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Immunological and Biological Characterization of *Coxiella burnetii*, Phases I and II, Separated from Host Components

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Coxiella burnetii, phase I and II, cells cultivated in the yolk sac of chicken embryos were separated from host cell components by two cycles of isopycnic Renografin gradient centrifugation. Initial steps in the purification of viable *C. burnetii* involved differential centrifugation and sedimentation through an aqueous solution of 30% sucrose and 7.6% Renografin. After the first, but not the second, cycle of Renografin gradient centrifugation, the cells were passed through microfilter glass filters which facilitated the removal of host components. The integrity of morphologically different cell variants was maintained during purification procedures by suspending highly purified *C. burnetii* in phosphate-buffered saline-sucrose solutions. *C. burnetii*, phases I and II, obtained by these methods appeared to be free from host cell components by serological methods while retaining morphological integrity and infectivity for yolk sacs and experimental animals. Average yields of *C. burnetii* were 2.83, 1.5, and 0.84 mg (dry weight) per yolk sac of the Ohio strain (phase I), 9 Mile strain (phase I), and 9 Mile strain (phase II), respectively. Recovery of phase I cells averaged about 70%, whereas the recovery of phase II cells was approximately 40%. The temporal sequence of phase I and II antibody response was demonstrated in infected and vaccinated animals. Also, no antibody response in mice and guinea pigs to yolk sac antigens was detectable after two injections of vaccine or viable cells. Importantly, this is the first report of the separation of viable phase II cells of *C. burnetii* free of host components.

Coxiella burnetii is a gram-variable (13) bacterium which parasitizes eucaryotic cells. This obligate intracellular organism progresses through its developmental cycle in the phagolysosome of the host cell (3). Although a variety of eucaryotic cells can be parasitized by *C. burnetii*, experimentally infected yolk sacs (YS) of embryonated eggs usually provide the best yield of organisms. Published methods for the separation of *C. burnetii* from YS cells of embryonated eggs include differential centrifugation (5, 26, 31, 37), centrifugation in 1 M KCl solutions (5, 26, 31), ion-exchange adsorption (18, 22), Celite and trypsin treatment (5, 22, 26), dextran sulfate precipitation (10), and gradient centrifugation in sucrose, glycerol, and Renografin (4, 31, 37). These methods have been applied to both formaldehyde-inactivated and viable cells of *C. burnetii*. Although an accumulated body of evidence suggests that the above-described techniques preserve most of the properties of *C. burnetii*, the effects of the various treatments on the antigenic, biochemical, and morphological integrity of the rickettsiae have not been clearly elucidated.

Purification of viable *C. burnetii* has received less attention and, to date, four methods have been described (11, 22, 26, 31, 41). All of the methods have been applied to phase I *C. burnetii*. The method described by Wiebe et al. (37) used Renografin gradient centrifugation as a final step in the analysis of the pleomorphic cells of *C. burnetii*. Since Renografin has been very useful for the purification of *Rickettsia typhi* (36), *R. prowazekii* (8), *Chlamydia trachomatis* (17), and *R. rickettsii* (2), we were interested in developing a standard technique for the purification of viable *C. burnetii*. The viability of *C. burnetii* after purification has never been a major consideration since this bacterium is clearly the most physiologically stable member of the rickettsiae. A more important objective was the separation of viable *C. burnetii*, phase I and II cells, from host components. Previous studies had shown that phase I cells were easily separated from host components, whereas phase II cells adhered tenaciously to host materials (11, 12, 34).

In this paper, we report that viable *C. burnetii* phase I and II cells separated from host components in isopycnic Renografin gradients retain their morphological integrity. Initial steps in the

procedure subject the rickettsiae to mild treatments which do not appear to alter the biological and antigenic properties of the cells. The final step using Renografin density gradients is essential for the separation of phase II cells from host componets.

MATERIALS AND METHODS

Bacteria. *C. burnetii* strains used were in various passage levels in guinea pigs (GP), chicken embryo fibroblast tissue culture (TC), and chicken embryo YS. Previous studies have shown that phase I *C. burnetii* are selected when strains are isolated from the spleens of infected guinea pigs during the febrile response, whereas phase II strains are obtained by repeated egg passage (EP) in YS (34). Clones used in this study were isolated by selecting plaques on chicken embryo fibroblast monolayers (23). Phase determinations of each strain were carried out as previously described (34). The Ohio (29) strain (5EP/2GP/2EP [indicates number of passages in each culture]) contained undetectable phase II antigens, whereas the 9 Mile (7, 9) strains were phase I (307GP/1TC/1EP, clone 7) and phase II (90EP/1TC/4EP, clone 4); these strains are hereafter referred to as CBOI, CB9MI, and CB9MII, respectively. *R. typhi* and *R. rickettsii* were propagated in chicken embryo YS as previously described (2, 36).

Buffers and chemicals. Phosphate-buffered saline (PBS), pH 7.4, of the following composition was used as the diluent: Na_2HPO_4, 53.9 mM; KH_2PO_4, 12.8 mM; and NaCl, 72.6 mM. PBS-sucrose (PBSS) was prepared at a final concentration of 0.25 M sucrose (ultrapure sucrose; Schwarz/Mann, Orangeburg, N.Y.). The osmolarities of PBS and PBSS were 301 and 544 mosmol/kg, respectively. Renografin-76 (E. R. Squibb & Sons, Princeton, N.J.) and metrizamide (Accurate Chemical and Scientific Corp., Hicksville, N.Y.) were used as the density gradient materials. Density gradient marker beads were obtained from Pharmacia Fine Chemicals, Inc., Piscataway, N.J.

