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An Electron-Dense Stain for Isolated Fragments of Plasma and Acrosome Membranes from Porcine Sperm[1] (37736)

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(Introduced by W. R. Featherston)

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Isolation and biochemical characterization of surface membranes from mammalian sperm have been hampered by a lack of reliable marker constituents or components to identify and distinguish between acrosome membranes and plasma membranes. The membranes of the acrosome and the plasma membrane vesiculate during cell fractionation, and these vesicles are difficult to identify in isolated cell fractions where positional relationships characteristic of intact sperm are lost. This report concerns the use of a phosphotungstic acid (PTA)–chromic acid staining procedure, developed for plant cells (12), to identify and differentiate between plasma and acrosome membranes in cell-free preparations from porcine sperm.

Materials and Methods. Preparation of cell fractions. Ejaculated boar sperm were removed from the sperm-rich semen fraction by filtration through glass wool and low speed centrifugation (15 min, 600*g*). Sperm were either fixed directly for electron microscopy or fractionated according to the procedure outlined by Lunstra *et al.* [(5) and unpublished data] prior to fixation. For fractionation, sperm were disrupted by gentle sonication (Branson Model W140 with ⅛ in. microtip; 15 sec at 8 W) in 5 ml of a medium containing 0.075 *M* ethylenediaminetetraacetic acid. Unbroken heads were removed by centrifugation at 600*g* for 10 min and retained for preparation of acrosome membranes. Tails and contaminating cytoplasmic droplets were removed by centrifugation at 6000*g* for 10 min. The supernatant, rich in plasma membranes, was then centrifuged on a discontinuous sucrose gradient ($d = 1.14$ to 1.20; 100,000*g*, 90 min). The plasma membrane-rich subfraction was obtained from the $d = 1.15/1.17$ interface and collected by dilution and centrifugation at 20,000*g* for 30 min. The acrosome-rich fraction was prepared by treating the plasma membrane-depleted sperm heads (600*g* pellet from above) with 0.1% Hyamine 2389 (1) for 30 min, followed by sonication and sucrose gradient centrifugation as for plasma membrane. All operations were at 0 to 4°.

Electron microscopy. Cell fractions or whole sperm were fixed in buffered 2% glutaraldehyde (0.1 *M* sodium cacodylate, pH 7.3) for 1 hr at 4°. The preparations were rinsed with buffer and postfixed for 1 hr at 26° with buffered 1% osmium tetroxide, rinsed with buffer, dehydrated through a graded acetone series, and embedded in Epon (4). Thin sections were stained with alkaline lead citrate (10) or by the PTA–chromic acid procedure (12). For the latter, sections were collected in a plastic loop and destained on the surface of a 1% aqueous solution of periodic acid for 30 min followed by five rinses with distilled water for 10 min each. Specific staining was achieved by treatment for 10 to 30 min with 1% phosphotungstic acid (PTA) in 10% chromic acid followed by one transfer to 0.1 *N* HCl for 10 min and five transfers of 10 min each to distilled water.

