ISOLATION OF *COWDRIA RUMINANTIUM* BY CELLULAR AFFINITY CHROMATOGRAPHY AND DETECTION BY AN ENZYME-LINKED IMMUNOSORBENT ASSAY

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ABSTRACT

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The isolation of *Cowdria ruminantium* by means of wheat germ lectin affinity chromatography as described in this paper permits the recovery of partially purified viable organisms under mild conditions in short time. These conclusions are based upon results of analyses of column fractions by intravenous inoculation into sheep, protein determination, electronmicroscopy and enzyme-linked immunosorbent assay. The entire purification procedure could be completed in 4–5 hours using only either infected sheep tissue or nymphae as starting material.

INTRODUCTION

The purification of *Cowdria ruminantium* is important for several reasons. Of prime importance is the need for a preparation free from extraneous antigens to provide a suitable vaccine (Wilson, 1967). Furthermore, investigations into the biochemical, antigenic and immunogenic properties of the organism require the availability of pure preparations. Through such studies, methods for sensitive, specific serodiagnosis could possibly be developed, the nature of the immunity to heartwater disease elucidated, the taxonomic position of the organism more accurately described and morphological studies extended (Pienaar, 1970; Du Plessis, 1970; Du Plessis(in press); Uilenberg, 1981). In addition, information concerning the vector and host specificity of the organism could be gained. The study of the developmental cycles and distribution in the vertebrate and invertebrate hosts would also be facilitated. Pure preparations are also essential for the study of the presumed toxin produced by these pathogens (Neitz, 1968).

The isolation of sufficient amounts of viable pure *C. ruminantium* has been hampered for many years by their extremely labile nature and the difficulties encountered in the cultivation of the organism in chicken yolk sacs and tissue culture (Uilenberg, 1983). The propagation of this pathogen in laboratory animals has also met with problems (Du Plessis, 1982). Recently, Du Plessis (1982) and Mackenzie & McHardy (1984) have succeeded in the propagation of certain strains of *C. ruminantium* in mice thus providing an alternative source of the organisms for further purification.

Various methods for the purification of rickettsial organisms have been reported. These include differential centrifugation (Bell & Theobald, 1962), sucrose (Wang & Grayston, 1967), Renografin (Howard, Orenstein & King, 1974), or Percoll (Tamura, Urakami & Tsuruhara, 1982), density gradient centrifugation, continuous flow zonal certrifugation (Anacker, Gerloff, Thomas, Mann, 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) and cation exchange chromatography (Hara, 1958). Many of these methods are time-consuming and have detrimental effects on the organisms (Weiss, Cool-

baugh & Williams, 1975). Apparently no attempts utilizing these or alternative techniques for the purification of *C. ruminantium* have been described in the literature.

Affinity chromatography with specific lectins is a quick and mild procedure for the isolation of a variety of cells (Sharma & Mahendroo, 1980). Since *C. ruminantium* organisms show staining characteristics similar to those of gram-negative bacteria (Cowdry, 1925), an attempt was made to purify viable *C. ruminantium* by means of wheat germ lectin cellular affinity chromatography (Sharma & Mahendroo, 1980). These lectins show specificity towards N-acetyl-glucosamine (Nagata & Burger, 1974), which is a characteristic constituent of the cell wall of gram-negative microorganisms (Salton, 1964). As a source of antigen, *C. ruminantium*-infected sheep brains or engorged *A. hebraeum* nymphae were used.

A further objective of the present research was to develop an enzyme-linked immunosorbent assay (ELISA) for the detection of *C. ruminantium*.

MATERIALS AND METHODS

Analytical quality reagents were used in all the experiments. Glassware and equipment were sterilized with 70 % (v/v) ethanol and the buffers by filtration through 0,22 μm filters[1].

Preparation of crude brain and nymph extracts

To obtain *C. ruminantium*-infected brain material, 5 sheep were infected by intravenous inoculation with the Onderste poort Ball 3 heartwater blood vaccine. The disease was allowed to run its course. Immediately after the death of the animals their brains were removed, frozen in liquid nitrogen and stored in dry ice. In an attempt to increase the number of organisms in the brains, 2 sheep were treated with a tonic containing arsenic[2] (Neitz, 1940) for 18 days prior to inoculation. The dosage regime was 5 mℓ i.v. every 3rd day.

