REGIONAL VARIATIONS IN THE COMPOSITION OF THE PERINUCLEAR THECA OF THE RAT SPERMATOZOA

by

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Je dédie cet ouvrage
à ma mère,
Pour son amour et sa devotion
To know that we know what we know, and to know that we do not know what we do not know, that is true knowledge.

(Confucius)
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ABSTRACT

The perinuclear theca is a rigid cytoskeletal structure which encapsulates the nuclei of mammalian spermatozoa. It is composed of two structurally continuous regions, the perforatorium which underlies the acrosomic system and the postacrosomal sheath, which begins where the acrosomic system ends and directly underlies the plasmalemma. Utilizing an alkaline treatment the perforatorial part of the perinuclear theca of rat spermatozoa was detached from the nucleus and fractionated on a sucrose gradient. The isolated perforatoria were denatured and used to raise antibodies or run on polyacrylamide gels. Such gels revealed many polypeptide bands of which seven were prominent. The main objective of this study was to determine the distribution of these perforatorial polypeptides in the head of the rat spermatozoon.

Anti-perforatorium serum reacted with the entire perforatorium of fixed spermatozoa but, in addition, it reacted with the ventral spur of the postacrosomal sheath and with the a portion of the "outer periacrosomal layer" lying between the plasma membrane and the outer acrosomal membrane. In order to localize individual perforatorial proteins, polyclonal antibodies were affinity purified from these polypeptides and tested 1) for their distribution on electron microscope sections of late spermatids and spermatozoa by immunogold labeling and 2) for their specificity on Western blots of denatured perforatorial polypeptides by immunoblotting. Immunoblotting demonstrated that seven
perforatorial polypeptides tested in this study, had epitopes in common. Immunogold labeling of spermatozoa showed that the distribution of the 34, 43, 57 and 63 kDa polypeptides corresponded exactly to the regions of the perinuclear theca immunolabeled with the whole perforatorium serum. However, antibodies affinity purified against the 13, 13.4 and 16 kDa polypeptides were restricted in their localization to the thicker apical portion of the perforatorium and to the inner layer of the ventral spur.

These results suggest 1) the perforatorium is biochemically distinct from the postacrosomal sheath with the exception of a restricted region referred to as the ventral spur 2) there are regional differences in protein composition of the perforatorium, of the outer periacrosomal layer and of the postacrosomal sheath; and 3) that perforatorial polypeptides may not be necessarily be restricted to the subacrosomal region, but may also compose portions of the outer periacrosomal layer, and postacrosomal sheath. and 4) Actin is not part of the perforatorium in mature rat spermatozoa.

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RESUME

La Thèque périnucléaire est une structure rigide qui encapsule le noyau du spermatozoïde des mammifères. Elle est composée de deux régions distinctes, le perforatorium qui est sous-adjacent au système acrosomique et la couche postacrosomale, sous-adjacente à la membrane cytoplasmique. Des perforatoria isolés par traitement à la soude ont été soumis à une analyse sur gel de polyacrylamide. Les résultats ont montré plusieurs bandes sur gel dont sept proéminentes qui ont servis à la production d'anticorps. Le but principal de la présente étude a consisté à montrer par immunocytochimie la distribution de ces protéines cytoskelettiques dans la tête du spermatozoïde de rat. Le serum anti-perforatorial total réagit avec tout le perforatorium du spermatozoïde. De plus, il réagit avec l'éperon ventral de la couche postacrosomique et une portion de la couche externe où periacrosomale se trouvant entre la membrane plasmique et la membrane acrosomale externe. Des anticorps polyclonaux ont été purifiés par affinité à partir de ces protéines et analysées 1) pour leur distribution sur des sections de spermatides et spermatozoïdes à l'aide du microscope électronique et 2) par "immunoblotting" pour déterminer leur spécificité sur des Western blots de protéines perforatoriales dénaturées. Les résultats ont démontré que sept des protéines perforatoriales étudiées possèdent des épitopes communs. Les études
immunocytochimiques ont montré que les protéines de 34, 43, 57 and 62 kDa se trouvent dans tout le perforatorium. Par contre, les anticorps contre les protéines de 13, 13.4 et 16 kDa montrent une distribution limitée à la région apicale du perforatorium et à la couche interne de l'éperon ventral. Les résultats montrent de plus que: 1) Le perforatorium est biochimiquement distinct de la couche postacrosomique à l'exception de l'éperon ventral. 2) Il existe des différences dans la distribution des protéines dans le perforatorium, de la couche externe périacrosomale et de la couche postacrosomique. 3) Les protéines perforatoriales ne sont pas exclusivement associées à la région sous acrosomique mais peuvent se trouver dans la couche externe périacrosomale et dans la couche postacrosomique.

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I. INTRODUCTION

The spermatozoon is a functionally unique cell required to perform multiple and highly complex tasks prior to delivering its DNA into the oocyte. The spermatozoon must be capable of vigorous motility, penetrate the zona pellucida and fuse with the plasma membrane of the oocyte. Furthermore, it must contribute to a normal genomic complement necessary for the development of the embryo. For each of these requirements, as well as for its own metabolism, the spermatozoon is served by specialized organelles.

1. Ultrastructure

a) Structure of the Spermatozoon

The spermatozoon consists of a motile tail and a head which includes the nucleus with its genetic material and the acrosomic system providing the spermatozoa with enzymes to penetrate the egg. The wide variety of sperm head shapes which exist in mammals can generally be divided into two major categories: Falciform (Fig.1A) and Spatulate (Fig.1B) (Clermont et al., 1991). However, all species share common structures; For example, all spermatozoa are characterized by a condensed nucleus delineated by a double nuclear membrane and an apical oriented acrosomic system which contains the enzymatic machinery necessary for the fertilization of the oocyte. Overlying the nuclear membrane is a cytoskeletal structure now referred to as the perinuclear theca. This structure
has been visualized by light microscopy (L.M.) (Clermont et al., 1955; Hancock, 1957; Hancock and Trevan, 1957), by electron microscopy (E.M.) (Fawcett and Ito, 1965), by ultrarcryotomy (Courtens et al., 1976) and freeze fracture (Fléchon, 1974). This electron dense material does not, however, cover the perifossal zone located at the base of the nucleus around the insertion site of the tail (Fawcett, 1970; Lalli and Clermont, 1981). The perifossal zone or implantation fossa is lined by the basal plate. The actual connection between head and flagellum is mediated by an articular structure consisting of filamentous proteinaceous material filling the narrow space between the basal plate and the capitulum (Fawcett and Phillips, 1969; Zamboni and Stefanini, 1970; Fawcett, 1975; Zamboni, 1991). During spermatid development, the posterior part of the nucleus is surrounded by a ring of microtubules referred to as the manchette. However, the manchette microtubules disappear at the end of nuclear condensation (Fawcett and Bedford, 1979).

b) Structure of the Perinuclear Theca

The perinuclear theca can be divided into two structurally continuous parts: the perforatorium, apically, underlying the acrosomic system and caudally, the postacrosomal sheath underlying the plasma membrane (Clermont et al., 1955; Hadeck, 1963, 1969; Stefanini et al., 1969; Fawcett, 1970; Franklin et al., 1970a; Yanagimachi et Noda, 1970 a,b; Zamboni et al., 1971).

The term perforatorium was first introduced in the beginning of the century to designate the structure on the anterior tip of rodent
sperm head which was thought to have a cutting and penetrating function. For several years investigators mistakenly considered the perforatorium and the acrosomic system to be the same structure (Leuhosek, 1898; Duesberg, 1908; Bowen, 1924; Brokelman, 1963). The perforatorium, a characteristic feature of falciform spermatozoa, is composed of dense amorphous material that lies within the cytoplasmic region between the acrosome and the nucleus; it has been referred to as the subacrosomal layer, especially as it pertains to spatulate spermatozoa (Courtens et al., 1976, 1980; Bellvé and O'Brien, 1983). In the sickle shaped rat sperm head, as well as in pig, chinchilla and squirrel, the acrosome extends considerably beyond the apex of the nucleus. In the rat, in particular, it extends in a curved and pointed fashion (fig. 1A) (Fawcett and Hollenberg, 1963; Fawcett, 1965; Fawcett and Phillips, 1969; Clermont et al., 1991). Consequently in falciform spermatozoa, the subacrosomal layer or perforatorium, located between the inner acrosomal membrane and the outer nuclear membrane, is shaped like a curved triangular rod. As the perforatorium passes over the apex of the nucleus, it splits into three interconnected prongs, one dorsal and two ventral which eventually taper off to a thin collar that becomes continuous with the postacrosomal sheath (Blandau, 1951; Lalli and Clermont, 1981; Oko and Clermont, 1988). The perforatorium in falciform spermatozoa represents a greater percentage of the perinuclear theca material than does the postacrosomal sheath.
The earliest report concerning the postacrosomal sheath, made by Zelfen (1957), Nicander and Bane (1962), described this structure as an amorphous layer attached to the inner aspect of the plasma membrane. The postacrosomal sheath was later described, using ultrastructural analysis, as consisting of two adjacent layers of material: 1. The calyx, the inner layer closely applied to the nucleus and continuous with the subacrosomal layer and 2. The postacrosomal lamina, a paracrystalline sheath of ridges or filaments connecting the plasma membrane to the calyx (Wooding and O'Donnell, 1971; Pedersen, 1972; Olson and Winfrey, 1985; 1988; Olson et al., 1983, 1987). The connection between the subacrosomal layer (perforatorium) and the calyx, was first described by Jones (1970, 1971). The structural continuity of the subacrosomal layer and the postacrosomal sheath is now recognized as a common feature of mammalian spermatozoa (Franklin et al. 1970; Jones, 1971; Calvin and Bedford, 1971; Gordon, 1972; Bedford 1974; Courtens et al., 1976). Interestingly, Clermont and Lalli (1981) among others have observed that both regions of the perinuclear theca tightly bind membranes.

