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Purification of the erythrocytic stages of *Babesia bigemina* from cultures

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Abstract. Exposure of erythrocytes infected with *Babesia bigemina* to glycerol-enhanced osmotic shock yielded preparations containing infected erythrocyte ghosts, free parasites, and some intact erythrocytes. The released parasites were purified and concentrated by centrifugation in Percoll gradient. Recovered free parasites were shown by the fluorescein acetate technique to be metabolically active, but their infectivity in vitro was low. It was demonstrated by electron microscopy that most of the released parasites had intact plasma membranes. There was only slight contamination of the free-parasite preparation with host erythrocyte debris.

Mahoney (1967) was the first to attempt the release of *Babesia bigemina* from bovine erythrocytes. He used a hypotonic saline solution to lyse the infected red blood cells and then separated the released parasites by centrifugation in a sucrose density gradient. However, the parasites were damaged, as determined by light microscopic examination. In 1972, this investigator reported that methods developed to release *Plasmodium* from red blood cells have not been useful for separating *B. bovis* and *B. bigemina* from bovine erythrocytes. These methods included lysis by saponin, anti-erythrocyte serum and complement, mechanical pressure in a French pressure cell, and digestion of stroma with enzymes. Although some parasites were released by these techniques, the preparations were considered to be unsuitable because the *Babesia* were contaminated with erythrocytic materials. O'Donoghue et al. (1985) reported the isolation of *B. bigemina* merozoites after lysis of infected erythrocytes with hypotonic saline solution and differential centrifugation. Their claim that the preparation was free of host-cell contaminants was based exclusively on examination with the light microscope.

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When *B. bovis* cultures are deprived of CO_2, there is accumulation of extracellular merozoites in the culture medium (Levy and Ristic 1983). These merozoites can then be separated from contaminating red blood cells and stroma by centrifugation in Percoll gradient (Rodriguez et al. 1986). Carbon dioxide deprivation was also reported to cause accumulation of merozoites in the supernatant medium of *B. bigemina* cultures used in cryopreservation studies (Vega et al. 1985b). However, a large proportion of parasites remained within the erythrocyte stroma, leaving few of the organisms free for separation purposes (Vega, personal communication).

Human and rodent red blood cells infected with *Plasmodium falciparum* and *P. chabaudi*, respectively, have been reported to be lysed by glycerol-enhanced osmotic shock (Hall et al. 1983; Wunderlich et al. 1985), a procedure reported to provide for the release of intraerythrocytic parasites. These findings prompted us to test the suitability of such osmotic shock for *B. bigemina*, because a parasite preparation free of host red-blood-cell contaminants would be highly desirable for biochemical, physiologic, and immunologic investigations. The present study deals with the separation of *B. bigemina* from glycerol-lysed, infected bovine erythrocytes and their concentration by a density-gradient centrifugation technique.

Materials and methods

Source of parasites

Seed, an original Mexican isolate of *B. bigemina*, was continuously cultivated in 24-well culture plates as previously described (Vega et al. 1985a). Cultures were expanded in 75-cm^2 flasks as a 10% suspension of bovine erythrocytes in a medium containing 40% normal bovine serum in M 199 (pH 7.0). Cultures were incubated in an atmosphere comprising 5% O_2, 5% CO_2, and 90% N_2 at 37° C, and the spent medium was exchanged after 24 h. Leukocytes were removed from normal bovine red blood cells (nRBC) by passing blood through a cellulose column (Vega et al. 1986).

Parasite concentration

The Percoll-Renografin density-gradient centrifugation technique for the concentration of cultured *B. bigemina* was as previously described (Vega et al. 1986), except that 30 × 90 mm round-bottom, polycarbonate tubes containing 35 ml Percoll-Renografin gradient were used. In all, 5 ml washed, packed, infected red blood cells (iRBC) from 48-h cultures of the parasite was added. The tubes were rotated end-over-end for complete mixing, then centrifuged at 25300 *g* for 12 min at 4° C. After centrifugation, a broad band at the top of the tubes was collected and washed three times in Vega y Martinez (VYM) solution (Vega et al. 1985a) to eliminate the Percoll-Renografin material. Bands of concentrated iRBC from several tubes were pooled. Thin blood films were made from the concentrated material and percentages of parasitized erythrocytes (PPE) were calculated after Giemsa staining, as previously described (Vega et al. 1986). The total number of RBC was estimated by counting a 1:200 dilution of the suspension in a hemacytometer.