Preparation of rickettsiae. *C. burnetii* were propagated in specific-pathogen-free type IV, antibiotic-free, fertile hen egg YS (H and N Hatchery, Redmond, Wash.). Stock cultures of *C. burnetii* were stored at −70°C as 50% YS suspensions in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) YS suspensions at −70°C. Stock cultures were prepared by injecting *C. burnetii* into the YS of chicken embryos on day 5 postincubation at 36°C. Incubation was carried out at 35°C in a humidity-controlled Jamesway incubator. On the day 7 postinfection, the YS from live embryos were harvested, and a 50% YS suspension in BHI was prepared by blending (Waring, model F.C. 114) for 30 s at a powerstat setting of 100. Stock cultures were shell frozen as 2-ml portions in sealed glass ampoules. Inocula for YS were 1.1×10^9 plaque-forming units (PFU) for CBOI, 1.5×10^7 PFU for CB9MI, and 5.6×10^7 PFU for CB9MII. Mean survival time of the infected embryos was 7.4 ± 0.2 days at 35°C. Cultures destined for the separation of *C. burnetii* from host material were frozen in batches of 25 to 35 YS and stored at −70°C, or they were used as fresh YS without freezing.

Separation of rickettsiae from YS components. Purification procedures consisted of several high- and low-speed centrifugation steps in various buffered media at pH 7.4. All procedures were carried out according to established contaminant protocols for class III agents. Unless otherwise specified, all manipulations were carried out at 4°C. Collectively, the purification of *C. burnetii* free of host components was divided into three steps as follows.

For step 1, infected YS previously harvested and frozen at −70°C were thawed under running tap water, placed in a stainless-steel sealed blender with 300 ml of PBS (approximately one YS per 10 ml of PBS), and blended two times at 1-min intervals at a powerstat setting of 100. The suspension was placed in two centrifuge bottles with an additional 300-ml rinse of the blender. The bottles were sealed with leakproof closures, and centrifugation was carried out at 11,000 × *g* for 45 min. Fat adhering to the walls of the bottles and supernatant was removed by aspiration, and the pellets were resuspended in 200 ml of PBS and centrifuged at 400 × *g* for 20 min. Supernatant fluids were saved, and the pellets were transferred to a blender, resuspended in 300 ml of PBS, and processed as above. Supernatants from the two low-speed centrifugations were combined, and the rickettsiae were sedimented at 11,000 × *g* for 30 min. Supernatants were removed by aspiration, and the pellets were resuspended in 50 ml of PBS.

For step 2, suspension volumes of 25 ml were layered over 180 ml of a mixture of 30% sucrose and 7.6% Renografin with PBS as the diluent. Centrifugation was carried out at 11,000 or 18,000 × *g* for 60 or 30 min, respectively. After centrifugation, the supernatants were removed by aspiration and the pellets were suspended in 25 ml of PBSS.

For step 3, linear density gradients of Renografin were prepared with PBS as the diluent, using a conical-bore, triple-outlet gradient maker connected to a polystaltic pump (Buchler Instruments Div., Nuclear-Chicago Corp., Fort Lee, N.J.). Six cellulose nitrate tubes (Beckman Instruments, Inc., Fullerton, Calif.; 8.9 by 2.5 cm) containing 30 ml of 20 to 45% linear Renografin gradients were used for the first cycle. The second cycle was treated the same way except that the Renografin gradient was from 25 to 45%. Five-milliliter volumes of rickettsial suspension were layered over the gradients and centrifuged (SW27 rotor) in a Beckman L5-75 ultracentrifuge at 85,000 × *g* for 45 min. After the first gradient centrifugation cycle, areas of the gradient above and below the rickettsial band were removed with a syringe and 15-gauge cannula. The rickettsial bands from six tubes were removed, placed in 100 ml of PBSS, and filtered through one type AP20 microfilter glass filter (47 mm, 50 μm; Millipore Corp., Bedford, Mass.); the filters were then washed with 30-ml volumes of PBSS. This suspension of rickettsiae was centrifuged at 17,300 × *g* for 25 min, and the pellets were suspended in 25 ml of PBSS. Rickettsiae were banded in a second Renografin gradient (25 to 45%); the organisms were collected without filtration, centrifuged as above, and suspended in 30 ml of PBSS or the appropriate diluent. Organisms purified by this technique were designated as Renografin-purified rickettsiae (RPR).

Tests of biological activity and immunogenicity. Guinea pigs (Hartley strain) and mice (C57BL/10) were inoculated in groups of 5 to 10 animals intraperitoneally with log dilutions of the live purified organisms. Guinea pigs were monitored for fever response for 2 weeks, and deaths in both guinea pigs and mice were recorded for 30 days. The sera collected after 30 days postinfection were evaluated for seroconversion by complement fixation (CF) (25, 27) and microagglutination (MA) (11). Particulate whole-cell antigen used in the above tests was prepared by fixing RPR in 1% formaldehyde (see below) and by a previously described technique (21).

RPR were inactivated with 1% formaldehyde for 24 h at room temperature. Formaldehyde was removed by dialysis, using cellulose dialysis sacs with a molecular weight cutoff of 2,000, against three changes of 1-liter volumes of sterile deionized distilled water (SDDW). The final cell suspension in water was lyophilized and weighed to determine the dry weight yield per YS and per gram of YS. Protein determinations were carried out as previously described (19), using bovine plasma albumin as a standard. This material was evaluated for serological reactivity by microimmunofluorescence (28), CF (25, 34), and MA (11) assays, using antisera derived from vaccinated guinea pigs (Hartley strain) on 7, 11, and 21 days postvaccination (34). Hyperimmune (HI) guinea pig sera were obtained a minimum of 28 days after challenge or vaccination with viable or killed-whole cells of *C. burnetii*.

Enumeration of microorganisms. PFU were determined by a previously described method (23). The number of bacteria per milliliter of sample was determined by the differential staining technique described by Silverman et al. (32).

