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PURIFICATION OF *COWDRIA RUMINANTIUM* BY DENSITY GRADIENT CENTRIFUGATION

A. W. H. NEITZ[1], N. M. J. VERMEULEN[1] and G. J. VILJOEN[2]

ABSTRACT

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The isolation of *Cowdria ruminantium* by differential and isopycnic density gradient centrifugation is reviewed with special reference to the suitability of Percoll as density gradient medium. Infected sheep brain, *Amblyomma hebraeum* nymphae and various mouse organs were used as starting material. By these methods, partially purified viable populations of the organism with distinctly different densities were obtained. The conclusions are based upon results of analyses of density fractions by inoculation into sheep or mice, protein determination, electron microscopy and enzyme-linked immunosorbent assay. Morphological differences were observed in the density fractions obtained from infected brain tissue and *A. hebraeum*.

INTRODUCTION

The answers to many current questions in cell biology depend on methods of cell separation. These methods are designed to isolate from a heterogeneous mixture of cells and biological components a population of cells which is significantly enriched in a specific cell type. This is generally a difficult task since cells which are distinctly different in terms of biological function are often very similar with respect to their physical characteristics and *vice versa* (Catsimpoolas & Griffith, 1979).

Ironically, the purification of discrete cell populations has lagged behind the purification of their subcellular particles, macromolecules and other cellular components. Clasically, whole tissues were homogenized and treated as if their cellular constituents were all identical. The existence of developmental cell cycles and different populations of cells were ignored.

The reason for the interest in developing techniques for cell purification stems from the recognition of these factors and from the realization that in order to accurately describe the biochemical characteristics of a particular cell type, pure preparations are essential.

The purification of *Cowdria ruminantium* is important for several practical and analytical reasons. The identification and characterization of immunogenic and antigenic determinants and their use in the production of a specific vaccine through recombinant DNA technology could become feasible once purified organisms become available (Brown, 1986). Purified organisms are also of importance for the development of specific serodiagnostic methods, ideally through the production of monoclonal antibodies. Furthermore, the taxonomic position of the organism could be described more accurately and its developmental sequence in the vector and vertebrate host studied (Du Plessis, 1975), as well as the relationship between developmental stages and pathogenicity. Pure preparations are also essential for the study of the presumed toxin produced by these pathogens (Neitz, 1968) and for extending morphological studies (Pienaar, 1970; Du Plessis, 1970; Uilenberg, 1981).

Numerous methods have been devised for the isolation of cells. Many parameters utilized for the fractionation of molecules and subcellular particles are also applicable to cells (Edelman & Rutishauser, 1974). These include methods which depend on physical properties, such as size, density, shape, adsorption or adherence and chemical or biochemical properties, such as charge and specific affinity for antigens, antibodies, lectins or receptors. As in the case of the fractionation of molecules, successful purification of cells may require a combination of methods. Such combinations, however, may be time consuming and lead to loss of viability particularly in the case of obligate intracellular organisms, such as *C. ruminantium*. A summary of methods for the purification of cells are shown in Table 1.

Various methods for the purification of rickettsial-like organisms have been reported. These include differential centrifugation (Bell & Theobald, 1962), sucrose (Wang & Grayston, 1967), Renografin (Howard, Orenstein & King, 1974; Weiss, Coolbaugh & Williams, 1975) or Percoll (Tamura, Urakami & Tsuruhara, 1982; Neitz, Bezuidenhout, Vermeulen, Potgieter & Howell, 1983; Viljoen, 1983) density gradient centrifugation, continuous flow zonal centrifugation (Anacker, Gerloff, Thomas, Masun, Brown & Bickel, 1967), celite-treatment (Weiss, Rees & Hayes, 1967), fluoro-carbon extraction (Dubois, Cutchins, Berman, Lowenthal & Timchak, 1972), anion (Hoyer, Bolton, Ormsbee, Le Bouvier, Ritter & Larson, 1958), cation (Hara, 1958) and cellular affinity chromatography (Viljoen, Vermeulen, Oberem, Prozesky, Verschoor, Bezuidenhout, Putterill, Visser & Neitz, 1985).

Whatever the biological material to be isolated, it is necessary to define a criterion of purity. Because of the extreme complexity of cells compared to macromolecules, it is difficult to define the purity of a cell isolate. Thus the cells may be homogeneous with respect to several properties but heterogeneous with respect to numerous others.

An idealized diagrammatic representation of the strategy for the purification of *C. ruminantium* organisms is shown in Fig. 1.

Some of the methods that have been employed for the purification of *C. ruminantium* are reviewed in accompanying articles in this journal. In this review centrifugal methods with special attention to isopycnic density gradient centrifugation are discussed.

Differential and rate-zonal centrifugation

Differential centrifugation (differential pelleting) is an inefficient method for the fraction of cells in a heterogeneous cell population. This can be deduced from the following considerations.

It can be shown that the sedimentation rate, v of a spherical particle in a centrifugal field is given by (Bowen, 1970):

