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In Vitro Studies on Rickettsia-Host Cell Interactions: Intracellular Growth Cycle of Virulent and Attenuated *Rickettsia prowazeki* in Chicken Embryo Cells in Slide Chamber Cultures

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Upon entry into the cytoplasm of irradiated chicken embryo cells in slide chamber cultures infected over a 2-h period, yolk sac-grown virulent (Breinl strain) and attenuated (E strain) *Rickettsia prowazeki* underwent indistinguishable reproducible intracellular growth cycles. They promptly entered an exponential growth phase, without detectable lag and without microscopic evidence for any unusual early replicative phase. The generation time for both strains was 8.8 to 8.9 h at 34 C. During most of this period, the state of the organisms and growth were very similar from one cell to another. The exponential-growth phase continued for at least 36 to 48 h, when the rickettsiae became too numerous to count by microscopic examination. Between about 36 and 48 h, cells packed with rickettsiae began irregularly to break down and release organisms. These began to initiate new infection cycles in previously uninfected cells over many hours, as demonstrated by the rise in percentage of cells infected, yielding a highly disordered infected culture with different cells containing rickettsiae in diverse stages of growth. The organisms underwent regular minor changes in morphology, similar to those seen in bacterial cultures, in the first infection cycle. As the cells became packed with rickettsiae, the microorganisms regularly diminished in size to become minute coccobacillary to coccoid forms. However, the rickettsiae in the second and subsequent infection cycles in aging cultures often assumed filamentous or swollen bizarre forms. Only the first infection cycle conformed closely to the concept of a one-step growth cycle. A set of terms is proposed and defined for the infection cycle.

Although typhus rickettsiae have been grown in tissue culture by various investigators from the very early days of tissue culture, first in tissue or organ explants (32, 33, 45, 55) and then in agar slant or Maitland-type cultures of minced tissues (36-40, 59), these methods did not permit easy quantitation of rickettsiae or systematic sequential examination of cell populations infected at about the same time. Much later, Smadel and his associates (6, 10, 46), who concentrated on *Rickettsia tsutsugamushi* but who also made some observations on *Rickettsia prowazeki* as well as several other rickettsial organisms, began to apply modern cell culture methods in a quantitative way to the study of the rickettsial infection cycle. They used colchicine to prevent host cell division which improved the accuracy of direct microscopic rickettsial counts in growth experiments. Weiss and Dressler (50) then showed that cultures of nondividing, X-irradiated cells would also support the growth of *R. prowazeki*.

We have adapted improved culture systems for the quantitative study of the infection cycle of *R. prowazeki*. Another study (C. L. Wisseman, Jr., and A. D. Waddell, manuscript in preparation) describes the kinetics of the process of infection ("uptake") of chicken embryo cells (CE) by virulent and attenuated strains of *R. prowazeki*. The present paper describes kinetic and light-microscope morphological aspects of the intracellular growth cycle of both virulent and attenuated strains of *R. prowazeki* in CE cells in slide chamber cultures and proposes a tentative set of terms for the rickettsial infection cycle.

MATERIALS AND METHODS

General. Eight-chambered slide cultures (Lab Tek Products, no. 4808) of well-separated secondary X-

irradiated CE cells, prepared as previously described (53, 54), were used throughout this study, except that the medium was Dulbecco's modification of Eagle minimum essential medium with Earle salts (48), containing 10% fetal calf serum (GIBCO). They were infected by incubating for 2 h at 34 C with an appropriate dilution in tissue culture medium of yolk sac-adapted, yolk sac-grown seeds of either virulent Breinl strain (yolk sac seed 72-1; 7.8×10^7 plaque-forming units [PFU]/ml) or attenuated E strain (271 yolk sac passage; 3.7×10^7 PFU/ml) of *R. prowazeki*. The unadsorbed inoculum was removed, the chambers were washed two to three times with medium, and, finally, 0.3 ml of complete medium was added to each chamber. The slide cultures were incubated at 34 C in a humid atmosphere of air containing 5% CO_2.

Measurement of growth. Immediately after the 2-h adsorption period (time zero) and at specified intervals thereafter, three slides were removed for staining by Giménez method (14) or Giemsa stain as previously described (53, 54). A total of 800 cells was counted for each time period, using the oil immersion lens. As in previous studies, any microorganism falling within the borders of the host cell was arbitrarily counted. In this study, the percentage of cells infected and the average number of rickettsiae per infected cell were the basic data employed. Measurements of the percentage of rickettsiae dividing and average length of the rickettsiae were made from prints (8 by 10 inches [14.3 by 25.4 cm]) of photomicrographs (Kodak panatomic X film, Zeiss photomicroscope) of representative areas of the slide cultures.