Glycerol-enhanced osmotic shock

The procedure described by Hall et al. (1983) was adopted for the release of *B. bigemina* from bovine erythrocytes. Briefly, to 0.5 ml washed, concentrated iRBC was added 10 ml RPMI 1640 culture medium with 7% (v/v) anhydrous glycerol. The suspension was equilibrated at room temperature for 20 min and the material, pelleted by centrifugation at 1,544 *g* for 10 min. After removal of the supernatant fluid, the RBC were lysed by rapid resuspension in 10 ml RPMI 1640 medium without glycerol. The lysate was spun down at 3,953 *g* for 10 min and washed twice in VYM solution before separation of the suspension by density-gradient centrifugation.

Separation of cell-free B. bigemina

Pellets obtained after the glycerol osmotic shock treatment were resuspended in 0.5 ml VYM solution and mixed with 7.5 ml of an iso-osmotic Percoll solution prepared at 50% concentration in VYM solution. The preparation was transferred to 14 × 70 mm round-bottom, polycarbonate tubes and centrifuged at 25300 *g* for 36 min in a superspeed Sorvall RC2-B (Ivan Sorvall Inc., Newtown, Conn.) centrifuge (Rodriguez et al. 1986). Separated fractions were carefully collected from the top to the bottom of the gradient using a Pasteur pipette and then washed three times in VYM. Density of samples was determined by comparing their position with that of density-marker beads (Pharmacia Fine Chemicals, Uppsala, Sweden).

Test for metabolic activity

Recovered free *B. bigemina* or infected erythrocyte ghosts were quantitated in a hemacytometer using phase-contrast microscopy. Metabolic activity of the parasites was estimated by the fluorescein diacetate (FDA) technique (Mishell et al. 1980). The number of metabolically active parasites was expressed as the percentage of fluorescing organisms (metabolically active) vs the total number of *Babesia*.

In vitro infectivity test

Glycerol-released parasites were tested for their ability to infect nRBC under standard culture conditions (Vega et al. 1985a). Cultures were prepared in 24-well culture plates by the addition of 1 ml complete culture medium containing 10% (v/v) suspension of nRBC and were then preincubated for 1 h at 37° C in an atmosphere comprising 5% O_2, 5% CO_2, and 90% N_2. The 1-ml cultures were estimated to contain about 1.61×10^9 nRBC. Aliquots of preparations with different numbers of parasites were added to the 1-ml cultures (in duplicate) to yield various ratios of iRBC or free *Babesia*:100 nRBC.

The elapsed time from the start of the parasite concentration procedure to the start of the in vitro infectivity test was 5 h. Cultures were monitored for 6 days, with medium exchange carried out every 24 h and a subcultivation, at 72 h by 1:2 or 1:5 dilution of old cultures with fresh nRBC-containing complete medium. Giemsa-stained smears were prepared every day and the PPE was calculated to ascertain whether the number of iRBC was high enough.

Scanning electron microscopy

Samples containing approximately 2×10^8 free parasites or 1×10^9 infected erythrocyte ghosts were fixed with 5 ml 2.5% glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.2) for 2 h at 4° C. They were then washed (4 × 10 min) with the same buffer at 4° C, resuspended, and pelleted by centrifugation at 3,953 *g* in each wash. Postfixation was carried out by resuspending the pellets in 1% osmium tetroxide in the phosphate buffer for 1 h at 4° C. After fixation, the organisms were washed three times in distilled water and prepared for scanning electron microscopy (SEM) following the technique of Sanders et al. (1975). Coverslips were coated with poly-L-lysine solution prepared at 1% (w/v) in phosphate buffer, then rinsed in distilled water and placed in a petri-dish humid chamber. In all, 50 µl of the parasite suspension was pipetted onto coated coverslips, which were kept at 4° C for 14 h to enable parasite sedimentation.