$$v = \frac{V(\rho - \rho_o)\omega^2 x}{f} \qquad (1)$$

[1] Department of Biochemistry, University of Pretoria 0002

[2] Veterinary Research Institute, Onderstepoort 0110

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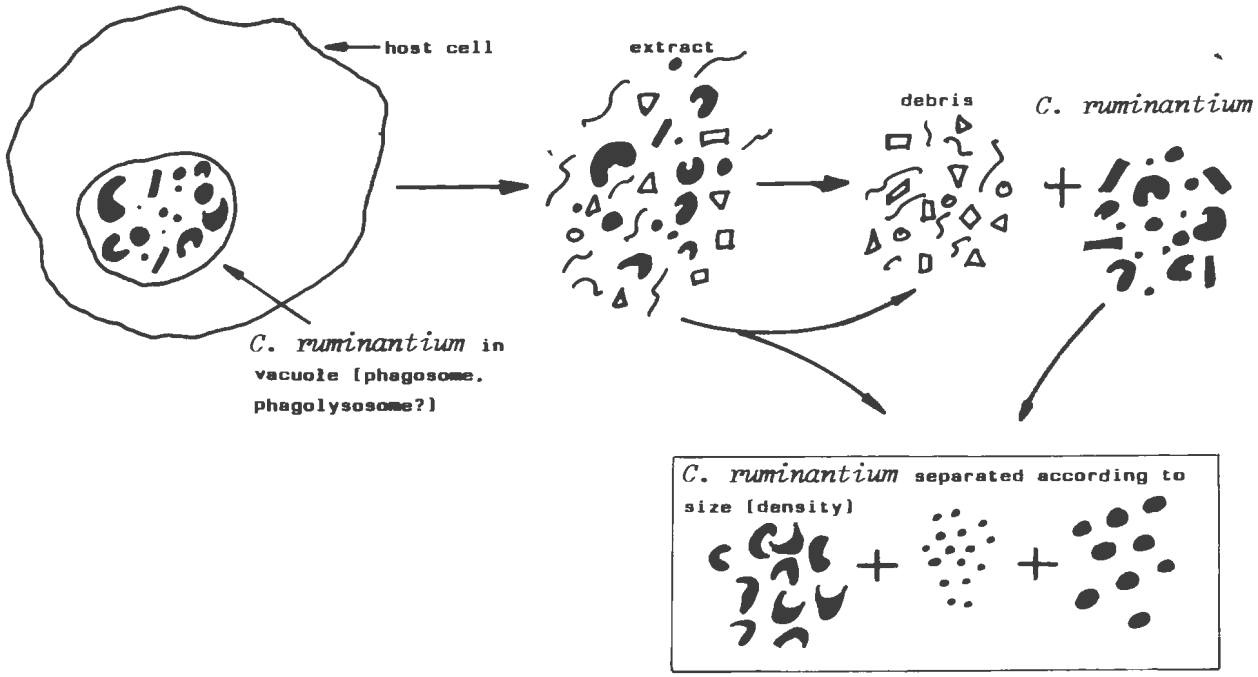


FIG. 1 Idealized purification strategy for the isolation of *Cowdria ruminantium*

TABLE 1 Some methods used for the purification of cells

Method	Main property exploited	Reference
Selective cell lysis	Differences in membrane/cell wall stability	Evans, 1982
Centrifugation:		
differential	Size, shape	Chambers & Rickwood, 1978
rate—zonal	Size, shape, density	Anderson, 1966
isopycnic	Density	Birnie & Rickwood, 1978
elutriation	Size	Lindahl, 1956
sedimentation field flow	Size, density	Kirkland & Yau, 1982
Electrophoresis	Electrostatic charge	Shortman, 1973
Affinity columns	Specific interaction with immobilized lectins, antibodies etc.	Robins & Schneerson, 1974
Two phase partitioning	Density, surface properties	Albertsson, 1962
Fluorescence selection	Development of fluorochromasia	Hullet, Bonner, Barrett & Herzenberg, 1969

in which $\omega^2 x$ is the centrifugal field, V is the volume of the particle, ρ and ρ_o are the density of the particle and suspending medium respectively, and f is the frictional coefficient of the particle which is a function of its shape. This theoretical treatment demonstrates that the sedimentation rate is proportional to the centrifugal field as well as the size and shape of the particle. As is shown later, density variations have a relatively minor influence on the sedimentation rate, provided that the density of the suspending medium does not approach the density of the cells (Shortman, 1972).

Because of the high sedimentation coefficients and relatively narrow size distribution of cells in general, they have similar sedimentation rates, resulting in pelleting of all cells in a short time at relatively low centrifugal forces.

Differential centrifugation is however extremely useful for the removal of host cells, cell debris, intracellular particles and soluble intra-and extracellular material during the initial isolation steps of *C. ruminantium*. Table 2 (adapted from McCall & Potter, 1972 with modifications) summarizes the usefulness of this method for isolation of *C. ruminantium* from biological material. It can be seen that eukaryotic whole cells, cellular debris, nuclei and mitochondria can be removed by low speed centrifugation whereas centrifugation at 30 000 *g* may be used to pellet *C. ruminantium* organisms leaving microsomes and smaller cellular components in the supernatant.

It should be noted that after differential pelleting the pellet is enriched in the larger particles but always contains a mixture of all the different species particles. It is only the most slowly sedimenting components which may be purified by a single centrifugation. The contami-

TABLE 2 Summary of the behaviour of cells and cellular components during centrifugation

RCF (g)	Approximate sedimentation coefficient	Component
<200	$>10^7$	Eukaryotic cells, large debris
700	7×10^6	Nuclei
7000	5×10^4	Mitochondria, bacteria
30000	10^4	*Cowdria ruminantium*
105000	10^2–$1{,}5 \times 10^4$ 30–80	Microsomes Ribosomes
105000	up to 120	DNA
400000 >400000	4–50 2–25	RNA Soluble proteins

nation of the pellet can be reduced by washing (resuspending the pellet and centrifugation). This is time consuming and reduces the yield. The efficiency of the fractionation of particles according to size and shape can be improved substantially by using rate-zonal centrifugation through a density gradient. However, the sample capacity of the centrifuge tubes is greatly reduced.

The separation of cells according to size and shape may be achieved by employing rate-zonal sedimentation in a shallow density gradient at 1 *g* (unit gravity) to slow down sedimentation. The gradient is made as shallow as possible to ensure that separation is on the basis of differences in sedimentation rather than differences in density (Miller, 1984).

The theoretical considerations are outlined below. For spherical particles, the frictional coefficient is given by Stokes's law.

$$f = 6\pi nr \qquad (2)$$

where n is the coefficient of viscosity.

The volume, V is

$$V = \frac{4\pi nr^3}{3} \qquad (3)$$

in which r is the radius of the particle. Substituting equations (2) and (3) in equation (1) and noting that the sedimentation is under the action of the earth's gravitational field, equation (1) becomes:

$$V = \frac{2(\rho-\rho_o)gr^2}{9n} \qquad (4)$$

For *C. ruminantium* the organisms have been shown to vary from 0,2 μm to 0,8 μm in diameter (Neitz, 1968) and in exceptional cases larger organisms with diameter more than 2 μm have been observed. Their densities have been found to vary from approximately 1,05 g/mℓ to 1,10 g/mℓ (Neitz, Viljoen, Bezuidenhout, Oberem, Putterill, Verschoor, Visser & Vermeulen, 1986).