Calculations. The following conventional bacterial growth equations and derived characteristics (11, 21, 34, 35) were employed:

$$N = N_0 2^{Rt} \quad (1)$$

$$\log_2 N = \log_2 N_0 + Rt \quad (2)$$

$$R = \frac{\log_2 N_2 - \log_2 N_1}{t_2 - t_1} \quad (3)$$

$$g = \frac{1}{R} \quad (4)$$

where $N_{0, 1, 2 \ldots t}$ = average number of rickettsiae per infected cell at time 0, 1, 2 . . . or t hours; t = time in hours; R = growth rate (i.e., divisions per hour); and g = generation time in hours.

Equation (2) was subjected to linear regression analysis (3, 7) over the linear or exponential portion of the growth curve, with r = correlation coefficient. The graphic method of Monod (35) was used to examine for the presence of a "lag" phase of growth.

RESULTS

Growth of virulent Breinl strain of R. prowazeki in slide chamber cultures. The organism underwent a well-defined, reproducible intracellular growth cycle. Figure 1 presents in graphic form the growth cycle data from one of three similar experiments carried out with the virulent Breinl strain of *R. prowazeki*. The photomicrographic morphology at various points in the growth cycle is shown in Fig. 2 to 4.

From the end of the adsorption period onward, morphologically identifiable organisms of typical structure, easily and characteristically stained, were continuously present in increasing numbers. The percentage of cells infected remained constant, within narrow limits of experimental error, throughout the entire first cycle of infection.

The organisms began to grow (increase in average length), to divide (increase in percentage of dividing forms), and to increase exponentially in number per infected cell almost immediately after entry into the host cells, without a graphically measurable lag or adaption phase (35). This exponential ("log") growth phase continued at least until the organisms became too numerous to count, which occurred between 36 and 48 h after infection. Over the measurable period of exponential growth, the generation time was 8.8 h, and the organisms were remarkably comparable from cell to cell.

Between 36 and 48 h after infection, the cytoplasm of the CE cells became so packed with rickettsiae that they could not be counted. Because of this, it is not known if the rate of growth remained constant or if it slowed to approach a "stationary" phase. The possible existence of a stationary phase and a phase comparable to the phase of exponential death of bacterial cultures will be explored in other systems. However, during this period (36 to 48 h) some infected cells began to break up ("burst"), thus marking the beginning of the end of the first infection cycle. Rickettsiae were liberated into the medium and the percentage of infected cells began to rise sharply as previously uninfected cells became infected.

Cells of the first infection cycle appeared to burst over an extended period of time, some apparently remaining more or less intact for more than 72 to 96 h. Moreover, there appeared to be considerable variation in the rate and completeness of escape of rickettsiae from CE cells. In some, the rickettsiae seemed to be liberated rapidly and completely, sometimes leaving a damaged nucleus and a trace of cytoplasmic debris attached to the slide. At the other extreme, in some CE cells, whose cytoplasm was almost totally replaced by rickettsiae and whose plasma membrane appeared to have been destroyed, very large numbers of rickettsiae remained in densely packed masses around the nucleus. Thus, cells of the second cycle were being infected over a very long period of time and, hence, different cells were in markedly