Projecting ventrally from the postacrosomal sheath of falciform spermatozoa, is a thicker accumulation of material, referred to as the ventral spur (Fig. 1A) (Stefanini et al., 1969). Whether the ventral spur is biochemically distinct from the rest of the postacrosomal sheath is not known. The ventral spur is absent in spatulate- shaped sperm heads such as in the bull. Therefore, the major structural differences between falciform spermatozoa (eg.
rat, squirrel, hamster) and spatulate spermatozoa (e.g. bull, human, rabbit) lie in the shape and size of the subacrosomal layer (perforatorium) and in the presence or absence of a ventral spur (Fig. 1A,B). These structural differences raise the possibility that the perinuclear theca vary in composition as well as in function in falciform versus spatulate-shaped spermatozoa. In order to make this determination, a thorough study of the biochemical composition of perinuclear theca in different species is necessary. As a first step towards this end, this study makes an attempt to identify the proteinaceous composition of the perinuclear theca in the rat, a species displaying typical falciform morphology in its spermatozoa. Based on the methods and results contained herein, the perinuclear theca composition in other species may be eventually studied and compared.
FIGURE 1

Diagrams illustrating side and cross sectional views of A) a falciform head of a rat spermatozoon and in B) a spatulate head of mammalian spermatozoa.

Labels: PM, plasma membrane; N, nucleus (black); AS, acrosomic system (stippled area); A, acrosome; HC, head cap; HCS, separated head cap segment; VS, ventral spur; P, perforatorium; PL, postacrosomal dense lamina; PT, perinuclear theca; ES, equatorial segment. The dotted white line on the nucleus demarcates the area of the postacrosomal dense lamina (dorsally) from the perifossal zone (PZ) (ventrally).

Also labeled: CP, connecting piece with its striated columns (Sc) and capitulum (C); MP, middle piece with its axoneme (Ax) associated with outer dense fibers (ODF) and mitochondria (M) (Clermont et al., 1991).
c) Structure of the Acrosomic System

The acrosomic system, which overlies the perforatorium and covers the anterior part of the sperm head, generally shows two distinct regional areas: The acrosome, a thicker portion of the acrosomic system projecting dorsally along and beyond the apical margin of the nucleus, and the head cap, continuous with the acrosome extending over the sides of the anterior half of the nucleus (Fig. 1). A thin extension extending from the caudal end of the head cap in spatulate spermatozoa is referred to as the equatorial segment (Fig. 1B) (Fawcett, 1970; Lalli and Clermont, 1981; Eddy, 1988). The structural heterogeneity of the acrosomic system has been suggested to imply that each segment of the acrosomic system may have a different functional role during fertilization (Bedford, 1968, 1970; Moore and Bedford, 1975; Pedersen, 1972). Whether this heterogeneity is reflected in the composition of the underlying subacrosomal layer or perforatorium is unknown.

This study, involves the isolation, identification and localization of perinuclear proteins using immunocytochemical techniques. In so doing, the heterogeneous or homogeneous character of the perinuclear theca may be determined.
2. Biochemical Composition of the Perinuclear Theca

The biochemical properties of the perinuclear theca proteins are still unknown. However, the way in which these polypeptides react under certain conditions provide some information about their amino acid composition. For example, given their resistance to solubilization in sodium dodecyl sulfate (SDS) (an ionic detergent) one may suspect a high content of cysteine (Calvin and Bedford, 1971; Calvin 1976; Olson et al., 1976a). Also many of these proteins are highly phosphorylated reflecting a high content in serine (Pruslin and Rodman, 1985). It is further known that a 13 kD protein discovered in the rat's perforatorium by Olson et al., (1976) is rich in cysteine, proline, glutamic acid and serine. Finally, lysine residues were shown to be contained in the postacrosomal region of the perinuclear theca by Courtens and Loir, (1975), using hydroquinone-ferricyanide-copper phosphotungstic acid (HQQ-fc staining). A prerequisite for determining the biochemical composition of the perforatorium is to isolate this structure and separate the proteins by gel electrophoresis.

A controversy exists in the literature as to whether or not the perinuclear theca is homogeneous. It was suggested that the perforatorium and postacrosomal sheath are chemically similar because these two regions of the perinuclear theca, in both human and guinea pig spermatozoa, stained equivalently with phosphotungstic acid (Nicander and Bane, 1962, 1966; Gordon, 1969). In addition, the perinuclear theca's resistance to commonly
used protein solubilizing agent like SDS (Calvin, 1976; Nicander and Bane, 1966) suggested biochemical homogeneity. However silver staining clearly differentiated between a stained postacrosomal sheath and an unstained perforatorium (Elder and Hsu, 1981). In addition, at least in falciform spermatozoa, these two regions of the perinuclear theca have been shown to assemble at different times during spermatogenesis supporting the notion of regional differences within the perinuclear theca (Lalli and Clermont, 1981). Most convincingly perhaps are the immunocytochemical studies which demonstrate a difference in composition between the perforatorium and the postacrosomal sheath of rat spermatozoa (Longo et al., 1987; Olson and Winfrey, 1988; Oko and Clermont, 1988).

a) Biochemical Composition of the Perforatorium

Biochemical studies on the composition of the perforatorium have demonstrated that the structure is resistant to commonly used protein solubilization agents such as SDS, Triton x-100 and DTT, Urea and DTT, Guanidine-HCl and DTT, and strong alkali treatment. This may be due to the keratin like nature of cysteine rich component proteins of the perforatorium (Olson et al., 1976). Initial extraction attempts on rat spermatozoa by Olson et al., (1976), using a combination of SDS and DTT followed by analyzing the extracted sperm pellet, revealed a single protein of 13 kD which was attributed to the perforatorium. Using this treatment, different spermatozoa structures solubilized sequentially. Prolonged intervals under these conditions induced swelling of the nucleus
and allowed visualization of the perforatorium as a structural unit. Combining a reducing agent and a solubilizing agent, such as SDS and DTT, can eventually solubilize all sperm proteins, thus it can not be dismissed, in the above study, that other perforatorial proteins were extracted. Furthermore in this experiment, the swollen nucleus was not separated from the perforatorium.

In contrast, Oko and Clermont (1988), using an alkaline isolation technique, succeeded in completely detaching the perforatorium from the nucleus and isolating it on a sucrose gradient. They showed that several proteins ranging from 13 to 63 kDa were major constituents of the perforatorium. They also demonstrated an immunological distinctiveness between the postacrosomal sheath and the perforatorium, a finding sharply contrasting with the homogeneity hypothesis proposed by Nicander and Bane (1962, 1966) and Gordon (1969).

**b) Biochemical Composition of the Postacrosomal Sheath**

Longo et al. (1987) attempted to isolate the postacrosomal sheath and study its protein composition. The approach undertaken consisted in treating demembranated sperm heads with high salt buffer and DTT which caused the nucleus to swell. DNase was concomitantly used in order to digest the dispersed chromatin and separate it from what they claimed to be an intact postacrosomal sheath. However, under these identical conditions we have shown that the postacrosomal sheath completely solubilizes. Nevertheless, based on their unsubstantiated isolation
of the postacrosomal sheath, Longo et al., (1987) reported that it was composed of a major 60 kDa protein and a group of minor basic proteins (MBP), ranging from 56 to 74 kDa. The MBPs were immunolocalized to the postacrosomal sheath but very poorly represented in the perforatorium. Based largely on immunofluorescent techniques, Longo and co-workers proposed that the 60 kDa protein, referred to as calcin, may be the major and exclusive component of the postacrosomal sheath (Longo et al., 1987). This finding lends support to the notion of a heterogeneous perinuclear theca. Interestingly, however, neither light nor electron microscopic immunocytochemical techniques were conclusive since 1. Structures were indistinguishable on immunogold labeled electron microscope sections, 2. the acrosome system covering the anterior part of the sperm head may have possibly hid potential perinuclear sites on immunofluorescent treated sections. Thus it would be interesting to know whether the perforatorium is also composed of a major 60 kDa protein. Because the isolation protocol could not be substantiated it is conceivable that the postacrosomal sheath is composed of several major proteins.