If these cells are sedimenting through an aqueous medium with a density of 1,0 g/mℓ it is evident that size variations can give rise to approximately a sixteenfold variation in terminal velocity, whereas density variations give rise to only about a twofold variation in terminal velocity. Consequently, variations in the sedimentation-rate will primarily be as a result of variations in size.

Equation (4) holds true for non-spherical particles with an error of less than 10 % for both prolate and oblate spheroids with axial rations a/b <3 when r is taken to be that of an equivalent sphere (Miller, 1984).

When sedimentation is carried out at 1 *g* with ρ_o = 1,01 g/mℓ and n = 1,567 cP equation (4) becomes

$$v = 5{,}0(\rho-1{,}01)r^2 \qquad (5)$$

in which v is in mm/h, ρ is in g/mℓ and r is in μm. A cell with ρ = 1,06 g/mℓ will exhibit a velocity shown in equation (6)

$$v = \frac{r^2}{4} \qquad (6)$$

C. ruminantium cells will thus sediment with velocities of between 0,0025 mm/h and 0,04 mm/h. On the other hand, host cells migrate with velocity of approximately 6 mm/h. These calculations indicate that *C. ruminantium* cells may efficiently be resolved from host cells. They also show, however, that *C. ruminantium* organisms migrate extremely slowly and that the separation of these organisms according to size are impracticable by velocity sedimentation at unit gravity.

Consequently, it seems appropriate to resolve *C. ruminantium* organisms according to differences in cell density which is roughly inversely proportional to cell size (Miller, 1984).

Isopynic density gradient centrifugation

In contrast to rate-zonal density gradient centrifugation, in which the highest density of the gradient is lower than that of the particles, isopycnic density gradient centrifugation involves sedimentation in a gradient whose maximum density exceeds that of the particles. During centrifugation, a situation is thus reached during which the density of the particles equals that of the medium and, according to equation (1) when $\rho = \rho_o$, the sedimentation rate is zero (Bowen, 1970).

The method is capable of very high resolution and the buoyant density of a given cell type can be reproduced to $\pm$ 0,0003 g/mℓ over a period of years (Shortman, 1984). It is an equilibrium technique in which cells are separated on the basis of their respective buoyant density. The latter is a reflection of a cell's average chemical composition with the major determinant being the relative water content or relative dry mass of the cell (Shortman, 1972). The separation is independent of the time of centrifugation, after equilibrium is reached, and of cell size and shape. These parameters do however determine the rate at which equilibrium is reached and the width of the zones.

The sample may be layered on a pre-formed gradient or on a gradient forming material or mixed with the latter beforehand. In the 2 last mentioned methods the gradient is formed *in situ*.

The limitations of isopycnic gradient centrifugation are often due to the physical characteristics of the gradient medium. At the density required for ideal resolution, many media have characteristics which are detrimental for living cells. Thus, a compromise between resolution and maintenance of biological integrity has often to be made.

An ideal gradient medium should meet the following criteria (Birnie & Rickwood, 1978).

It should: 1. be stable in solution and cover a sufficient density range for isopycnic banding of all biological particles of interest; 2. possess physiological ionic strength and pH; 3. be iso-osmotic throughout the gradient; 4. be of low viscosity; 5. be non-toxic; 6. be totally inert towards biological particles, that is, it should not be surface-active or interact with them, disaggregate or aggregate them or otherwise alter their physical or chemical structure or composition; 7. be sterilizable; 8. not be hydrated in aqueous solution, that is the water activity of aqueous solutions should be unity; 9. be able to form self-generated gradients by centrifugation at moderate g-forces; 10. have no adverse effect on assay procedures; 11. be completely and easily separable from fractioned particles; 12. have no quenching effects on radioactivity; 13. not have adverse effects on rotors, tubes, tube caps, seals, etc.

Many compounds have been explored for the creation of density gradients, and very few have all the properties of an ideal solute for a particular application. None of the salts used in isopycnic density gradient centrifugation satisfies the criteria with respect to cell separation. Thus, CsCl, which is commonly used for the isolation of DNA, RNA, viruses and HCHO-fixed nucleoproteins, is unsuitable for the separation of cells, because the high osmolarity and ionic strength of the dense salt solutions are detrimental to cells. For cell separations, various non-ionic gradient media are available. Some of these are shown in Table 3 (Pertoft, Laurent, Laas, & Kagedal, 1978; Birnie & Rickwood, 1978; Shortman, 1984) to-