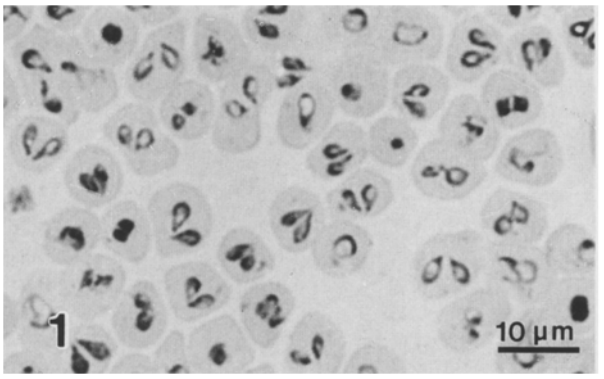
The specimens were dehydrated in a graded ethanol series, then dried by the critical-point method using liquid CO_2. The coverslips with the dried samples were mounted on specimen stubs and coated with gold/palladium in a sputter coater. Samples were examined in a JEOL-SM35 scanning electron microscope operating at 20 kV.

Transmission electron microscopy

Pellets of infected ghost erythrocytes and cell-free *B. bigemina*, obtained after 50% iso-osmotic Percoll centrifugation of glycerol-treated iRBC, were processed for transmission electron microscopy (TEM) according to the procedure of Langreth et al. (1978), with minor modifications. The pellets were resuspended in 5–10 ml 2% (v/v) glutaraldehyde in 0.1 *M* sodium cacodylate buffer (pH 7.4) containing 0.12 *M* sucrose and then fixed for 1 h at 4° C. After four washes in the buffer, the samples were postfixed in buffered 1% (w/v) osmium tetroxide for 1 h at 4° C. After fixation, the pellets were washed three times with buffer and rinsed six times with distilled water. Pellets were then embedded in 1% water agar, cut into blocks measuring about 2 mm^3, dehydrated in a graded ethanol series, cleared in propylene oxide, and embedded in Epon-Araldite resin. Ultrathin sections were cut with a diamond knife and stained with uranyl acetate and lead citrate. Sections were examined in a Hitachi (H-600) transmission electron microscope operating at 100 kV.

Results

B. bigemina-infected RBC (iRBC) obtained from cultures were concentrated by centrifugation in the Percoll-Renografin gradient. The concentrate contained all the developmental stages observed in vitro, i.e., small dense bodies and ring and ameboid forms, as well as dividing and mature piriform organisms (Fig. 1). Samples of concentrated *B. bigemina* iRBC that had been exposed to



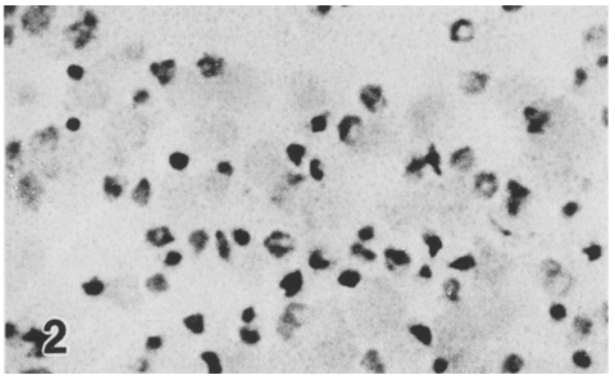


Fig. 1. Intact bovine erythrocytes infected with *B. bigemina*. **Fig. 2.** Bovine erythrocytes lysed by glycerol-enhanced osmotic shock and free *Babesia* released from these host cells. Scale in Fig. 1 also applies to Fig. 2

glycerol (7% v/v in RPMI medium) and then washed in glycerol-free RPMI medium contained free parasites, uninfected and infected erythrocyte ghosts, and very small numbers of unlysed erythrocytes (Fig. 2).