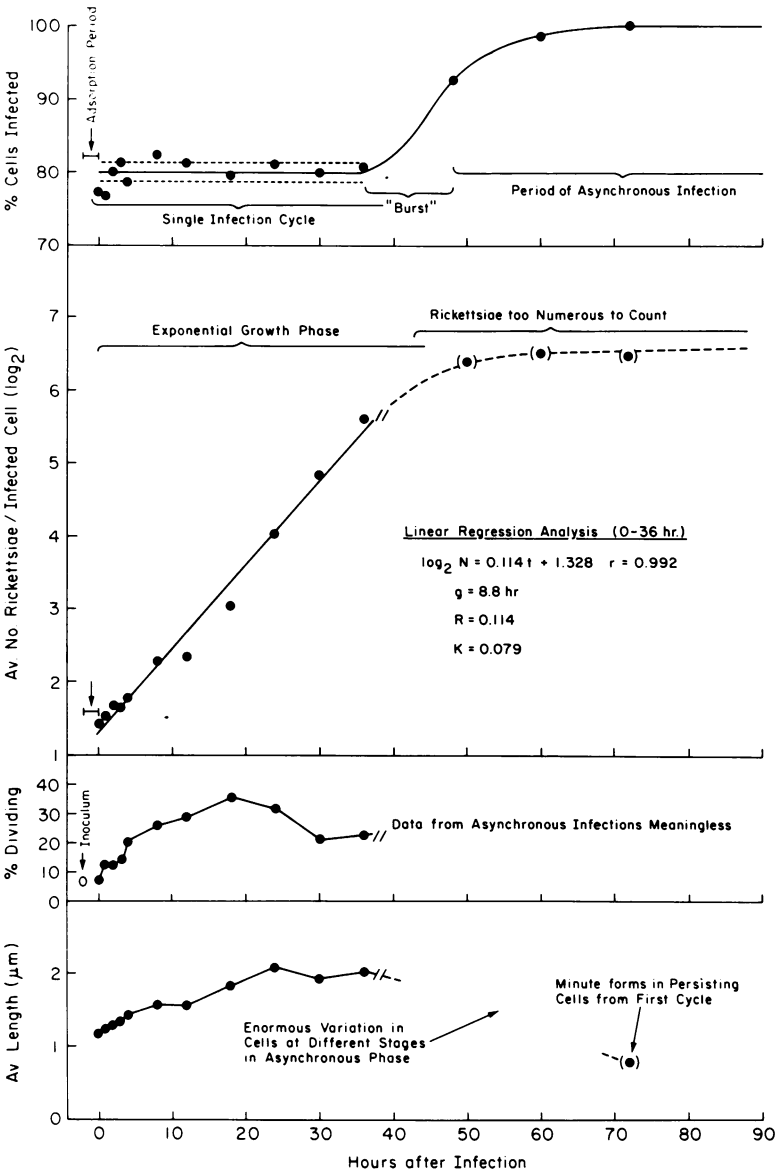

FIG. 1. *Growth characteristics of the virulent Breinl strain of R. prowazeki in secondary irradiated CE cells in slide chamber cultures. Between 0 and 36 h, linear regression analysis yielded results which were consistent with the hypothesis that there was no significant change in the percentage of cells infected with respect to time (correction coefficient, 0.21; slope, 0.057; y intercept, 79.3%). There is no systematic deviation of experimentally derived points from the mean value ±1 standard deviation of the values between 0 and 36 h which would suggest a transient "invisible" or eclipse phase.*

diverse stages of infection at any given time—a highly disordered state, in contrast to the marked uniformity observed in the first infection cycle.

During the first infection cycle, the organisms underwent regular changes in morphology reminiscent of those which certain bacteria undergo in fluid culture (see Fig. 1 to 3). Thus, after entry into the host cell, the rickettsiae enlarged somewhat, taking a strong clear stain, elon-

