

Purification on Renografin Density Gradients of *Chlamydia trachomatis* Grown in the Yolk Sac of Eggs

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Chlamydia trachomatis grown in the yolk sac of embryonated eggs was purified by centrifugation on continuous isopycnic Renografin density gradients. A band of chlamydial particles with a buoyant density of 1.20 contained 70% of the starting particles, and electron microscopy revealed the virtual absence of contaminating egg material. Centrifugation on Renografin gradients caused only a moderate decrease in infectivity. For large-scale purification, infected yolk sac was centrifuged through Renografin solutions, resulting in greater than 60% recovery of starting chlamydial particles, but less than 1% recovery of the dry weight and protein.

Quantities of *Chlamydia trachomatis* sufficient for large-scale production of vaccine can be grown in the yolk sac membranes or chorioallantoic membranes of embryonated eggs. Chlamydial strains isolated from clinical trachoma patients infect cells in culture very inefficiently, so vaccine production by this method is not practical at this time. The yolk sac of eggs provides the most abundant source of trachoma particles and would be an excellent medium for vaccine production if contaminating egg material could be removed by a simple and efficient procedure.

Published methods for purification of chlamydia from yolk sac include differential centrifugation (2, 19), centrifugation in 1 M KCl (17), ion exchange chromatography (19), and sucrose gradient centrifugation (19). Although fluorocarbon extraction has been commonly used, this procedure was reported to cause loss of infectivity of *C. trachomatis* (8, 19) and could destroy important antigens. Digestion of contaminating egg protein with trypsin caused destruction of antigenicity involved in the mouse toxicity prevention test (20).

In this paper, we report that trachoma agent propagated in yolk sac can be highly purified by centrifugation on isopycnic Renografin density gradients by a procedure in which recovery of chlamydial particles is routinely greater than 60% and viability is retained.

MATERIALS AND METHODS

Organism. *C. trachomatis* strain TRIC/2/SAU/HAR-36/OT (1) in the 8th passage in yolk sac was used throughout these studies.

Preparation of infected yolk sac. The infected yolk sac, which was harvested from living eggs (2),

was diluted with an equal weight of phosphate-buffered saline, pH 7.2 (PBS) (18), and the suspension was homogenized in an Omnimix (Ivan Sorvall, Inc.) homogenizer for 30 s at 0 C. This 50% yolk sac suspension was stored at -60 C and was the starting material in all purification experiments. Before use the thawed yolk sac again was homogenized in an Omnimix homogenizer for 3 to 5 min. Normal yolk sac was harvested from 12- to 14-day-old eggs and was treated in the same manner as infected yolk sac.

Renografin. Renografin-76 (3, 7) was supplied as a sterile solution by E. R. Squibb & Sons, Inc. (Princeton, N.J.). All diluted Renografin solutions contained PBS, and density measurements of Renografin solutions included the weight of the PBS.

Linear Renografin gradients. Linear 5 to 20% Renografin gradients were formed in 34-ml cellulose nitrate tubes (2.5 × 7.6 cm) by use of a model 570 gradient former (Instrumentation Specialties Co., Lincoln, Neb.). Gradients had a volume of 28 ml and were overlaid with 2.5 ml of either 50%-infected or uninfected yolk sac. Tubes were centrifuged in a Spinco SW25.1 rotor at 20,000 rpm (40,700 × g) at 4 C for 2 or 4 h. The bottoms of the tubes were punctured, and fractions of approximately 1.5 ml were collected. A 0.2-ml sample was removed from each tube for density measurements, and the remainder of each fraction was diluted with 5 ml of PBS and centrifuged at 17,000 × g for 15 min. The precipitated material was washed twice in PBS and suspended in 0.2 ml of PBS for particle counts and protein determinations.

Recovery of viable particles from discontinuous gradients. A discontinuous gradient consisting of 20 ml of 20% Renografin on top of 3 ml of a 50% solution was overlaid with a 5-ml sample of infected yolk sac and centrifuged for 1 h. The conditions of centrifugation were the same as described for linear gradients. The band of turbidity at the 20 to 50% interface was collected, diluted to 40 ml, sedimented by centrifugation, and suspended to 5 ml in PBS for measurement of chlamydial particles and viability. Viability was

measured by egg infectivity and is expressed as egg infectious doses using a 50% endpoint (EID_{50}) (13).

