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Fine Structural Localization of Concanavalin A Binding Sites on Hamster Spermatozoa

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The plasma membrane of epididymal spermatozoa of the golden hamster (Mesocricetus auratus) exhibits morphological differences over various parts of the head and tail as detected by air-dried replicas and freeze-etching techniques. In an attempt to ascertain whether any topographical differences exist in the number or distribution of carbohydrate moieties associated with the cell surface, cells were labeled with Concanavalin A and marked with hemocyanin.

It was found that while the plasma membrane over the acrosomal region differed from that of the postacrosomal region in membrane components revealed by freeze fracturing, there was no apparent difference in the distribution or density of Con A binding sites detectable by hemocyanin localization. The tail regions exhibited differences in both fracture face appearance and the distribution of detectable carbohydrate moieties.

It was also found that binding sites for Concanavalin A exist on the inner and outer acrosomal membranes in addition to those on the plasma membrane.

Key words: hamster spermatozoa, Concanavalin A, cell surface, acrosome

INTRODUCTION

It has been recognized for several years that the plasma membrane of mammalian spermatozoa exhibits a high degree of regional specialization, both functionally and morphologically (1–3). The head region is of particular interest since two processes of great importance to fertilization occur here, the acrosome reaction and fusion with the egg. In addition to general fine structural studies of sperm membrane architecture (4–7), there have been a number of efforts to study specific membrane components by means of lectins, which have been used to localize certain carbohydrate residues on the cell surface. These studies have indicated that differences in surface carbohydrates occur between species and, in some cases, between different regions of the same cell (8–13).

The purpose of this investigation was to study the plasma membrane of spermatozoa from the golden hamster (Mesocricetus auratus) by the freeze-fracture technique and to localize, at the ultrastructural level, surface carbohydrate that will bind the lectin Concanavalin A. These observations regarding the organization of this functionally complex membrane will provide a baseline for similar studies currently being conducted with capacitated spermatozoa.

MATERIALS AND METHODS

Cells

Spermatozoa were obtained from the cauda epididymides of adult golden hamsters and suspended in a modified Tyrode's medium containing 0.3 mM sodium phosphate, pH 6.5–6.8. These cells were routinely washed twice in approximately 100 vol of medium to remove epididymal fluid before being used for further experimentation.

Microscopy

Specimens for thin-section microscopy were fixed in 2.5% glutaraldehyde in 0.25 M sodium cacodylate, pH 7.2, for at least 1 hr, washed in buffer, and postfixed with 1% osmium tetroxide in 0.25 M sodium cacodylate. Specimens for air-dried replicas were further washed with distilled water, dried onto Parlodian-coated grids and shadowed as previously described (15). Specimens for thin sectioning were dehydrated with a graded ethanol series and embedded in Epon 812 (16). For visualization of hemocyanin, sections were stained with uranyl acetate for 2 hr at 65°C.

Freeze-etching was performed with both glutaraldehyde-fixed and unfixed cells. Fixed cells were washed in buffer and slowly transferred to distilled water containing 25% glycerol for several hours before freezing. Live cells in Tyrode's medium were pelleted and frozen quickly from 37°C with no cryoprotectants added. Instrumentation used for the freeze-fracturing has been described previously (17).

Lectin Labeling

Cells were labeled with Concanavalin A (Con A) which was then localized by secondary addition of hemocyanin (18). Con A (Sigma Chemical Co.) was purified by affinity chromatography according to the method of Agrawal and Goldstein (19) and dialyzed extensively to remove the hapten sugar. The protein was then dialyzed into labeling medium (glucose-free modified Tyrode's) or cacodylate buffer on the day before use, and protein content was determined by Lowry assay using bovine serum albumen as a standard. Hemocyanin was obtained from Busycon canaliculata (Woods Hole Biol. Supply) as previously described (15) and dialyzed overnight into the labeling medium or buffer.

The plasma membrane of these cells is very fragile and labeling of unfixed cells resulted in a high proportion of damaged cells, making morphological analysis very difficult. Better results were obtained with fixed cells; however, both methods will be described here.

Labeling of glutaraldehyde-fixed cells was done as follows. Fixed cells were washed in cacodylate buffer and then incubated in two changes of 2% glycine in 0.1 M cacodylate for a period of 1 hr, washed in buffer, and brought to a concentration of 1×10^6 cells/ml. A 0.3 ml portion of this suspension was added to 3.0 ml of buffer containing 1 mg/ml Con A. These conditions (low cell concentration and high lectin concentration) were found necessary to prevent massive agglutination of the cells which would result in extensive membrane damage upon subsequent washing. This mixture was incubated for 5 min at 37°C, washed twice with buffer, and resuspended for 20 min at 37°C in buffer containing 8 mg/ml hemocyanin, washed twice, and fixed fixed with glutaraldehyde.

Labeling of live cells was done in the same way as fixed cells except that all solutes were in glucose-free Tyrode's solution, and the glycine incubation was omitted. These labeling conditions were found to result in maximal labeling of the cells.

Controls

Controls to test whether or not the labeling was specific – that is, whether it represented binding to carbohydrate (primarily glucose and mannose) – included the following:

1. Cells, both live and fixed, were incubated with hemocyanin, 8 mg/ml for 20 min at 37°C, washed twice, and fixed with glutaraldehyde. No hemocyanin was detectable upon examination of sections and replicas. This indicates that hemocyanin does not bind to the cell in the absence of lectin.