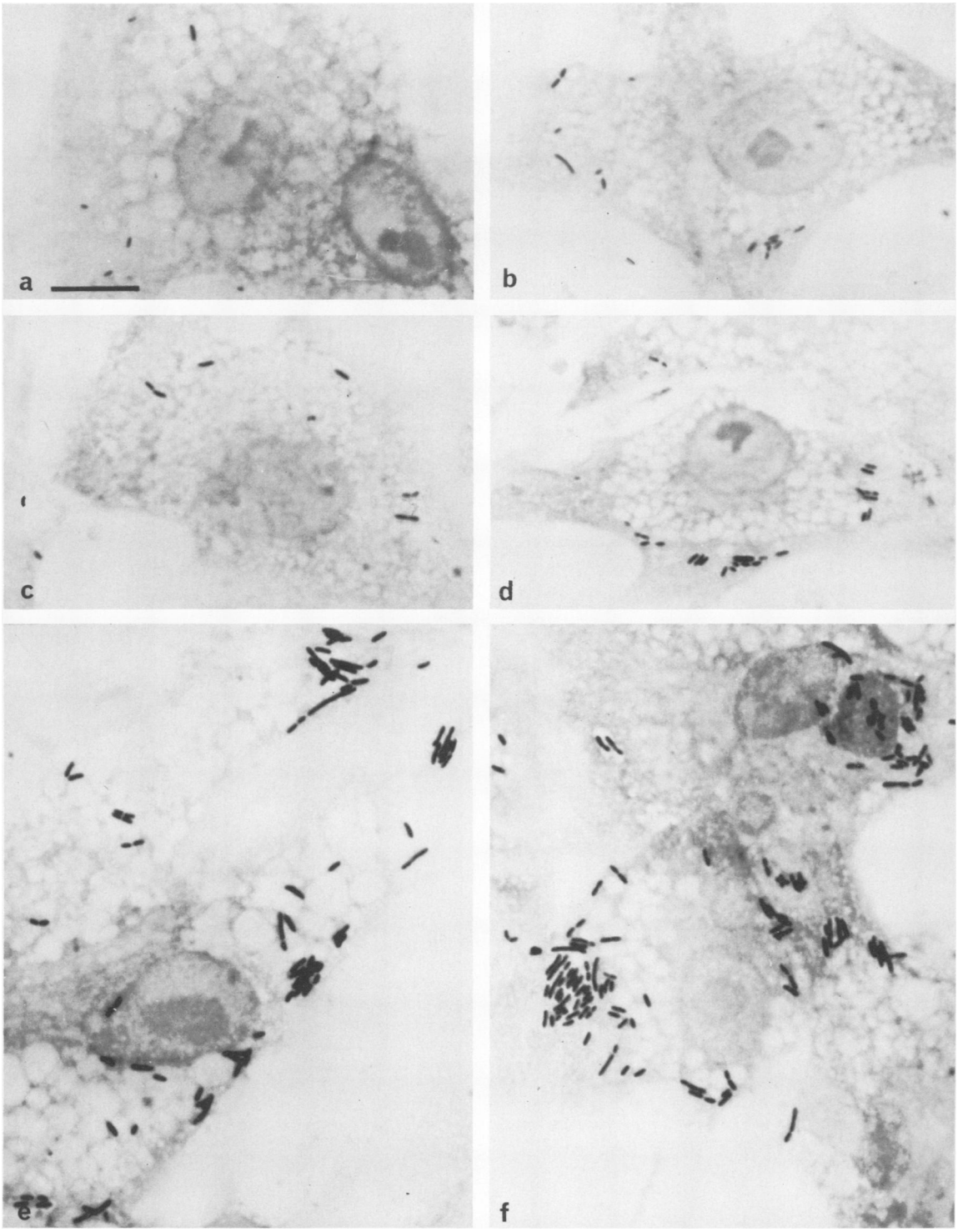

FIG. 2. *Early growth phases of R. prowazeki (Breinl) in chicken embryo cells. (a) Time zero (after 2 h of absorption); (b) 4 h; (c) 8 h; (d) 12 h; and (e to f) 24 h. Note enlargement, elongation and dividing forms. In addition to typical binary forms, some elongated forms at 24 h show unequal division, as though in the process of forming chains. Bar in (a) = 10 μm (original magnification ×1620).*