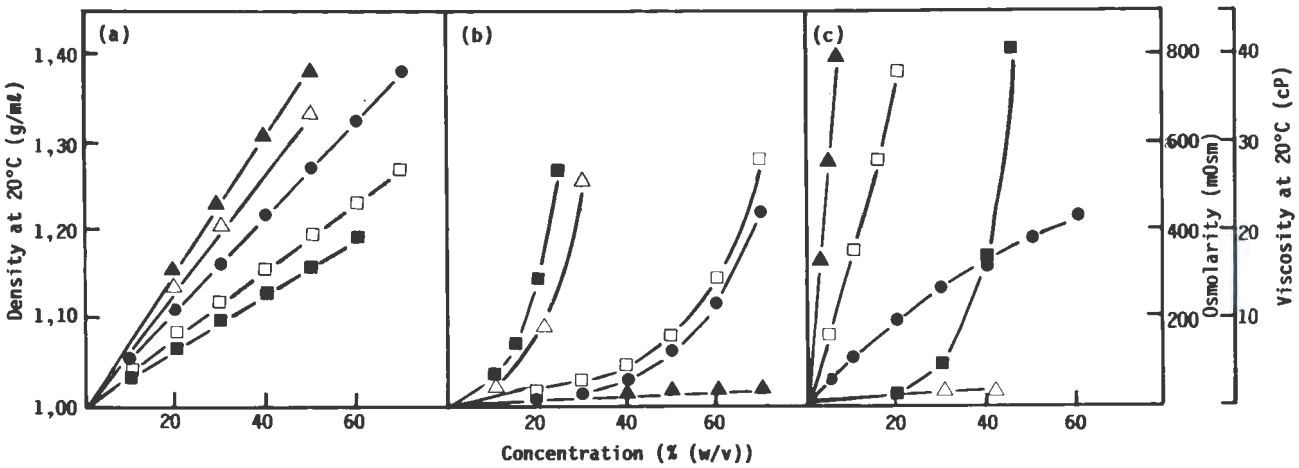


FIG. 2 Physicochemical properties of CsCl and non-ionic gradient media: the variations of (a) density, (b) viscosity and (c) osmolarity with the concentration of solutions of sucrose-□-□-; Ficoll-■-■-; metrizamide-●-●-; Percol-△-△- and CsCl-▲-▲-. Percoll solutions containing 0,15 M NaCl have much lower viscosity than Percoll alone. The data are taken from Birnie & Rickwood (1978) and Rickwood (1978)

TABLE 3 Uses of main types, non-ionic, density-gradient, media in comparison with caesium chloride

Medium	Uses				
	Macro-molecules	Nucleo-proteins	Cell organelles	Viruses	Cells
Albumin					+
Mono- and disaccharides		Some	+	+	
Polysaccharides			+	+	+
Iodinated compounds	+	+	+	+	+
Colloidal silica (Ludox)			+	+	+
Percoll			+	+	+
CsCl	+	Some			

gether with their uses in comparison to CsCl. Their physical properties are shown in Fig. 2 (Birnie & Rickwood, 1978; Rickwood, 1978).

It is evident from Fig. 2 that sucrose solutions have a high osmolarity and that solutions above 9 % (w/w) are hypertonic with the result that a maximum density of approximately 1,03 g/mℓ can be obtained for the separation of osmotically sensitive cells. This density is too low for the isopycnic banding of most cells. A number of polysaccharides, such as glycogen, dextran and Ficoll, are available, as density gradient media. Ficoll, is a copolymer of sucrose and epichlorhidrin. This polymer, at concentrations below 20 % (w/w), equivalent to a density of 1,07 g/mℓ are fairly osmotically inert. At higher concentrations, the osmolarity rises sharply (Fig. 2). Ficoll solutions have a high viscosity and gradients must be prepared with a gradient mixer.

Iodinated gradient solutions, which are derivatives of tri-iodobenzoic acid form gradients *in situ*. With the exception of metrizamide, they are all partially ionic. Metrizamide has proved to be exceptionally inert as a gradient medium. There are indications, however, that ribonucleoproteins are partially dissociated in this medium.

Proteins have been used to separate cells by density gradient centrifugation. The commonest protein employed is bovine serum albumin. Solutions of up to 40 % (w/v) can be prepared. However, the solutions absorb strongly in the U.V.-region and are quite viscous, though they are fairly inert osmotically. Removal of the protein from separated particles may be difficult.

Colloidal silica (Ludox) is useful as gradient medium but is unstable in salt solutions, sticks to membranes and is toxic to biological materials. A new gradient medium (Percoll), consisting of colloidal silica particles which have been firmly coated with a layer of polyvinylpyrrolidone, has been prepared (Pertoft *et al.*, 1978). Percoll shows minimum interaction with biological material and is non-toxic towards cells. Furthermore, it exhibits very

TABLE 4 Characterization of density gradients of Percoll generated in various solvents and centrifuges (Pertoft *et al.* 1978)

Type of rotor	Angle of tube to axis	Volume of centrifuge tube (mℓ)	Solvent	Starting densities (g/mℓ)	Slope of the density gradient (g/mℓ/cm × 10^3) in the middle of the centrifuge tube after centrifugation at 10^6 g × min
Beckman rotor 65	23,5°	13,5	0,25 M sucrose	1,05–1,16	3,5
Beckman rotor 65	23,5°	13,5	0,15 M NaCl	1,05–1,14	7,5
Beckman rotor 35	25°	94	0,25 M sucrose	1,08–1,16	2,0
Beckman rotor 40.2	40°	6,5	0,25 M sucrose	1,05–1,12	1,1
Beckman rotor 40.2	40°	6,5	0,15 M NaCl	1,06–1,13	6,0
Beckman rotor SW 36	90°	13,5	0,15 M NaCl	1,06–1,13	0,7
MSE rotor 10 × 100	18°	100	0,25 M sucrose	1,08–1,16	3,0
MSE rotor 8 × 14	29°	14	0,25 M sucrose	1,05–1,16	1,8
MSE rotor 8 × 14	29°	14	0,15 M NaCl	1,05–1,14	7,0
MSE rotor 6 × 100	30°	100	0,15 M NaCl	1,06–1,13	5,5
MSE rotor 6 × 100	30°	100	0,25 M sucrose	1,08–1,12	1,5
MSE rotor SW 29	90°	38	0,25 M sucrose	1,08–1,16	0
Sorvall rotor SS34	34°	50	0,15 M NaCl	1,06–1,13	6,0

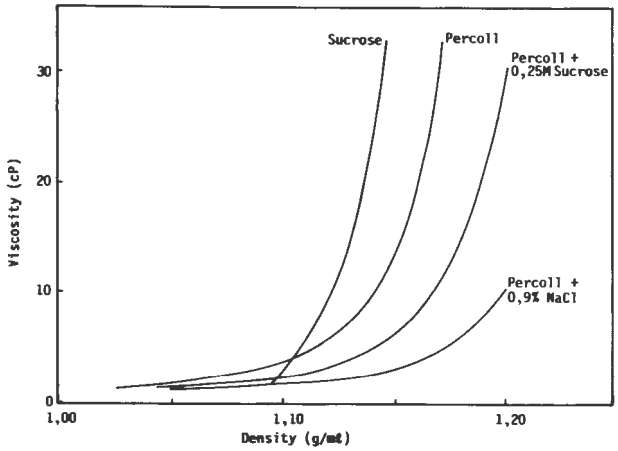


FIG. 3 Relationship between the density and viscosity of sucrose, Percoll, Percoll-sucrose and Percoll-NaCl solutions (Pharmacia Fine Chemicals, 1977)

low viscosity and low osmolarity, which therefore changes little across the gradient. The viscosity is much less than that of sucrose and the viscosity decreases with the addition of saline (Fig. 3). It is also more stable, especially in physiological salt solutions. Gradients ranging from 1 to 1,3 g/mℓ are rapidly formed within 10–30 min, and particles with sedimentation coefficients higher than 60S can be successfully banded (Pharmacia Fine Chemicals, 1982).