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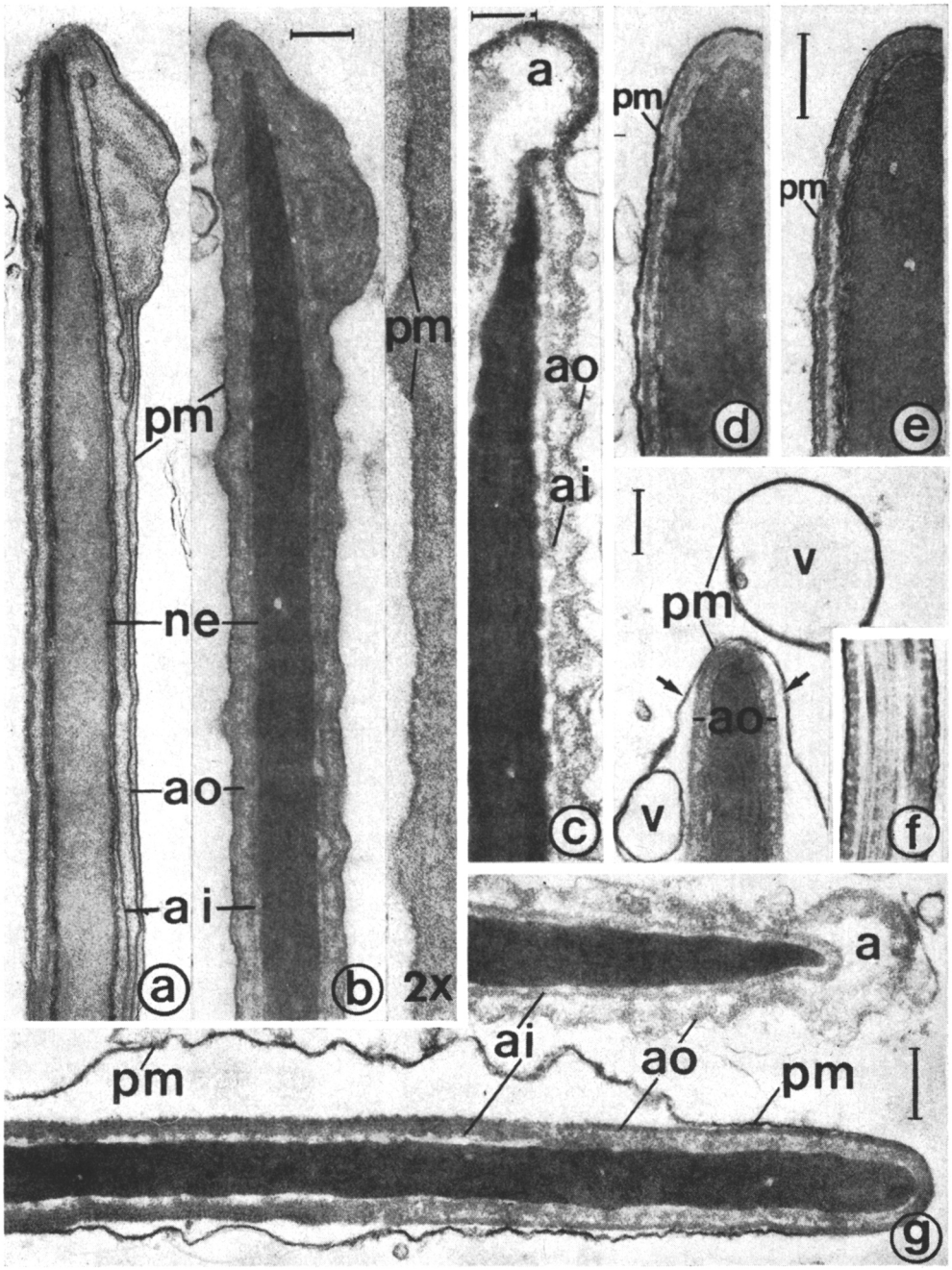

FIG. 1. Electron micrographs of portions of whole porcine sperm. Glutaraldehyde–osmium tetroxide fixation. (a) Lead stained. (b) As in (a) but PTA–chromic acid procedure (10 min). Outer (ao) and inner (ai) acrosome membranes and nuclear envelope (ne) are unstained and obscured by fibrous components of the acrosome contents. *Inset* is enlarged photographically 2× to detail staining of plasma membrane and acrosome contents. (c) PTA–chromic acid procedure (60 min) to show fibrous nature of material adhering to inner surfaces of the acrosome (a) membranes (ao, ai). (d) As in (b) except near base of sperm head. (e) Lead stained for comparison. (f) Staining of plasma membrane (pm) by PTA-chromic acid (20 min) is retained where the

After the final water wash, the sections were transferred to parlodion- and carbon-coated grids. Specimens were viewed and photographed with a Philips EM-200. Proportions of plasma and acrosome membranes in the cell-free preparations were estimated according to the morphometric procedure of Loud (3) as outlined by Lembi *et al.* (2).

Results. Sperm. When stained with alkaline lead citrate in the usual manner, all membranes of the sperm stained with equal intensity (Fig. 1a, e). When stained by the PTA–chromic acid procedure, only the plasma membrane was intensely stained (Fig. 1b, d). Acrosome membranes, nuclear envelope and mitochondria (not illustrated) were unstained (Fig. 1b inset, c, d, f, g). Nuclear and acrosomal contents were stained, however, by PTA–chromic acid. With the PTA–chromic acid stain, the acrosome contents appeared as a distinctive fibrous material that adhered to the inner surface of the acrosome membranes and was most clearly revealed in sperm with swollen acrosomes (Fig. 1c, g). The specificity of the stain was retained or even enhanced with preparations of broken sperm (Fig. 1f, g). Ruptured plasma membrane vesicles were stained (Fig. 1f), while swollen or vesiculated acrosome membranes did not stain intensely even with sperm in which the plasma membrane was lost during specimen preparation (Fig. 1g).