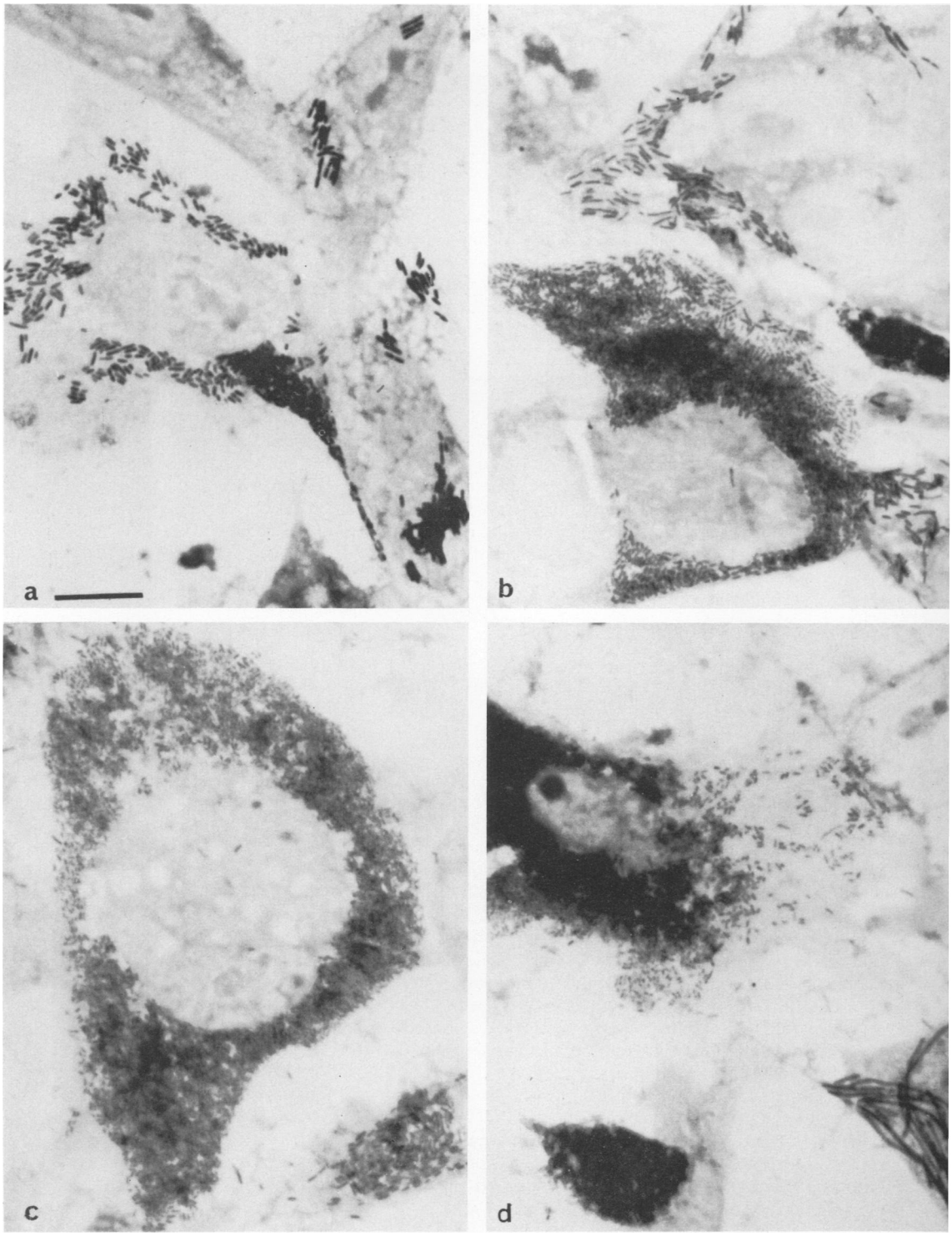

FIG. 3. *Late growth phases of R. prowazeki (Breinl) in chicken embryo cells. (a) 36 h; (b) 48 h; (c to d) 60 h. The 36-h specimen (a), near the end of the first growth cycle, shows typical rickettsial morphology in cells especially selected to permit individual rickettsiae to be seen but is not representative of the average number of organisms per infected cell. The 48-h specimen (b) shows a large cell persisting from the first growth cycle packed with rickettsiae which are smaller than at 36 h but still bacillary in shape, as well as newly infected cells well into the second growth cycle. The 60-h specimens (c to d) show degenerating CE cells packed with diminutive rickettsiae, now largely coccobacillary to coccoidal in shape, and large filamentous ("spaghetti") forms of the second growth cycle (d). Bar in (a) = 10 μm (original magnification ×1620).*

gated, and underwent division. Although the most common dividing form was the classical binary form, occasional rickettsiae showed substantial elongation with multiple division points. Organisms in this actively growing state dominated up to the end of the measurable exponential growth phase, about 36 h.

In infected cells which did not break down and liberate their organisms immediately, the organisms showed a significant diminution in size, clearly evident by 48 h and extreme at 72 to 96 h (Fig. 3 and 4). Although the diameter of the organisms did not appear to change greatly under the light microscope, the organisms became very short, coccobacillary to almost coccal in shape. Indeed, the extreme was an almost spherical organism approaching the size and appearance of the elementary bodies of chlamydiae. The existence of these forms no doubt indicates some change in growth characteristics and perhaps in structure, metabolic activity, viability, and possibly antigenic composition in the heavily infected cell, which must await the completion of experiments of a different design for evaluation. Although it is impossible to count by microscopy the number of rickettsiae in those CE cells whose cytoplasm appeared almost totally replaced by a dense mass of minute forms, the number in such a cell must have been enormous, possibly approaching 10^3 per cell.

Cells infected in the second cycle showed organisms with much greater morphologic diversity than those of the first cycle. Elongated, "spaghetti" forms with and without multiple division points, and swollen atypical forms without visible division points, were common. These were reminiscent of the aberrant, involutional or megalomorphic forms of certain bacteria in old cultures and may reflect either changes in the medium or in the host cell which produced suboptimal nutritional conditions (the medium was not changed during the observation period). Thus, in these aging cultures, a great heterogeneity existed not only in stages of growth cycle but also in morphology of the organisms, a situation which may reflect also a great heterogeneity in other properties.

Growth of attenuated E strain of R. prowazeki in slide chamber cultures. The results of a similar study performed with the attenuated E strain of *R. prowazeki* in CE cells (Fig. 5) are indistinguishable from those just described for the virulent Breinl strain. The generation time of 8.9 h in the exponential growth phase is well within experimental error of that obtained with the virulent Breinl strain. Thus, once within the host cell, the E strain is equally as "virulent" for irradiated CE cells as the Breinl strain.

Examination for "eclipse phase" or alternative modes of replication. Once within CE cells, both strains of rickettsiae were continuously present in conventional morphologically identifiable forms. The percentage infected and the average number of rickettsiae per infected cell never declined throughout the first infection cycle with either strain. The slight increase in percentage of cells infected over the first cycle may have been the result of "leakage," i.e., the escape of occasional rickettsiae from cell processes as has been described for *R. rickettsii* (46), or of increasing ease of identifying infected cells as the number of rickettsiae increased. Hence, there is strong evidence against an eclipse phase involving any significant proportion of the rickettsial population. Moreover, no form of replication other than conventional transverse fission was detected by microscopic examination of stained smears of any stage of the infection cycle. For example, nothing resembling the "initial body" of the chlamydiae (8) or the large homogeneous forms (15–17, 24) was observed.

DISCUSSION

The growth cycle of *R. prowazeki* has been examined in the present study on a quantitative basis in one host cell system by direct microscopic examination. Although ideas about the adsorption of the rickettsiae onto the CE cells and their penetration into the cytoplasm of these cells in another study (Wisseman and Waddell, manuscript in preparation) draw strongly on concepts elucidated most precisely with viruses, an analogy with viruses no longer holds once the rickettsiae are within the host cell cytoplasm. Instead, a typhus rickettsia was found to behave, from this time on to the escape of its progeny from the host cell, as a bacterium multiplying in a fluid culture. Indeed, each infected cell behaved as an independent miniculture going through its individual growth cycle and an infected cell culture, as a large population or collection of minute independent rickettsial cultures.

