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Simplified Specimen Preparation for Electron Microscopic Studies of an Intra-erythrocytic Parasite, *Anaplasma marginale*. (27572)

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Hemoglobin and the cytoplasmic matrix of the intact bovine erythrocyte preclude direct electron optical observation of the morphology of its associated intra-cellular parasite, *Anaplasma marginale*.

Various methods of dehemoglobinization of *anaplasma* infected erythrocytes have been previously reported. These include freezing and thawing(1), distilled water lysis of blood smears(2), distilled water lysis of washed and packed cells(3), and lysis induced by saponin(1) or specific hemolysin(1). Transfer of the hemolyzed stromata to specimen grids has been effected by several technics: pseudo-replication from glass(2) or from agar(1), nebulizing(3), collodian stripping of a positive replica prepared by pressure molding polyethylene(3), and evaporation of a droplet of the stroma suspension directly onto a filmed grid(1). In most instances the final preparations were lightly shadowed with metal prior to microscopy.

For routine electron microscopy of this intra-erythrocytic parasite the above methods involved one or more of the following disadvantages: (1) lengthy or tedious procedure, (2) obscuring of morphological detail by addition of extraneous mass to the specimen, (3) derangement or alteration of anatomical structures as a result of an applied physical stress, and (4) separation of the organism from its host cell stroma.

Attempts were made in the present study to hemolyze *anaplasma* infected bovine erythrocytes and to prepare specimen grids under conditions which would retain sufficient structural integrity of the parasite to facilitate its recognition directly within the erythrocyte stroma.

Materials and methods. Blood samples from splenectomized calves in the acute phase of the disease were collected in tubes containing heparin.

Dehemoglobinization. Preparation of the bovine erythrocyte stromata containing the parasites was carried out by a dialysis procedure similar to that devised by Danon, Nevo and Marikovsky(4). These workers have shown that human stromata prepared by dialyzing an erythrocyte suspension against hypotonic solution consisted of thin membranes with a granular surface structure and were free of rents, "craters" or ruptures. Two or 3 drops of infected whole blood were added

to 12 ml buffered saline (pH 7.3 phosphate), mixed and transferred to a dialyzing casing (1 inch flat width). The casing was suspended in 1.5 l of aqueous 0.25% ammonium acetate. With the aid of gentle magnetic stirring, dialysis was allowed to proceed until maximum hemolysis was obtained (45 to 60 minutes at room temperature). The hemoglobin-free stromata and other blood cells were then lightly packed by centrifugation at 3000 rpm in a Clay-Adams clinical centrifuge, Model CT-1002, (1100 × *g*, 15 minutes) and the supernatant fluid discarded. For transfer to specimen grids, the stromata were resuspended in a small volume (1 to 5 ml) of the ammonium acetate solution. Additional handling of the stromata, *e.g.*, washing and centrifuging, served only to disrupt the organisms and enhance clumping of the erythrocyte membranes. Fixation of the stroma suspension with osmium tetroxide as used by Danon, *et al.*(4) was unnecessary.

Specimen grid preparation. Suitable droplets (*ca.* 0.025 ml) of the stroma suspension were applied by syringe or micro-pipette to Formvar-coated grids held by clamping tweezers. Sedimentation of the stromata and other blood cells onto the substrate film was allowed to proceed for 1 to 3 minutes after which the droplet was removed by touching a pointed piece of filter paper to the surface of the droplet. The thin residual liquid film was similarly removed at the grid margin while tilting the tweezers. Care was taken to remove any fluid trapped in the tweezer jaws. The specimens were shadow-cast with Germanium at an angle of about 5:1 and backed with a film of Carbon to minimize beam damage. Electron micrographs were taken with an RCA EMU-3C at initial magnifications of 5,600 × to 22,000 ×.

Reliability of the method. To estimate the effectiveness of this procedure in retaining recognizable forms of the parasite within the erythrocyte stroma, a sample of infected blood containing an average of one parasite per 6 erythrocytes (determined by hematological examination of Giemsa stained thin films) was diluted approximately 20-fold with fresh uninfected erythrocytes and carried through the described procedure. Two grids were examined for occurrence of the parasite within the stroma. From the dilution it was expected that one organism would be seen in about 120 erythrocyte membranes. Six readily recognizable organisms were observed in approximately 900 membranes scanned which was in fair agreement with the anticipated number, eight. A further 20-fold dilution of this synthetic mixture was similarly prepared and examined. In this instance 4 organisms were observed in about 8,000 membranes as compared with an expectation of 5.