Isolated fractions. The characteristic staining patterns of acrosome and plasma membranes with the PTA–chromic acid stain were retained in isolated preparations (Figs. 2 and 3). As plasma membranes were removed from the sperm, the characteristic staining behavior was retained or even enhanced in vesicles continuous with plasma membrane in normal positional relationship (Fig. 1f, g). Membranes of vesicles derived in similar fashion from acrosomes, nuclear envelopes, or mitochondria were not specifically stained. In this way plasma membranes were shown to be the only membrane components of either intact or broken sperm preparations specifically stained by PTA–chromic acid (Fig. 3a). With fractions rich in acrosome membranes, the fibrous appearance of the material adhering to the membranes and absence of intense membrane staining with PTA–chromic acid provided definitive markers (Fig. 3b).

The relationship between time of exposure to the PTA–chromic acid stain and the appearance of acrosome and plasma membranes is illustrated in Fig. 2. When stained with alkaline lead citrate (Fig. 2a), many of the profiles could not be identified. Destaining with periodic acid (no lead staining) left only faint membrane profiles (not illustrated). Subsequent PTA–chromic acid treatment gradually restained the membranes with the plasma membrane staining most intensely (Fig. 2b–d). With 20 or 30 min of PTA–chromic acid staining (Fig. 2c, d, Fig. 3), plasma and acrosome membrane fragments were clearly differentiated.

Other mammalian tissues, cells and cell fractions. Plasma membranes of cells of rat, bovine and porcine liver and skeletal muscle, bovine and rat mammary gland, and somatic cells of porcine testis did not stain under the conditions reported here, nor did plasma membrane fractions isolated from rat liver. Thus far, the intense staining of plasma membranes with the PTA–chromic acid procedure is restricted to sperm and plants.

Discussion. During cell homogenization, large membranous cell components such as plasma and acrosome membranes are broken into smaller vesicles. The origins of these vesicles are obscured in cell fractions as the positional relationships characteristic of whole cells are lost. This problem is particularly critical in mammalian sperm where specific membrane characteristics to identify plasma and/or acrosome vesicles are unclear.

The PTA–chromic acid staining procedure,

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plasma membrane is separated from the outer acrosome membrane (ao) by swelling (arrows) or vesiculation (v); see also (g). Inset = tail. (g) Acrosome (a) membranes (ao, ai) do not stain intensely with PTA–chromic acid (20 min) even in swollen sperm where the intensely stained plasma membrane (pm) is widely separated from the acrosome (below) or lost entirely (above); see also (c). Bar = 0.25 μm.

developed for identification of plasma membranes isolated from plant cells (12), permits positive identification of plasma and acrosome membrane fragments in cell fractions from porcine sperm (Fig. 3). With PTA–chromic acid staining for approximately 20 min, plasma membranes are stained intensely while acrosome membranes are unstained but readily identified from a fibrous material of the inner acrosomal surface detailed by the stain.

The chemical basis for the stain is unknown. Phosphotungstic acid at low pH was used originally to detect complex carbohydrates and glycoproteins (6–9, 11) but, except for portions of the Golgi apparatus, se-