C. ruminantium infected, engorged *A. hebraeum* nymphae were obtained by feeding the larvae on sheep showing a positive reaction to inoculation with the Onderstepoort Ball 3 vaccine, as described by Bezuidenhout (1981). The nymphae were then fed on either heartwater susceptible sheep or on sheep reacting to vaccination, as described above. The Spes Bona strain of *A. hebraeum* was used in all cases, as it has been found to be free of any rickettsial organisms other than Wolbachia-like symbionts. Nymphae were used within 3 days after dropping.

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[1] Millipore, South Africa (Pty) Ltd

[2] Acetarsonic acid (5 %), Vetoquinol, France

TABLE 1 Properties of heartwater infected and non-infected crude extracts and column fractions

Source	Amount	Protein content (mg)			
		Crude extract	Peak 1	Valley	Peak 2
Nymphae:	2400+	3600[1]	3582[1]	0	13,5[1]
	400+	1919[3]	1860[3]	0	12,6[3]
	200+	843[1]	828[1]	0	11,7[1]
	200−	758[2]	750[2]	0	2,7[2]
	200−	712[3]	678[3]	0	2,1[3]
Sheep brain:	224 g+	1728[2]	1716[2]	0	11,0[5]
	220 g+	1560[2] } 6	1548[2] } 6	0	11,2[2] } 6
	209 g+	1492[2]	1479[2]	0	11,9[2]
	207 g+	1476[2]	1452[2]	0	12,3[5]
	122 g+	864[2]	846[2]	0	12,7[2]
	214 g−	1452[3]	1446[3]	0	2,6[3]
	197 g−	1164[3]	1158[3]	0	2,7[3]
Total volume/fraction		10 mℓ	60 mℓ	30 mℓ	9 mℓ

+ =Heartwater infected brain and nymph material
− =Heartwater non-infected brain and nymph material
[1] =Heartwater infective (organisms viable)
[2] =Heartwater non-infective (organisms non-viable)
[3] =Not biologically tested
[4] =Died within 24 hours after injection
[5] =Showed a high body temperature for one day, but no protection against heartwater when challenged
[6] =Brain material was pooled and half of the sample was injected intravenously and the rest subcutaneously into different sheep

All further work on these sources of *C. ruminantium* was performed at 4 °C. The crude brain extracts were prepared, using 200–300 g of frozen, infected or control non-infected brain. These were quickly thawed and homogenized at 4 °C at low speed for 5 min in a Waring Blender[1] in 100–150 mℓ of a 0,05 M HEPES—0,154 M NaCl buffer, pH 7,4 (hereafter referred to as HEPES buffer).

Infected and non-infected *A. hebraeum* nymphae were homogenized for 10 min in the same buffer and blended at a dilution of 10 nymphae per 5 mℓ of buffer.

The brain and tick homogenates were centrifuged for 30 min at 1 000 × g in a Rotor 19 in a Beckman L5-65 ultracentrifuge. This centrifuge was also used in all subsequent centrifugations with half maximum acceleration and braking. The supernatants were then centrifuged for 30 min at 10 000 × g with a Rotor 30. The resultant supernatants were centrifuged at 30 000 × g for 30 min in a Rotor 30. The sediment was resuspended in 12 mℓ of HEPES buffer.

Wheat germ lectin chromatography

Wheat germ lectin Sepharose 6MB[2] (10 mℓ) was packed into a Pharmacia[2], column C10/20 (1,0 × 13,5 cm). The binding capacity of the column was 1 mg ovomucoid per mℓ bed volume. The void volume of the column was *c.* 10 mℓ.

The gel was regenerated with 100 mℓ of 0,1 M Tris-HCl, 0,5 M NaCl, 0,02 % (w/v) NaN₃, pH 8,5, followed by 100 mℓ of 0,1 M sodium acetate, 0,5 M NaCl, 0,02 % (w/v) NaN₃, pH 4,5, and equilibrated with 100 mℓ of HEPES buffer containing 0,02 % (w/v) NaN₃. The column was also stored in the latter buffer. Before use, the column was washed with 500 mℓ of HEPES buffer to remove the azide. The resuspended sediments of the crude extracts (10 mℓ) were applied to the column and incubated for 2 h. The non-adsorbed material was eluted from the column with HEPES buffer before a pulse of N-acetyl-D-glucosamine was applied (20 mℓ of HEPES buffer containing 2 g of carbohydrate). The column fractions were analysed for their protein content, infectivity and antigenicity. Fractions were also investigated electronmicroscopically.