The slope of the gradients can be determined *in situ* by means of density indicator beads. The use of Percoll thus offers numerous advantages and has been applied to the isolation of a variety of cells with complete retention of morphological integrity and biological activity (Schumacher, Scheifer, Holtstein & Hitz, 1978).

Rotor geometry and the size of the tubes have a substantial effect on gradient slopes, as shown in Table 4 (Pertoft *et al.*, 1978).

The length of the liquid column in the tubes in the direction of the centrifugal field decreases progressively as the angle of the tube decreases from 90° (in swing-out rotors) to 0° (in vertical-tube rotors). The time required to form a density gradient is given by equation (7) (Birnie & Rickwood, 1978).

$$t = k(x_b - x_t)^2 \qquad (7)$$

where t is the time in hours, x_t and x_b are the distances in cm of the top and bottom, respectively, of the density gradient from the axis of rotation, and k is a constant which is inversely proportional to the diffusion coefficient of the solute which forms the gradient. Thus the time required for an equilibrium gradient to form depends on the length of the liquid column in the centrifugal field and is independent of the angular velocity (ω) of the rotor. However, the time required for sample particles to reach equilibrium is inversely proportional to the 4th power of ω.

The slope of the gradient ($d\rho/dx$) is proportional to ω^2. Thus the slope formed at low speeds results in sample zones further apart. However, since the width of the zones is inversely proportional to ω^2, the zones are wide at low speed and may overlap. Conversely, at high speed the zones are narrow, but closer together.

From the short discussion on the various centrifugal methods available for cell separation it is evident that a combination of differential and isopycnic centrifugation with Percoll as gradient medium in a fixed angle rotor should be ideally suited for the isolation of *C. ruminantium* from various sources.

Our attempts to isolate *C. ruminantium* by these methods are summarized below. Details of these investigations have been published (Neitz *et al.*, 1986; Viljoen, 1983; Viljoen, 1985). In addition, some unpublished experimental results are furnished.

MATERIALS AND METHODS

Prepration of crude sheep brain, mouse liver and spleen and nymph extracts

Sheep brain and *Amblyomma hebraeum* nymphae (Spes bona strain) were infected as described by Viljoen *et al.*, (1985) with the Onderstepoort Ball 3 strain of *C. ruminantium*.

Tissue homogenates were prepared as described previously (Neitz *et al.*, 1986). All isolation steps were performed at 4°C.

The *C. ruminantium* Kümm strain infected mouse liver and spleen tissue (Du Plessis & Kümm, 1971; Du Plessis, 1982), were homogenized at low speed, immediately after dissection, with a Waring blender in buffered lactose peptone (BLP), consisting of 0,6 g (w/v) Na_2HPO_4, 0,08 g (w/v) KH_2PO_4, 2 % (w/v) Difco peptone and 10 % (w/v) lactose for 5 min.

All homogenates were centrifuged for 30 min at 1 000 *g*, followed by centrifugation at 10 000 *g* for 30 min and 30 000 g for 30 min. The final pellet was resuspended in 0,5 mℓ or 15 mℓ 0,154 M NaCl. This represented the crude extract.

Percoll density gradient centrifugation

An iso-osmotic Percoll[1] stock solution (SIP) with density of 1,123 g/mℓ was prepared by adding 9 mℓ of Percoll to 1 mℓ of 1,54 M NaCl. The density of this solution (ρ_i) was calculated from equation (8) (Pharmacia Fine Chemicals, 1982),

$$\rho_i = \frac{V_o\rho_o + V_x\rho_x}{V_x + V_o} \qquad (8)$$

in which V_x and V_o are the volumes of the diluent and Percoll, respectively, and ρ_x and ρ_o are the densities of 1,54 M NaCℓ (1,058 g/mℓ) and Percoll, respectively.

The volume of further diluent (V_y), required to produce a final Percoll solution with density of 1,066 g/mℓ, was calculated from equation (9) (Pharmacia Fine Chemicals, 1982).

$$V_y = V_i \frac{(\rho_i - \rho)}{(\rho - \rho_y)} \qquad (9)$$

in which V_i is the volume of SIP, ρ_y is the density of the diluent and ρ is the density of the diluted Percoll solution.

The resuspended crude extracts (0,5 mℓ) were layered on top of the gradient-forming material, prepared by mixing 15 mℓ SIP with 14,5 mℓ of 0,154 M NaCl. In some cases, the crude extract was suspended in the diluent prior to mixing with SIP. As reference, a tube containing density marker beads[1] was used. Centrifugation was performed for 15 min at 30 000 × g in a Rotor 30 employing maximal acceleration and half maximal braking. Hereafter, the gradient was collected from the bottom of the tube with a fraction recovery system (Beckman Intruments[2]). Ten fractions of 3 mℓ were collected and either used immediately or stored at −30 °C.