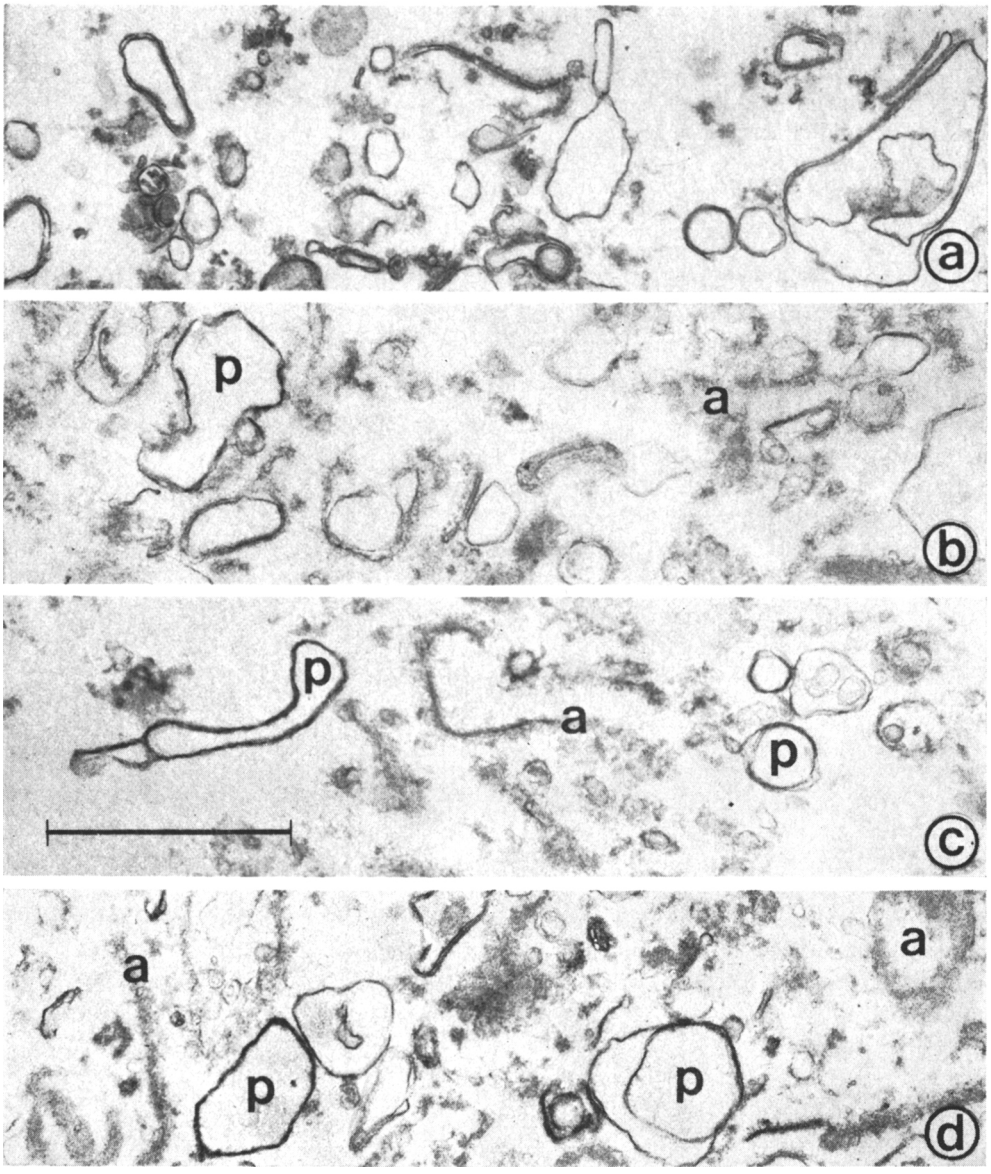

FIG. 2. Portions of an acrosome membrane-rich preparation selected to contain both plasma membrane (p) and acrosome (a) fragments. (a) Lead stained. (b–d) PTA–chromic acid procedure showing changes with increasing time of exposure to the PTA–chromic acid mixture. (b) 10 min; (c) 20 min; (d) 30 min. Bar = 1 μm.