Separation of free parasites from infected erythrocyte ghosts was accomplished by centrifugation in an iso-osmotic 50% Percoll gradient. Three major fractions were collected from the gradient for light microscopic examination (Fig. 3). Fraction I consisted of two bands, which were difficult to collect individually, and was located 2–6 mm from the meniscus. This fraction contained the bulk of uninfected erythrocyte ghosts and numerous parasites trapped among erythrocyte membranes. Fraction II was located 13–19 mm from the meniscus and did not form a distinct band; it could be discerned by the unaided eye only due to the formation of "clumps" in the gradient. This fraction was composed of a small number of free parasites, either entangled or agglutinated with host erythrocyte membranes. At a point located 44–49 mm from the meniscus, fraction III appeared as a diffuse, whitish granular band with a density of 1.064–1.074 g/ml; it consisted primarily of free ameboid and single or paired piriform organisms.

Hemacytometric quantitation of the fractions obtained after 50% Percoll centrifugation revealed that the number of free parasites collected from fraction III accounted for only 9%–10% of the total number of iRBC counted before exposure to glycerol and lysis. The remainder of the parasites were not released by the chemical treatment. Therefore, they were enveloped by the erythrocyte membrane (fraction I) or trapped in the host erythrocyte membrane debris (fraction II) and could not be freed by centrifugation.

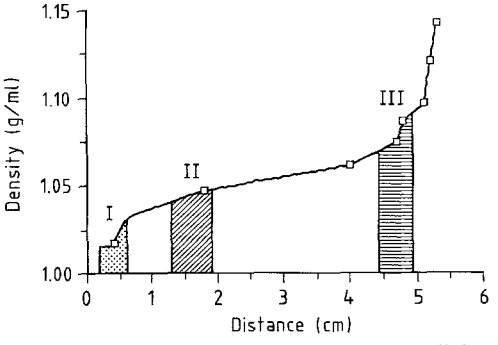


Fig. 3. Graphic representation of 50% Percoll density-gradient separation of *B. bigemina* freed from bovine erythrocytes by glycerol-enhanced osmotic shock

Attempts were made to increase the yield of free *B. bigemina* by increasing the concentration of glycerol in the medium and by incubating the concentrated iRBC with the glycerol solution for longer periods or at a higher temperature (37° C). However, these procedures did not increase the number of free parasites. Prolonged exposure of iRBC to glycerol concentrations of $\geq 7\%$ may have osmotically damaged the organisms, since both free forms and those still within host-cell membranes appeared to be swollen (not shown).

Metabolic activity tests, as determined by hydrolysis of fluorescein diacetate, revealed that from 77% to 87% of the parasite were metabolically active for 5 h at 4° C; however, only 40%–45% remained active after 9 h at this temperature. The remaining parasites were active after 12 h but not after 24 h of storage at 4° C.

The in vitro infectivity of *B. bigemina* that were free from host cells, as assessed by the invasion of normal bovine RBC, is presented in Table 1. The data indicate that exposure of concentrated iRBC to a solution of 7% glycerol decreases the parasites' ability to infect nRBC. The medium in cultures initiated with iRBC that had been exposed to glycerol was darker (a result of lysis of the host cells) than that in cultures initiated with unexposed iRBC. Giemsa-stained smears of erythrocytes from cultures incubated for 20 h contained clumps of infected erythrocyte ghosts and few free parasites, but no iRBC were found after a 5-min examination of these preparations. After a 44-h incubation, only very few iRBC (with ring forms) were observed. A further 24-h incubation increased the PPE to a level corresponding to the number of initially added iRBC.