An exponential phase of growth was identified during which the generation time at 34 C was 8.8 to 8.9 h. This figure is of the same order of magnitude as the three divisions per 24 h at 37 C reported in the early tissue culture studies on *R. tsutsugamushi* by Bozeman et al. (6). Sequential morphological changes similar to those occurring in bacterial cultures were observed. By limiting the initial infection to a short time span, and working within the estab-

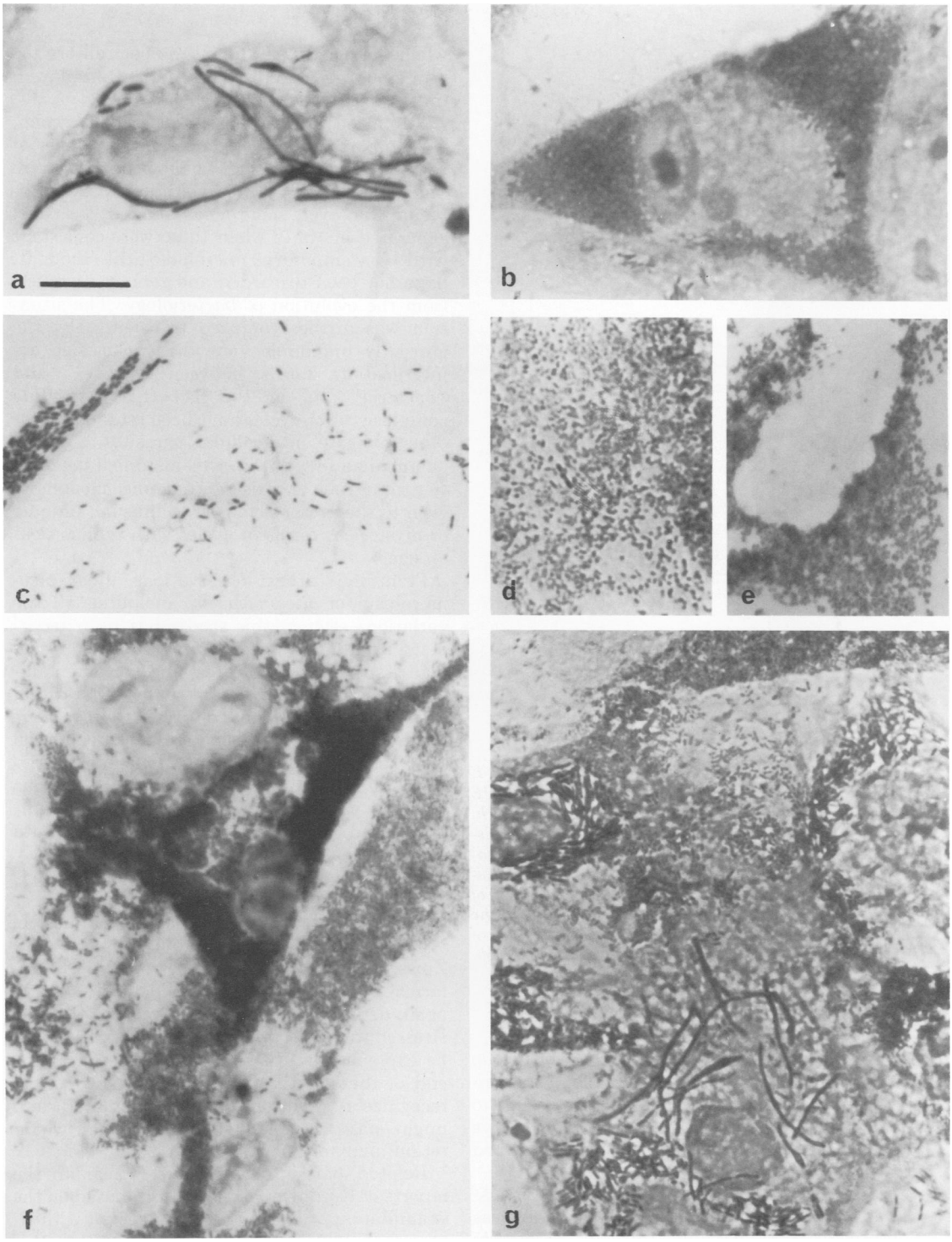

FIG. 4. *Various forms of R. prowazeki (Breinl) in late chicken embryo cultures. (a) Filamentous and swollen forms in second growth cycle at 48 h. (b) Typical distribution of R. prowazeki in CE cells (60 h). (c to e) Diminutive coccobacillary and coccoid forms in cells of first growth cycle persisting at 72 to 96 h. (f) Cytoplasm of cell persisting from first growth almost completely replaced with diminutive rickettsiae. (g) Cells in different infection cycles at 72 h showing enormous pleomorphism: coccoid, bacillary, filamentous, swollen, and bizarre. Bar in (a) = 10 μm (original magnification ×1620).*

