

Purification of Large Quantities of *Coxiella burnetii* Rickettsia by Density Gradient Zonal Centrifugation

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The purification of large quantities of inactivated, phase II *Coxiella burnetii* by isopycnic zonal centrifugation for use as diagnostic antigen and as a vaccine is described. The fractionation of egg yolk sac-derived *C. burnetii* vaccine resulted in the separation of two distinct populations of organisms, each devoid of microscopically and serologically recognizable components of egg yolk sac. One population of organisms, characterized by an equilibrium density of 1.240, was rod shaped (1.0 by 0.5 μ mole) with a thick, densely strained wall and prominent central body. The second population, with an equilibrium density of 1.280, had a coccobacillary shape (approximately 1 μ mole in diameter), granular, sometimes fibrillar cytoplasm, thin cellular walls, and lacked a prominent nucleoid.

In recent years, zonal centrifugation techniques have been employed widely for large-scale preparation of subcellular particles and microorganisms (1). Despite reports of successful application of the methodology to commercial production of purified, inactivated influenza vaccine (5, 8), apparently little consideration has been given to evaluating the feasibility of utilizing these procedures in the production of other viral and rickettsial vaccines, particularly those associated with egg products. In addition to the desirability of minimizing extraneous antigenic material, improvement in the homogeneity of preparations would allow better characterization of their immunogenic and allergenic properties and increased sensitivity and specificity of diagnostic reagents. The present study describes purification of large quantities of inactivated *Coxiella burnetii* by zonal centrifugation for use as diagnostic antigen and as vaccine.

MATERIALS AND METHODS

A large quantity of Formalin-inactivated, liquid Q fever vaccine, lot FP2313, was obtained from Walter Reed Army Institute of Research. This vaccine was prepared in 1964 from egg yolk sac (EYS) membranes infected with (Phase II) *C. burnetii*, Henzeling strain, as previously described (2). Two liters of vaccine was centrifuged at 95,500 $\times g$ for 30 min and the resulting pellet, suspended in 50 ml of saline

solution, was subjected to isopycnic centrifugation in a Spinco B XIV zonal rotor. With the rotor spinning at 3,000 rev/min, the sample was layered via the core line on a 29 to 56% linear sucrose gradient containing 0.15 M NaCl which was followed by a 20-ml saline overlay. The rotor was accelerated to 35,000 rev/min and centrifuged for 150 min. After deceleration to 3,000 rev/min, 33 20-ml fractions were collected by displacement of the gradient with 59% sucrose. Samples of pooled samples were prepared for electron microscopy, analyzed for nitrogen content by an automated Technicon procedure, and titrated for complement fixation activity with anti-EYS sera and with guinea pig antisera obtained during convalescence from an infection with *C. burnetii*. The anti-EYS sera employed could detect 0.12 μ g of EYS protein nitrogen per ml.

Serial fivefold dilutions of selected preparations were employed in assays for immunogenicity. Hartley strain guinea pigs of both sexes weighing 300 to 400 g were segregated into groups of 10 and injected intraperitoneally (ip) with 1 ml of an appropriate dilution of the selected fraction on day 0. Rectal temperatures were recorded daily from day 18, and 1,000 median guinea pig intraperitoneal fever doses of virulent Phase I *C. burnetii*, Henzeling strain, were injected ip on day 21. Animals developing fever of 40 C or greater for 2 consecutive days were considered to be unprotected. Titration end points were calculated by the method of Reed and Muench (7). The median guinea pig intraperitoneal immunizing dose (ED₅₀) was expressed as the amount of protein nitrogen (μ g) that prevented development of a febrile response in 50% of the challenged guinea pigs.

RESULTS

High-speed centrifugation of Q fever vaccine resulted in stratification of sedimentable particulate components into three different layers (Fig. 1). The bottom of the pellet consisted of *C. burnetii* organisms in which two distinct morphological types were recognized. One type had a prominent electron-dense central body and thick cell walls. The second type was rounder and lacked both a prominent central body and thick cellular walls. The middle

layers of the pellet were composed of mixtures of organisms, electron-dense globules, membrane fragments, and amorphous material. A homogeneous mixture of small vesicles, membranes, and amorphous substances formed the upper portions of the pellet.