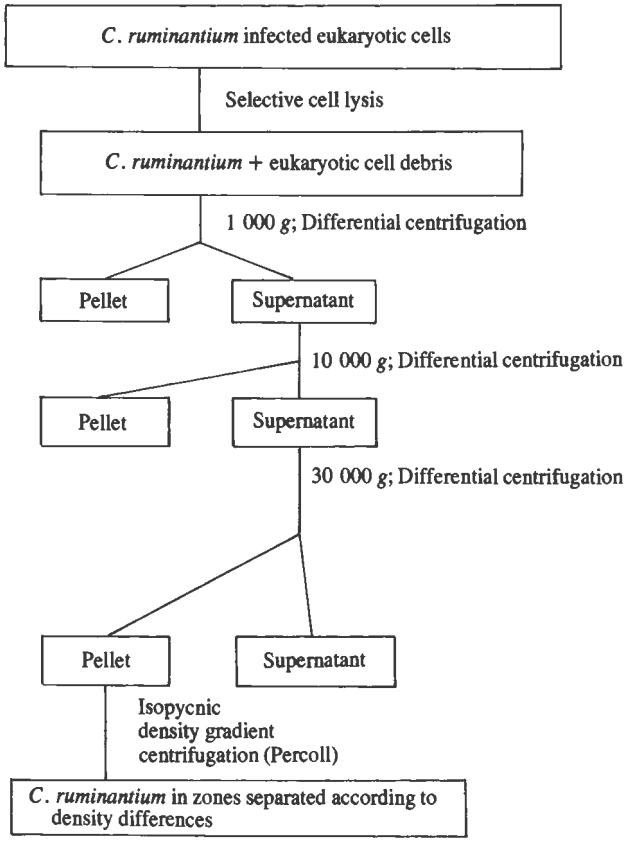
Analysis of fractions

Protein determinations, ELISA and electron microscopic and infectivity investigations were performed as described previously (Neitz *et al.*, 1986).

[1] Pharmacia Fine Chemicals, Uppsala, Sweden
[2] Beckman Instruments, Palo Alto, USA

The isolation method is summarized in Scheme 1.

Scheme 1 Purification of *Cowdria ruminantium* by differential and isopycnic density gradient centrifugation



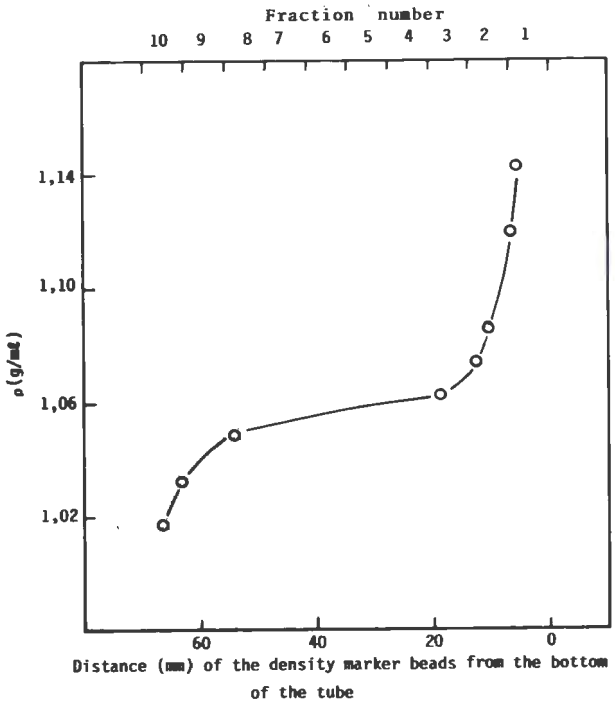


FIG. 4 Typical distribution of density marker beads in a 50 % Percoll gradient after centrifugation at 30 000 *g* for 15 min

RESULTS

A typical density gradient profile as determined with density marker beads is shown in Fig. 4.

Protein determinations of fractions obtained after Percoll density gradient centrifugation revealed proteins in all the fractions with the content significantly higher in the heartwater infected material in comparison to the controls. In all cases the protein content showed 3 peaks in the lower, middle and higher density regions, respectively (Table 5).

All infected Ball 3 strain crude brain extracts and subsequent gradient fractions were infective after i.v. inoculation (Table 6) into sheep. The Kümm strain infected crude liver and spleen extracts and density gradient fractions were infective after i.p. inoculation. The Kümm strain fractions were titrated using mice. For practical reasons the original 10 Percoll density gradient fractions were pooled (Table 6). The infectivity (LD_{50}) of the starting material, titrated at 1/790 and 1/10 000, correlated well with the infectivity of the isolated density gradient fractions (Table 6).

After Percoll density gradient centrifugation of crude heartwater suspensions, *C. ruminantium* organisms and antigen were dispersed widely in the gradient according to biological tests (Table 6) and ELISA methods (Table 7). With ELISA, the highest infected to non-infected (P/N) ratios were observed at densities of 1,109 g/mℓ, 1,058 g/mℓ and 1,050 g/mℓ.

Suspected *C. ruminantium* organisms were seen in all density gradient fractions according to electron-micro-

TABLE 5 Protein content of heartwater infected and non-infected brain or nymph material after Percoll density gradient centrifugation

Percoll fractions	Protein content (mg)			
	Brain material		Nymph material	
	P[1]	N[2]	P[1]	N[2]
Crude extracts	9,0 ± 0,2	1,5 ± 0,15	8,0 ± 0,3	0,7 ± 0,1
1	1,3 ± 0,1	0,08 ± 0,02	0,9 ± 0,1	0,02 ± 0,01
2	0,2 ± 0,1	0,06 ± 0,03	0,03 ± 0,1	0,01 ± 0,01
3	0,7 ± 0,15	0,09 ± 0,03	0,4 ± 0,05	0,02 ± 0,01
4	0,4 ± 0,05	0,07 ± 0,03	0,4 ± 0,03	0,03 ± 0,01
5	1,1 ± 0,1	0,10 ± 0,03	0,9 ± 0,05	0,08 ± 0,02
6	1,2 ± 0,2	0,11 ± 0,04	1,2 ± 0,1	0,1 ± 0,01
7	0,6 ± 0,15	0,05 ± 0,03	0,7 ± 0,07	0,05 ± 0,01
8	0,5 ± 0,2	0,04 ± 0,03	0,6 ± 0,03	0,02 ± 0,01
9	1,4 ± 0,15	0,28 ± 0,04	1,1 ± 0,1	0,1 ± 0,02
10	1,5 ± 0,2	0,42 ± 0,04	1,3 ± 0,2	0,2 ± 0,03

P—Heartwater infected (experiment II and V from Table 6)
N—Heartwater non-infected

[1] All fractions obtained from heartwater infected fractions contained *Cowdria ruminantium*-like organisms according to electron-microscopy

[2] No heartwater suspected organisms were found in the non-infected material according to electron-microscopy.