Determination of infectivity

Sheep of *c.* 40 kg body mass of either sex were injected intravenously at a dosage rate of 2 mℓ per animal with either the resuspended sediments of the crude extracts or 6 mℓ per animal with the column fractions. In the case of crude extracts, the needle was dipped into a 1 % adrenalin solution[1] prior to injection. This reduced the initial shock of the injection. Daily rectal temperatures were taken and the animals were kept under observation for at least 24 days. In the case of no reaction, the sheep were challenged 21 days after the initial inoculation with 5 mℓ (1 dose) of Onderstepoort heartwater Ball 3 vaccine. Reacting sheep were allowed to die, after which a complete necropsy was performed.

A diagnosis of heartwater was made only after Giemsa-stained brain smears were found to be positive for typical *C. ruminantium* colonies (Purchase, 1945).

Protein determinations

The protein content was determined according to the high temperature Biuret-Folin method described by Dorsey, McDonald & Roels (1977). The Folin-Ciocalteau[2] reagent was diluted 1:1 with distilled water. The colour development was monitored at 660 nm on a Beckman Model 25 spectrophotometer against a blank, containing 0,1 mℓ of HEPES buffer. The protein content served as a means of estimating sample concentration. In some cases, the Folin method (Lowry, 1976) was compared to the Dorsey *et al.* (1977) method.

Enzyme-linked immunosorbent assay (ELISA)

A modification of the ELISA method described by Notermans, Timmermans & Nagel (1982) was used. Crude extracts from sheep brain or nymphae and the column fractions obtained were screened for *C. ruminantium* specific antigenic properties.

Sera from heartwater-infected and non-infected sheep were used. Blood samples (10 mℓ) were left to clot and after 2 hours the coagulated blood was centrifuged for 10 min at 300 *g* in a Piccolo bench top centrifuge at room temperature. The sera were siphoned off, divided into 1 mℓ batches and stored at −30 °C. A serum solution was
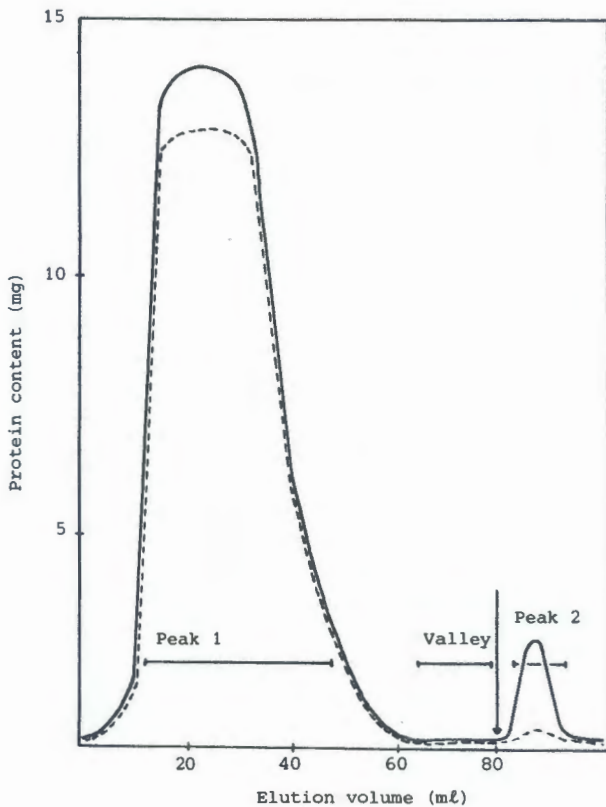
FIG. 1 Representative chromatogram of nymph crude extracts on a wheat germ lectin Sepharose 6 MB column (void volume: 10 mℓ). The column was equilibrated at 4 °C with a 0,05 M HEPES; 0,154 M NaCl, pH 7,4 buffer. Crude extracts (10 mℓ) were applied to the column and left for 2 h to bind. The column was then washed with equilibrating buffer. The arrow indicates the application of elution buffer (*c.* 20 mℓ) containing N-acetyl-D-glucosamine (1 g/10 mℓ). Infected nymph crude extracts (————), non-infected nymph crude extracts (- - - - -). Flow rate was 30 mℓ/h

made up as follows: 1 mℓ of serum was diluted 1:30 with a 0,05 M Tris, 0,1 M NaCl, pH 7 buffer. IgG was isolated from antiserum by the method of Deutsch (1967).