Zonal centrifugation of reconstituted pellets resulted in fractionation of the particulate material into three distinct peaks. Figure 2 shows a typical distribution pattern of material in the gradient as determined by light ab-

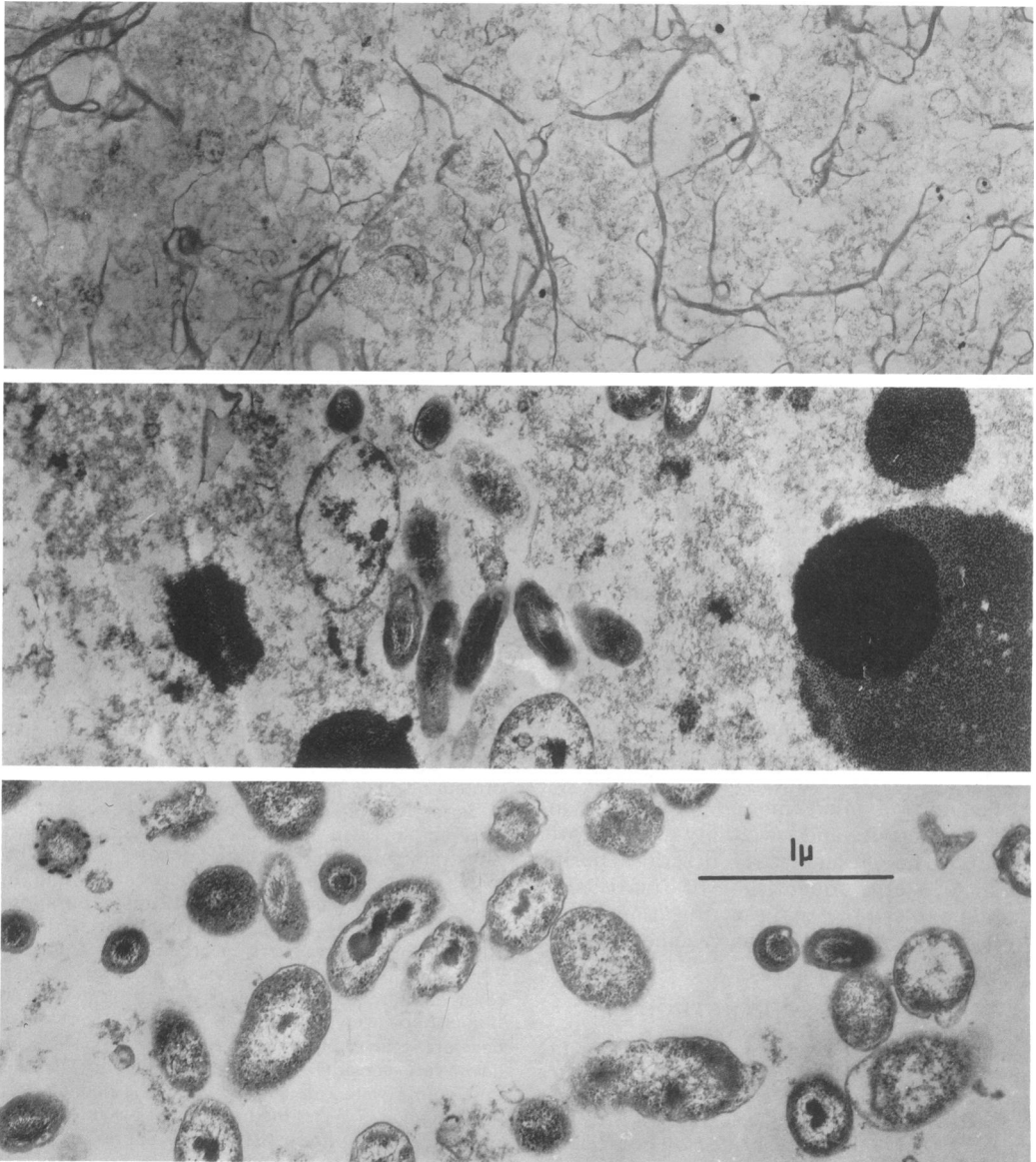


FIG. 1. Electron micrograph of particulate components of crude Q fever vaccine showing the top, middle, and bottom sections of a pellet obtained after high-speed centrifugation.

sorption at 280 nm. Fractions pooled prior to chemical and morphological examinations are indicated in Fig. 2 by line segments labelled A through F.

