0.13
Late (stationary phase)		5.51–5.81		0.19

[a] Chances of collision per effective collision, calculated on total RLB count or PFU. Since plaquing efficiency is probably substantially <1, uptake efficiencies of <1 on a PFU basis are possible.

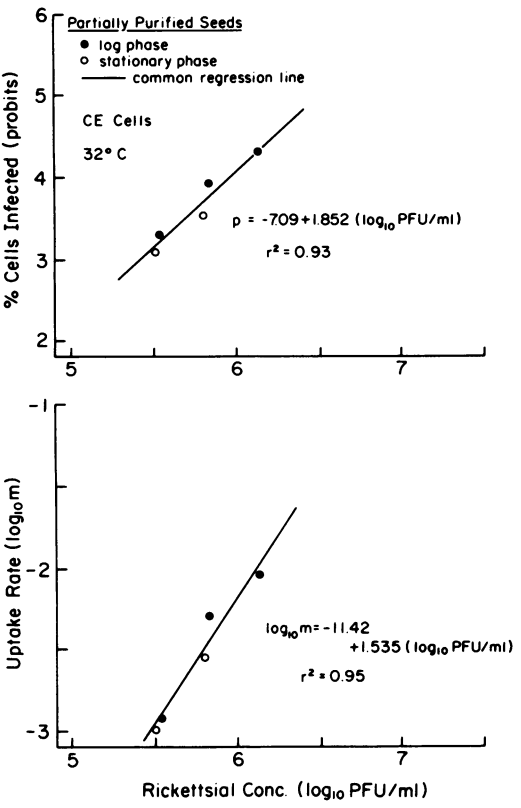

FIG. 9. *Dose-response uptake relationships in suspended CE cell system of membrane-depleted tissue culture log- and stationary-phase R. prowazeki seeds.*

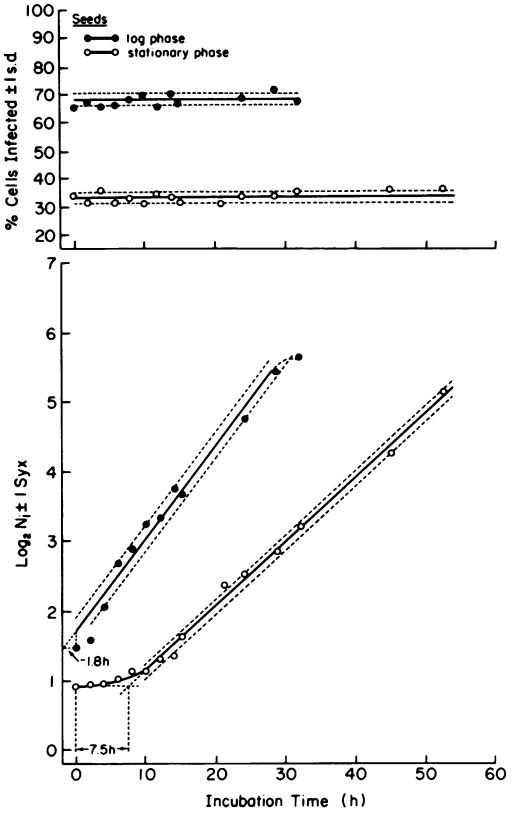

FIG. 10. *Comparison of early growth stages of log- and stationary-phase R. prowazeki seeds in first infection cycle in CE cells in slide chamber cultures. Demonstration of a lag phase with stationary-phase seed inoculum. The observations were confined to the first infection cycle, as indicated by the constant value of percent cells infected. In neither instance did the observed points form a line whose slope differed significantly from zero; hence, the means of all observations (solid lines) ± 1 standard deviation (s.d.) (dotted lines) were drawn to represent the data. The best-fit straight line to fit the points on the exponential phase of growth was determined by the method of least squares and is represented by a solid line ± 1 standard error of determination (Syx) (dotted lines).*

seeds (Table 3) were very much greater than those of the crude preparations. These results strongly suggest that host cell membrane fragments compete with the plasma membranes of intact cells for the rickettsiae, as postulated above.

Intracellular growth: demonstration of a lag phase with a "late" seed. In experiments specifically designed to detect a lag phase with rickettsial seeds prepared from organisms in the two defined stages of growth, the following results were obtained (Fig. 10; Table 4).

(i) The rickettsiae that had been harvested from cultures in the log phase of growth, despite the interlude of freezing, storage and thawing, readily penetrated CE cells in culture, the organisms actually penetrating having a similar morphology to the dominant form in the seed (Fig. 1c). They began to grow and divide without a detectable lag phase. Indeed, a slight negative value was calculated for the lag phase (Table 4), which probably reflects the

TABLE 4. *Lag phase and growth rate of early and late R. prowazeki seeds in CE cell slide chamber cultures*

Seed	Lag-phase length (h)		Log-phase generation time (h)
	Graphic method	Mathematical method	
Early (log phase)	(−1.6)	(−1.85)	7.46
Late (stationary phase)	+7.5	+7.47	10.75

experimental error of the methods used. The results obtained with this seed were comparable to those reported in the original description of the intracellular growth cycle (19). The light-microscope morphology of the intracellular organisms after 12 h of growth, well into early exponential phase, was similar to that of the organisms at zero time and in the seed (Fig. 1d).