3. Other cytoskeletal proteins localized in the spermatozoa

Structural characteristics of specific cell types usually involve a complex set of protein fibers found in the cytoplasm. In somatic cells, cytoskeletal elements function to generate motility, maintain polarity, direct organelle movements and to regulate the
distribution of specific membrane proteins (Lazarides and Woods, 1989; Rodriguez-Boulan and Nelson, 1989; Schilwa, 1986). Electron microscopy has shown that all eukaryotic cells contain three major classes of cytoskeletal fibers: actin, intermediate filaments and microtubules (Darnell et al., 1986).

Actin, one of the most abundant proteins in living cell, might be one cytoskeletal protein common to both the perforatorium and the postacrosomal sheath since it was shown to be in these two regions of developing spermatids: the perforatorium (Campanella, 1979; Bacceti, 1980; Flaherty, 1987; Russell et al., 1979; Fouquet, 1988; Camatini et al., 1987) and the postacrosomal sheath (Clarke and Yanagimachi, 1978; Talbot and Kleeve, 1978; Campanella, 1979; Tambly, 1980; Clarke et al., 1982; Greenberg, 1987; Castellani, 1986; Jamil, 1986). Actin was found to be in its filamentous form and concentrated between the nuclear and acrosomal membranes of the spermatid but seems to disappear from this location at the end of spermiogenesis in most species (Olson and Winfrey, 1991). At the present time it is clear that actin is present in spermatid, but unclear whether it is present in spermatozoa. By isolating the perforatorium and separating the polypeptides forming this part of the perinuclear theca, the presence of actin in the perforatorium of rat spermatozoa can be ascertained. The presence of actin would furthermore imply the coexistence of actin binding proteins such as villin, fimbrin, calmodulin, profilin and cytokeratins. These proteins should also be sought among the polypeptide bands obtained from the isolated perforatorium.
In most animal cells, intermediate filaments, which are very resistant to high and low ionic strength, are present around the nucleus. They can be of various types, each with common and characteristic sets of amino acids. However, these intermediate filaments (i.e. vimentin, keratin, desmin, neurofilaments, etc...) have never been localized in spermatids let alone spermatozoa (Longo et al., 1987; Virtanen et al., 1984; Ochs et al., 1986; Olson et al., 1985).

Microtubules surround the caudal head region of spermatid heads but disappear well before the maturation of spermatozoa (Zamboni, 1971; Hadek, 1969). An exception may be present in the rays and spikes of crustacean spermatozoon which are reactive to antibodies against tubulin (Perez et al., 1991). The function of these spikes lies in the actual extrusion of the acrosome during fertilization in this species.

4. Formation

From a developmental viewpoint, the perforatorium and postacrosomal sheath in falciform spermatozoa appear to form independently from one another (Lalli and Clermont, 1981; Oko and Clermont, 1991). Such independance is theoretically consistent with biochemical heterogeneity observed between these two structures despite their apparent structural continuity.
The outstanding question, however, of whether or not the perinuclear theca proteins have the same origin still remains to be answered. In order to study the biogenesis of perinuclear theca proteins, it is essential therefore to 1. effectively isolate the structure 2. separate the various proteins forming the structure and 3. using immunocytochemistry, follow their individual progression through spermatogenesis. It is thus the intention of this study to determine the protein composition and distribution of these within the perinuclear theca, in order to expedite the study of its biogenesis.

5. **Functions Attributed to the Perinuclear Theca**

The perinuclear theca represents the principal cyto/karyo/skeletal structure of the mammalian sperm head (Bellvé et al., 1975; Bellvé and O'Brien, 1983; Longo et al., 1987). The mammalian sperm heads have been shown to vary greatly from one species to the next, while maintaining a certain degree of fundamental structural homogeneity. It has also been demonstrated that the perinuclear theca displays equally wide morphological diversity among various species. These two observations, taken together, have led many researchers to believe that the perinuclear theca is involved in determining the shape of the sperm head (Cloney, 1966; Longo and Anderson, 1968; Clarke and Spudich 1977; Bestor and Schatten, 1981). Furthermore it has been suggested that the perinuclear theca is an important membrane
binding element of the sperm head, involved in sperm development as well as in fertilization.

a) Membrane interactions

Because membranes are closely apposed to the perinuclear theca, the latter may function as a "cement" between the nuclear envelope and the acrosome apically, and between the nuclear envelope and the plasmalemma caudally. In the acrosomal region, this cement hypothesis is substantiated by the persistence of the inner acrosomal membrane on spermatozoa that have undergone the acrosome reaction (Yananagimachi, 1970). These general notions about the binding function of the perinuclear theca have been proposed by a multitude of investigators (Franklin and Barros, 1970; Yanagimachi and Noda, 1970; Courtens et al., 1976; Courtens and Courot, 1980; Fawcett and Ito, 1965; Fléchon, 1973; Franklin, 1974; Morstin and Courot, 1973; Brown et al., 1975; Courtens and Loir, 1975; Lalli and Clermont, 1981; Bellvé and O'Brien, 1983; Longo et al., 1987).

It has been observed that different regions of the sperm plasma membrane have unique surface properties and antigens (Myles et al., 1981; Primakoff and Myles, 1983). Therefore components of the perinuclear theca may be involved in establishing the topology and properties of sperm membranes, determining specific domains within the plasma membrane (Virtanen et al., 1984) and regulating intracellular calcium binding during the acrosome reaction (Ruknudin, 1989).
The plasma membrane is connected to the postacrosomal sheath by periodic substructures that emerge from the sheath. These periodic substructures form what has been called the paracrystalline sheath (Longo et al., 1987; Olson and Winfrey, 1988) which could affect the properties of the overlying plasma membrane because of its intimate relationship with it (Toshimori et al., 1991). These periodic substructures are thought to be sperm cytoskeletal components because of their resistance to extraction with detergent and high salt buffer and given their attachment to the plasma membrane (Longo, 1987; Olson and Winfrey, 1988).

b) Fertilization

Fertilization is the process by which the sperm initiates and participates in the development of the egg. In order to fulfill this role, the ejaculated spermatozoa undergo a number of morphological changes, some of which have been postulated to involve perinuclear theca proteins. Mammalian spermatozoa must undergo chemical and physical alterations which prepare them to fertilize the ovum. Modifications of the acrosome, nucleus and plasma membrane are among the changes occurring at capacitation. Coating materials from the sperm surface are removed or altered (Clegg, 1983; Koehler, 1981; Kopecky and Fléchon, 1981; Oliphant et al., 1985), the nucleus increases in stability (Lelannou et al., 1985) and the contour as well as the texture of the acrosome changes (Cummins and Yanagimachi, 1985). The rearrangements of intramembranous particles may be modulated to some extent by
perinuclear theca proteins (Fawcett, 1975; Olson et al., 1983; Lee and Storey, 1985; Primakoff and Myles, 1983).

Capacitation is a prerequisite for the acrosome reaction which in turn is an obligatory event prior to sperm penetration of the zona pellucida of the egg (Austin and Bishop, 1958). The acrosome reaction is a sequence of events starting with multifocal fusions of the plasma membrane and the outer acrosomal membrane (Barros, 1967). The absence of membrane fusions in the equatorial segment region may be due to stabilizing interactions between the acrosomal membranes and perforatorial proteins (Oko et al., 1990).

The fusion of the plasma and the outer acrosomal membrane, results in their vesiculation and consequent release of hydrolytic enzymes; The end result is that the inner acrosomal membrane (Bacceti and Afzelius, 1976), which covers the perforatorium, becomes exposed. This inner part of the acrosomal membrane remains tightly attached to the perforatorium during its penetration through the zona pellucida and is continuous with the plasmalemma covering the intact equatorial segment. It is believed that the perforatorium’s pointed blade-shaped structure which underlies the inner acrosomal membrane facilitates the mechanical penetration of the sperm through the zona pellucida of the oocyte following the acrosome reaction (Yanagamachi and Noda, 1970; Yanagamachi, 1988; Clermont et al., 1991).
c) Development

The perforatorium may be implicated in the formation of the acrosome during spermiogenesis because it directly underlies this structure. It could function in attaching and spreading the developing acrosome over the nucleus. In this context it is postulated that proteins in the subacrosomal space (perforatorium) attach to transmembrane proteins on the edges of the acrosomic vesicle as the acrosomic vesicle grows and spreads over the spermatid nucleus. In fact, it is well documented that "acrosomeless" infertile round-headed spermatozoa are devoid of the perinuclear theca, that spermatozoa presenting perinuclear theca defects are highly correlated with acrosome anomalies and that many of these acrosome defects have as yet an uncharacterized genetic component (Courtot et al., 1987; Bacetti, 1989b; Escalier, 1990; Courtot, 1991).