Fraction A. This fraction had a protein nitrogen concentration of 29 $\mu\text{g}/\text{ml}$ and contained very light density material that floated from the sample zone to the top of the overlay solution.

Fraction B. Forty-two per cent of the protein nitrogen in the sample applied on the gradient was found in this fraction. Electron microscopy demonstrated that fraction B was comprised largely of membrane fragments, small vesicles, and numerous electron-dense granules about 150 nm in diameter. An occasional rickettsial organism, probably trapped in the sample zone, was seen (Fig. 3). Reaction with specific anti-EYS sera indicated that a substantial concentration of EYS antigens was present in this fraction.

Fraction C. This fraction contained particulate matter that equilibrated in the gradient at a density of 1.195 to 1.205. The particulate constituents of this portion of the gradient included granules and globules of high electron density, membrane fragments, amorphous substances, and rickettsiae (Fig. 4).

Fraction D. *C. burnetii* organisms having a mean equilibrium density of 1.240 were found in this fraction. The predominant rickettsial form was rod shaped with a thick, densely staining wall and prominent central body (Fig. 5). A second, but minor, component of this fraction was a larger coccobacillary form characterized by granular, sometimes fibrillar, cytoplasm, thin walls, and small nucleoids. No reaction occurred with specific anti-EYS sera. The ED_{50} of this material was 0.016 μg of protein nitrogen.

Fraction E. This fraction had a mean equilibrium density of 1.260 and a protein nitrogen concentration of 19 $\mu\text{g}/\text{ml}$. Electron microscopy confirmed that this fraction contained a mixture of organisms found in fractions D and F.

Fraction F. The predominant forms in this fraction had a mean equilibrium density of 1.280 and were coccobacillary-shaped organisms approximately 1 μm in diameter with thin cellular walls and without prominent nucleoids (Figs. 5 and 6). Some *C. burnetii* organisms characteristic of fraction D were also seen. This fraction, like fractions D and E, was devoid of microscopically and serologically recognizable components of EYS.

A summary of the fractionation procedure and analysis is given in Table 1. Fractions D,

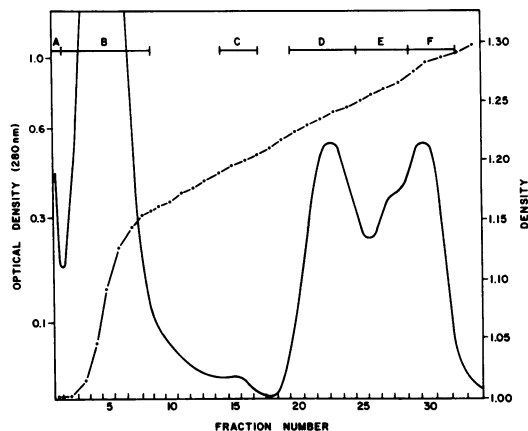


FIG. 2. Elution pattern of optically dense material in sucrose gradient after zonal centrifugation of particulate components in crude Q fever vaccine. Lettered line segments indicate portions of sucrose gradient pooled for analyses.

E, and F accounted for 1.88% of the protein nitrogen content of the crude vaccine.

DISCUSSION

Fractionation and purification methods for *C. burnetii* have been previously reported (3, 4). These published methods have, however, been subject to minimal yields and time-consuming procedures. The methods described in this study overcome these shortcomings in that substantial quantities of purified organisms can be isolated from present vaccine preparations with relative ease.

Electron microscopic and complement fixation tests showed that rickettsial fractions were free of detectable EYS material. In addition, only $\frac{1}{100}$ as much protein of fraction D material, as compared with the crude vaccine, was required to protect guinea pigs against a live Q fever challenge.

C. burnetii is known to exhibit pleomorphism. This phenomenon could explain the presence of the two rickettsial forms. The rod-shaped form that predominated in fraction D electron micrographs measured approximately 1.0 by 0.5 μm and was similar in size and morphology to the classical bipolar forms described by Ormsbee in Horsfall and Tamm (4) and also to the forms studied by Stoker et al. (10). In fraction F, the predominant coccobacillary form was larger (1.0 by 1.25 μm) and denser than any Phase II organisms thus far described in the literature. Although this may reflect pleomorphism, we cannot rule out the possibility that the latter population of rickettsiae may have been derived from rod-shaped