Electron microscopy. Suspensions of RPR of both phase I and phase II cells were fixed by adding 10 volumes of a primary fixative containing 2.5% glutaraldehyde (Polysciences, Warrington, Pa.), 2.0% formaldehyde, which was prepared from paraformaldehyde (J. T. Baker Chemical Co., Phillipsburg, N.J.), and 2.5 mM $CaCl_2$ in 66 mM cacodylate buffer (pH 6.8) overnight at 4°C. The suspensions in 1.5-ml microsample tubes were centrifuged in a Beckman Microfuge B after each stage of both fixation and rinsing. Primary fixation was followed by three 30-min rinses in cacodylate buffer (66 mM, pH 6.8) at 4°C. Secondary fixation was carried out for 1 h with 1% osmium tetroxide in 66 mM cacodylate buffer at 4°C. Excess osmium was removed by a second cacodylate buffer wash. After further centrifugation, the rickettsiae were embedded in 2% Noble agar (Difco) (14), cut into blocks, and dehydrated through ascending serial concentrations of methanol. The blocks were stained with 0.5% uranyl acetate for 1 h at room temperature during dehydration in 30% methanol. The agar blocks were embedded in Spurr resin (33), using Beem capsules. Sections were cut on a Reichert OMU2 microtome, collected on uncoated Pelco 300 grids, stained with lead citrate (30), and examined in an EM HU-11E-1 (Hitachi) operating at 75 kV.

Osmolarity determinations. Osmolarity of buffers was determined by the freezing point depression method, using an osmometer (model 3W; Advanced Instrument, Needham Heights, Mass.).

RESULTS

Separation of *C. burnetii* (phase I) from host components. Both CBOI and CB9MI were purified by the procedure described in Materials and Methods. In a typical experiment, 137 YS, weighing 497 g, which had been infected with CBOI and harvested on the day 7 postinfection were subjected to the purification procedure. In step 1, the differential centrifugation steps providing low- and high-speed pellets were characterized by a central white area consisting of organisms and a great abundance of host material. In step 2, separation of rickettsiae from host components was extremely effective. The resulting white pellet was covered with a thin film of host components. Although several combinations of Renografin and sucrose were tested, the most effective concentrations of sucrose and Renografin were 30 and 7.6%, respectively. At these concentrations, both *R. typhi* and *R. rickettsii* were easily pelleted, thereby eliminating the use of bovine plasma albumin as described by Weiss et al. (36). In step 3, the first 20 to 45% linear gradient revealed that the rickettsiae from step 2 were still heavily contaminated with host material (Fig. 1A, phase I). Host components were visualized at the top of the gradient, immediately above and below the band of rickettsiae, and a small pellet was observed at the bottom of the tube. Density gradient marker beads above (1.159 g/ml) and below (1.191 g/ml) the rickettsial band (1.187 g/ml, at the center) marked the variation in distribution of the organisms in the gradient. Filtration of the collected rickettsial band through a single AP20 filter removed material with a pale yellow color. The second 25 to 45% linear gradient gave a centrally located white band of rickettsiae (Fig. 1B, phase I) which was delimited by the density marker beads.

In an experiment using 31 infected YS weighing 96 g, the distribution of the YS and rickettsial antigens was monitored by the CF test. The sensitivity of the test was described previously by Peacock et al. (27). Those studies indicated that the CF test detects as little as 16 to 32 μg of particulate antigen and <1 μg (M. G. Peacock and J. C. Williams, unpublished data) of soluble antigen per ml of sample. A comparison of the fractions indicated that *C. burnetii* antigen was present in the supernatant of the 30% sucrose–7.6% Renografin step. This antigen was identified as a particulate material which adhered to host components in the supernatant fluid. After removal of the antigen by centrifugation at $100{,}000 \times g$ for 1 h, no rickettsia-soluble antigen was detectable via double diffusion against HI guinea pig sera or the CF test (data not shown). The first density gradient step was sufficient to

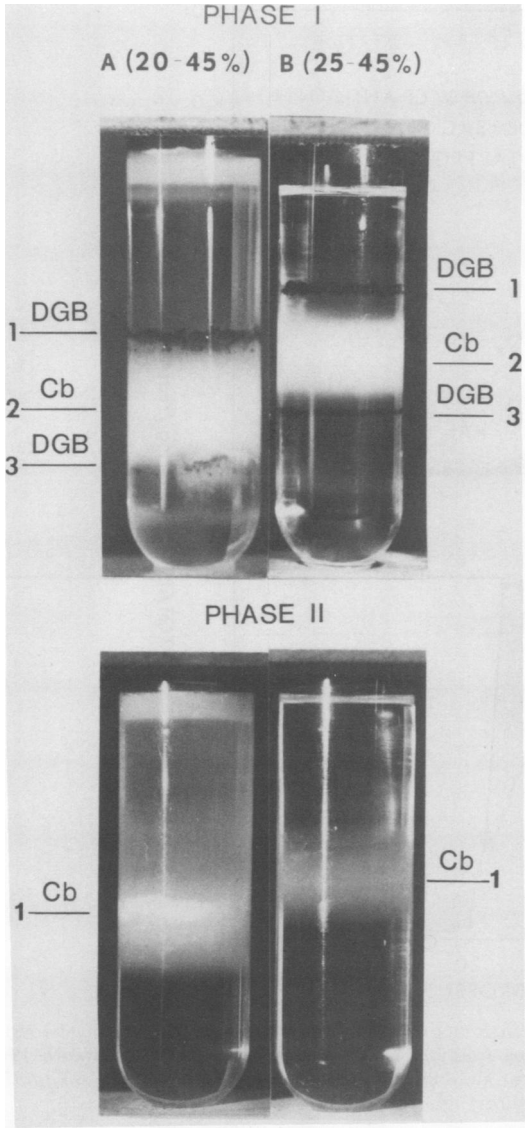

FIG. 1. *Separation of C. burnetii cells from host components in Renografin density gradients. (A) Purification of CBOI in Renografin density gradients. (B) Banding profile of CB9MII in Renografin density gradients. Density gradient beads (1 and 3) banded at 1.159 and 1.191 g/ml, respectively. Rickettsiae (2) banded at 1.187 g/ml at the center. Each gradient tube contained about 5 mg (phase I) and 2 mg (phase II) (dry weight) of bacteria. DGB, Density gradient beads; Cb, Coxiella burnetii.*