Whole antiserum was used for screening nymph and brain material, while the purified IgG fraction was used for detection of antigen from brain material only.

Microtitre plates[1] were coated with 100 $\mu\ell$ per well at an antigen concentration of either 3,10 or 100 μg protein per mℓ (Conradie, Vorster & Kirk, 1981) of a 0,05 M glycine, 0,1 M NaCl, 0,05 M Tris buffer pH 7. To determine the effect of sonication on antigen adsorption, the antigen, 10 μg protein/mℓ, was sonified with a Branson cell disruptor B-30[2] for 5 seconds with a micro tip.

The plates were incubated for 2 h while being gently shaken on a Titertek[1], washed twice with 0,05 M Tris, 0,1 M NaCl, 0,05 % Tween 20 (v/v), pH 7, and 3 times with distilled water. The washing solution was siphoned off, and the plates were dried on a vacuum line after the final wash. The blocking buffer, 3 % (w/v) bovine serum albumin (BSA) in Dulbecco's phosphate buffered saline (PBS), was applied in 200 $\mu\ell$ quantities.

After gentle shaking for 1 h, the blocking buffer was siphoned off and the serum containing 0,1 mg of protein or 0,1 mg isolated IgG in 25 mℓ of 0,05 M Tris, 0,1 M NaCl, pH 7, was then added to the microtitre plate in 100 $\mu\ell$ quantities to each well and gently shaken for 1 h. The plate was washed with 0,05 % (v/v) Tween 20 in PBS and with 1 % BSA (w/v) in PBS. A stock solution of 0,5 mg of Protein A-alkaline phosphatase[1] in 50 mℓ of distilled water was prepared. Of this solution, 5 $\mu\ell$ was diluted to 7,5 mℓ with 1 % BSA (w/v) in PBS, 50 $\mu\ell$ of which was added to each well and incubated at room temperature for 40 min while shaking. The plate was finally washed 6 times with 1 % BSA (w/v) PBS solution.

P-nitro phenylphosphate (35 mg) was added to 10 mℓ of freshly made substrate buffer, containing 3,16 g of 2-amino-2-methyl-1:3-propanediol and 40,6 mg MgCl$_2$. 6H$_2$0 in 100 mℓ of distilled water (pH 10,25). Of the substrate solution, 50 $\mu\ell$ was added to each well and the colour development was monitored with a Titertek Multiscan MC[1] at 690 nm and 405 nm. Colour development was stopped after 120 min. Signal to background ratios was calculated from absorbancies obtained for infected and corresponding non-infected fractions.

Electronmicroscopy

Column fractions (peaks 1 and 2) of 3 separate batches of heartwater-infected, *A. hebraeum* nymphae and infected brain material of 3 sheep were thawed and diluted with 10 mℓ 0,154 M NaCl. The material was centrifuged at 30 000 *g* for 60 min at 4 °C. After the supernatants were siphoned off, *c.* 0,1 mℓ 2 % (w/v) of agar at 45 °C was added to the sediment (pellet) of each fraction and siphoned into a glass capillary tube. The contents of the tubes were blown onto filter paper, which was cut into small blocks *c.* 1 mm in diameter and fixed in 2 % glutaraldehyde (Karnovsky, 1965) for 1 h at room temperature, followed by 2 % osmiumtetroxide for 1 h. Centrifugation of the peak 2 fraction of 1 batch of infected nymphae did not result in a pellet large enough to be processed, as outlined. In this case the supernatant was decanted and the last few drops of fluid in the tube were used to resuspend the faintly visible pellet.

The contents of the tubes were siphoned into a micro-haematocrit tube and centrifuged at 12 000 *g* for 15 min. The pellet so obtained was embedded in glutaraldehyde and processed as outlined for the other specimens. Specimens were dehydrated in graded ethanol series (50–100 %), passed through propylene oxide as the intermediate solvent and embedded in Polaron 812[2] (Kay, 1965).

Column fractions of non-infected *A. hebraeum* nymphae and non-infected brain material of sheep processed as outlined for the infected material, served as controls.

Thin sections were stained for 45 min at room temperature in saturated aqueous solution of uranyl acetate and for 10 min in lead citrate.

RESULTS

Isolation of Cowdria ruminantium

The brains of the animals injected with arsenic were not notably more infected with heartwater organisms than those where no arsenic was injected.