Only very low infectivity of *Babesia* was noted in samples of concentrated iRBC that had been exposed to glycerol and lysed by being rapidly suspended in glycerol-free medium. The same was true of erythrocyte-free parasites obtained after iso-osmotic 50% Percoll density-gradient centrifugation. Increasing the number of parasites used to start the cultures (1 infected ghost or

Table 1. In vitro infectivity of *B. bigemina* freed from bovine RBC by glycerol-enhanced osmotic shock

Treatment of iRBC[a]	Ratio parasite/nRBC[b]	Well	Percentage of iRBC at various times (h) after initiation of culture[c]					
			20	44	68	92	116	140
None	1:100	1	0.39	2.35	2.65	0.59	2.48	3.10
		2	0.48	2.65	2.36	0.57	1.66	3.42
Glycerol[d]	1:100	1	–	<0.1[h]	0.76	0.97	3.09	3.06
		2	–	<0.1	1.17	1.12	1.39	3.34
Glycerol	1:200	1	–	<0.1	0.48	0.81	2.18	2.76
		2	–	<0.1	0.45	1.81	2.39	1.90
Glycerol+ Percoll[e] fraction I[f]	1:25	1	–	<0.1	<0.1	<0.1	0.39	0.92
		2	–	–	<0.1	<0.1	0.28	0.66
Glycerol+ Percoll fraction I	1:50	1	–	<0.1	<0.1	0.37	1.24	2.33
		2	–	<0.1	<0.1	0.39	1.27	2.76
Glycerol+ Percoll fraction III[g]	1:25	1	–	<0.1	<0.1	<0.1	0.44	0.75
		2	–	<0.1	<0.1	<0.1	0.28	0.78
Glycerol+ Percoll fraction III	1:50	1	–	<0.1	<0.1	<0.1	0.39	0.74
		2	–	–	<0.1	<0.1	0.39	0.82

[a] Concentrated *B. bigemina*-infected RBC
[b] iRBC, infected erythrocyte ghosts or freed parasites/nRBC
[c] Based on counts of at least 1000 RBC
[d] 7% (v/v) anhydrous glycerol in RPMI 640 medium
[e] 50% Percoll density gradient (see Materials and methods)
[f] Infected erythrocyte ghosts and parasites trapped among RBC membranes
[g] Free ameboid and single or paired parasites
[h] At least 1 iRBC found in a 5-min search

parasite/25 or 50 nRBC) did not enhance infectivity. As early as 44 h after the initiation of cultures, iRBC were observed in Giemsa-stained smears; however, it took at least 5 days (including subcultivation at 68 h) to reestablish the normal multiplication rate. When a culture was initiated with free parasites and nRBC at 1:100–1:200 ratios, up to 15 days were required to reestablish continuous growth (data not shown).

Electron microscopy

Only fractions I and III from the 50% Percoll gradient provided enough material for examination by electron microscopy. SEM micrographs of these fractions are shown in Figs. 4 and 5. Fraction I contained parasites trapped in erythrocyte membranes (Fig. 4); consequently, the parasites were not clearly discernible. Fraction III contained large numbers of parasites, either single or in small or larger groups (Fig. 5), which appeared to be free of erythrocyte membranes and intact. Intact erythrocytes were infrequently observed in both fractions.