remove most of the host components (Fig. 2A), which were readily observed at the top of the gradient solution. Analysis of YS and rickettsial antigens present in all of the fractions indicated that the band of rickettsiae (fractions 9 through 16) was free of detectable YS antigen by the CF test. Only three fractions (7, 8, and 9) immediately below the rickettsial band contained measurable YS antigen, whereas fractions at the top (23, 24, and 25) of the gradient solution contained measurable rickettsial and YS antigens. Interestingly, fractions 19, 20, 21, and 22 contained a strong anticomplementary activity which we have not characterized. When the second cycle of the Renografin gradient was analyzed, no YS antigens or anticomplementary activity was detectable, whereas protein determinations suggested that a small amount of protein was leached from the rickettsiae (Fig. 2B). Again, minor amounts of rickettsial antigen were detected in the upper fractions. When the organisms were suspended and filtered in the presence of PBSS rather than PBS alone, neither protein nor rickettsial antigen was detected in the upper fractions (data not shown).

Density determinations from three separate gradients indicated that the rickettsiae banded at an average density of 1.187 g/ml. This average density agrees with the densities for CB9MI reported by Wiebe et al. (37) in 30 to 41% Renografin gradients.

Purification of *C. burnetii* (phase II). The purification of CB9MII from 88 YS weighing 458 g was carried out by the standard procedure used for phase I organisms. Each substep in the step 1 procedure reflected the tenacity of phase II organisms for host components. Pellets of rickettsiae generated by low- or high-speed centrifugations were clearly unlike those of phase I organisms in that no white central area in the pellet was visible with phase II organisms. Separation of phase II organisms from host components in step 2 was greatly reduced due to the aggregation of rickettsiae with remaining host components. In step 3, separation of phase II organisms from host components was greatly facilitated by the 20 to 45% linear Renografin gradient (Fig. 1A, phase II). Aggregation of rickettsiae with host components was visibly distributed through the gradient. However, some of the aggregated material cannot be seen in the photograph (Fig. 1A, phase II). Removal of this material with syringe and cannula was required before the rickettsiae would pass through the AP20 filter. In the linear 25 to 45% Renografin gradient, the phase II cells appeared to be evenly distributed in the band (Fig. 1B, phase II). The gradients containing phase II cells were not fractionated as described for phase I. However, the final preparation of rickettsiae autoagglutinated, but significant YS antigen was not detected by the CF test. Again, suspension and filtration of organisms in PBSS rather than PBS alone prevented the appearance of rickettsial antigen and protein in the upper fractions of the gradient (data not shown).

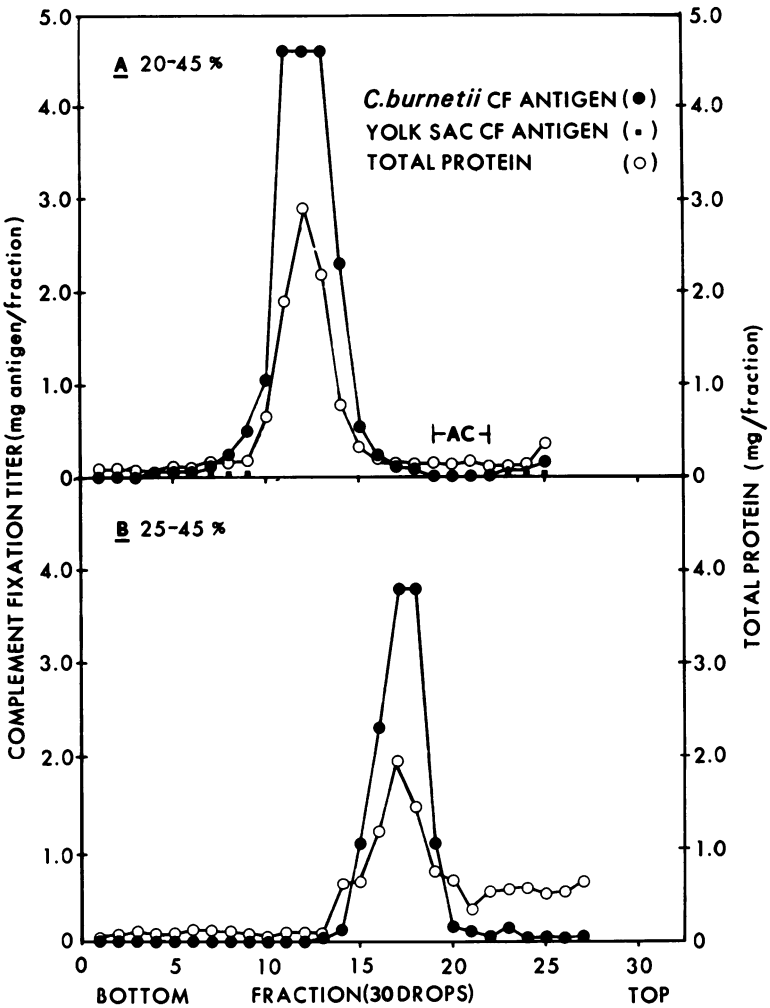

FIG. 2. *Fractionation of CBOI cells and YS antigen in Renografin density gradients. (A) First cycle; (B) second cycle. Gradients were fractionated into 30 drops/fraction. Each fraction was inactivated with 1% formaldehyde, dialzyed against SDDW, and analyzed for serological reactivity by the CF test and for total protein (see Materials and Methods). AC, Anticomplementary (25).*

Comparison of density gradient material. Metrizamide was found to be as effective as Renografin in the purification procedure. Although metrizamide is more expensive, we recommend its use when samples of organisms are required for nucleotide analysis or molecular sieve chromatography since metrizamide does not adhere to the cells (16). Sucrose or glycerol gradients were not as effective as Renografin in removing contaminating materials (data not shown).