No difference could be observed between the chromatograms (except for protein quantity) of sheep or nymph crude extracts after chromatography on a wheat germ

TABLE 2 ELISA signal to background ratios of absorbance values for determination of the effect of sonication on adsorption of the antigens [1] to limbro microtiter plate

Fraction	Origin of antigen material			
	Brain		Nymph	
	Non-sonified	Sonified	Non-sonified	Sonified
Crude extract	1,39 ± 0,38	1,74 ± 0,47	1,21 ± 0,31	1,46 ± 0,32
Lectin column Peak 1	1,64 ± 0,34	1,97 ± 0,37	1,43 ± 0,37	1,70 ± 0,29
Lectin column Peak 2	2,06 ± 0,42	2,55 ± 0,41	1,81 ± 0,34	2,28 ± 0, 31

[1] n = 6
Sample concentrations taken at 10 μg protein/mℓ

TABLE 3 ELISA signal to background ratios of absorbance values for the determination of optimal antigen adsorption [1] to a linbro microtiter plate

Fraction	Origin of antigen material					
	Brain (μg protein/mℓ)			Nymph (μg protein/mℓ)		
	3	10	100	3	10	100
Crude extract	1,42 ± 0,37	1,74 ± 0,47	1,53 ± 0,41	1,22 ± 0,37	1,46 ± 0,32	1,32 ± 0,42
Lectin column Peak 1	1,62 ± 0,32	1,97 ± 0,37	1,72 ± 0,44	1,47 ± 0,33	1,70 ± 0,29	1,60 ± 0,34
Lectin column Peak 2	2,23 ± 0, 39	2,55 ± 0,41	2,40 ± 0,43	2,02 ± 0,38	2,28 ± 0,31	2,14 ± 0,38

[1] n = 5

TABLE 4 ELISA signal to background ratios of absorbance values for specific determination of *Cowdria ruminantium* organisms in infected tissue [1]

Fraction	Origin of antigen material				
	Brain Source of antibody			Nymph Source of antibody	
	IgG fraction of antiserum	Antiserum	Normal serum[2]	Antiserum	Normal serum[2]
Crude extract	1,82 ± 0,43	1,74 ± 0,47	1,00 ± 0,10	1,46 ± 0,32	1,00 ± 0,10
Lectin column Peak 1	1,89 ± 0,38	1,97 ± 0,37	1,00 ± 0,10	1,70 ± 0,29	1,00 ± 0,08
Valley	1,00 ± 0,05	1,00 ± 0,04	1,00 ± 0,04	1,00 ± 0,05	1,00 ± 0,05
Lectin column Peak 2	2,80 ± 0,47	2,55 ± 0,41	1,00 ± 0,10	2,28 ± 0,31	1,00 ± 0,10

[1] n = 5
[2] Serum from a heartwater susceptible sheep

lectin Sepharose 6 MB column. A significant difference in the amount of protein bound to the column (peak 2) was observed, however, between extracts from infected and normal material (Fig. 1).

The high temperature Biuret-Folin protein assay of Dorsey *et al.* (1977) was found to be as reliable as the method of Lowry (1976). The protein content of heartwater-infective and non-infective material is shown in Table 1.

Biological tests

Sheep injected with infected crude brain extracts showed no heartwater symptoms during the entire observation period of 24 days. Sheep injected with crude fractions or column fractions from infected brain material (Fig. 1) showed a high body temperature in 2 cases only, one on the 8th and one on the 20th day after injection. The high temperature lasted just 24 h in both cases (Table 1). These animals showed no protection against heartwater when challenged. However, the crude extracts and column fractions from infected nymphae (peaks 1 and 2) showed a high heartwater infectivity (Table 1). These nymph fractions showed a constantly high body temperature (40 °C) from as early as on the 10th day after injection. Typical heartwater colonies were observed with Giemsa stain in the endothelial cells of brain smears from animals injected with infective tick material. Sheep, injected with the valley fractions obtained from infective as well as non-infective nymph crude extracts and column fractions (Table 1), failed to show a temperature reaction, and they succumbed when challenged.

Enzyme-linked immunosorbent assay (ELISA)

The signal to background ratios obtained by ELISA screening of antigen preparations from *C. ruminantium* infected and non-infected brain and nymph material, was initially low, even to the level of insignificance when anti-IgG-immunoglobulin-peroxidase was used as indicator reagent. However, with the use of Protein A-alkaline phosphatase as indicator reagent, as well as optimizing the coating efficiency of antigen by lectin affinity enrichment, sonication and concentration manipulation, it improved the ELISA results to a point where absorbance signals were obtained of more than twice background value with 1/30 dilutions of antiserum (Tables 2 & 3).