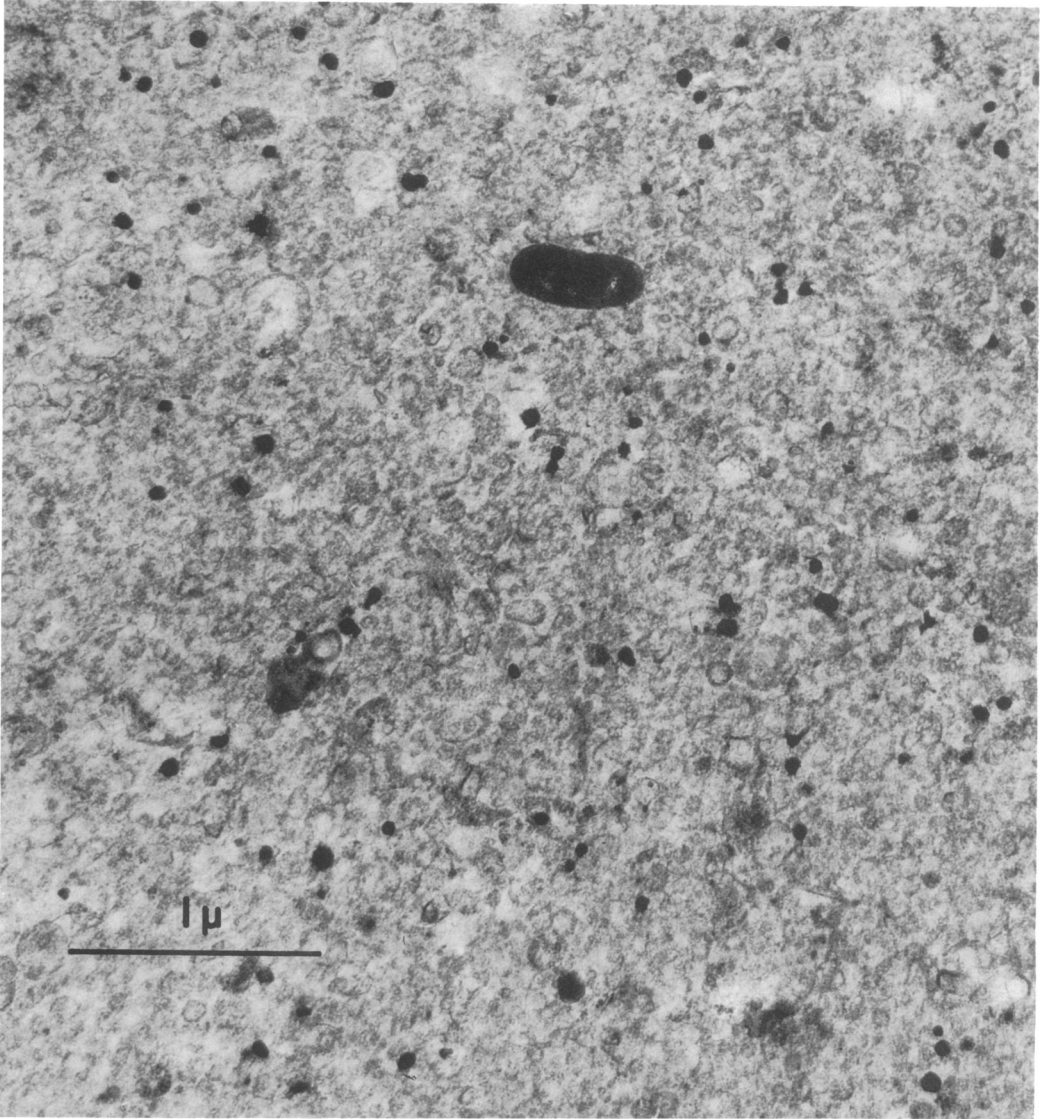


FIG. 3. *Electron micrograph of particulate components of fraction B.*

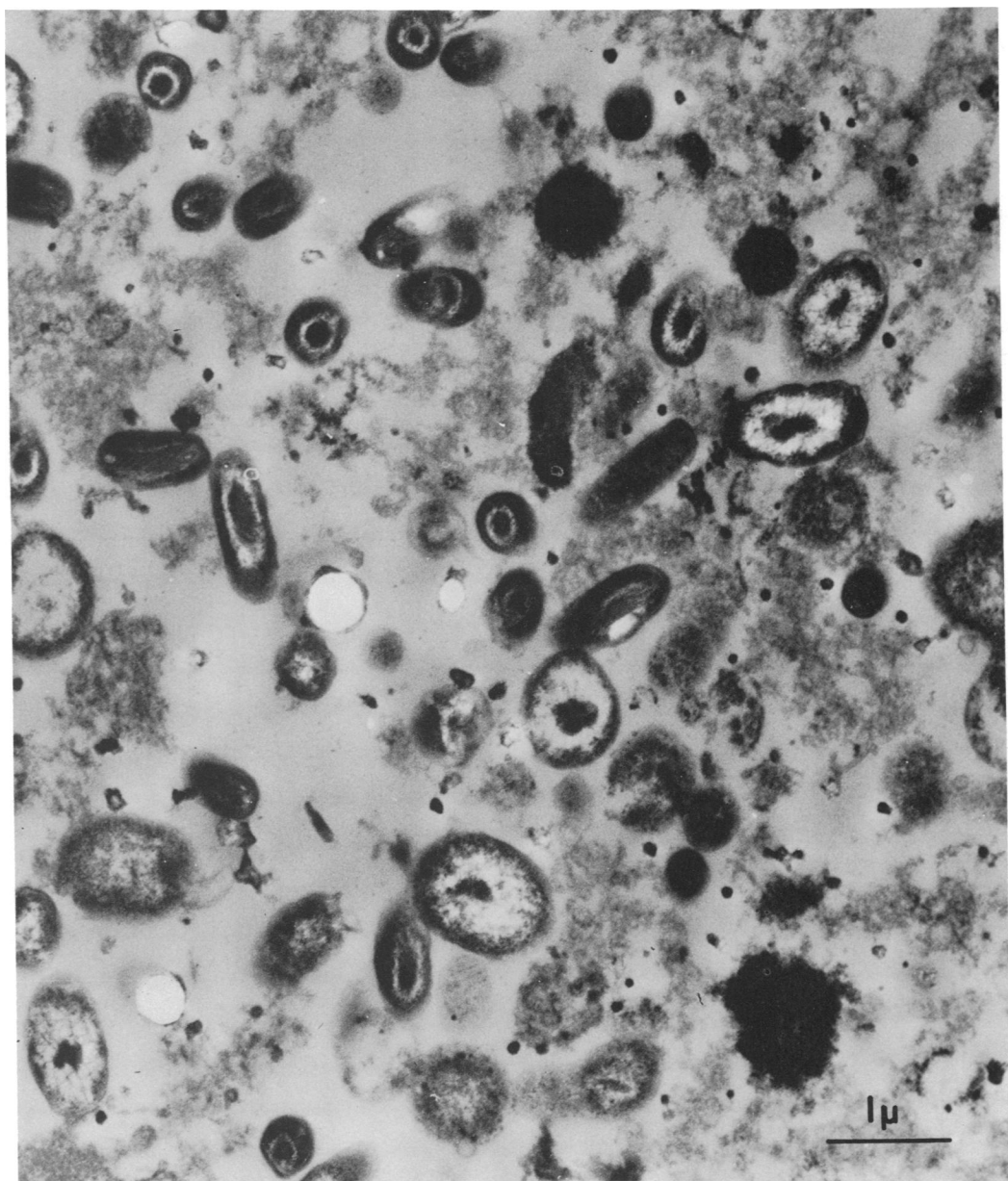


FIG. 4. *Electron micrograph of particulate components of fraction C.*

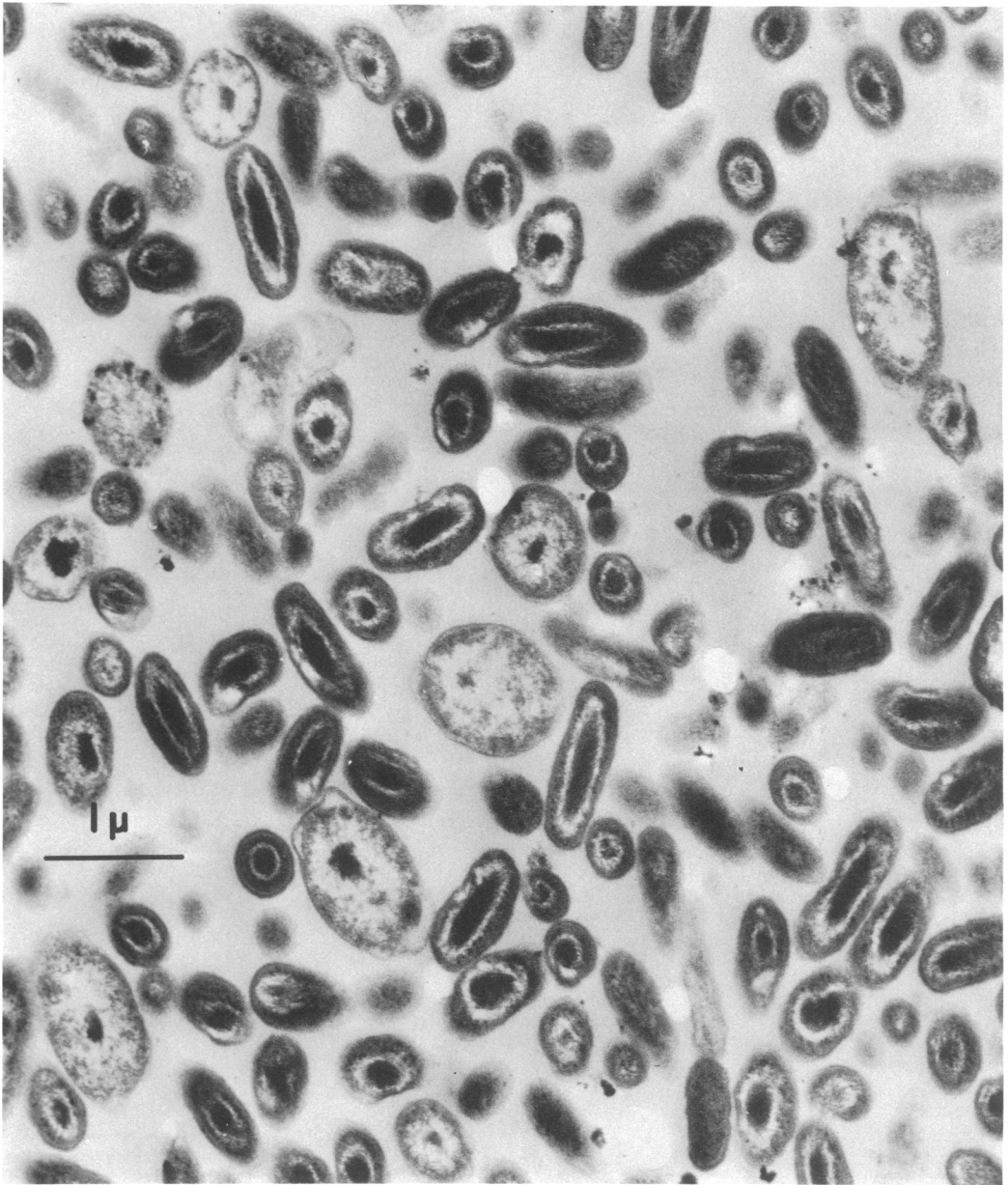


FIG. 5. *Electron micrograph of particulate components of fraction D.*

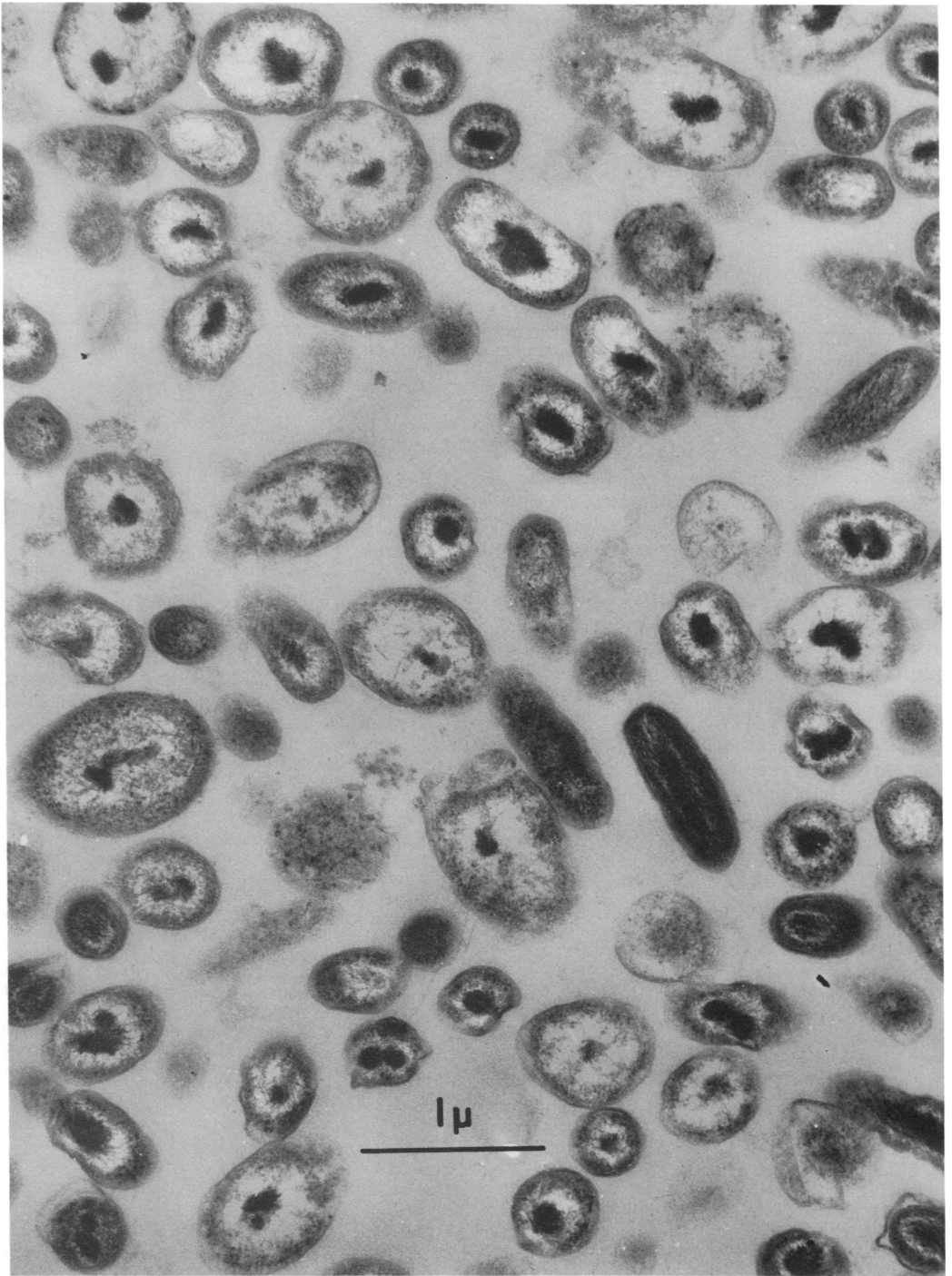


FIG. 6. Electron micrograph of particulate components of fraction F.