6. Objectives

The prerequisite to understanding the formation and function of the perinuclear theca is the identification of its proteins. Thus the objectives of the present study are to isolate the perinuclear theca from rat spermatozoa, study its protein composition and describe the distribution of its proteins within the sperm head. It is hoped that this study will constitute a methodological foundation for the analysis and identity of perinuclear theca proteins in other mammalian species and that the identity of the proteins will
facilitate future studies on their amino acid composition; biogenesis, and function.
II. MATERIALS and METHODS

1. Isolation of Spermatozoa

Epididymides were removed from twelve adult male Sprague-Dawley rats weighing between 450 and 500 grams. To facilitate isolation of sperm heads, the epididymides were divided into 12 equal samples. These were finely minced with razor blades and suspended in 15 ml of 0.02 M phosphate-buffered saline (PBS, pH 7). All the following procedures were carried out at 4°C unless otherwise indicated. Each suspension was stirred for 15 minutes and filtered through a 150 μm Nitex netting (Thompson, Montréal, Québec). The filtrate was divided into six tubes and centrifuged at 400 Xg for 10 minutes at 4°C on an IEC Centra-8r centrifuge. The supernatant containing fat, blood vessels and organelles was discarded. Phenylmethylsulfonyl fluoride (PMFS) (0.02mM) was added to all subsequent steps, to prevent protease digestion. The sperm pellet was washed twice in 15 ml of PBS buffer and after the last centrifugation at 400 Xg it was then resuspended in 5 ml of PBS containing 25 mM EDTA.
2. Isolation of Sperm Heads

The suspension was sonicated on ice with a Browill Biosonik IV, VWR sonicator (Scientific, San Francisco, CA) set at 100% output for four 15 second bursts at 30-40 second intervals. This step detached sperm heads from tails assuring 95% decapitation as verified by phase microscopy. The suspension was mixed in 10 ml of PBS which was subsequently centrifuged at 400 Xg for 10 minutes. The pellet was resuspended in 8 ml of 65% (w/v) sucrose containing 0.02 M PBS layered over a sucrose step gradient composed of 8 ml fractions of 65%, 70%, and 75% sucrose in 0.02 PBS (Calvin, 1976). This sucrose step gradient was spun in a Beckman SW 28 swinging bucket rotor at 100 000 Xg for 1 hour. The sperm heads formed a pellet at the bottom of the tube. At the 65% - 70% interface was a mixture of heads and tails. At the 70% - 75% interface was a concentration of tails. The pellet was transferred to a new tube while avoiding contact with the sides of the tube. The sperm head pellet was delicately broken down with a glass rod, resuspended in 12 ml of 65% sucrose in 25 mM Tris-HCl (pH 9.0) and layered on another sucrose gradient composed of 70% and 75% sucrose gradient and centrifuged at 100 000 xg. The final pellet was washed in 50 mM Tris-HCl (pH 9.0) buffer.
3. Isolation of the Rat Perforatorium

Five ml of Tris-HCL with an equal amount of 2N NaOH was added to the sperm head pellet and incubated at room temperature (RT°) with mild shaking. The NaOH incubation was monitored by phase contrast microscopy to observe gradual results. Within 30 minutes, when the perforatorium seemed partially detached from the nucleus, the suspension was passed through a 22 gauge needle several times. The shear force thereby generated assured complete detachment. This treatment allowed the NaOH solution to dissolve the possible remaining tails and optimize the yield and purity of the components. Once the perforatoria were detached, 25 mM of Tris-HCl (pH 9) was added and the mixture was put on ice.

The suspension was then layered over a sucrose step gradient composed of 10 ml factions of 10% and 70% sucrose in 25 mM Tris-HCl (pH 9) and centrifuged at 100 000 Xg for one hour. The nuclei formed an annular pellet at the bottom of the tube while the perforatoria were located at the 10% - 70% interface. The visible layer of perforatoria was collected and resuspended in 35 ml of 25 mM Tris-HCl (pH 9) and either filtered to collect material for electron microscopy or centrifuged at 50 000 Xg for 17 minutes. The pellets were transferred into preweighed Eppendorf tubes and centrifuged at 15 000 Xg for 5 minutes. The resultant pellets were used for gel electrophoresis and immunization. Protein concentration was estimated by the Bio-Rad protein assay (Bradford 1976).
Rat spermatozoa

Sonicated

Sucrose gradient (100 000 xg)

65%
70%
75%

Heads

NaOH 30 mns
+ shear force

Sucrose gradient (100 000 xg)

10%
75%

PERFORATORIUM
+ Wash/ 50 000 x g

Isolated Perforatorium

SDS gel Immunization Western blot analysis Electrophoresis Electron microscopy analysis
4. **SDS Polyacrylamide Gel Electrophoresis**

Isolated perforatoria were homogenized and then solubilized in 2% SDS and 5% β-mercaptoethanol for 5 minutes at 100°C. Particulate matter that did not solubilize was pelleted and only the supernatant was run on gradient preparative polyacrylamide gels (1.5 X 140 X 160 mm) using the SDS-discontinuous system originally developed by Laemmli (1970). Gels were assembled in a 2001 LKB electrophoresis system (LKB, Montréal, Canada). Approximately 25-50μg of perforatorial protein, obtained from extracted spermatozoa of 24 mature adult rat epididymides, were loaded in each preparative gel. Apparent molecular weights were determined from the mobility of low-molecular-weight standards (Pharmacia Electrophoresis Calibration Kits, Piscataway, NJ) which were run on side lanes. Preparative gels of actin (100μg/gel) were also prepared.

5. **Western Blotting**

Electrophoretic transfer of proteins to nitrocellulose paper (0.45 μm pore size; Schleider and Schuell Inc., Keene, NH) was executed immediately after SDS gel electrophoresis in order to avoid diffusion of proteins in the gel. The transfer was carried out on a Hoefer Transphor Apparatus (Hoefer Scientific Instruments, San Francisco, CA) according to the protocol of Towbin et al. (1979) and
Towbin and Gordon (1984). The electrophoretic transfer employed a buffer solution of 25 mm Tris-base, 192 mM glycine, and 20% methanol (pH 8.3) set at 0.5-0.6 Amps for 3-5 hours. Finally the nitrocellulose sheet containing the perforatorial proteins was soaked into washing buffer made of 10 mM Tris-base, 0.9% NaCl (TBS) and 0.5% Tween-20 (pH 7.4) (TWA) at RT°.

6. **Immunoblotting**

Vertical nitrocellulose strips of transferred perinuclear proteins were saturated in 5% normal goat serum (NGS) in TBS-Tween (TWA) for 2 hours at RT° or overnight at 4°C, in order to prevent non-specific binding. The strips were then incubated for 2 hours at RT° or overnight at 4°C with the polyclonal antibodies raised in rabbits (primary antibody) diluted 1/250 in TWA with 0.1% bovine serum albumin (BSA) and 1% NGS. One equivalent strip, representing the controls was not soaked in the primary antibody but instead in the rabbit immune serum. All strips were subjected to five rinses in TWA each for 5 minutes, followed by a 30 minutes wash with 5% NGS in TWA. The strips were then incubated at 37°C for two hours in 1/1000 dilution of the alkaline phosphatase-conjugated F(ab1) goat anti rabbit immunoglobulin G [IgG] (secondary antibody), Cappel-Cooper biomedical Inc., Malvern, PA. After washing the strips five times in TWA each for 5 minutes, they were rinsed for 2 minutes in 50 mM Sodium-Glycinate and 0.5 % Tween-20 (pH 9.6). The phosphatase reaction was developed according to McGadey.
It consisted of a final incubation of 10-15 minutes in the dark at RT° in a solution made up of 50 mM Sodium-Glycinate (pH 6), 0.1 mg/ml p-nitroblue tetrazolin chloride, 0.05 mg/ml of 5-bromo-4-chloro-3-indolyl and 4mM MgCl2.

7. Preparation of Immune Serum Against the Perforatorium

a) Surgical preparation

Anti-perforatorium serum against the isolated perforatoria was raised in New Zealand female virgin white rabbits following a popliteal lymph node procedure (Newbould, 1965). Prior to the surgical procedure, the rabbits were bled: this pre-immune serum was used as a control.