2. Cells, both live and fixed, were labeled according to the usual procedure except that the labeling medium or buffer contained 0.1 M α-methyl-D-mannoside (Sigma) in addition to the Con A (1 mg/ml). They were then washed once with α-methyl-D-mannoside-containing buffer or labeling medium and once with standard buffer or medium and then incubated with hemocyanin, 8 mg/ml, for 20 min at 37°C. Cells so treated had very little or no detectable labeling in replicas or sections, indicating that the active sites of the Con A molecule are necessary for labeling.

3. In order to rule out the possibility in the above experiment that Con A bound nonspecifically to the cell would not bind hemocyanin because it had been exposed to the hapten inhibitor (in which case nonspecific binding would not be detectable), the following experiment was done. Fixed cells washed with glycine were labeled with Con A (1 mg/ml) for 5 min at 37°C, then washed once with 0.1 M α-methyl-D-mannoside for 10 min. They were then washed once with buffer and labeled with hemocyanin for 20 min at 37°C. The rationale is that if α-MM binds so tightly and irreversibly to Con A on the cell surface that hemocyanin could not bind to it during the incubation period, then these cells would have no detectable hemocyanin.

These cells exhibited labeling that was much higher than in control (2), although not maximal. This indicates that the inhibitor monosaccharide does not remain irreversibly bound to the Con A molecule during the labeling procedure and that binding sites are available to hemocyanin under these conditions. Further indication that nonspecific labeling would be detectable as such comes from the observation that we were totally unable to inhibit labeling of fixed cells unless the hour-long wash in glycine-containing buffer was carried out.

RESULTS

Carbon-platinum replicas of air-dried cells reveal large areas of membrane for study and allow easy identification of the portion of cell underlying the membrane. In Fig. 1, showing a normal hamster spermatozoon, one can identify the acrosomal region constituting the hook-like, expanded portion of the head and ending in the flat, cresentic equatorial segment. The postacrosomal region extends caudally from the equatorial segment to the neck region. Close examination of the membrane overlying the postacrosomal region reveals a finely granular appearance. This is also evident in freeze-fractured and deep-etched cells (Fig. 2).

The junction between the postacrosomal region and the equatorial segment is marked by a crescent-shaped ridge (Fig. 1). In specimens in which the plasma membrane is torn away, it is evident that this ridge is actually a structure lying under the plasma membrane and not a part of it.

The plasma membrane over the acrosomal region also has a finely granular appearance as seen in air-dried replicas as well as in deep-etched specimens (Fig. 3). It is not clear

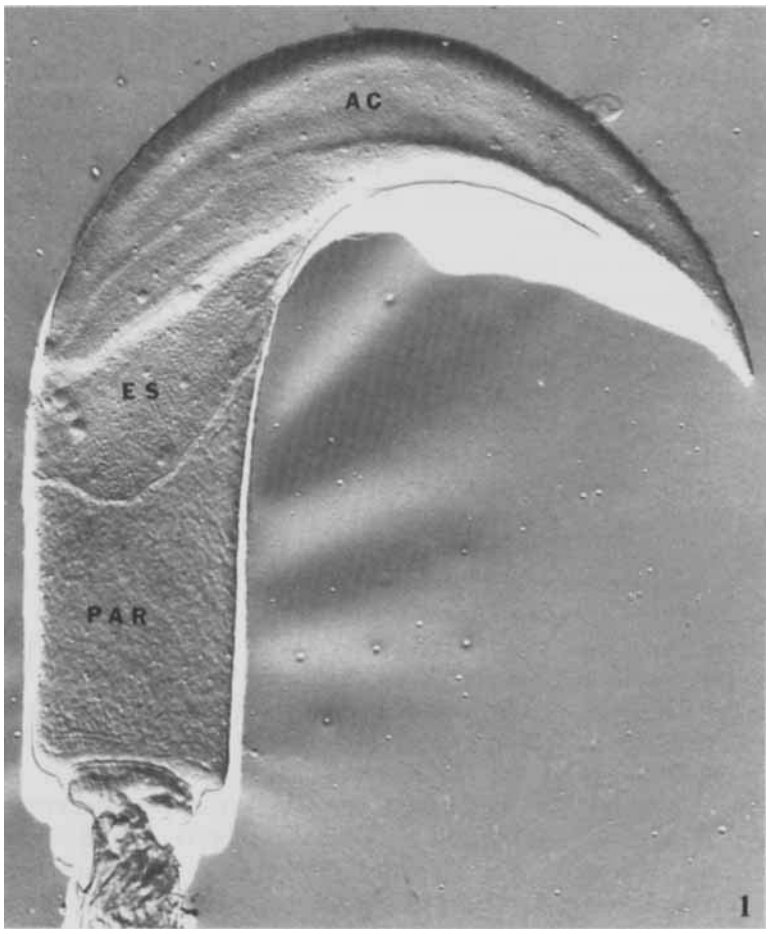


Fig. 1. Carbon-platinum replica of an epididymal hamster spermatozoon. The different regions of the head are easily distinguished. Acrosomal cap (AC), equatorial segment (ES), postacrosomal region (PAR). ×12,400.

whether this surface granularity represents material on the outer surface of the membrane or structures within or underneath it that project through to influence the surface topography.