Particle counts. Chlamydial particles were estimated by dark-field examination of Giemsa-stained preparations according to the method of Reeve and Taverne (14). Normal yolk sac at a final concentration of 1% was added to preparations before application to the glass slide in order to promote sticking.

Batch purification of trachoma agent by using 20 or 32% Renografin. A 200-ml sample of 50%-infected yolk sac was diluted with an equal volume of PBS and centrifuged at $12,000 \times g$ for 1 h. The pellet was suspended to 70 ml and mixed in an Omnimix homogenizer for 5 min. The suspension was layered in equal volumes on top of 25 ml of 20% Renografin in each of six centrifuge tubes. The tubes were centrifuged at $24,000 \times g$ for 2 h in a Sorvall SS-34 rotor, and the pellets were suspended to 32 ml and mixed in the Omnimix homogenizer for 1 min. One-half of the chlamydial suspension was layered on 25 ml of a 20% Renografin solution in each of two tubes, and the other half of the suspension was similarly layered on 32% Renografin solutions. The tubes were centrifuged as described in the previous step. The pellets from the suspension which sedimented through 20% Renografin were suspended to 10 ml in PBS. Pellets obtained by centrifugation through 32% Renografin were suspended in a similar volume of PBS.

Electron microscopy. Samples for electron microscopy were fixed by 1% glutaraldehyde prepared in 0.1 M phosphate buffer at pH 7.2. After fixation for a minimum of 5 h, a drop of the sample suspended in fixative was spread as a thin film on a clean glass slide and allowed to air-dry. To remove the dried buffer salts and glutaraldehyde, the slide was rinsed thoroughly by gentle immersion in distilled water and was air-dried. It was then coated with a 0.75% solution of Formvar in ethylene dichloride and placed in a vertical position to drain. After air-drying, the portion of the film overlying the sample was floated off onto the surface of distilled water (10). Copper grids of 200 mesh were placed on the floating film, and the film containing the grids was then picked up on a glass cover slip with the film uppermost. The cover slip containing the dried replica overlaying the copper grids was shadowed with a thin film of 80% platinum and 20% palladium. Grids containing the shadowed replicas were examined with an RCA EMU 3H electron microscope.

Quantitative methods. Protein was measured by the method of Lowry et al. (9) by using bovine serum albumin as a standard. Dry-weight measurements were made on samples which had attained constant weight in a vacuum oven at 70 C. Material to be assayed for cholesterol was treated with alcoholic KOH and extracted with Ligroine (4). The solvent was evaporated, and the residue was dissolved in absolute ethanol for cholesterol assay by the method of Glick et al. (6).

RESULTS

Centrifugation on continuous Renografin gradients. A 2.5-ml sample of 50%-infected yolk sac, containing 1.08×10^{10} trachoma

particles and 130 mg of protein, was centrifuged on a continuous 5 to 50% Renografin gradient for 2 h. Chlamydial particles were present in a major band at a density of 1.20 and in a minor band at a density of 1.14 (Fig. 1a). Total recovery of particles was 84%, with approximately 70% of the starting particles present in the three fractions centered at a density of 1.20. The presence of chlamydia in this band was confirmed by the staining of the particles with fluorescein-labeled antibody against the chlamydial group antigen (12). Centrifugation for 4 h under the same conditions also gave a band of trachoma particles centered at a density of 1.20 and a minor band corresponding to the lighter band present after the 2-h run, thereby demonstrating that equilibrium of chlamydial particles had been achieved in the shorter run.

Both protein determinations and particle counts were made after twice washing the contents of each fraction in PBS. This eliminated Renografin, which yielded a blue color in the protein determinations. The major band of chlamydial particles at a density of 1.20 corresponded to a small peak of protein which