Rabbits were weighed and their temperature taken. They were anesthetized with an Acepromazine (1mg) / Ketamine (10 mg) by intramuscular injection. Evan's blue dye-saline (0.2 ml) was injected and after 45 minutes 10 mg/Kg of Ketamine was administered. After one hour the rabbit was asleep. The hind legs were shaved below and above the knees. 0.3 ml of xylocaine was injected subcutaneously in each leg. An incision was made in order to inject the sample directly into the lymph nodes.
b) Sample preparation

Purified perforatorial fractions were solubilized in 0.5 M Tris-HCL (pH 6.8) containing 2% SDS-5% β-mercaptoethanol and left at 100°C for 5 minutes. The denatured protein were then emulsified in an equal volume (0.5 ml) of Freund's complete adjuvant and 100 μl of the resulting solution (1μg protein/μl) was injected into each popliteal lymph node (Newbould 1965). The remainder was injected in various sites along the neck and back. Three weeks later rabbits were boosted with the same treated sperm head proteins but this time mixed 1:1 with incomplete Freud's adjuvant. Rabbits were bled 7-10 days after each boost.

Rabbit blood obtained before the first injection and collected after each boosts was left at RT° for 1 hour. The resulting clot was carefully detached from the inner surface of the tubes and tubes refrigerated overnight. The next morning, the clot was discarded and the serum was poured to 15 ml glass centrifuge tubes. The serum was centrifuged in a Sorvall centrifuge for 20 minutes at 10000 Xg and a second time for 10 minutes then aliquoted. The pre-immune serum and immune serum were stored frozen at -70°C with protease inhibitors added.
8. **Immunoelecting**

The antisera were diluted 1/50 in a solution containing 20 mM Tris-HCl, 0.9% NaCl, and 0.1% bovine serum albumin (BSA) (TBS), pH 7.4 and adsorbed onto portions of preparative Western blots containing the perforatorial polypeptides of interest. Each of the seven polypeptide bands, having molecular masses of 13, 13.4, 16, 34, 43, 57 and 63 kDa, were cut out from six preparative Western blots, pooled together and utilized for immunoadsorption. The bands were detected by staining the Western blots with 0.2% Ponceau rouge in 3% trichloroacetic acid and by staining vertical strips on each side of the blot with a colloidal gold solution adjusted to pH 3.

Prior to immunoadsorption, the respective bands were washed and destained in three 15 minute changes of TBS containing 0.5% Tween-20 (TWA). They were then chopped up into 3 mm pieces, inserted into a 10 ml syringe, and saturated for 2 hours in 5 ml of TBS + Tween containing 10% goat serum (GS) at RT° or refrigerating overnight. Following incubation with antisera for two hours, the respective blot pieces were subjected to five 5 minute changes of TWA with continual shaking to remove nonadsorbed or nonspecific antibodies. Elution of the adsorbed or specific antibodies was similar to a procedure described by Talian (1983). After extruding the solution from the last wash, the blot pieces were incubated and stirred for 3 minutes in 5 ml of 0.2 M glycine-HCl, pH 2.8. The acid solution containing the eluted antibodies was then
immediately extruded out of the syringe, neutralized with a predetermined amount of 1N NaOH and mixed with 5 ml of buffer containing 40 mM Tris-HCl, 1.8% NaCl, and 0.1% BSA, pH 7.4. The eluate was concentrated to ~2-3 ml with a Centriprep 30 concentrator (Amicon, Danvers, MA) and utilized directly for immunoblotting. After immunoblotting, the eluate was further concentrated with a Centricon 10 microconcentrator (Amidon, Danvers, MA) to as little as 50μl and utilized directly for immunocytochemistry. Control solutions were obtained by following the same procedures of adsorption and elution of antiperforatorial serum on Western blots of low-molecular-weight standards (Pharmacia Electrophoresis Calibration Kits, Piscataway, NJ.)

A C4 anti-actin mouse monoclonal antibody, obtained from Dr. James L. Lessard (Children's Hospital research Foundation, Cincinnati, OH), was also utilized in this study. This monoclonal antibody was also known to react with all six known vertebrate isoactins, either on immunoblots or by immunofluorescence and is also known to compete for the G-actin binding sites of DNAse I (Lessard 1988). Furthermore it has been shown to react with actin isolated from guinea pig spermiogenic cells, but it does not detect actin in purified epididymal spermatozoa (Halenda et al. 1987).
9. **Lowicryl Embedding**

Epididymal and testicular tissues of 5 adult Spague-Dawley rats were fixed by perfusion for 20 minutes with 1% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer containing 15 M lysine at ph. 7.4. Tissues were immersed in this solution and then washed in 4% sucrose, in PBS, at ph. 7.4 and a second time in PBS containing 50 mM NH₄Cl for 1 hour at 4 °C. Before dehydration in methanol, tissues were washed in PBS. Tissues were embedded in Lowicryl K4M (Grant et al., 1985) without passage through osmium tetroxide. Tissues were deposited into resin filled beam capsules which were pre-cooled and covered tightly. To polymerize the resin, the capsules were positioned 20-30 cm away from a U.V. light. Polymerized blocks were stored in the freezer in a sealed box containing a drying agent. Sections of Lowicryl embedded epididymides and testes were immediately cut using glass knives. Thin sections were mounted on 200μ Formvar coated nickel grids.

10. **Immunocytochemistry**

Lowicryl sections on Formvar-coated grids were saturated for 30 minutes with 5-10 % normal goat serum (NGS) before being incubated for one hour with the primary antibody, diluted 1/100 at 4°C. As controls, grids were incubated on preimmune serum from the same rabbits. After incubation, grids were washed four times for 5 minutes each in 0.1 % TBS containing 0.1% Tween and then
saturated with 5-10% NGS for 15 minutes. The grids were then incubated for 30 minutes with the secondary antibody. The secondary antibodies were either 10-15 mm gold-conjugated goat anti-rabbit IgG particles or goat antimouse IgG (1/20 dilution) (Janssen Pharmaceutica, Olen, Belgium), and the immunolabeling protocol was adapted from information given by Janssen Life Science Products. Following incubation in secondary antibody, the sections were washed several times with TBS + 0.1% Tween and finally with distilled water. The dried grids were stained with 4% uranyl acetate containing 30% ethanol and lead citrate and then they were examined on a Philips 400 electron microscope.
III. RESULTS

1. Morphology of the Perinuclear Theca and Outer Periacrosomal Layer

The resolution obtained on Lowicryl embedded testes allowed us to differentiate between the membrane systems and the perinuclear theca of spermatozoa. The fine structure analysis is presented below.

At the apex of the falciform head of the rat spermatozoon, the perforatorium is in the shape of a curved triangular rod which is covered by the acrosomic system (Figs. 2,3). As the perforatorium extends over the apex of the nucleus, it splits into three interconnected prongs, one dorsal and two ventral (Figs. 2,3). The ventral prongs of the perforatorium gradually taper off caudally into a thin layer underlying the acrosomal head cap portion of the acrosomic system (Figs. 2,3 b-d). The dorsal prong persists along the dorsal edge of the nucleus as a narrow triangular crest (figs 2,3 b-d). Ventrally and caudally, at the termination of the acrosomic system, the thin layer of the perforatorium is continuous with the postacrosomal dense lamina (or sheath), which directly underlies the plasmalemma (figs. 2,3 c-d). In a confined area at the ventral extremity of the sperm head, the perinuclear theca thickens to form a ventral prominence or spur. This ventral spur is composed of two layers: a thinner outer layer continuous with the dense lamina and a thicker inner layer (Figs. 2,3 d).
Sandwiched between the plasmalemma and the outer membrane of the acrosomic system lies yet another distinct thin layer, "the outer periacrosomal layer" (Figs. 3 a-d). At the caudal edge or extremity of the acrosomal head cap, both the outer periacrosomal layer and the perforatorium are continuous with each other and with the postacrosomal dense lamina (Figs. 3 a-d). There is thus a distinct layer of dense cytoplasmic material interposed between the plasma membrane and the acrosomic system.

2. Isolation of Rat Perforatorium

Rat epididymal spermatozoa were fractionated according to the scheme on page 22 a. The sperm tails, plasma membrane, nuclei acrosomal membranes and acrosome contents were removed by this protocol, leaving an isolated perforatorium as verified by phase contrast and electron microscopy (Figs. 4 a-d).

The first step in the isolation protocol was to separate rat sperm heads and tails by sonication and to subfractionate them on a discontinuous sucrose gradient. The sperm heads obtained were washed and subsequently exposed to a timed alkaline treatment which was monitored by contrast phase microscopy. After isolated sperm heads were exposed for 15 minutes to 1N NaOH, the perforatorium began to separate from the nucleus. Within 30 minutes of NaOH treatment, the perforatorium detached from the condensed nucleus but remained attached to the base of the nucleus.
by a fine thread of material. Passing the suspension through a 22
gauge needle several times it insured complete detachment. The
ensuing sucrose gradient guaranteed a complete separation of the
perforatorium from the nucleus (Fig. 4 b). Electron microscopy
confirmed that the perforatorial fraction was free of nuclei and
other contaminants (Figs. 4 c,d).