Standard concentration of *C. burnetii*. In two separate experiments, a correlation was made between the turbidity reading obtained with a Klett-Summerson photoelectric colorimeter at 420 nm (blue filter), PFU, and direct counts (Table 1). Subsequently, a standard curve was constructed which correlated turbidity of viable or formaldehyde-fixed cells with direct count and dry weight (Fig. 3). The curves constructed with CBOI, CB9MI, and CB9MII showed no significant differences among strains or between phase I and phase II of the same strain.

Recovery of phase I and II rickettsiae from YS. Recovery of *C. burnetii* from YS material was examined by particle counts, PFU, and dry weight. Dry weight determinations were carried out on whole cells which had been fixed with 1% formaldehyde, dialyzed, and then lyoph-

ilized (see Materials and Methods). Particle counts and PFU determinations were carried out before purification from a 50% (wt/vol) suspension of infected YS and at the end of the procedure. The dry weight yields of rickettsiae from three strains based on milligram per YS or milligram per gram of YS were markedly different (Table 2). The yield of *C. burnetii* (phase I) was consistently greater than the yield of cells in phase II. The average percentage yield from three experiments based on total PFU at the initiation of purification as compared with the final suspension of purified bacteria was 70% for phase I and 40% for phase II.

The recovery of CBOI from the YS of fertile hen eggs was followed by harvesting 96 YS on each successive day postinfection (Table 3). Day 3 postinfection was the first day that cells could be visualized in the Renografin gradients. The yield of cells increased during successive days of harvest until day 8, at which time 68% of the remaining embryos had died (Table 3). The harvest on day 8, 24 h postmortem, indicated that recovery of cells from intact YS was greatly

TABLE 1. *Quantitation of C. burnetii (CBOI) after purification in Renografin gradients*

Expt[a]	Bacteria/ml[b]	
	PFU	Direct count
1	2.6×10^{10}	1.3×10^{10}
2	1.9×10^{11}	1.5×10^{11}

[a] Two separate purifications of fresh viable cells on day 7 postinoculation of 1.1×10^9 cells per embryo.

[b] Turbidity of the final suspension was measured in a Klett-Summerson photoelectric colorimeter at 420 nm. Serial dilutions of the whole cells were compared with a standard curve (see Fig. 3). Direct counts and PFU were determined by previously described techniques (see references 23 and 32).

TABLE 2. *Recovery of C. burnetii from the YS of fertile hen eggs*

C. burnetii strain	Amt inoculated[a] (μg/YS)	Recovery[b]	
		mg/YS	mg/g of YS
CBOI	29.1	2.83 ± 0.15	0.91 ± 0.05
CB9MI	0.4	1.50 ± 0.10	0.58 ± 0.04
CB9MII	1.5	0.84 ± 0.05	0.16 ± 0.01

[a] YS were inoculated 5 days after fertilization. The YS from live embryos were harvested on day 7 postinfection and stored at −70°C until subjected to the purification procedure.

[b] Recovery of whole cells was based on a standard curve comparing dry weight with Klett units and direct rickettsial counts (i.e., 1 mg [dry weight] = 3.78×10^{10} organisms; see Materials and Methods and Fig. 3). Values are mean ± standard error of three determinations.

TABLE 3. *Growth and recovery of C. burnetii (CBOI) from the YS of fertile hen eggs*

Day postinfection[a]	YS[b] (g)	% Embryo lethality	Yield of rickettsiae[c] (mg)
3	115	0	0.8 (1)[d]
4	117	0	5.4 (7)
5	191	9	38.8 (51)
6	240	20	147.0 (193)
7	300	41	274.8 (362)
8	123[e]	68	20.2 (26)

[a] Each YS was inoculated with 1.1×10^9 PFU or 29.1 μg of organisms.

[b] Weight of intact YS recovered from 96 embryos.

[c] Determinations were made from a standard curve correlating dry weight with Klett units (absorbancy at 420 nm) (see Fig. 3).

[d] Numbers in parentheses indicate fold increase in yield as compared with day 3 postinfection.

[e] Harvest from dead embryo yolk sacs (24 h postmortem).

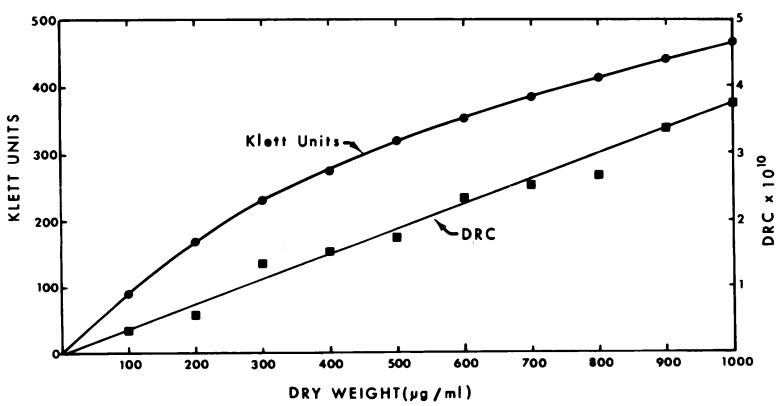

FIG. 3. *Comparison of formaldehyde-killed CBOI cells based on turbidity, direct count, and dry weight. DRC, Direct rickettsial counts (33). Klett units, Absorbancy at 420 nm.*

decreased after death of the embryo. Note also that grams of YS increased from day 4 to 7, which roughly paralleled the increase in yield of *C. burnetii*. There was a 3.8-fold increase in the weight of organisms from day 5 to 6 and a 1.9-fold augmentation from day 6 to 7, whereas the recovery of YS was enhanced by 1.3-fold at each time period.