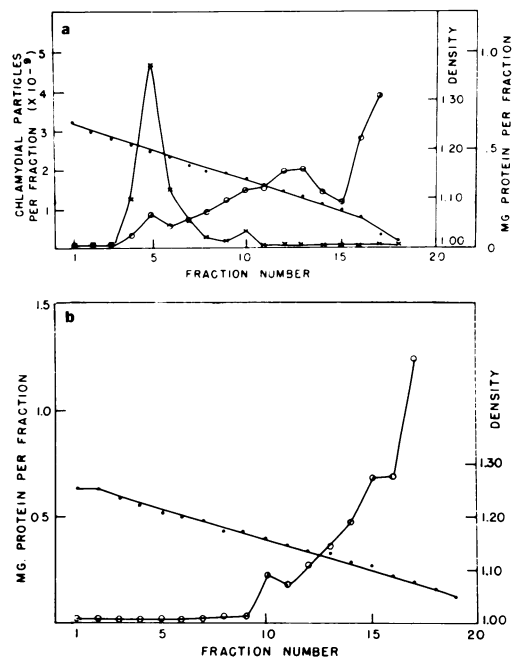


FIG. 1. (a) Centrifugation of infected yolk sac on a 5 to 50% continuous Renografin gradient. Symbols: X, chlamydial particles; O, protein; ●, density. (b) Centrifugation of uninfected yolk sac on a 5 to 50% continuous Renografin gradient. Symbols: O, protein; ●, density.

contained 0.3 to 0.5% of the total protein applied to the gradient (Fig. 1a). The top 3 to 5 ml of the gradient consisted of a yellow band on top of a broader red band, both of which were difficult to collect because of clogging of the collecting device. These bands were not examined further.

A 2.5-ml sample of uninfected yolk sac, containing a total of 155 mg of protein, was centrifuged on an identical gradient for 2 h, and the resulting distribution of insoluble protein is presented in Fig. 1b. Fractions 1 through 9, covering a density range of 1.25 to 1.17, contained 75 μ g of protein or approximately 0.05% of the protein applied to the gradient.

Electron microscopy of purified trachoma agent. The major band of chlamydial particles was collected in the first 12 ml, corresponding to a density of 1.26 to 1.17, from another gradient which had been centrifuged for 2 h as described in the previous experiment. This material was washed twice in PBS and suspended in 1 ml of 1% glutaraldehyde for examination by electron microscopy.

The sample consisted almost entirely of spherical particles of two distinct types which were present either as individual particles or aggregates. One morphological type was approximately 375 to 500 nm in diameter and contained a centrally located dense mass typical of chlamydial elementary bodies (Fig. 2) (11). The other type was approximately 600 to 1,000 nm in diameter, did not have an electron-dense center, and resembled the noninfectious chlamydial reticulate body (11). There was little or no contaminating egg material visible in the sample.

Viability of trachoma after centrifugation

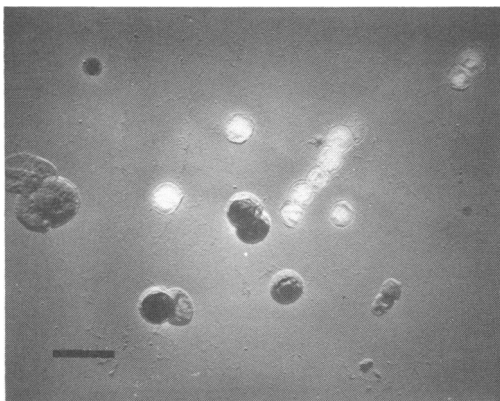


FIG. 2. Electron micrograph of a shadowed preparation of chlamydial particles purified on a 5 to 50% continuous Renografin gradient. Bar represents 1.0 nm.

on Renografin gradients. A 5.0-ml sample of infected yolk sac was centrifuged for 1 h on a 20 to 50% discontinuous Renografin gradient. The band of turbidity at the 20 to 50% Renografin interface was collected, and the material was sedimented, washed once, and suspended to 5.0 ml. Particle counts and EID₅₀ were determined on the starting material and on the band from the gradient. Centrifugation on the gradient resulted in a 0.6 to 0.7 log₁₀ decrease in EID₅₀ (Table 1).