3. Polypeptide Composition of the Rat Perforatorium

Polyacrylamide Sodium Dodecyl Sulfate (SDS) gels of isolated
rat perforatorial fractions revealed many polypeptide bands of
which the 16, 34 and 43 kDa bands were the most prominent (Fig. 5
a)

4. Immunostaining of Western Blots

a) Perforatorial Antibodies

Immune serum raised against the whole rat perforatorium after
two successive immunization boosts with the perforatorial fractions
reacted with the 16, 24, 34, 43, 57 and 63 kDa perforatorial
polypeptides transfered onto a Western blot (Fig. 5b, lane 3). The
strongest reactions were with the 16, 24 and 34 kDa bands.
However, after repeated immunization boosts it was found that the
anti-perforatorial sera reacted strongly with most of the transfered
perforatorial polypeptides including the 13, 13.4, 16, 34, 43, 57 and
63 kDa bands (Fig. 6, lane 3). Affinity purified antibodies prepared against each of the above perforatorial polypeptides reacted strongly with their respective polypeptides on Western blots but also cross-reacted with all the other major polypeptides (Fig. 6).

b) Actin Antibodies

The C4 anti-actin mouse monoclonal antibody (which recognizes all actin isoforms) did not react with any of the perforatorial polypeptides (Fig. 6, lane 6) but did react strongly with a Western blot preparation of actin (not shown).

5. Immunogold Labeling of Mature Rat Spermatids

a) Rat Perforatorial Antibodies

Anti-perforatorium serum immunolabeled the perinuclear theca as far distally as the limits of the acrosomic system (Fig. 3). The antiserum also labeled a substance found between the plasmalemma and outer acrosomal membrane of the head cap that together with the perforatorium, on the inner aspect of the acrosomic system, appeared continuous with the postacrosomal dense lamina with the exception of a confined area located on the inner aspect of the ventral spur of the spermatozoa head (Fig. 3)

Antibodies, affinity purified (using antiperforatorial serum) from either the 34, 43, 57 or 63 kDs perforatorial polypeptide bands, characteristically immunolabeled the entire perforatorium, the part of the outer periacrosomal layer adjacent to the acrosomal
head cap, and the inner layer of the ventral spur (Figs. 7, 9). No labeling was found over the postacrosomal sheath with the exception of the inner layer of the ventral spur.

In contrast, antibodies affinity purified from either the 13, 13.4, or 16 kDa perforatorial polypeptide bands, immunolabeled only the thicker apical portion of the perforatorium, i.e. the apical triangular rod (Fig. 8a) and the dorsal and ventral prongs (Fig. 8b, 9b). The inner layer of the ventral spur also reacted (Fig. 8d). No labeling was found over the caudal, thinner part of the perforatorium, which underlies the head cap (Figs. 8d, 9b). The outer periacrosomal layer was unreactive.

b) Actin Antibody

The C4 anti-actin mouse monoclonal antibody did not react with the perforatorium or any other cytoskeletal element of the head of the mature step 19 spermatid (Figs. 10a,b). However, the C4 antibody did immunolabel the material in the subacrosomal space of spermatids before step 19 of spermiogenesis (Figs. 10c-d), as did all the other seven antiperforatorial antibodies (Fig. 10e).
FIGURE 2

Drawing of a side view of the head of a rat spermatozoa accompanied by representative cross sections (a-d). The stippling on the diagram represents the extent of the perinuclear theca. Except for the perifossal zone (PZ) of the nucleus, the perinuclear theca covers the entire nucleus which is outlined on this diagram by the dashed line. On the side view, the lighter stippling conveys that this part of the perinuclear theca is covered directly by the acrosomic system, whereas the darker stippling conveys that this part of the perinuclear theca, the basal region, is only covered by the plasmalemma. The perforatorium (P), underlies the acrosomic system and is lightly stippled on the side view. The basally located postacrosomal dense lamina (DL), is darkly stippled in side view. The ventral spur (VS) refers to the ventral prominence of the sperm head which is due to the ventral thickening of the perinuclear theca. The acrosomic system is composed of the acrosome (A), head cap (HC) and displaced head cap segment (HCS) and covers the perinuclear theca in the anterior and dorsal regions of the sperm head. Labels: N, nucleus: PM, plasma membrane (Oko and Clermont 1988). Cross sections labeled a, b, c and d are represented by electron micrographs in figure 3.
FIGURE 3

Electron micrographs of Lowicryl-embedded step 19 spermatids or spermatozoa representing four different cross sectional views through the spermatozoon head.

a, b. Apical sections of the head through the thickest part of the perforatorium showing the interconnected dorsal prong (DP) and two ventral prongs (VP) of the perforatorium. The acrosomic system, composed of the acrosome (A) and head cap (HC) encompass the perforatorium. The former represents the triangular rod (TR). The latter section, which comprises the apical part of the nucleus (N) and the head cap segment (HCS), is visible as well.

c, d. Two consecutively caudal sections showing the thinner part of the perforatorium underlying the head cap portion of the acrosomic system and its continuity with the postacrosomal dense lamina (PDL) at the end (arrowhead) of the head cap. The ventral spur (VS) seen in the latter section appears to be composed of an inner and outer layer.

In all three sections, note the continuous layer of material termed the outer periacrosomal layer (OPL) found between the cell membrane and the outer acrosomal membrane. In Lowicryl-embedded material, the membranes appear as light unstained lines. The outer periacrosomal layer is also continuous with the postacrosomal dense lamina at the caudal edge of the head cap (arrowhead).

a, b, c, d, X 72 500.
**FIGURE 4**

Micrographs of different steps involved in the isolation of rat perforatorial fractions.

a. Phase contrast micrograph, after 30 minutes in 1N NaOH. X 800.

b. Phase contrast micrograph of gradient separated perforatoria following repeated extrusion through a 22 gauge-needle. X 800.

c. Electron micrograph (longitudinal section) of isolated perforatorium fractions. X 10 000.

d. Electron micrograph (cross section) of isolated perforatorium fraction. X 40 000.
FIGURE 5

a. Coomassie Brilliant Blue stained 8% - 18% linear gradient sodium dodecyl sulfate (SDS) polyacrylamide gel. Molecular masses of the polypeptides are denoted by numbers x103 in this and subsequent figures.

Lane S. Low molecular weight polypeptide Pharmacia standards of 14, 20, 30, 43, 67, 94 kDa.

Lane 1. Isolated rat perforatorial fraction.

b. Preparative Western blot of perforatorial polypeptides.

Lane 1. Colloidal gold stain of transferred perforatorial polypeptides.

Lane 2. Preimmune serum control.

Lane 3. Perforatorial polypeptides immunostained with antiserum raised against the whole perforatorium.
**FIGURE 6**

Western blot of rat perforatorial polypeptides.

**Lane 1.** Colloidal gold stain of transferred rat perforatorial polypeptides.

**Lane 2.** Respective preimmune serum control.

**Lane 3.** Rat perforatorial polypeptides immunostained with antiserum raised against the whole perforatorial fraction.

**Lane 4,5.** Perforatorial polypeptides immunostained with antibodies affinity purified from the 16 kD and 63 kD perforatorial polypeptides respectively. Although not shown, antibodies purified from the 13, 13.4, 34, 43, 57 and 63 kD polypeptides immunostained identically shown in these two lanes.

**Lane 6.** Rat perforatorial polypeptides immunostained with a C4 anti-actin mouse monoclonal antibody. No reaction is evident.
FIGURE 7

Cross sections through the head of step 19 spermatids showing the immunogold labeling obtained with antibodies which were affinity purified from either the 34, 43, 57, 63 kDa perforatorial polypeptides. Distributions obtained with the antibodies against the 34, 43, 57, 63 kDa polypeptide and the anti-perforatorium serum labeled identically.

The immunolabeling is distributed over the entire perforatorium (P), over the part of the outer periacrosomal layer (OPL) adjacent to the acrosomal head cap (HC), and over the inner part of the ventral spur (VS), d. Little or no labeling is found over the postacrosomal dense lamina (PDL), c, d.

a, X 60 000; b, X 52 000; c, X 55 000; d, X 69 000
Cross sections through the heads of step 19 spermatids representing the characteristic pattern of immunogold labeling obtained with antibodies affinity purified from either the 13, 13.4 or 16 kDa perforatorial polypeptides. These figures represent the results obtained with the antibody to the 16 kDa polypeptide; however the three antibody preparations immunolabeled identically.