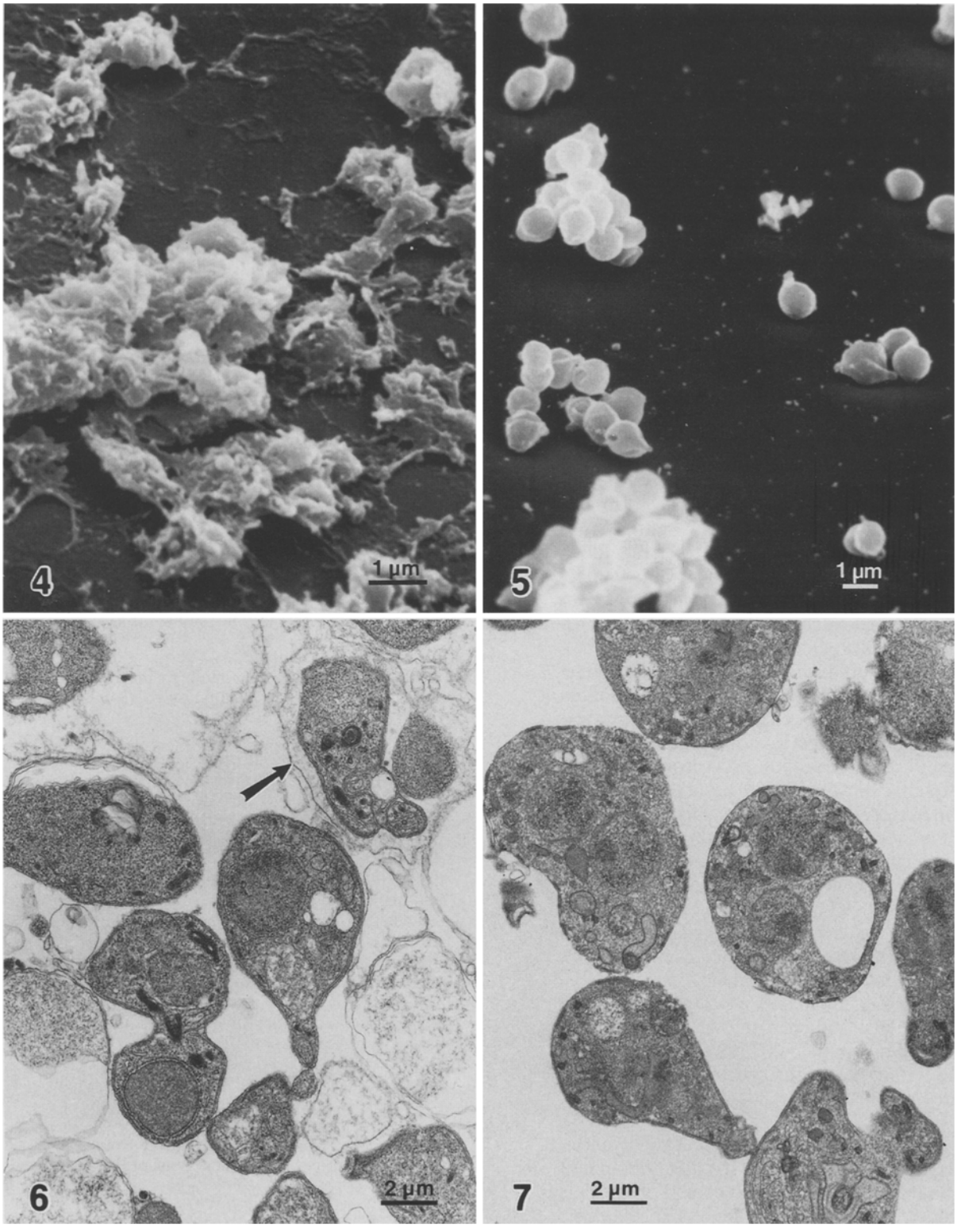
As observed by TEM, fraction I contained primarily infected erythrocyte ghosts and a few free parasites intermingled with erythrocyte membranes (Fig. 6). The majority of glycerol-released *Babesia* in fraction III were free of RBC membranes (Fig. 7). The parasites appeared to retain their structural integrity; they were neither highly vacuolated, swollen, nor disrupted. The parasite membrane was intact and the cytoplasm contained nuclei and various organelles.

Discussion

Not all of the intraerythrocytic parasites were released in our preparations; this finding is similar to results reported by Hall et al. (1983). Only 10% of the total number of parasites were recovered as free organisms after the purification and concentration steps. Increased glycerol concentration or incubation time of *B. bigemina*-infected erythrocytes did not yield more free parasites; indeed, higher concentrations of glycerol in the medium led to disruption of the parasites after they had been resuspended in glycerol-free medium (not shown). A 10% (v/v) glycerol solution has been reported to induce the release of *Plasmodium chabaudi* from up to 80% of parasitized erythrocytes (Wunderlich et al. 1985), and up to 80% of these are recovered in the Percoll-gradient centrifugation step.