(ii) Rickettsiae of the seed that had been harvested late in the growth cycle, equivalent to the stationary phase or perhaps even phase of exponential death, also penetrated the CE cells, with minute coccobacillary forms well represented (Fig. 1g). However, in contrast to the log-phase seed, there was a lag of about 7.5 h before they entered the exponential phase of growth (Fig. 10; Table 4). During this lag phase the organisms increased in size and eventually assumed (at 12 h) a typical bacillary morphology (Fig. 1h) in the early log or exponential growth phase. Thus, as with other bacteria, the minute stationary-phase organisms increase in size before they begin to divide and enter the maximal, exponential growth phase.

Cultures infected with each seed were observed for a substantial portion of the exponential growth phase of the first infection cycle to establish rate of growth (Fig. 10; Table 4). Despite the fact that the two growth experiments were carried out at the same time on the same slides using the same lot of medium, the same batch of CE cells, and the same incubator, the generation time of the rickettsiae during the exponential growth phase was numerically longer in cells infected with the stationary-phase seed (10.75 h) than in the cells infected with the log-phase seed (7.46 h). The reasons for this difference are unknown at the present time.

DISCUSSION

Certain characteristics of the two tissue culture-grown *R. prowazeki* seeds, i.e., the early or log-phase and the late or stationary-phase seeds, differed substantially. They displayed differences in size and ultrastructure that were consistent with those of certain other bacteria in comparable growth phases (3, 7, 8, 11, 16, 19). Of particular interest is the restriction of the vacuoles, presumably representing storage granules or inclusions that have been noted previously (2, 14), to organisms in the later phases of growth. Moreover, the RLB/PFU ratio of the late or stationary-phase seed was greater by an order of magnitude than that of the log-phase seed, suggesting that the former contained a large proportion of dead or noninfectious organisms. Although physiological studies were not performed on these organisms, another study (Wisseman et al., in preparation) indicated that late rickettsiae are much less susceptible to spheroplast formation than are log-phase rickettsiae in the presence of penicillin, which suggests that the late-growth-phase rickettsiae, like many bacteria, no longer are in an active growth state. The present study examined the influence of the growth phase of the inoculum, represented by these two seeds, on two stages of the infection cycle of *R. prowazeki*, as previously defined (19): (i) uptake (or penetration), i.e., the entry of the rickettsia into the host cell; and (ii) the intracellular growth cycle.

One of the properties of typhus rickettsiae that sets them apart from most other bacteria, aside from the dependence upon another cell for growth, is the capacity to penetrate through the plasma membrane of the host cell (Wisseman and Waddell, in preparation). Rickettsiae with the characteristic light-microscope morphology of both log- and stationary-phase organisms had the capacity to penetrate into CE cells in suspension. Dose-response experiments with the quantitative suspended-cell uptake system (Wisseman and Waddell, in preparation), with standardized rickettsial seeds prepared from infected yolk sacs (i.e., 20% yolk sac) that contained more or less constant amounts of host cell fragments, had shown that the uptake of three different seed preparations fell remarkably close to a common regression line when dose was expressed as PFU per milliliter. This was not the case with the crude tissue culture seeds of the early and late growth phases in the present study in which the concentration of host cell fragments differed between the two preparations. However, when the seeds were depleted of host cell fragments, the uptake of early and late seeds fell close to a common regression line with a slope that was steeper than that with the yolk sac seeds of previous experiments or the crude tissue culture seeds of the present study. The difference was even more striking when the efficiency of uptake was calculated. We interpret this to mean that host cell fragments, presumably membranes, compete with intact host cells for rickettsial attachment.

In intracellular growth experiments with the two seeds, the size of the organisms that actually gained entrance into the CE cells appeared to be representative of the organisms in the respective seed, suggesting that despite their morphological differences, both log-phase and stationary-phase organisms retained the capacity to penetrate host cells in the slide chamber system as well (Wisseman and Wad-

dell, in preparation). The two seeds, however, differed strikingly in their behavior during the first few hours of the intracellular growth cycle. Organisms from the log-phase seed appeared to begin division immediately at a maximum constant rate without a lag phase measurable by the methods used; i.e., it entered the log or exponential phase immediately, as was observed in the previous study (19). The organisms did not show any striking changes in size or staining characteristics in this early growth period. On the other hand, the late- or stationary-phase seed exhibited a classical lag phase of about 7.5 h under similar growth conditions. In spite of the fact that the stationary-phase inoculum may have contained a significant proportion of dead organisms, the probability that the observed lag phase was real instead of apparent (8, 9) is greatly increased by the facts that (i) the organisms actually observed for growth had actively penetrated into their host cells, (ii) the organisms that penetrated into the CE cells were of the same minute coccobacillary morphology characteristic of the majority of organisms in the stationary-phase population, and (iii) these intracellular organisms increased in size and staining intensity during the first few hours of incubation before they began to divide. At this point, they were morphologically indistinguishable from the organisms derived from the log-phase seed. Thus, it is clear that the presence or absence of a measurable lag phase in the intracellular growth cycle of *R. prowazeki* depends upon the growth phase or physiological state of the inoculum, a phenomenon which strengthens the conformity of the intracellular growth cycle of *R. prowazeki* to the classical bacterial growth cycle.

In addition to providing information on the basic infection cycle of *R. prowazeki* (19), this study indicates means by which rickettsial preparations of predetermined, highly predictable characteristics can be prepared. It also suggests practical means by which seeds with highly efficient uptake characteristics can be prepared and by which interference by tissue fragments with quantitation of rickettsiae can be minimized. Moreover, it suggests improved experimental approaches to the study of the interaction between rickettsiae and host cell membranes, especially as related to uptake and cytotoxic phenomena.

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