Results. Several dissimilar morphological forms and a variety of deranged organisms were observed in each preparation. The parasites found within their stroma were distinctly outlined against the cell membrane and some internal features could be discerned (Fig. 1 to 5). The most prominent characteristics of the intact parasite were its terminal round electron dense structure and an associated sac-like projection.

The round structures were frequently subdivided into a variable number of smaller units (Fig. 1, 2, 3 and 4). Some organisms possessed these round structures on both ends of the sac. The double-ended forms of the parasite were generally not symmetrical, one end being frequently differentiated into granules (Fig. 4).

The sac-like projection had approximate dimensions of 1 μ flat width and 5 μ length (length ranged from 1 to 8 μ). In most instances this structure was tapered and appeared to have lysed in a manner similar to the erythrocytes (Fig. 1, 3, 5, 6). Occasionally, one extremity of the sac was sharply tapered (Fig. 2).

Fig. 5 illustrates the appearance of 2 organisms within the same stroma.

Although most of the parasites were retained within their host cell stroma, about one in 3 was completely separate. Structural complexities of some developmental forms of this parasite were most clearly demonstrated in these separated organisms as illustrated in Fig. 7 and 8. In Fig. 7 is shown an array of small rectangular extensions and a single knob-like projection on opposite ends of the sac. When the organisms were not severely deranged the small sac extensions were found