Gravely and Kreier (1974) suggested that electron microscopy be used for confirmation that free parasites are devoid of host-cell contaminants. In addition, the quality of released parasites should be evaluated in terms of morphologic integrity, viability, and antigenic activity (Schimizu et al. 1988). According to McElwain et al. (1987), after thawing from liquid N_2, merozoites in *B. bigemina*-infected blood stabilates could be purified by a continuous Percoll gradient. However, TEM revealed that the merozoites were still surrounded by the host erythrocyte membrane.

On electron microscopic examination, *B. bigemina* purified by the procedure described in the present report appeared to be morphologically normal and devoid of RBC membranes. However, some free parasites ap-



Figs. 4–7. Electron micrographs of fractions I and III of *B. bigemina* obtained in 50% Percoll density gradient of a preparation containing parasites freed from bovine erythrocytes by glycerol-enhanced osmotic shock

Figs. 4, 5. Scanning electron micrographs of fractions I and III, respectively. **Figs. 6, 7.** Transmission electron micrographs of fractions I and III, respectively. Scale in Fig. 6 also applies to Fig. 7

peared to be slightly larger and more rounded than intraerythrocytic organisms. Similar results were reported by Gravely and Kreier (1974), who found that *B. microti* became rounded on their removal from erythrocytes by continuous-flow ultrasonication. Whether the rounding of released *B. bigemina* is due to physical osmotic stress or whether it merely reflects the absence of host-cell restraint cannot be determined at this time.

Viability of 70%–85% was routinely recorded from the fraction of free parasites with the aid of the fluorescein diacetate technique, as long as the test was carried out within 4–5 h after purification; however, the infectivity of this fraction was very low. This could well be the result of low numbers of infective merozoites in the preparations. *B. bigemina* develops asynchronously in vitro (Vega et al. 1985a) and the concentration of iRBC as determined by the Percoll-Renografin technique reveals parasites in all stages of development. Thus, the presence of higher numbers of immature stages (trophozoites) in the sample would lead to delayed development once these organisms were returned to culture. Alternatively, only a reduced number of mature, infective parasites could survive the treatment, which would account for the 3- to 4-day delay in reinfection of fresh RBC. Indeed, Dalgliesh (1972) has previously shown low infectivity of *B. bigemina* iRBC that had been exposed to a 4 *M* glycerol solution and then inoculated intravenously into nonsplenectomized cattle.

Dalgliesh (1972) could simulate in vitro the effect of sudden dilution with bovine plasma of glycerinated blood – >80% of the erythrocytes were lysed. However, the suspension was not infective for cattle as a result of the osmotic effect of intracellular glycerol. Therefore, the exposure to this substance may be highly toxic for the parasite, affecting the organism's ability to invade erythrocytes. Parasites still enclosed within the erythrocyte membrane remained slightly more infective than did free *Babesia*. Even at lower numbers, infected ghost erythrocytes needed 68–92 h incubation to reach a PPE of 1%. This was considerably shorter than the 140-h period required by the free parasites to reach 1% PPE, even when used at 2- or 4-fold higher concentrations. Thus, RBC membranes appeared to confer protection to the parasite against sudden osmotic shock. It was demonstrated by McElwain et al. (1987) that *B. bigemina* contained within the erythrocyte membranes could still cause acute babesiosis when inoculated into splenectomized calves.

With the aid of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent Western blotting analysis, Figueroa et al. (1990) have shown that the free parasites retained their antigenic characteristics and that they were contaminated by only a small amount of host-erythrocyte material. Hence, despite all the limitations of the procedure, preparation of free parasites by glycerol-enhanced shock offers a source of organims that is relatively free of host-cell contamination. These preparations have been used for the antigenic identification and characterization of parasite-surface components with potential importance as immunogens (Figueroa et al. 1990).

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