More detailed information regarding the organization of the membrane can be obtained by the freeze-fracture technique. Figure 4 shows that the plasma membrane over the postacrosomal region of the head contains a dense population of intramembranous particles. The P face exhibits particles of varying size but generally falling into two size classes: "large" (approximately 100 Å) and "small" (approximately 40 Å) (see Figs. 4 and 5). These are distributed densely and evenly over the entire postacrosomal region. The E face exhibits particles of roughly two size classes also; the larger ones are sparsely but uniformly distributed while the smaller ones are much more numerous (Figs. 6 and 7).

The junction between the equatorial segment and the postacrosomal region is usually evident as a slight shelf following the cresentic outline of the equatorial segment. At this point, a change in particle distribution is apparent in the fractured surface of the

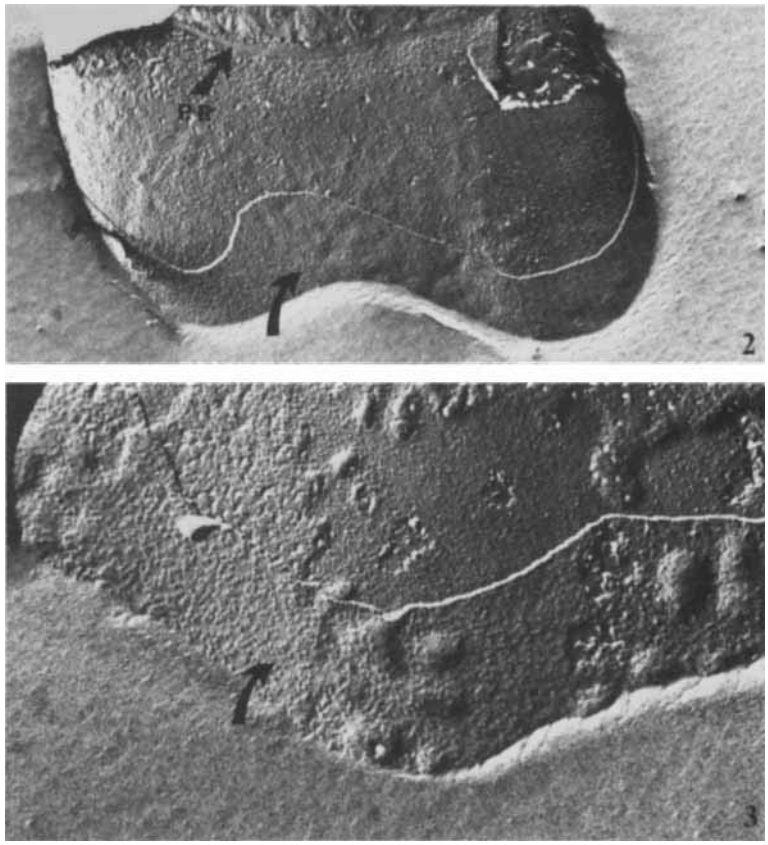


Fig. 2. Freeze-fractured and deep-etched hamster spermatozoon. The etched surface over the post-acrosomal region has a finely granular appearance (arrow). The posterior ring is also seen (PR). X 32,000.

Fig. 3. Deep-etched hamster spermatozoon. The granular appearance of the etched surface of the plasma membrane is also apparent over the acrosomal cap (arrow). X48,000.

membrane. On the P face, the large particles undergo a sharp reduction in density of distribution as one moves from the postacrosomal region to the acrosomal region (Figs. 4 and 5). The large E face particles, already rather sparse on the postacrosomal region, become more sparsely distributed over the acrosomal region (Figs. 6 and 7). The smaller particles do not appear to change in density or distribution over the entire head region on either E or P faces.

The posterior ring, which separates the head region from the neck and middle piece, is seen to be entirely devoid of intramembranous particles (Fig. 2), although in favorably shadowed fractures there is some hint of the striation seen in other species (3). The plasma membrane over the middle piece has a very dense population of intramembranous particles fairly homogenous in size and distribution (Figs. 8 and 9). The P face (Fig. 8) has a more dense distribution than the E face (Fig. 9). At the junction with the principal piece, the annulus, this pattern changes. Here the fracture face reveals several tightly packed rows of particles running circumferentially around the annulus (Fig. 10). The plasma membrane