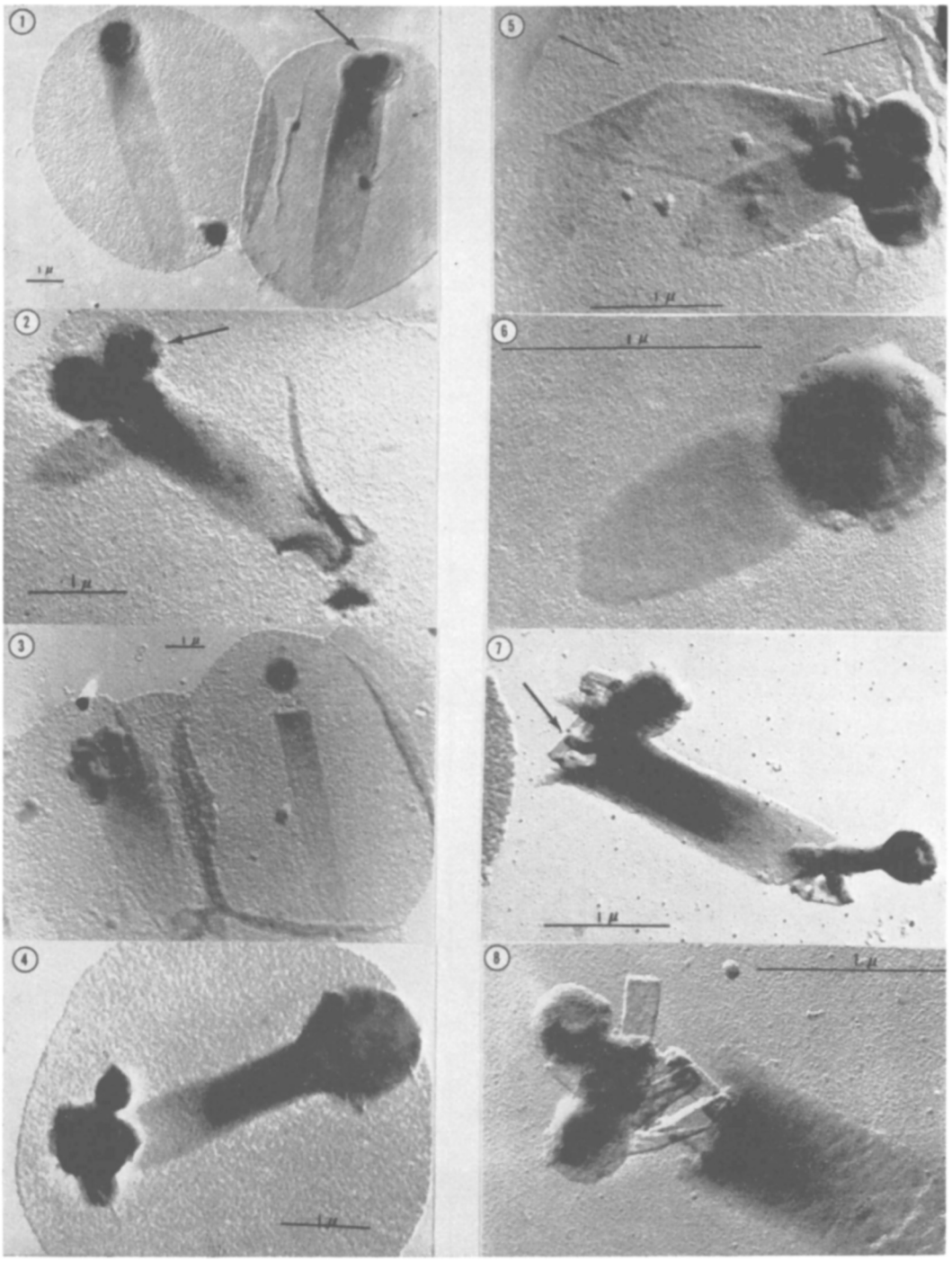

FIG. 1. Individual organisms in host cell stroma showing apparent lysis of the sac-like structure and possible subdivision of a terminal round body (arrow).

FIG. 2. Incompletely lysed organism with sharp tapered sac and divided terminal body (arrow).

FIG. 3. Dislocations of terminal round bodies from the sac-like structures.

FIG. 4. Double-ended organism showing dissimilarity of terminal bodies.

FIG. 5. Two organisms within a single stroma. Stroma margin indicated by arrows.

FIG. 6. Small organism, apparently intact, separated from its host cell stroma.
FIG. 7. Double-ended organism separated from its host cell stroma. The derangement reveals small rectangular extensions of the sac (arrow).
FIG. 8. Cluster of small organisms associated with the terminus of a sac.

to be associated with the terminal round bodies (Fig. 8). A small but apparently intact organism is shown in Fig. 6. The thinness of this organism's sac is indicated by the ability to perceive the substrate granularity when viewed through its flattened membrane.

Specimens from healthy uninfected calves did not exhibit forms similar to those described.

Discussion. The method described here for preparing electron microscope specimens of *A. marginale* is simple and rapid. Most of the parasites were recovered intact although some were deranged as a result of handling. The sac-like projection commonly observed on this organism appeared to have undergone lysis during the course of dialysis. After preparation approximately one-third of the organisms were completely separated from their host cells. In spite of these artifacts, the semi-quantitative retention of recognizable morphological forms of the organism within its host cell stroma suggests that the method may aid in diagnosing chronic cases of infection.

The variety of forms of *A. marginale* seen in these preparations is in accordance with the observations of Espana, Espana and Gonzalez(1), who demonstrated the complex structure of this organism and discussed the difficulties encountered in preparing suitable specimens for electron optical examination.

The present study has provided additional morphological detail of at least one form of this parasite by the disclosure of small rectangular extensions of its sac-like projection (Fig. 7) and has suggested an intimate relationship between these sac-extensions and the small round electron dense bodies (Fig. 8). Other unpublished micrographs taken during this work support this conclusion.

Espana *et al.*(1) have observed numbers of the smaller "tailed" forms resembling the larger organisms, but were unable to determine their nature. The results of the present study (Fig. 6, 7 & 8) suggest that the small "tailed" forms observed by these workers were in fact *anaplasmata* and were related to the typical parasites through a natural process of development at the terminus of the organism.

Summary. A rapid and simple method is described for preparing specimens of an intra-erythrocytic parasite for morphological examination in the electron microscope. The technic includes dehemoglobinization of the erythrocyte by dialysis against a hypotonic solution of a volatile salt. The hemolyzed stromata are transferred to specimen grids by gravity sedimentation. Three principal artifacts associated with the method of preparation are noted. The complex structure of *A. marginale* as reported by previous workers is confirmed and extended by these results. A possible relationship between certain observed forms and the parasite is suggested.

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