TABLE 1. Fractionation analysis of killed *Q* vaccine

Fraction	Vol. (ml)	Nitrogen		Recovery (%)	CF titer ^a		ED ₅₀ (μg)
		μg/ml	Total (mg)		Vaccine	EYS	
Unfractionated vaccine	2,000	263	526.0		40	256	1.606
Supernatant fluid	1,943	258	501.4	95.3	10	256	
Pellet	42	780	32.8	6.2	200	80	
A	27	29	0.79	0.15 (2.4) ^b	10	64	
B	190	73	13.77	2.65 (42.0)	10	64	
D	225	19	4.25	0.81 (13.0)	40	Neg	0.016
E	207	19	3.91	0.74 (11.9)	20	Neg	
F	140	17	2.24	0.43 (6.8)	40	Neg	
(Total)			24.96	4.75 (76.1)			

^a Reciprocal values: titered with antiunfractionated vaccine and anti-egg yolk sac (EYS) sera. CF = complement fixation.

^b Percent of sample placed on gradient.

organisms altered by the chemical treatment employed in the preparation of the original vaccine.

The small-sized dense granules, without visible membrane structures, noted in fractions B and C may be derived from fragmentation of morphologically altered rickettsiae. Plotz et al. (6) described similar small, dense granules which he thought represented cellular debris, salt crystals, or portions of disintegrating rickettsiae.

Antigenic and allergenic characteristics of the rod- and coccobacillary-shaped rickettsiae are currently under investigation. Such studies may help to discern the nature and origin of the two rickettsial structures.

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