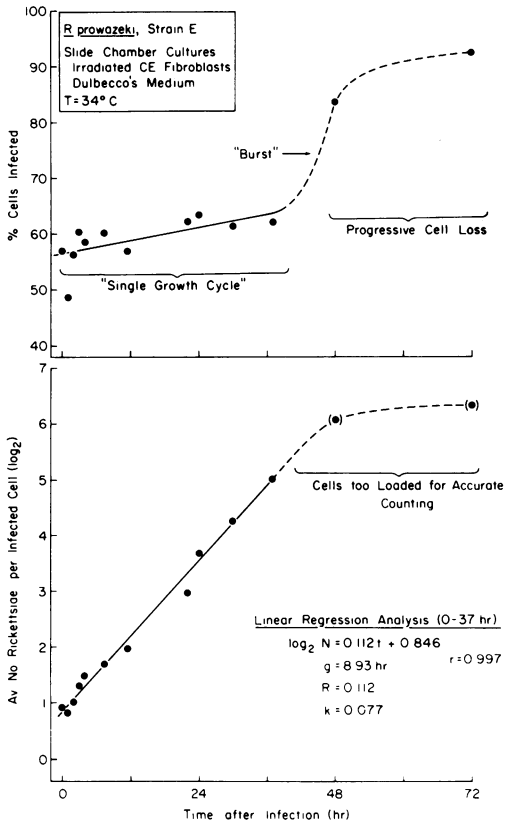

FIG. 5. *Growth characteristics of the attenuated E strain of R. prowazeki in secondary irradiated CE cells in slide chamber cultures. Between 0 and 37 h, the percentage cells infected gave the visual impression of a slight increase with respect to time and indeed linear regression analysis yielded a slightly positive slope (0.22) but with a correlation coefficient of only 0.46. Moreover, only two points fell outside the limits of ±1 standard deviation around the mean of the individual observations. No evidence was obtained for an eclipse or invisible stage.*

lished time frame of the first infection cycle, however, something similar to a "one-step infection cycle" is achieved. Thus, it is possible to infect cells in a predictable way and to observe the organisms in any defined predetermined growth phase desired.

This preliminary study has not been exhaustive. For example, although no lag phase was observed, it is possible that *R. prowazeki* adapted to one host cell system might require a period of adaptation if introduced into another host cell system. The influence of medium composition, pH, etc., has not yet been explored. The later phases of the growth cycle, when the number of organisms per cell are too numerous to count by the method employed, must be defined by other means.

Published observations on *R. prowazeki* span, with prolonged gaps, a substantial portion of the period over which ideas about bacteria were undergoing a series of evolutionary developments; however, some ideas about rickettsiae, generated at times when these were consistent with contemporary microbiological thought, have not been discarded and remain as relicts from the evolution of bacteriology. The situation was further confused transiently by the currently untenable view that rickettsiae are intermediate forms between "viruses" and "bacteria" and by the fact that bona fide complex growth cycles have been established for other obligate intracellular parasites, e.g., the chlamydiae (8). Within this historical perspective, it is not surprising that one encounters even to the present claims for unusual, unique, or mysterious forms or cycles with typhus rickettsiae.

From the earliest observations, great pleomorphism of *R. prowazeki*, including all the variations seen in this study, has been recognized in different host systems, such as arthropod tissues, tissue cultures, and various mammals inoculated by different routes (4, 9, 16, 25, 29, 32, 33, 36–44, 47, 52, 55–57, 59). Since none of these systems permitted a careful sequential study of growth in cell populations simultaneously infected, the relationship of the various forms to a growth cycle and to growth conditions was difficult to recognize and organize into a set of growth patterns as was subsequently done with other bacteria in cell-free media (11, 19–22, 34, 35).

The old controversy over the "filterability" of typhus rickettsiae, persumably laid to rest by Zinsser and Batchelder (58), revived ("infraforms") in recent years by Golinevich (18), and again denied by Pospisil and Brezina (41), who stressed the importance of filter integrity, might be explained by the minute forms seen at the end of the growth cycle. They are difficult to recognize as rickettsiae by light microscopy and might pass through some filters that would retain many ordinary bacteria.

Related, but not necessarily identical, are the reports of Kordoý et al. (26–28, 30) based on the examination of L-cell cultures infected with *R. prowazeki* which led her to conclude that this organism undergoes a change very early in the infection to yield forms which are not detectable by a variety of methods and which suggested to her the possibility of some unusual form of reproduction or an eclipse phase. We have

found no evidence of this in our system. Two observations may help explain her results. In other studies, D. D. Dalton and C. L. Wisseman, Jr. (manuscript in preparation) have found that it is sometimes difficult to stain and recognize the few rickettsiae which gain entrance into certain cell types, including L cells which Kordová et al. used. With care and diligence, however, they can be stained, and patterns of growth similar to those described here for CE cells can be established. Moreover, if her inoculum had contained a significant fraction of dead rickettsiae, it is conceivable that their destruction by host cells shortly after ingestion might also have caused a substantial transient fall in morphologically rocognizable forms and have given the illusion of an invisible early growth phase.