Extraction of *C. burnetii* antigens by 1% formaldehyde treatment. Renografin-purified CBOI cells were inactivated in a 1% formaldehyde-PBS solution for 24 h at room temperature. The suspension was fractionated as follows: (i) the suspension was washed with SDDW three times by centrifugation (13,300 × *g* for 30 min), and the pellet was resuspended in SDDW; and (ii) the supernatants were pooled and dialzyed against SDDW, using dialysis tubing with molecular weight cutoff of 2,000. Both the residue and supernatant fractions were lyophilized and weighed. Of the total dry weight yield, 90.5% was residue and 9.5% was in the supernatant fraction. Analysis of these two fractions for serological activity by the CF test using standard anti-*C. burnetii* sera indicated that both fractions contained antigens (Table 4). Indeed, the low-speed supernatant fraction was 3.9-fold more reactive in the CF test with 20-day serum than were the whole cells, whereas the reactivity with HI sera was not greatly altered. Fractionation of this low-speed supernatant fraction into a high-speed pellet and supernatant (75,000 × *g*) revealed that the reactivity with 20-day sera could be partially sedimented, whereas the reactivity with HI sera was fractionated into soluble and particulate components. Therefore, the low-speed supernatant contained both soluble and particulate antigens. These results suggest that the low-speed supernatant fractions contained antigens which would be discarded if *C. burnetii* were inactivated with 1% formaldehyde in a YS suspension and then subjected to purification procedures.

TABLE 4. *Extraction of C. burnetii (CBOI) antigens by 1% formaldehyde inactivation of live cells*

C. burnetii (CBOI) antigen[a]	CF titer[b]		
	12-day serum	20-day serum	HI serum
Whole cells	0	4	64
Low-speed supernatant	0	16	64
High-speed supernatant	0	T	8
High-speed pellet	0	16	32

[a] Antigen suspended at 1 mg/ml of PBS. Low-speed supernatant, 13,300 × *g* centrifugation for 30 min; high-speed supernatant and pellet, 75,000 × *g* centrifugation for 4 h, generated from the low-speed supernatant.

[b] Reciprocal titers as determined by the CF block test (25), using 12-day, 20-day, and HI anti-*C. burnetii* serum from guinea pigs (11). Expressed as endpoint dilution of antigen (i.e., 1:4, 1:16, etc.) reacting with antiserum diluted 1:8. The larger number indicates enhanced activity. T, Trace.

Infectivity of the purified *C. burnetii*. Inoculation of mice with log dilutions of the purified preparations indicated that seroconversion was obtained by as few as two to seven microorganisms (J. L. Cantrell and J. C. Williams, submitted for publication). The number of organisms was calculated from direct counts (32) and PFU (23) (Table 1). Our data are in general agreement with the previously published results of Ormsbee et al. (24). Inoculation of guinea pigs with log dilutions of RPR showed the typical pyrogenic response and seroconversion described by Ormsbee et al. (24).

Purity of *C. burnetii*. Purified *C. burnetii* were analyzed for the presence of contaminating host material by electron microscopy, the CF test for YS antigen, and ability of guinea pigs to raise antibody directed against YS antigen. With the CF test (25, 28), YS antigen was not detected in phase I preparations at 1 mg (dry weight) per ml. However, this test indicated that trace amounts (<1.6%) of YS antigen were detectable in phase II preparations. Guinea pigs injected intraperitoneally with 100 μg of phase I cells in PBS did not show an antibody response against YS components, whereas the temporal development of phase antibodies against rickettsial antigen was demonstrated (Table 5) (12, 34). An attempt was made to elicit an antibody response against the apparent trace YS antigen in phase II cells by injecting 200 μg of phase II cells intraperitoneally into guinea pigs, 100 μg per week at 7-day intervals. Seroconversion against YS or phase I antigens was not detected, whereas the MA titer against phase II antigens at 28 days postvaccination was 512.

Electron microscope results showed the general appearance of *C. burnetii* cells in thin sections of purified preparations of both CB9MI and CB9MII (Fig. 4). The preparations contained a mixture of morphologically distinct cell variants. The small-cell variant was recognized by its uniform rod-shaped size and an extremely dense cytoplasm. The large-cell variant was more rounded, larger, and pleomorphic, with the nucleoid filaments being more dispersed. Whereas the cytoplasm of the small cell was extremely dense, the cytoplasm of the large cell was less dense. Contaminating host material (i.e., membrane, nuclei, and mitochondria) was not observed in these preparations.

Serological activity of purified *C. burnetii*. Serological activity of *C. burnetii* purified as viable cells or as described by Ormsbee (21) was compared by using 12-day, 20-day, and HI anti-*C. burnetii* sera (Table 6). CBOI cells purified

TABLE 5. *Temporal development of phase antibodies in guinea pigs against C. burnetii whole-cell antigens*

C. burnetti antigen[a]	Day post-inoculation[b]	MA titer[c]	
		Phase I	Phase II
CBOI	7	0	256, 16, 64 (64)
	14	8, 8, 2 (5)	2,048, 2,048, 2,048 (2,048)
	28	32, 128, 32 (50)	8,192, 4,096, 8,192 (6,502)
CB9MII	7	0	0
	14	0	128, 1,024, 1,024 (512)
	28	0	512, 512, 1,024 (645)

[a] Antigen was prepared from Formalin-killed whole cells of CBOI and CB9MII.

[b] Vaccine (CBOI or CB9MII) was administered subcutaneously suspended in PBS at 100 μg/ml. Dosages of vaccine (100 μg/animal) were given at time zero and again on day 6.

[c] Endpoint reciprocal dilutions of antisera, using CB9MI and CB9MI-II as antigen. The data for three guinea pigs are presented. Geometric mean (G) is given in parenthesis ($G = \sqrt[n]{X_1X_2X_3 \cdots X_n}$).

by our method were compared with standard antigens (21) by the MA test (11). This test was chosen because it has been shown to be more sensitive than the CF test (11). Inactivated whole cells of CBOI were compared after dialysis or centrifugation to remove the salts and perhaps other components (Table 6, experiments 1 and 2). A difference of two dilutions was observed with the 20-day antisera, whereas only a one-dilution difference was found with the HI antisera. Comparison of CBOI (experiment 2) and CB9MI (experiment 3) revealed no detectable differences between these preparations.