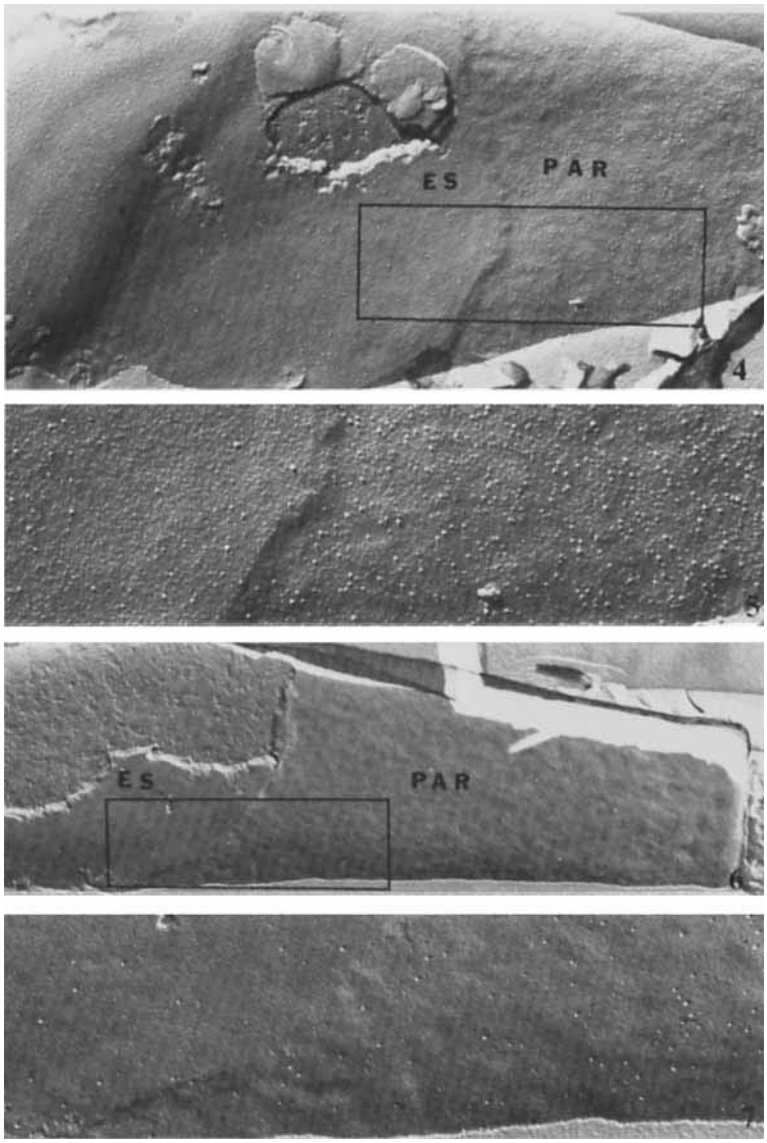


Fig. 4. Freeze-fractured hamster spermatozoon, P face. The boundary between the acrosomal and postacrosomal region is shown. It is evident that large P face particles are more densely distributed over the postacrosomal region than over the acrosomal region. Equatorial segment (ES), postacrosomal region (PAR). ×22,400.

Fig. 5. The rectangle shown in the previous figure is enlarged here. "Large" and "small" intramembranous particles can be distinguished. × 48,000.

Fig. 6. E face of freeze-fractured specimen showing the postacrosomal region. Large E face particles are more densely distributed over the postacrosomal region than over the acrosomal region. Equatorial segment (ES), postacrosomal region (PAR). × 23,200.

Fig. 7. The area of the rectangle shown in the previous figure is shown here at higher magnification. "Large" and "small" intramembranous particles are more easily distinguished. × 64,000.

over the principal piece exhibits a fractured appearance similar to that of the middle piece except that the particle density appears to be slightly lower. The etched surface of the principal piece exhibits rows of particles arranged in a staggered fashion (zippers) that are characteristic of the principal piece in a number of species (20,21). Often, these structures seem to be represented on the P face by a single or double row of intramembranous particles.

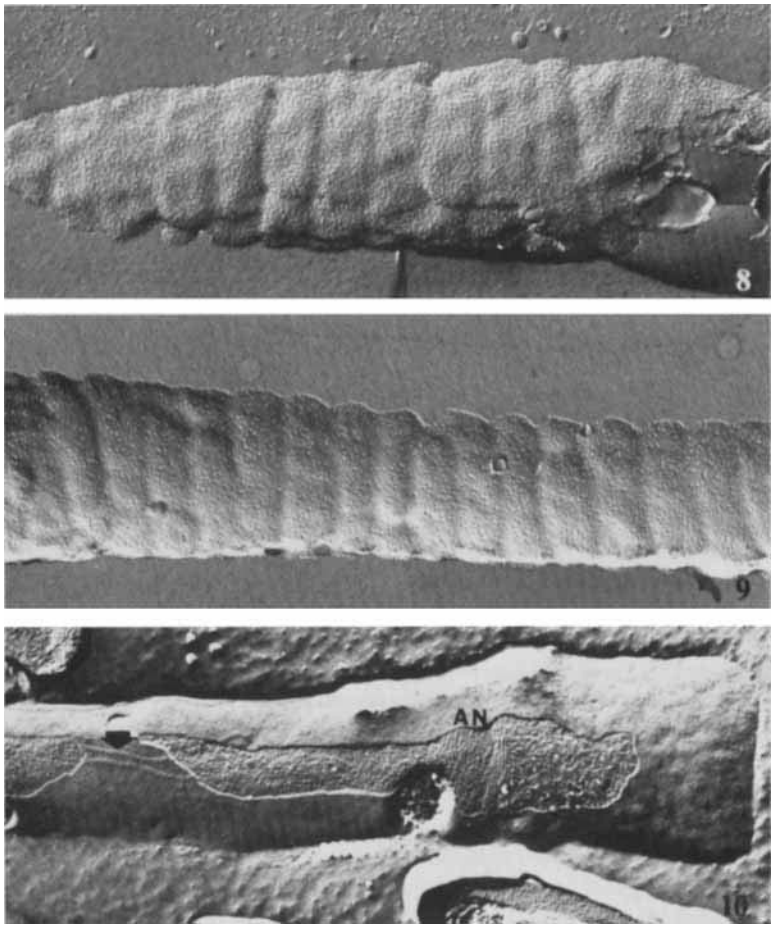


Fig. 8. Freeze-fractured middle piece (P face). A dense distribution of irregularly arranged particles is evident. ×32,000.