TABLE 6 Infectivity of Percoll density gradient fractions

Percoll fractions	Density g/mℓ	Ball 3 strain					Kümm strain	
		Sheep brain material		*Amblyomma hebraeum* Spes Bona strain			Mouse liver and spleen	
		I	II*	III	IV	V	VI[1]	VII[1]
Starting material		A	A	A	A	A	10 000	790
Crude extract		+(14)	+(17)	A	A	A		
1	1,134 ± 0,008	+(12)	+(11)	+(11)	+(18)	+(10)	2 512	166
2	1,109 ± 0,017	+(11)	+(10)	+(8)	+(18)	+(8)		
3	1,078 ± 0,013	+(13)	+(11)	+(8)	+(17)	+(7)		
4	1,062 ± 0,003	–	+(12)	+(10)	–	+(7)	3 163	398
5	1,058 ± 0,001	–	+(10)	+(10)	–	+(8)		
6	1,056 ± 0,001	–	+(11)	+(8)	–	+(9)	2 512	158
7	1,054 ± 0,002	–	+(12)	+(8)	–	+(8)		
8	1,050 ± 0,002	+(13)	+(12)	+(11)	–	+(9)	1 585	106
9	1,045 ± 0,004	+(10)	+(10)	0	+(17)	+(8)		
10	1,034 ± 0,007	+(11)	+(10)	0	–	+(8)		

The day on which the first heartwater symptoms (constant high rectal temperature) appeared, is given in parentheses

I to VII — Experiment number; I–V: tested in sheep as experimental animals, VI and VII: in mice as experimental animals

\+ — Positive reaction (raised temperature)

– — No reaction in sheep (temperature normal during incubation period). Heartwater positive reaction on challenge

A — Not biologically tested

0 — Sheep died c.15 min after injection, probably as a result of anaphylactic shock

* — Investigated electron-microscopically (see Table 5)

[1] — Fractions were pooled as follows:

Fractions 1, 2 and 3, fractions 4 and 5; fractions 6 and 7 and fractions 8, 9 and 10 and titrated in mice. Dilution factors of the pooled fractions which caused 50 % mortality in mice are shown in the table.

TABLE 7 ELISA detection[1] of heartwater organisms after Percoll density gradient centrifugation

Percoll fraction	Density g/mℓ	Brain material	Nymph material
		P/N ratio[2]	P/N ratio[2]
Crude extracts		1,53 ± 0,6	1,82 ± 0,5
1	1,134 ± 0,008	1,36 ± 0,3	1,48 ± 0,2
2	1,109 ± 0,017	2,30 ± 0,3	2,72 ± 0,5
3	1,078 ± 0,013	1,54 ± 0,4	1,84 ± 0,5
4	1,062 ± 0,003	1,70 ± 0,3	1,61 ± 0,4
5	1,058 ± 0,001	2,21 ± 0,5	2,36 ± 0,4
6	1,056 ± 0,001	1,1 ± 0,2	1,88 ± 0,5
7	1,054 ± 0,002	1,1 ± 0,1	1,55 ± 0,2
8	1,050 ± 0,002	2,1 ± 0,4	2,54 ± 0,4
9	1,045 ± 0,004	1,2 ± 0,4	1,55 ± 0,3
10	1,034 ± 0,007	1,2 ± 0,5	1,42 ± 0,6

[1] n = 6

[2] Heartwater infected to heartwater non-infected ratio

scopic investigations (Fig. 5 & 6 and Table 6). In non-infected brain material, very little or no background was observed electron-microscopically. In the case of non-infected nymph material, unidentified rickettsial organisms other than *C. ruminantium* were observed.

Although a limited number of organisms was studied, it appeared that different sizes of heartwater organisms were present at different densities: An increase in the size of the organisms was observed with a decrease of density, the smaller ones (0,45 ± 0,15 μm) at a density of 1,134 g/mℓ and the larger (1,00 ± 0,2 μm) at a density of 1,034 g/mℓ. No significant ultra-structural differences were observed between the organisms of different densities. It is interesting however, to note that ultra-structural differences were observed between the organisms present in infected brain and nymph material. The infected mouse material was not studied electron-microscopically. Organisms obtained from brain tissue demonstrated the presence of defined electron-transparent regions in the electron micrograph (Fig. 5), whereas, organisms isolated from infected nymph material displayed several dispersed electron-transparant areas (Fig. 6).

DISCUSSION

The isolation of *C. ruminantium* by means of Percoll density gradient centrifugation permits the recovery of partially purified, viable organisms possessing different densities in the range from 1,134 g/mℓ to 1,034 g/mℓ.

This exemplifies the pleomorphism of this organism, and the presence of organisms in different stages of development. Similar observations were made for *Rickettsia tsutsugamushi* (Tamura *et al.*, 1982).

Infected crude brain extracts were shown to be infective when inoculated i.v. Only on one occasion has this route of injection of brain material been proved to be successful in transmitting heartwater (Uilenberg, 1971). This is contrary to the results of Ilemobade & Blotkamp (1978), who were able to cause infection by infected brain homogenates through the s.c. route only. Inoculations i.v. either failed to cause disease or resulted in immediate fatalities. These contradictary findings could possibly be explained by the fact that our crude brain extracts were prepared by differential centrifugation of brain homogenates. Thus certain unknown components in brain homogentates may prevent infectivity by the i.v. route.