Earlier, the development of large homogeneous forms (whose nature has not yet been satisfactorily resolved), when typhus rickettsiae were introduced into certain unusual hosts, was regarded by some (but not other) investigators as evidence for the occurrence of some kind of life cycle (1, 4, 15–17, 24). Similar diverse views were expressed about other bacteria in earlier years (19–22, 49). In the present study, no evidence was obtained for a cycle in any way analogous to that of the chlamydiae or for any other unusual growth or reproductive cycle. Only in the older CE cell cultures did bizarre forms appear, consistent with changes seen in bacterial cultures under unfavorable conditions (11, 19–22, 34, 35, 49). We, therefore, suspect that many of the unusual rickettsial forms which have been encountered under various conditions do not represent different mechanisms of replication but rather represent the kinds of morphological changes which occur in many bacteria under suboptimal or hostile conditions.

One rickettsial variable was studied, namely, "virulence". The E strain of *R. prowazeki*, which is attenuated in its capacity to produce infections in man and certain small mammals (12) and to grow in human macrophages (13), but not in its capacity to grow in human body lice (5), grew equally well as the virulent Breinl strain in CE cells. Our findings are in contrast to those of Ignatovich and Gulevskaia (23), who reported that the E strain grew less well in CE cells than the Breinl strain. These investigators neither gave quantitative information about their seed preparations in terms of numbers of organisms nor provided evidence that they had infected their cultures with comparable numbers of viable organisms; hence, differences in input might explain their results, since the rate of uptake (Wisseman and Waddell, manuscript in preparation) of the E strain is also indistinguishable from that of the Breinl strain in CE cells when conditions are quantitatively similar.

As a result of these studies and the experience of others, we propose for *R. prowazeki* the following terms for the rickettsia-host cell interactions of the kind described here.

(i) "Infection cycle" is defined as the entire sequence of events from extracellular ricketsiae, through the act of entry into the host cell and the intracellular growth phase, to the final release of the progeny into the medium from degenerating host cell.

(ii) "Uptake" is used as a nonspecific term which refers to the entry of extracellular rickettsiae into the site of replication within the host cell by any mechanism: e.g., (a) direct penetration through the host cell membrane after the manner originally described by Cohn et al. (10) for *R. tsutsugamushi* and by us for *R. prowazeki* (Wisseman and Waddell, manuscript in preparation); (b) phagocytosis of the rickettsia by the host cell followed by their escape from the phagosome into the host cell cytoplasm, as described by Andrese and Wisseman (2). The dominant mode of entry of *R. prowazeki* may be a function of the degree of phagocytic activity of the host cell. (Other "rickettsiae" may differ in their capacity for direct penetration and escape from the phagosome, e.g., perhaps *Coxiella burneti*.)

(iii) "Growth cycle" refers to sequence of events taking place during the intracellular growth phase. This has been partially elucidated for *R. prowazeki* in the present study. Description of this phase should include a description of the location and dispersion within the cell, as well as presence or absence of a limiting host vacuolar membrane, since these may vary both with organism and host cell.

(iv) "Burst" is defined as the release of rickettsiae from the infected host cell at the end of the growth cycle. Kinetic and light microscope observations in this study indicate that individual host cells in a population initially infected over a brief period of time reach the burst stage at different times and release rickettsiae at different rates over a considerable span of time, i.e., the burst is highly asychronous, disordered, and prolonged. This burst at the end of the growth cycle is to be differentiated from the leakage of rickettiae from nondegenerating cells as described by Schaechter et al. (46) for *R. rickettsii*.

(v) A "one-step infection cycle" is defined as the sequence of events from uptake to burst during the first infection cycle in a population of

infected cells in which the majority of cells and their rickettsiae are in essentially the same growth state at any given point in time. Although Weiss et al. (51) suggest that their metabolic studies on *R. mooseri*-infected L cells in culture were essentially one-step growth experiments, insufficient data are given to ascertain if their observations were made in the first highly ordered cycle or if they were made in a subsequent, disordered cycle, as the peak activity between 4 and 6 days might suggest.

Undoubtedly, as more information is accumulated from further studies, these stages will be subject to more precise definition and descriptions, and perhaps to different definitions with different rickettsial agents. Nevertheless, they constitute an initial conceptual framework for increased precision in describing rickettsia-host interactions.

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ADDENDUM IN PROOF

Studies made since this manuscript was submitted have shown a classical "lag" phase after infection of CE cells with a seed prepared from *R. prowazeki* (Breinl)-infected CE cell cultures harvested in the late "stationary" phase, whereas a similar seed prepared from rickettsiae in the log or exponential growth phase caused infection without detectable lag phase (C. L. Wisseman, Jr., and A. D. Waddell, manuscript in preparation).

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