Fig. 9. Freeze-fractured middle piece showing the E face. Intramembranous particles appear to be somewhat less densely distributed than on the P face. ×30,400.

Fig. 10. Deep-etched specimen showing the region of the annulus (AN), middle piece to the right and principal piece to the left. The "zippers" present on the etched surface of the principal piece (arrow) are usually represented on the fracture face by a single or double row of intramembranous particles. ×32,000.

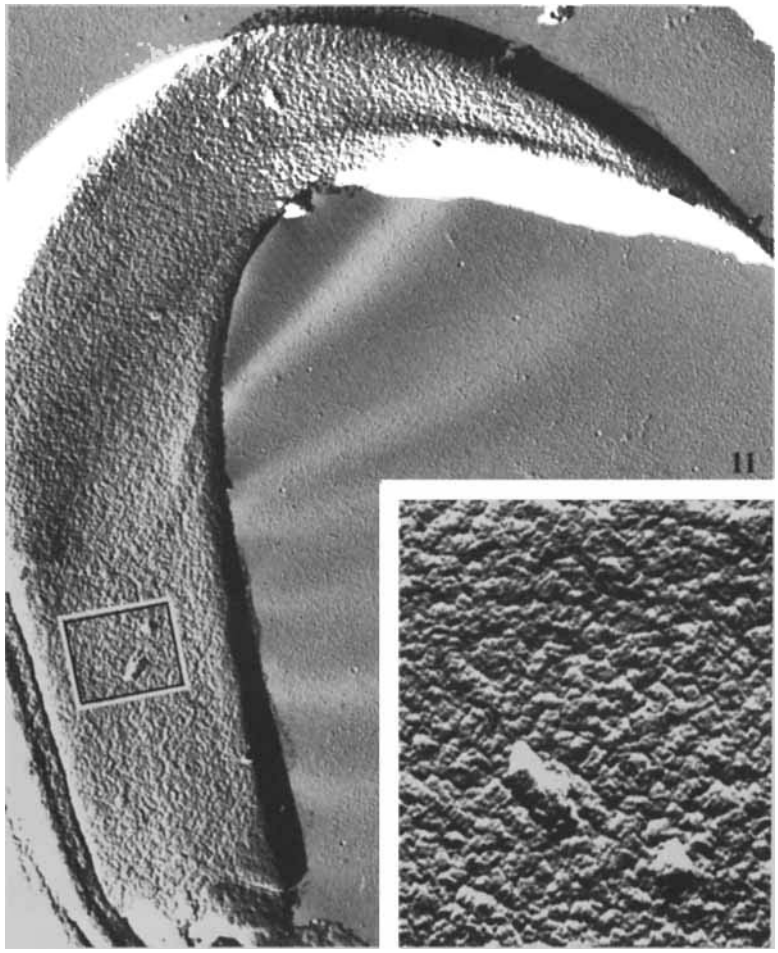


Fig. 11. Carbon-platinum replica of a hamster spermatozoon labeled with Con A and hemocyanin. Hemocyanin is very densely distributed over the entire cell surface. ×14,400. Inset: At higher magnification, individual hemocyanin molecules can be distinguished. ×48,000.

Con A Labeling

Surface replicas of air-dried cells were most useful as they allow one to observe about half of the surface of each cell. As seen in Fig. 11, the head region displays a heavy and uniform distribution of Con A-bound hemocyanin with no discernible differences in amount or distribution of label over the different parts of the head region. Sections of these cells confirm this result (Figs. 12 and 13). Occasionally, sections seem to show a slightly higher density of hemocyanin over the acrosomal region than over the postacrosomal region. This difference is always very slight and may be due to undulation of the plasma membrane, which often occurs over the acrosomal region.

The middle piece has a distribution and density of labeling similar to that of the head region (Fig. 14). This is more easily seen in sectioned material due to the poor preservation of the middle piece during the drying process for surface replicas. The principal piece is demarcated from the middle piece by the annulus. At this point there is an abrupt