Differences were observed when Renografin-purified CB9MII cells were compared with CB9MI cells which had been extracted with cold trichloroacetic acid. The acid extraction procedure has been shown to remove phase I antigens from the surface of *C. burnetii* cells, thereby exposing the phase II antigens (11). Indeed, Renografin-purified CB9MII cells were not useful as an MA antigen (i.e., autoagglutination was observed), whereas trichloroacetic acid-extracted CB9MI cells were again shown to be the best source of phase II antigens (11). Although the Renografin-purified phase II cells could not be used with the MA test, they were useful in the CF test (data not shown). The application of the microimmunofluorescence technique (28) allowed the antigens on the surface of the RPR to be labeled with fluorescein-conjugated antisera prepared from infected or vaccinated guinea pigs (data not shown).

DISCUSSION

Phase I and phase II cells of *C. burnetii* have been purified as viable organisms by procedures which maintain the integrity of this obligate intracellular parasite. Our procedures differ from previously described purification schemes which were designed by others (4, 5, 10, 21) for the production of large quantities of formaldehyde-fixed cells for vaccine development and for antigen used in serological assays (29). Particulate antigens prepared by our procedure and subsequently fixed with 1% formaldehyde have been shown to be effective as immunogens and serological reagents. Indeed, these antigens (i) elicit the temporal sequence of phase I and II antibody response (ii) are free of host material, and (iii) are good CF, MA, and fluorescent-antibody antigens. More important, we have shown that if *C. burnetii* are treated with formaldehyde and then purified, both particulate and soluble antigens may be released into the supernatant. Preliminary studies indicate that RPR whole-cell vaccines protect guinea pigs (J. C. Williams and M. G. Peacock, manuscript in preparation) and mice (J. L. Cantrell and J. C. Williams, submitted for publication) against lethal challenge doses of live *C. burnetii*. The response in C57BL/10 mice recently described by Cantrell and Williams (submitted for publication) showed that whole-cell vaccines induced liver necrosis, splenomegaly, and hepatomegaly, whereas chloroform-methanol-extracted cells did not induce toxic reactions. In summary, although killed whole cells induced immunity, they were toxic, whereas chloroform-methanol-extracted whole cells, which also induced immunity, were nontoxic (Cantrell and Williams, submitted for publication).

Studies on the physiology of *C. burnetii* were begun in 1958 by Paretsky et al. (26). Purification was initiated from 20% YS suspensions in sucrose-phosphate-glutamate buffer (41). Nine steps in the procedure were described, which included treatments with Celite, 6% bovine albumin, 1% trypsin, and, finally, sedimentation through anti-YS serum. The investigators were successful in demonstrating enzymatic activities associated with the rickettsiae, although complete purity of the preparation was not demon-

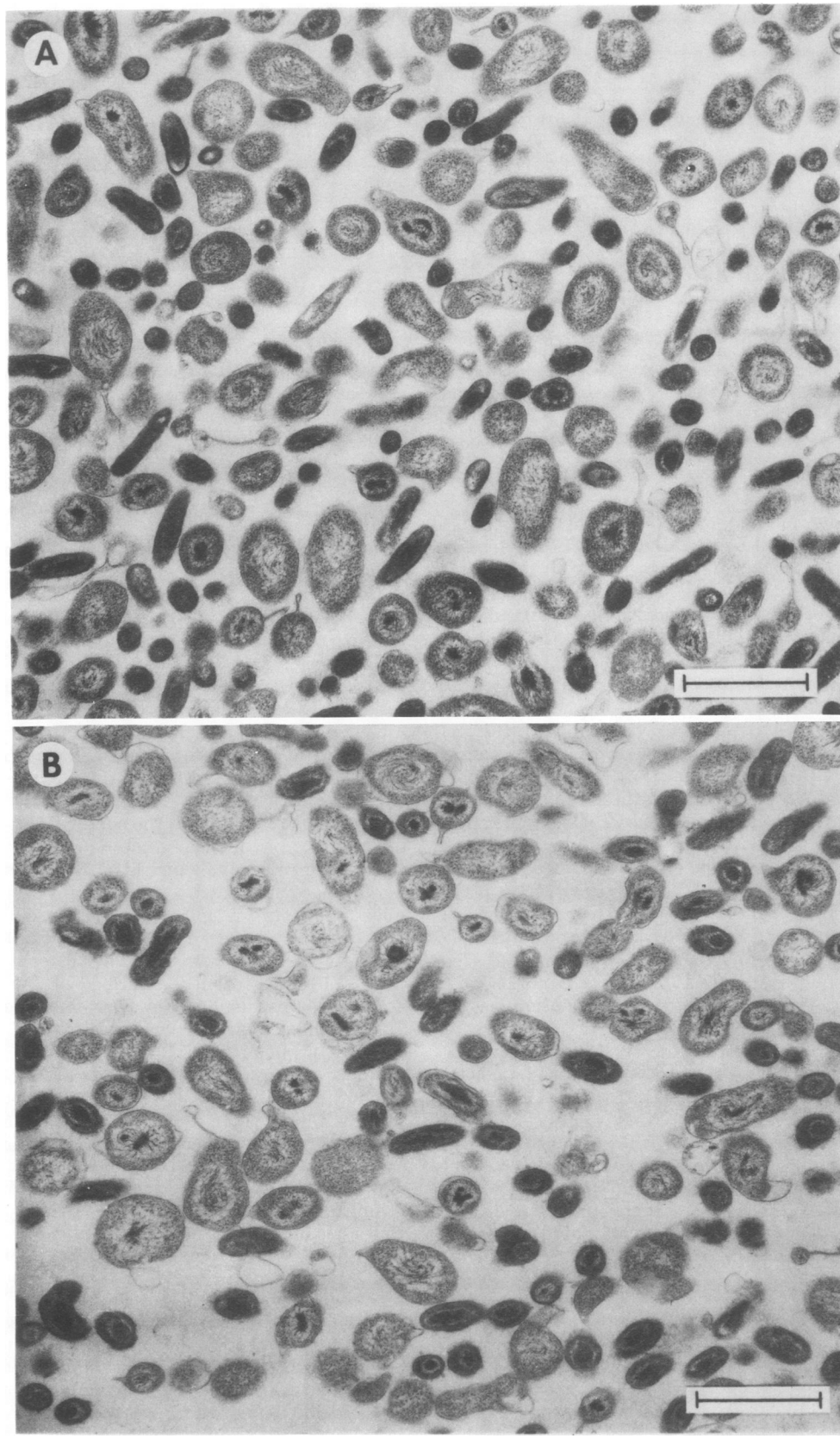

FIG. 4. *Electron photomicrographs of C. burnetii (phases I and II) separated from host components. (A) Phase I cells of CB9MI. (B) Phase II cells of CB9MII. Bar equals 1 μm.*