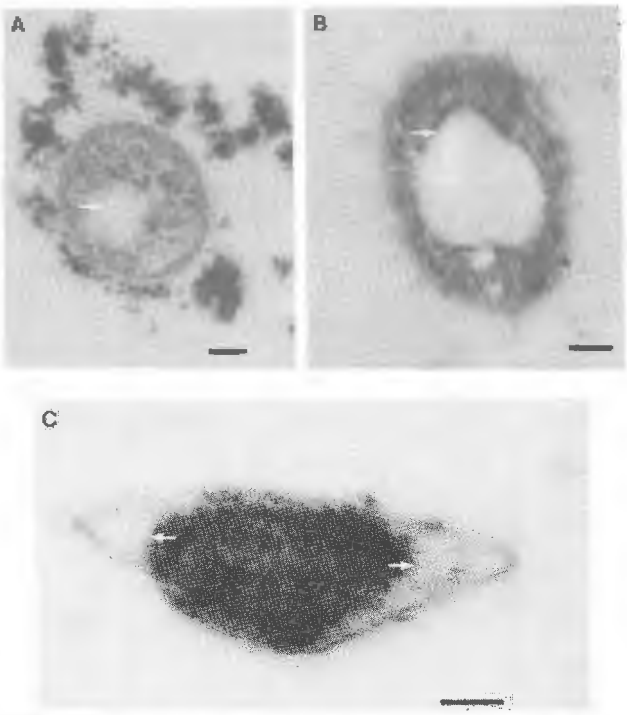


FIG. 5 Electron micrographs of suspected *Cowdria ruminantium* organisms isolated on a Percoll density gradient starting with heartwater-infected sheep brain material

(A) Fraction 2 (1,109 ± 0,017 g/mℓ)
(B) Fraction 5 (1,058 ± 0,001 g/mℓ)
(C) Fraction 8 (1,050 ± 0,002 g/mℓ)

Arrows indicate defined electron-transparent areas

Bar scale = 0,1 μm

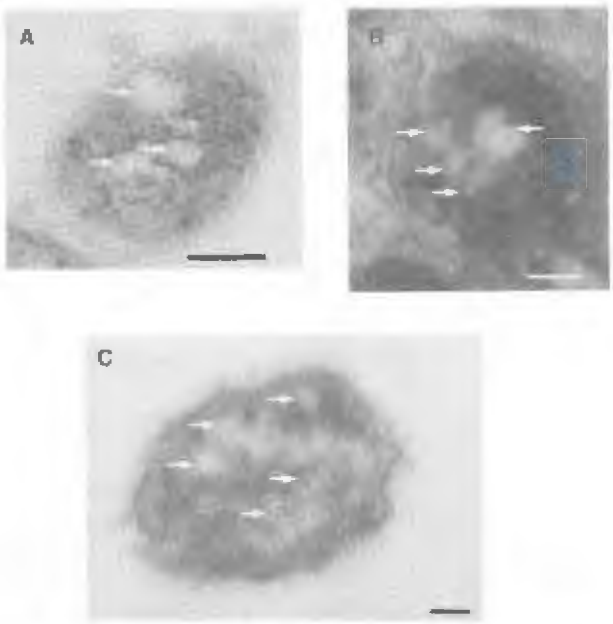


FIG. 6 Electron micrographs of typical *Cowdria ruminantium* (Ball 3 strain) organisms isolated on a Percoll density gradient starting with heartwater-infected *Amblyomma hebraeum* nymphae material

(A) Fraction 2 (1,109 ± 0,017 g/mℓ)
(B) Fraction 5 (1,058 ± 0,01 g/mℓ)
(C) Fraction 8 (1,050 ± 0,002 g/mℓ)

Arrows indicate some scattered electron-transparent areas

Bar scale = 0,1 μm

The location of defined electron-transparent areas observed in electron micrographs of *C. ruminantium*-like organisms from brain has also been reported by Prozesky & Du Plessis (1985). In organisms from nymph material, such defined, single electron-transparent areas were absent. Although caution should be exercised when identifying *C. ruminantium* on the basis of morphology of single organisms, the infectivity and ELISA and the absence of the organisms in the controls serve as additional evidence that the organisms are most probably *C. ruminantium*.

The fractions obtained at densities of 1,109; 1,058 and 1,050 g/mℓ, showed an ELISA infected to non-infected ratio of more than 2, suggesting that the antigenic material was more concentrated at these levels. The inability to count the individual organisms with standard procedures, for example, by means of a coulter counter or a light-microscope, made it difficult to establish the concentration of the organisms. Protein content was thus used as an indication of concentration.

The protein content was found to be a maximum at densities of 1,134; 1,078; 1,058; 1,056; 1,045 and 1,034 g/mℓ. Infectivity incubation periods could not be correlated with ELISA colour development or protein content. Protein determinations showed that the infected fractions had a higher protein content in comparison with the non-infected fractions, an indication that the protein content of these fractions relates to the infected state of the tissue. This observation is based on equal wet/frozen mass of brain and liver and spleen material and equal amounts of infected and non-infected nymphae. However, a simple relationship could not be revealed between organism concentration (protein content), antigenicity (ELISA) and pathogenicity (incubation period).

Electron-microscopy of the infected nymph material obtained from Percoll density gradient centrifugation showed, as was expected, contaminating micro-organisms, probably belonging to the Rickettsiaceae family described by Cowdry (1925). Brain material, in which no contaminating organisms could be detected, proved to be the better source of starting material for the purification of *C. ruminantium*. This stresses the importance of the source of the organisms used for their isolation. Infected endothelial tissue culture cells (Bezuidenhout, Paterson & Barnard, 1985) are at present being investigated as an alternative source. Other possibilities are various blood fractions of infected animals, notably the red blood cell fraction which has been shown to be associated to a high degree with *C. ruminantium* during a specific stage of infection (Neitz, Viljoen, Bezuidenhout, Oberem, Van Wyngaardt & Vermeulen, 1986). Mice tissue infected with the Kümm strain of *C. ruminantium* offers an ideal source of the organism, since the yields of separated fractions may be determined by infectivity titrations in mice. In addition, a combination of Percoll density gradient centrifugation and wheat germ lectin affinity chromatography (Viljoen *et al.*, 1985) may be required to obtain pure preparations from certain sources. This is at present being studied.

In conclusion, it seems justified to state that Percoll density gradient centrifugation represents a quick and mild procedure for the isolation of partially purified *C. ruminantium* organisms from various sources, as demonstrated by electron microscopic studies, ELISA and infectivity assays.

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