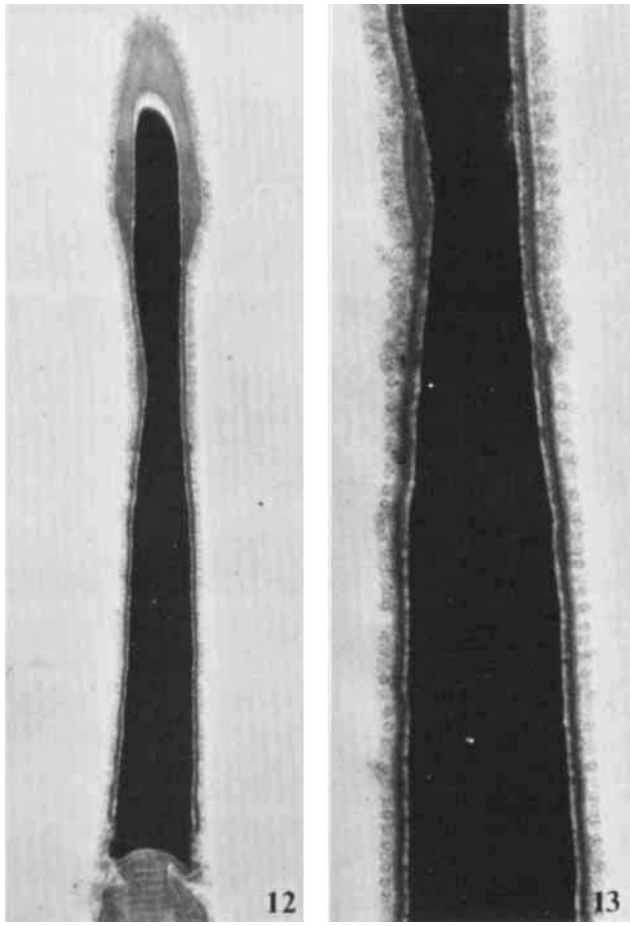


Fig. 12. Thin section of a hamster spermatozoon labeled with Con A and hemocyanin. Hemocyanin appears uniformily distributed over the plasma membrane. × 16,800.

Fig. 13. At higher magnification, individual hemocyanin molecules can be easily distinguished. ×40,800.

decrease in the density of detectable Con A receptor sites (Figs. 14 and 15). The middle piece and principal piece are the only parts of the cell that demonstrate a reproducible difference in the distribution of binding sites associated with fixation. Cells labeled before fixation exhibit a clumped distribution of binding sites, while those labeled after fixation have a more uniform distribution (Figs. 14 and 16). Whether the clumped distribution observed in cells labeled before fixation is due to loss of receptors from the membrane or redistribution of receptors within the membrane cannot be resolved by these data. In any case, we feel that the results obtained from the prefixed cells are more representative of the "native" situation; however, the possibility that glutaraldehyde might induce receptors originally in a clumped state to disperse cannot be ruled out entirely.

In the labeling procedures used here, a certain fraction of the cells underwent a type of "acrosome reaction." This involved loss of the plasma membrane from the acrosomal region and most of the postacrosomal region, exposing the postacrosomal dense lamina

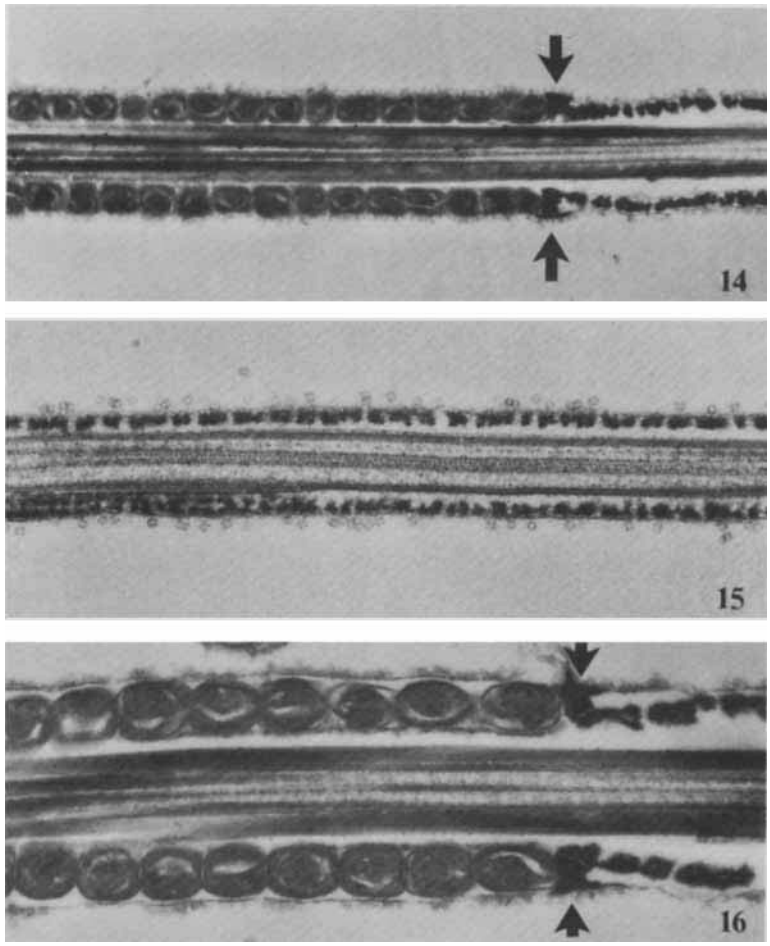


Fig. 14. Thin section of a sperm tail in the region of the annulus (arrows) labeled with Con A and hemocyanin. It can be seen that the density of labeling drops off as one moves from the middle piece (left) to the principal piece (right). ×17,600.

Fig. 15. Principal piece showing characteristic low density of labeling. ×48,000.