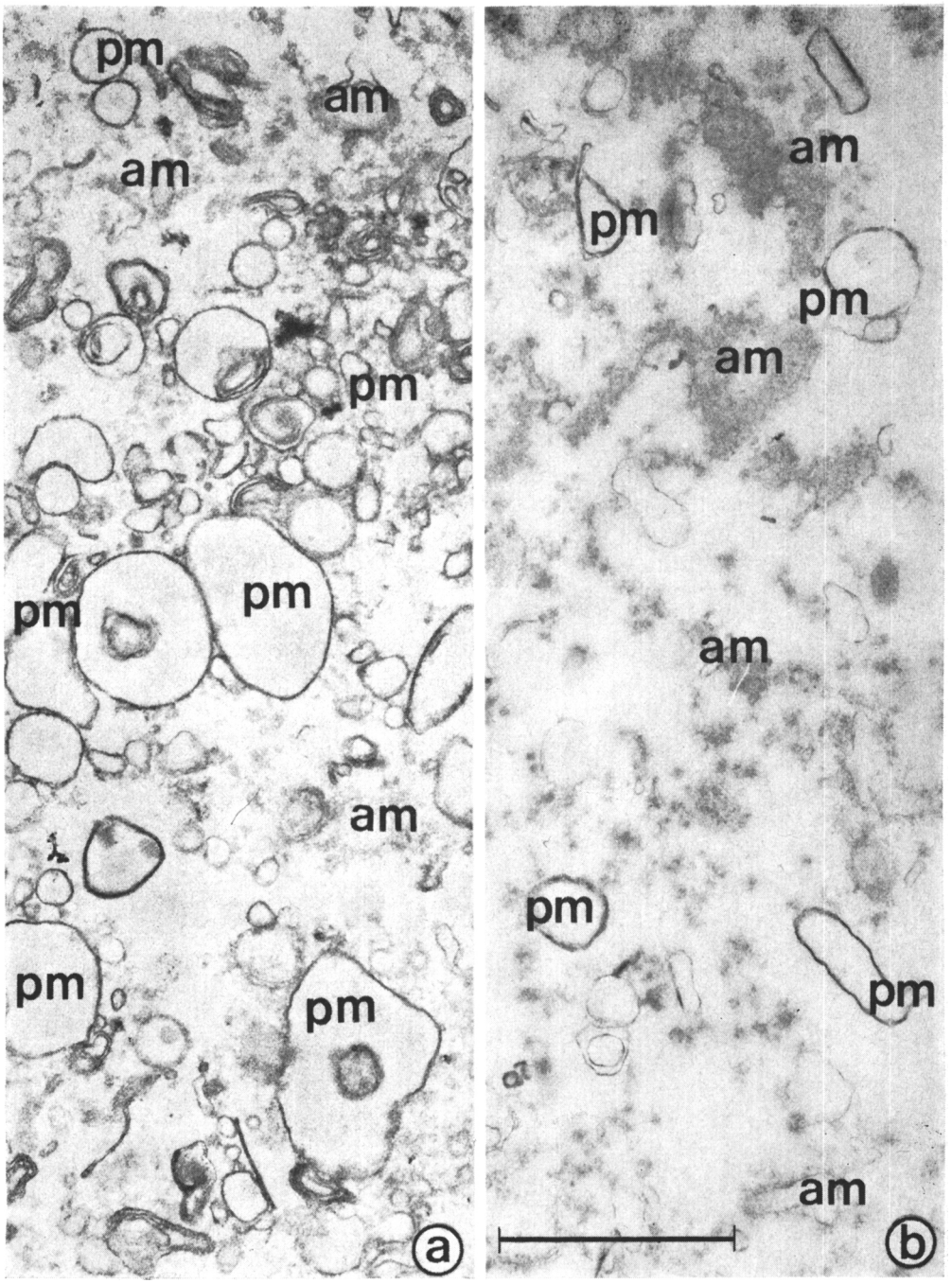

FIG. 3. Isolated sperm fractions stained for 20 min with PTA–chromic acid. Bar = 1 μm. (a) Plasma membrane-rich. From morphometry, this preparation contained 71% plasma membrane (pm), 22% acrosome membrane (am) and 7% unidentified membranes. (b) Acrosome membrane-rich preparation containing 79% acrosomal fragments, 16% plasma membrane vesicles and 5% unidentified fragments.

cretory vesicles and other types of transitional membrane elements involved in plasma membrane formation [(13), unpublished data], membranes other than plasma membranes do not stain even in plants.

Summary. A mixture of 1% phosphotungstic acid in 10% chromic acid selectively and differentially stains the plasma and acrosome membranes of ejaculated porcine sperm. Prior to staining, the cells or cell fractions are fixed with glutaraldehyde–osmium tetroxide, embedded in Epon, and, after sectioning, destained with periodic acid. Plasma membranes are stained intensely by the procedure while a fibrous component is revealed on the inner surface of acrosome membranes. Other sperm membranes do not show these staining characteristics. The specificity of the stain for plasma and acrosome membranes is retained in crude homogenates and purified cell fractions.

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