The immunolabeling was limited to the apical triangular rod (TR) of the perforatorium, a, and to the dorsal (DP) and ventral prongs (VP) of the perforatorium, b. The inner zone of the ventral spur (VS) was also labeled, d. No labeling was found over the thinner caudal layer of the perforatorium which lies subjacent to the head cap (HC) of the acrosomic system nor over the outer periacrosomal layer (OPL), which lies exterior to the acrosomic system, c,d. The postacrosomal dense lamina (PDL) was also unreactive, c,d.

a,b,c. X 58 000; d. X 41 000.
FIGURE 9

General view of immunogold labeling of Lowicryl embedded spermatids at step 19 with:

a. Antibodies affinity purified from either the 34, 43, 57 or 63 kDa perforatorial polypeptides labeling the entire perforatorium (P), outer periacrosomal layer (OPL) adjacent to the head cap (HC) and the inner aspect of the ventral spur (VS).

b. Antibodies affinity purified from either the 13, 13.4 or 16 kDa labeling exclusively the rod and prongs of the perforatorium and the inner aspect of the ventral spur (not shown).

Labels: PDL, postacrosomal dense lamina; DP, dorsal prong; VP, ventral prong.

a, X 55 000; b, X 60 000.
FIGURE 10

a, b. Cross sections through the head of step 19 spermatids immunolabeled with C4 anti-actin monoclonal antibody. Immunogold labeling is found over actin located in the ectoplasmic specializations (ES) of the Sertoli cell cytoplasm (see Vogl et al., 1986, for details regarding Sertoli ectoplasmic specializations). No labeling is found over the perforatorium (P) or other regions of the head of the spermatozoon. X 57 000.

c, d. Sections through the heads of elongating step 17 spermatids immunolabeled with a C4 anti-actin monoclonal antibody. Besides labeling the Sertoli cell ectoplasmic specializations surrounding the head of the spermatid, this actin antibody also labels material found in the subacrosomal space (arrows). X 52 000.

e. Sections through the head of a step 17 spermatid immunolabeled with antiperforatorial serum. The antiserum labeled material found in the subacrosomal space (arrows) of the elongating spermatid but, in contrast to the C4 anti-actin antibody, does not label the Sertoli cell ectoplasmic specializations (asterisks) surrounding the head of the spermatid. Note also the reactivity of the spermatid cytoplasm (arrowheads). X 59 000.
IV. DISCUSSION

In order to achieve an understanding of the morphology and protein composition of the sperm head, a systematic study of its substructures is required. This investigation involved the study of the rat perinuclear theca focusing primarily on the perforatorium and the analysis and localization of its protein constituents.

A procedure recently described for the isolation of the rat perforatorium was successfully used in this investigation (Oko, 1988). The apical region of the perforatorium, composed of rods and prongs, constituted the largest part of the isolated fraction. The isolated amount of the thinner posterior region, adjacent to the head cap was difficult to determine (Fig. 5). The postacrosomal sheath, the other component of the perinuclear theca, which is continuous with the perforatorium (Lalli and Clermont 1981), was not isolated by this procedure.

This method revealed that the perforatorium is composed of many polypeptides of which the 13, 13.4, 16, 34, 43, 57, 63 kDa are prominent and the 16 kDa is major. It would appear that our 16 kDa protein corresponds to the previously identified 13 kDa protein which was considered to be the only constituent of the perforatorium (Olson and al. 1976).
1. Interpretation of Immunoblotting

a) Perforatorial Antibodies

The results of the immunoblots, using antiperforatorial serum immunoeluted from individual polypeptide bands, showed that all seven of the more prominent perforatorial bands examined in this study (i.e., 13, 13.4, 16, 34, 43, 57 and 63 kDa bands) were immunocrossreactive and thus shared common epitopes. Yet, despite this apparent cross reactivity, the immunoreactivity of the perforatorium with the seven antibodies tested showed a distribution difference between the low and the high molecular weight proteins (Fig. 11) indicating that there are also distinctive epitopes between these two groups of polypeptides. This discrepancy between these two sets of results may be explained by the fact that in immunoblots the polypeptides are denatured; and, under this condition, there are possibly many more epitopes exposed and hence available for immunocross-reactivity than in the fixed, in situ, condition where the polypeptides presumably are in their native configuration.
b) Actin Antibody

The unreactivity of the C4 anti-actin mouse monoclonal antibody to any of the denatured perforatorial polypeptides provided evidence that actin was not an integral part of the perforatorial polypeptides. Correspondingly, the unreactivity of the head of the fully differentiated spermatid or spermatozoon to the C4 anti-actin antibody confirms these immunoblotting results. Indeed, this antibody has been convincingly shown to react with all known isoforms of actin both in immunoblots and in situ and to compete for the G-actin binding site of DNAse I (Lessard 1988). Actin has been detected in the subacrosomal space of spermatids in a variety of mammalian species (Russell et al., 1986; Halenda et al., 1987; Masri et al., 1987; Fouquet et al., 1989). The present study confirms this observation by showing immunoreactivity with the C4 anti-actin antibody in the subacrosomal space of steps 17-18 spermatids containing loose floculent material. But as soon as condensation of this material takes place to form the definitive perforatorium of the step 19 spermatid, this immunoreactivity is lost. Whether this actin is transformed or degraded during the last steps of spermiogenesis remains to be determined. This study also indicates that perforatorial polypeptides are colocalized with actin in the subacrosomal space before their condensation to form the definitive perforatorium.
2. **Regional Differences of Perforatorial Polypeptides in the Perinuclear Theca**

a) **Whole Perforatorium Serum**

Although the perforatorium and postacrosomal sheath form a continuous covering over the nucleus in falciform shaped sperm (Lalli and Clermont, 1981), our immunocytochemical analysis indicated that these two structures are biochemically distinct and also suggested that the perforatorium comprised the part of the perinuclear theca that directly underlies the entire acrosomic system (Fig. 12). The postacrosomic location of the postacrosomal dense lamina or sheath is obvious in spatulate sperm (Nicander and Bane, 1962, Bloom and Birch-Anderson, 1965, Wooding and O'Donnell, 1971) because it does not appear continuous with the perforatorium which is reduced to an inconspicuous structure at the apical end of the nucleus. However, the presence of a continuous subacrosomal layer is suggested in spatulate sperm that are partially disrupted in SDS (Calvin and Bedford 1971). Recently Longo et al. (1987) have identified a major 60 000 basic protein that, immunologically, appears exclusively localized to the postacrosomal sheath of both bull and rat spermatozoa. They also provided evidence that antibodies produced from a group of 56-74 kDa polypeptides, obtained from either whole bull sperm or heads extracted in 1M NaCl, reacted intensely with the postacrosomal sheath and less so with material in the subacrosomal space of these spatulate-shaped sperm. However, no definitive results were presented as to whether antibodies against the multi-band group of
polypeptides cross-reacted with the perforatorium. The apparent immunological distinctiveness of the perforatorium and the postacrosomal dense lamina or sheath is in agreement with the different modes of formation of these two structures. The postacrosomal sheath forms in the wake of the perinuclear ring as it descends over the caudal aspect of the bull spermatid nucleus from steps 9 to 12 of spermiogenesis (Oko, 1977), whereas the perforatorium appears to solidify in the subacrosomal space at the last step of spermiogenesis (step 19) of the rat (Lalli and Clermont, 1981).

The antibodies against the whole perforatorium not only labeled the perforatorium, but also labeled two additional areas: first, a thin layer of cytoplasm located between the plasma membrane and the outer acrosomal membrane which demarcates the head cap or equatorial segment of the acrosomic system (this layer was formerly considered as a space and not recognized as an actual structure); second, the inner zone of the ventral spur which by definition is considered a portion of the postacrosomal dense lamina (refer to diagramatical summary of fig. 12). This immunolabeling pattern suggests that the perforatorial polypeptides could either be constituents or resemble proteins of these two additional areas. Furthermore it raises questions about the actual origin of the ventral spur. Is the development of the ventral spur simultaneous with the postacrosomal sheath because of their structural continuity or with the perforatorium because of their common proteins? It is interesting to note that the thin
cytoplasmic layer overlying the acrosome or thicker dorsal part of the acrosomic system did not immunoreact with any of the perforatorial antibodies prepared in this study. Therefore, this so-called outer periacrosomal layer (i.e., the thin continuous layer of cytoplasm between the plasmalemma and the outer acrosomal membrane) shows two distinct regions with differing protein compositions, at least in terms of perforatorial polypeptides. The difference in composition of these regions of the outer periacrosomal layer may possibly be related to the well documented finding that during the acrosome reaction only the part of the outer acrosomal membrane that delimits the acrosome and the plasma membrane overlying it undergoes fragmentation and vesiculation; Whereas the part of the outer acrosomal membrane that delimits the acrosomal head cap, or equatorial segment and the plasma membrane that faces it, retain their integrity. The latter two membranes also remain stable during the migration of the spermatozoon through the zona pellucida until fusion with the oocyte (Moore and Bedford, 1978; Bedford, 1979; Bedford et al., 1979; Yanagimachi, 1988). It is thus entirely possible that the perforatorial polypeptides or proteins resembling the perforatorial polypeptides contribute to the stability of this particular region of the outer periacrosomal layer.

b) Individual Perforatorial Polypeptides

The immunoreactivity of the head components to the seven affinity purified perforatorial antibodies clearly showed a variation
in distribution that was related to the their molecular weights of the respective polypeptides. The four high molecular weight polypeptides (i.e., 34, 43, 57, 63 kDa) had a distribution that was clearly different from the one obtained with the low molecular weight polypeptides (i.e., 13, 13.4 and 16 kDa bands), as illustrated in the summary diagram (Fig. 11).