Fig. 16. Annulus (arrows) region of a cell labeled prior to fixation. Hemocyanin has a more clumped distribution than in cells fixed prior to labeling (Fig. 14). ×28,000.

and the external surface of the outer acrosomal membrane (Fig. 17). Usually the acrosomal cap was lifted, also exposing the inner acrosomal membrane. These cells were observed by phase contrast microscopy to be immobile and we feel that this reaction is a result of cell damage. It is useful in that it exposes different parts of the cell to Con A. Figures 18 and 19 show that the luminal surface of the inner acrosomal membrane as well as the external surface of the outer acrosomal membrane bear receptors for Con A, while the postacrosomal dense lamina has none or very few. This labeling as well as that described above for the plasma membrane is inhibitable by α-methyl-D-mannoside and is therefore considered to be specific labeling (Figs. 20 and 21).

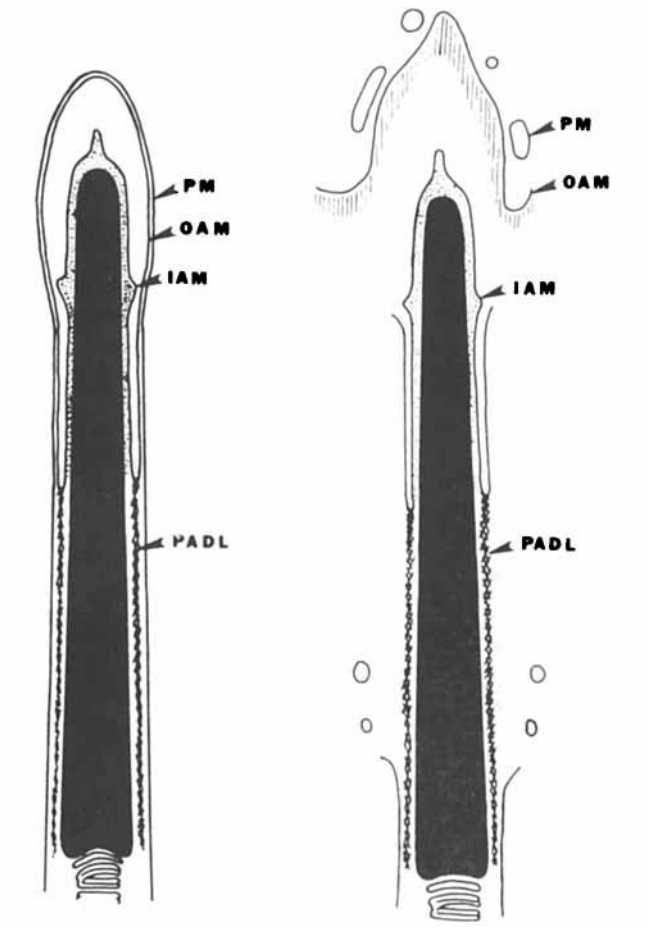


Fig. 17. Drawing showing the disposition of the acrosomal membrane in hamster sperm. The normal appearance of the sperm head is seen on the left portion of the figure. That portion of the acrosomal membrane in close apposition to the nucleus is termed the inner acrosomal membrane (IAM). That portion not immediately adjacent to the nucleus is the outer acrosomal membrane (OAM). Caudal to the acrosome, the postacrosomal dense lamina (PADL) is seen underlying the plasma membrane (PM). As a result of cell damage (right) the plasma membrane vesiculates and is lost over the anterior portion of the sperm head. This exposes the outer and inner acrosomal membranes as well as the postacrosomal dense lamina to the external environment and allows these surfaces to be probed with lectin. Results of such observations are seen in Figs. 18 and 19.

DISCUSSION

These results indicate that the plasma membrane of hamster spermatozoa differs regionally both in intrinsic components visualized by freeze fracture and in the distribution of surface carbohydrate detectable by the Con A-hemocyanin technique. Specifically, the plasma membrane over the postacrosomal region of the head has a much denser complement of large intramembranous particles on both the P and E faces than does the acrosomal region. However, it appears that there are no detectable differences in the density or distribution of glucose- and/or mannose-containing molecules over these regions, at least at the level of resolution obtainable with this technique.