Batch purification of trachoma agent. Based on studies with continuous gradients, centrifugation through 20% Renografin (ρ 1.10) would assure the sedimentation of all particles seen on continuous gradients whereas centrifugation through 32% Renografin (ρ 1.16) would sediment the particles seen in the major peak. On all continuous Renografin gradients, no protein was found in an area of the gradient more dense than the major band of chlamydial particles (fractions 1 to 3, Fig. 1a). Therefore a procedure, described in Materials and Methods, was devised for batch purification in which trachoma particles were sedimented first through 20% Renografin. One-half of the particles were again centrifuged through 20% Renografin, whereas the other half was centrifuged through 32% Renografin. Chlamydial particles, dry weight, protein, and cholesterol were measured in the starting 50%-infected yolk sac and in the material sedimented through the 20 and 32% Renografin solutions (Table 2). Centrifugation through 20% Renografin resulted in recovery of 71% of the starting particles along with 0.55% of the dry weight and 0.94% of the protein. Less than 0.12% of the cholesterol remained. Centrifugation through 32% Renografin resulted in recovery of 68% of the particles with slightly better purification as indicated by the protein and dry-weight values (Table 2).

DISCUSSION

Initial experiments in which 50%-infected yolk sac was centrifuged on continuous Reno-

TABLE 1. Recovery of infectious and total particles after centrifugation of infected yolk sac on a discontinuous Renografin gradient

50%-Infected yolk sac	EID 50 ^a	Total particles (no.)
Untreated	10 ^{7.56}	3.4 × 10 ¹⁰
After density gradient centrifugation	10 ^{6.92}	2.7 × 10 ¹⁰

^a Egg infectious dose using a 50% endpoint.

TABLE 2. Batch purification of trachoma particles by centrifugation through 20 and 32% Renografin

50%-Infected yolk sac	Trachoma particles		Dry Weight		Protein		Cholesterol	
	No.	Recov- ery (%)	Amount (mg)	Recov- ery (%)	Amount (mg)	Recov- ery (%)	Amount (mg)	Recov- ery (%)
Starting material (100 ml)	5.6×10^{11}	100	12,700	100	5,400	100	420	100
Centrifuged through 20% Renografin ^a	4.0×10^{11}	71	70	0.55	51	0.94	<0.5	<0.12
Centrifuged through 32% Renografin ^a	3.8×10^{11}	68	54	0.43	39	0.72	<0.5	<0.12

^a Values are expressed as quantities derived from 100 ml of starting 50%-infected yolk sac.

graffin gradients demonstrated that the chlamydial particles could be separated from most of the contaminating egg material in a one-step procedure. The greater part of the egg material did not enter the gradient. The remainder of the contaminating egg material is either less dense than the chlamydial particles or fails to attain equilibrium during the 2 h of centrifugation. When yolk sac from uninfected eggs was centrifuged on Renografin gradients only a very small quantity of protein was present at a density between 1.21 and 1.18, the density at which the major peak of particles was found when infected yolk sac was centrifuged. However, direct comparisons between uninfected and infected yolk sacs should be made with caution because of possible modification of egg material by the parasite. The density of 1.20 for *C. trachomatis* reported here is close to the 1.21 value observed for infective *Chlamydia psittaci* elementary bodies (5).

Electron microscopy of the trachoma particles in the major band revealed the presence of both the small electron-dense elementary body and the larger less electron-dense reticulate body. Organisms purified on Renografin gradients by the current procedure would contain antigens present in at least two stages of the life cycle. Separation of the two forms on continuous Renografin gradients may be practicable if small fractions are to be examined individually in the electron microscope; however, this was not attempted.

Electron microscopy of the major chlamydial band revealed little contaminating egg material. Chlamydial particles observed in Giemsa-stained preparations of the minor band were not seen by electron microscopy, perhaps because of the small number of particles and the presence of contaminating particulate matter.

The observation that Renografin did not cause a large decrease in viability is not surprising in light of the observation that competence for transformation in *Bacillus subtilis* was preserved during centrifugation on Renografin gra-

dients (3, 7). Likewise Friis (5) reported that *C. psittaci* grown in L cells retained infectivity after centrifugation on Renografin gradients. The viability of trachoma particles after centrifugation suggests the retention of antigenic structure which might be involved in the infectivity of the elementary body. Perhaps soluble chlamydial antigens, if present in infected yolk sac, could be recovered from the top fractions of the gradient and compared in immunogenicity and antigenicity to intact chlamydial particles.

A small scale one-step purification can be achieved on either continuous or discontinuous Renografin gradients. The purification procedure using Renografin solutions in the angle head rotor has been routinely used to process 1.5 liters of infected yolk sac per experiment. Continuous-flow zonal centrifugation (15, 16) using Renografin gradients would be useful in studies requiring large amounts of purified *C. trachomatis*.

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