Protein A-conjugate is more specific but less sensitive than anti-IgG-immunoglobulin-conjugates of peroxidase or alkaline phosphatase (Langone, Boyle & Borsos, 1978; Goding, 1978). By using Protein A-conjugate, some background interference observed by using an anti-IgG-peroxidase conjugate was eliminated. Thus, the signal to background ratio (being infected to non-infected material) obtained indicated with anti-IgG-peroxidase and infected nymph material (peak 2), as 1,87 ± 0,53, but by using Protein A-conjugate, this ratio was increased to the 2,28 ± 0,31 value reported in Table 2. Concentration-reconstituted, purified IgG fractions obtained from immune sera further improved this signal to almost three times the background (Table 4).

ELISA screening of fractions derived from the lectin column and its starting material, using all the abovementioned modifications, resulted in data which were in accordance with what can reasonably be expected from such an affinity purification (Table 4).
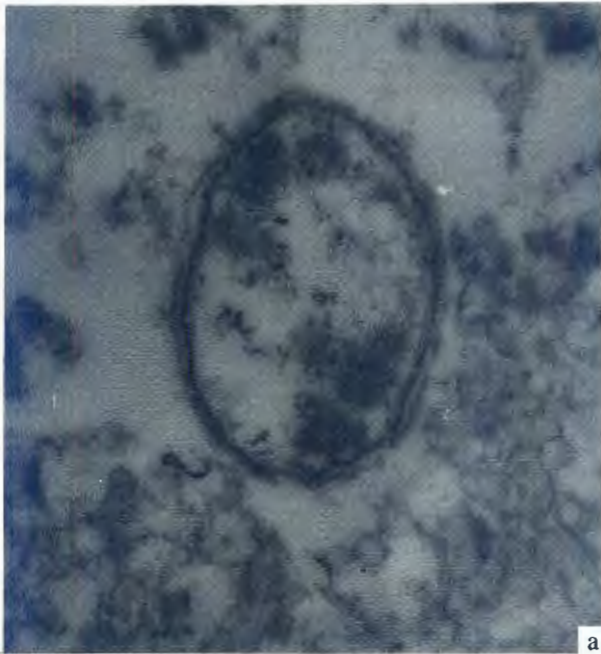
FIG. 2 a & b *Cowdria ruminantium*-like organisms: × 100 000

Electronmicroscopy

In the peak 1 fraction of 2 batches of infected nymphae and peak 2 fraction of 3 infected sheep brain, a low concentration of suspected *C. ruminantium* organisms was noted. Their organisms were oval to coccoid in shape, and ranged in size from 0,36–0,8 μm in diameter. Each organism was enveloped by an inner electron-dense membrane surrounded by an electron-transparent layer (*c.* 20–30 nm), which often had a rippled appearance [Fig. 2(a)]. The inner structure of the organisms consisted of electrondense and electron-pale areas, and no specific distribution pattern was evident. Occasionally, fine fibrillar material, in which small electron-dense granules

were suspended, was visible in the electron-pale areas. Organisms undergoing binary fission were seen infrequently [Fig. 2(b)].
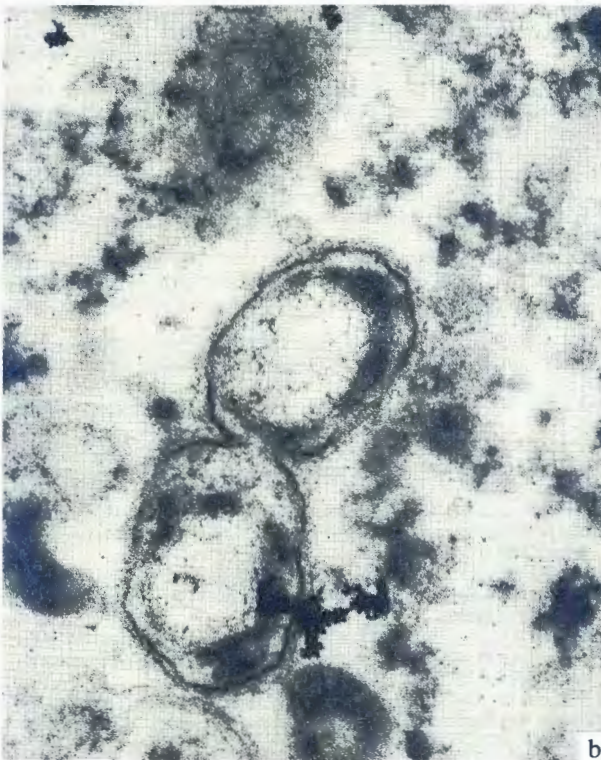
DISCUSSION