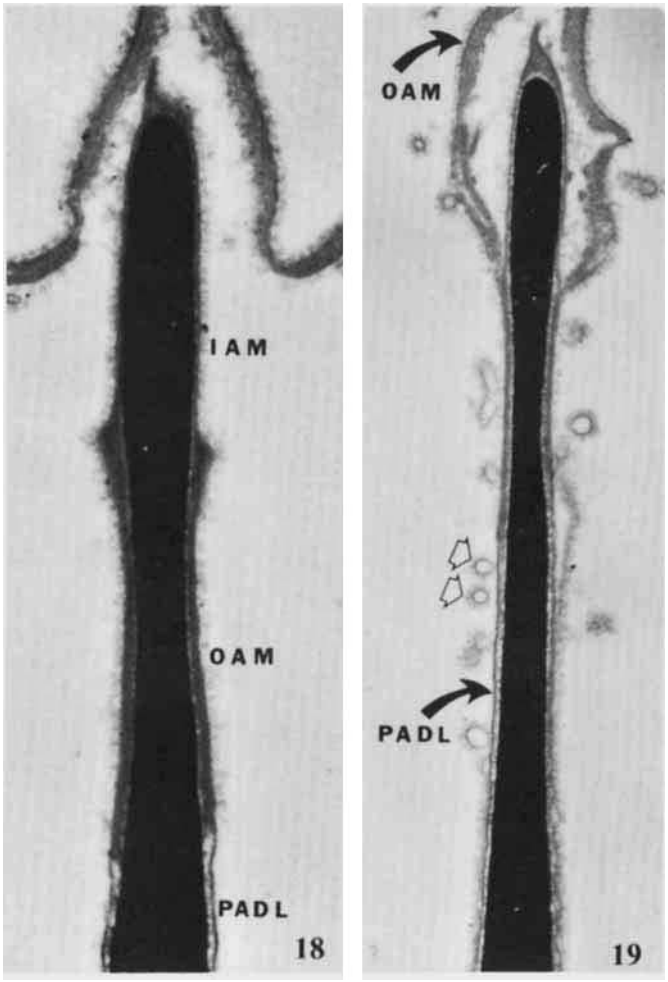
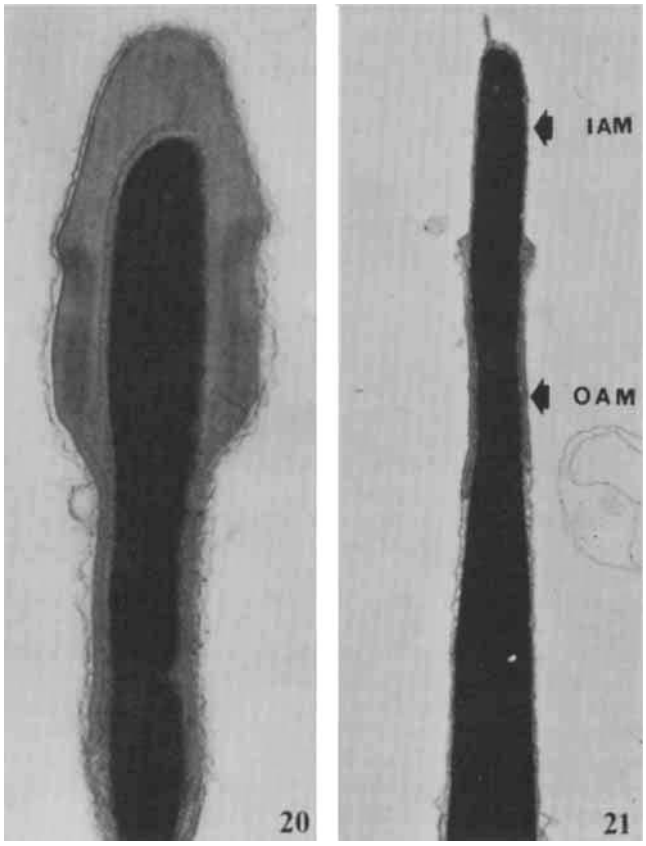


Fig. 18. In this cell the plasma membrane has been removed, exposing the postacrosomal dense lamina and the outer acrosomal membrane. In addition, the acrosomal cap has lifted, exposing the inner acrosomal membrane. The luminal surface of the inner acrosomal membrane as well as the external surface of the outer acrosomal membrane are labeled with hemocyanin, while the postacrosomal dense lamina is not. ×30,800.

Fig. 19. In this figure the plasmic membrane has vesiculated (open arrows) exposing the postacrosomal dense lamina and the outer acrosomal membrane. The postacrosomal dense lamina remains unlabeled, whereas the membranes show dense hemocyanin labeling. ×16,800.

The plasma membrane of the middle piece exhibits a fracture face containing a dense population of particles homogeneous in size and irregularly distributed. The distribution of detectable Con A binding sites is very similar to that of the head. The principal piece differs from the middle piece in that it exhibits linear arrays of surface particles (zippers) on the etched surface, and the intramembranous particles are less densely distributed. In addition, the amount of carbohydrate detectable with Con A is much less in this region than over the middle piece.



Figs. 20 and 21. These cells were labeled in the presence of 0.1 M α-methyl-D-mannoside. No Con A is detectable by hemocyanin localization on either the intact cell (left) or the cell that has shed its acrosomal cap (right). × 36,000 and 18,400 respectively.

One might speculate as to the mechanisms by which this nonrandom distribution of membrane components is maintained. One possibility is that diffusion of membrane components is limited either by the physical properties of the membrane itself or by interaction with structures underlying the membrane as hypothesized, for example, in the red blood cell system (22). Alternatively, there may be barriers to diffusion either within or outside the membrane that maintain the regional differences described here. The observations reported here do not allow us to distinguish between these possibilities. We can only say that regional differences exist and that structures observable within the membrane at the posterior ring and the annulus may serve to maintain these regional differences. No structures were observed between the acrosomal and postacrosomal region of the head that might serve such a barrier function.

The identification of lectin receptors on the inner and outer acrosomal membrane is a new finding. The acrosome is essentially a large, Golgi-derived, secretory vesicle, and it would be interesting to determine if membranes surrounding secretory granules in other cell types show lectin affinity. During the "physiological" acrosome reaction that accompanies or follows the capacitation process, the acrosomal cap is lost, exposing the luminal

surface of the inner acrosomal membrane, and it is apparent that "new" surface carbohydrate would be exposed at this time. The significance of this with respect to later events in the fertilization process remains to be determined.

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