TABLE 6. *Comparison of serological reactivity of C. burnetii by the MA technique*

Expt	*C. burnetii* antigen[a]	MA titer[b]		HI serum
		12-day serum	20-day serum	
1	CBOI; live purified, inactivated, and dialyzed	0	8	4,096
2	CBOI; live purified, inactivated, and washed	0	32	2,048
3	CB9MI; inactivated, purified, and washed	0	32	2,048
4	CB9MI-II; inactivated, purified, and washed	512	4,096	2,048
5	CB9MII; live purified and washed	AA[c]	AA	AA

[a] Cells were live purified or inactivated with Formalin before purification. After live purification, the cells were inactivated with Formalin and (i) dialyzed (membrane with molecular weight cutoff of 2,000) to remove salts (experiment 1) or (ii) washed by centrifugation (experiments 2 through 5). Conversion of phase I to phase II (CB9MI-II) was accomplished by trichloroacetic acid extraction (1; see Table 5).

[b] Serological MA test as described by Fiset et al. (11); reciprocal titers with 12-day, 20-day, and HI anti-*C. burnetti* sera from guinea pigs (23).

[c] AA, Autoagglutination.

strated. More recently, preparations such as the one described by Paretsky et al. (26) have been used by several investigators to study other enzyme systems of *C. burnetii* (35). Purification of viable organisms was carried out by Ormsbee and Peacock (22) by modification of the above procedure to include diethylaminoethyl (18)-cellulose adsorption of YS components. Tests of purity of microscope examination, serological assays, and density gradient centrifugation (22) revealed only slight contamination with host material. The adsorption of rickettsial material by this method was not determined. However, metabolism studies carried out on cells treated with diethylaminoethyl cellulose showed only minimal metabolic activity (22), possibly because the pH was well above the optimum (15). Metabolic studies carried out on cells purified by our method have shown that glucose and glutamate are metabolized optimally between pH 3.5 and 4.5, respectively (15; T. Hackstadt and J. C. Williams, submitted for publication).

Density gradient centrifugation in sucrose or glycerol was first used to purify *C. burnetii* by Ribi and Hoyer (31). Treatments such as Celite, trypsin, or diethylaminoethyl cellulose adsorption were not carried out in their procedure. Some cells fractionated from the gradients were easily disrupted by osmotic shock, creating cell walls; however, lysis was prevented if the cells were dialyzed or suspended in 0.02 M phosphate buffer containing 0.1 M KCl and 0.2 M sucrose. We have also demonstrated that osmotic shock caused lysis of the large-cell, but not the small-cell, variant (20). *C. burnetii* suspended in PBS (301 mosmol/kg) caused lysis of some morphologically large cells, as demonstrated by the appearance of antigen near the top of Renografin gradients (Fig. 2B), whereas resuspension in PBSS (544 mosmol/kg) prevented lysis; however, the cells were slightly plasmolyzed by this procedure (Fig. 4).

Application of the method developed by Ribi and Hoyer (31) was extended by Wiebe et al. (37) in the isolation and characterization of two cell types of *C. burnetii*. The buoyant densities of cells purified with and without trypsin treatment were compared in cesium chloride or linear gradients of sucrose and Renografin. Two cell types were described which differed in ultrastructure and density, with density ranging from 1.176 to 1.202 g/ml. The organisms recovered from the density gradients were infectious and free of host material. Although we observed two cell variants (Fig. 4), no attempt was made to separate them in density gradients.

The primary purpose of our investigation was to develop a method of purification that would provide a high yield of viable phase I and phase II cells suitable for biochemical, immunological, and genetic studies. Long-range objectives include the characterization of surface components, transport and enzymatic capabilities of whole cells and cell extracts, as well as genetic mechanisms underlying the phase transition of *C. burnetii*. The results presented herein strongly indicate that this purpose has been accomplished. The purification procedure offers several advantages over published methods because the organisms are not exposed (i) to perturbing extracellular reagents such as trypsin, Celite, or diethylaminoethyl cellulose, (ii) drastic osmotic shock procedures, or (iii) extraneous proteins such as bovine plasma albumin or anti-YS serum. With less purified preparations of phase I and phase II cells, identification of rickettsial surface components and enzymatic systems would require additional experimental techniques. The high yield and recovery obtained from the starting material (Table 2) indicate that the Renografin method is highly satisfactory. Recovery of rickettsiae from YS in the growth study showed that harvest from live embryo YS gave the greatest yield of organisms at about 41% embryo lethality (Table 3). We do not normally harvest organisms from dead embryo YS since in our studies the yield from such material is lower. A good relationship has been demonstrated between Klett units, dry weight, and particle counts (Fig. 3), which provides an easy method of determining the number of bacteria under investigation. Since the organisms

are highly resistant to external environments, we feel that PFU can be directly related to the turbidity measurements and direct counts (Table 1 and Fig. 3).

Renografin gradient centrifugation as a final step in the purification of rickettsiae from host components and organelles has been established as a common laboratory technique, using *R. typhi* and *R. prowazekii* (8, 36) and, more recently, *R. rickettsii* (2). This technique has been used as the first step before the analysis of antigenic structure (2, 36) and metabolic capabilities (6, 38–40) of the typhus and spotted fever rickettsiae.

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