Brain material from heartwater-infected sheep was initially used as source of *C. ruminantium*, since it was reasoned that this organism would be the only rickettsia-like organism present in infected brain. However, no viable organisms could be demonstrated by i.v. or, in one case, s.c. injection of brain crude extracts into sheep. In the latter case, no cryoprotectants were used (Uilenberg, 1983). Furthermore, these animals were not immune when challenged. Nevertheless, *C. ruminantium* organisms or derived antigens were shown to be present in these tissues by electronmicroscopic investigations and ELISA respectively. In an attempt to increase the proliferation of *C. ruminantium* in the brains of experimental animals, some test animals were treated with a tonic containing arsenic prior to inoculation (Neitz, 1940). No enhancement of infectivity through this treatment could be obtained.

Because of the lack of infectivity of the brain fractions, an alternative source of the organism was sought. Heartwater-infected nymphae proved to be suitable, since crude nymph extracts and column fractions obtained from this material caused typical heartwater symptoms when injected i.v. into sheep.

No difference was seen in the elution patterns of brain or nymph material after affinity chromatography, using wheat germ lectin as ligand. However, a large quantitative difference in protein content of the bound peak of infected and non-infected material was observed, which indicates that wheat germ lectin is suitable for the purification of *C. ruminantium*. This was further substantiated by the electronmicroscopic investigations. The morphology and size of the organisms in the peak 1 fraction of 2 batches of infected nymphae and the peak 2 fraction of 3 infected sheep brain closely resembled those of *C. ruminantium* described in sheep and mice (Pienaar, 1970; Prozesky & Du Plessis, 1985). Although caution should be exercised when identifying *C. ruminantium* on the basis of morphology of single organisms, the infectivity and ELISA of the nymphae fractions and the absence of organisms in the controls, serve as additional evidence that the organisms are most probably *C. ruminantium*. The difficulty encountered in demonstrating heartwater organisms by ultrastructural methods in fractions of the infective peak 2 from nymphae may be attributable to a too low concentration of organisms. Furthermore, the presence of material of variable electrondensity and morphology made the identification of suspected *C. ruminantium* organisms extremely difficult. The presence of *C. ruminantium* in the unbound peak is probably due to overloading of the column, because the protein content of the bound peak remained reasonably constant, irrespective of the number of nymphae used as starting material. Another possibility is that the omission of mechanical agitation during affinity absorption, which has been reported to optimize and enhance binding of cells to stationary phase, may have resulted in suboptimal binding (Kinzel, Richards & Kubler, 1977; Sharma & Mahendroo, 1980). Apart from inducing maximum contact between cells and the lectin, the time required for binding would possibly also be reduced. Such a modification in the purification procedure obviously warrants further investigation.

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The low signal to noise values and titres obtained with the ELISA-screening of infected tissues were to be expected because of the complexity of the antigen. Nevertheless, the values obtained were reproducible, and the technique was found to be useful as an additional and rapid assay for biological activity from the lectin affinity column. The results in Table 4 are evidence that at least some activity was retained on the column which could be eluted as an enriched fraction. In addition to the protein determinations which indicated overloading of the column, it is also possible that the column was unable to retain antigenic fragments lacking the carbohydrate moiety which is recognized by the lectin.

Lectin affinity purification of the infected tissue extracts therefore yields an improved antigen for use in the ELISA screening of sera for the detection of *C. ruminantium* specific antibodies. It could also be used for the development of monoclonal antibodies to single *C. ruminantium* specific determinants. Their application should further increase the specificity and sensitivity of this ELISA procedure to be used as a fast diagnostic identification when screening nymph material for their *C. ruminantium* infectivity, and to improve and speed up serodiagnostic identification of heartwater disease.

The suitability of the assay for serodiagnostic purposes has not been investigated. The earliest stage of detection of antibody in diseased animals and the determination of the persistence of antibody by this method as well as the specificity of the assay still need to be explored.

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