**High molecular weight perforatorial polypeptides**

The distribution of immunolabeling obtained with the antibodies prepared from the high molecular weight polypeptides corresponded exactly to that obtained with the anti-whole perforatorium serum (Fig. 11). The entire perforatorium was labeled as well as the outer periacrosomal layer and the inner layer of the ventral spur, however the antibodies against the high molecular weight proteins did not react with the postacrosomal sheath (fig. 11).

**Low molecular weight perforatorial polypeptides**

The low molecular weight perforatorial polypeptides, on the other hand, were not distributed throughout the entire perforatorium and were absent from the outer periacrosomal layer. The immunolabeling of the corresponding antibodies was limited to the thicker apical part of the perforatorium (which is made up of the apical triangular rod and dorsal and ventral prongs) and to the inner layer of the ventral spur, but it was absent from the thinner layer that bridges the prongs and more caudally from the thin perforatorial layer, which is seen under the head cap along the
lateral surfaces of the nucleus (Fig. 11). The reasons for such a distribution in the perinuclear theca remain unclear. However, if we consider that the triangular rod and prongs of the perforatorium as well as the ventral spur are seemingly rigid structures that contribute to shape the head of the spermatozoon, one may speculate that the low molecular weight polypeptides may contribute to consolidate or solidify these particular parts of the perinuclear theca. It would be interesting to know whether spatulate spermatozoa possess the low molecular weight perforatorial proteins because they do not have the apical perforatorial protuberance which characterizes falciform spermatozoa.

3. Functions of the Perinuclear Theca and Outer Periacrosomal Layer

The perinuclear theca and outer periacrosomal layer appear to function in giving the spermatozoon heads its apparent stable shape. One of the most striking features of these structures is that membranes are tightly bound to them. Indeed, the plasmalemma is fixed to the outer periacrosomal layer and to the postacrosomal sheath whereas it is loose and unattached at the level of the perifossal zone where the perinuclear theca is missing (Lalli and Clermont, 1981). One can imagine that if the cell membrane were loose and not tightly bound to the surface of the spermatozoon's
head, the hydrodynamic properties of this motile cell would be modified and the motion markedly hindered.

Although biochemically different, the perforatorium and the postacrosomal sheath, may act as bonding material between membranes at three possible locations in mammalian spermatozoa: 1. Between the inner acrosomic membrane and the nuclear membrane in the subacrosomal space. 2. Between the plasma membrane and the nuclear membrane. 3. The outer periacrosomal layer which is continuous with the perinuclear theca is another membrane cementing structure which connects the plasma membrane to the outer acrosomal membrane.

The binding properties of the perinuclear theca and outer periacrosomal layer appear essential to keep the head structures compact during the acrosome reaction and fusion of the sperm plasmalemma to the oocyte. From a functional standpoint, the regional variations in protein composition of the perinuclear theca and the outer periacrosomal layer, may influence the chemical properties of membranes directly overlying these regions whose integrity may play a crucial role in fertilization.

4. **General Summary and Conclusion**

This study implies that there are regional variations in protein composition of the perinuclear theca as summarized in diagramatrical forms in figures 11 and 12. Concerning the identity
of the seven perforatorial polypeptides examined in this study by
immunochemical means, we do not claim to know their biochemical
relationships other than they were found to have epitopes in
common. The exact relationship between them will ultimately
depend upon deriving their amino acid sequence. The
immunocytochemical results of this study however do argue against
the idea that the lower molecular weight polypeptides (13, 13.4 and
16 kDa components) are breakdown fragments of the higher
molecular weight group (34, 43, 57 and 63 kDa components). If
they were, one would expect antibodies prepared against the lower
molecular group, either to label the entire perforatorium, as did the
antibodies against the higher molecular weight group, or not to
label the perforatorium at all. This clearly was not the case and
since only the apical half of the perforatorium was labeled by the
lower molecular weight antibodies, it strongly suggests that there
are regional differences in protein composition of the
perforatorium. It must be emphasized that this study does not
stipulate or conclude as to what these differences are.

The cross reactivity, observed for the seven perforatorial
polypeptides on immunoblots suggests that the perforatorial
polypeptides are either derived from a larger protein or gene
product that has been proteolytically processed or from a closely
related group of proteins or genes. The latter possibility is partly
favored because of the difference in distribution found in this study
of the lower and higher molecular weight perforatorial
polypeptides.
It was also found in this study that antibodies prepared against all seven of the perforatorial polypeptides cross reacted with the inner aspect of the ventral spur, which by definition is part of the postacrosomal sheath. It must be emphasized that this "cross-reactivity" may only imply that the perforatorium and this region of the ventral spur have epitopes in common. Likewise it can be argued that the "cross-reactivity" observed with antibodies prepared against the high molecular weight perforatorial polypeptides and the outer periacrosomal layer may only be indicative of shared epitopes. However, whatever may prove to be the case, this cross-reactivity between the different cytoskeletal regions of the sperm head suggests a functional similarity between these different cytoskeletal regions.
FIGURE 11

Diagramatic representation of the regional differences in protein composition within the perinuclear theca, as revealed by affinity purified antibodies against individual perforatorial polypeptides. The distribution of high and low molecular weight perforatorial proteins in the rat sperm head was compared.

Diagrams showing cross sectional profiles of the head of a rat spermatozoon. On the left, the triangular profiles extend from the apical extremity of the head and on the right, the elongated profiles are from the caudal region of the head.

A. The dots represent the distribution of immunogold labeling obtained with antibodies prepared against the four high molecular weight perforatorial polypeptides (34, 43, 57, and 63 kDa). These proteins are seen in the perforatorium (P), outer periacrosomal layer (OPL), and inner zone of the ventral spur (VS).

B. The dots indicate the immunolabeling obtained with antibodies against the three low molecular weight perforatorial polypeptides (13, 13.4, and 16 kDa). The low molecular weight proteins are seen in the thicker parts (prongs) of the perforatorium and the inner zone of the ventral spur.

Labels: A, acrosome; N, nucleus; HC, head cap; HCS, head cap segment; PDL, postacrosomal dense lamina; PM, plasma membrane; OAM, outer acrosomal membrane; IAM, inner acrosomal membrane.
A) Regions immunolabeled with antibodies to 34, 43, 57 and 63 KDa perforatorial proteins

B) Regions immunolabeled with antibodies to 13, 13.4 and 16 KDa perforatorial proteins
**FIGURE 12**

Diagramatic representation of the regional variations in protein composition within the perinuclear theca as revealed by the anti-perforatorial serum.

Drawing of the head of a rat spermatozoa as seen from the side and accompanied by representative cross-sectional views (modified by Lalli and Clermont, 1981). The region of the perinuclear theca delineated in **smooth gray** and lying between the acrosomic system (A: **stippled in light gray**) and the nucleus (N: **dark black**) represents the perforatorium (P). The region of the perinuclear theca beginning where the acrosomic system ends and lying between the nucleus and the plasmalemma is the postacrosomal sheath (PS). The ventral spur (VS) refers to the ventral prominence of the sperm spur is colored in **smooth gray** to convey that this part of the ventral spur is similar in composition to the perforatorium. The acrosomic system is composed of the acrosome (A), head cap (HC) and separated head cap segment (HCS). The outer periacrosomic layer (OP), lying between the plasmalemma and the acrosomic system, is continuous, structurally, with the perforatorium and acrosomal sheath. The region of the outer periacrosomal layer adjacent to the acrosomic head cap is denoted in **smooth gray** to convey that this part of the outer periacrosomal layer is similar in composition to the perforatorium. The **dashed white line** at the base of the nucleus indicates the borderline of the perifossal zone.

Labels: PM, plasma membrane; A, axoneme; M, mitochondria; ODF, outer dense fibers.
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ORIGINAL CONTRIBUTIONS

1) Development of a technique to isolate perforatorium from falciform spermatozoa.

2) Confirmation that the perforatorium is biochemically distinct from the postacrosomal sheath.

3) Confirmation that there are regional variations in protein composition within the perforatorium and within the postacrosomal sheath of the perinuclear theca of falciform spermatozoa.

4) The demonstration that there are regional variations in protein composition within the perforatorium and within the postacrosomal sheath of the perinuclear theca of falciform spermatozoa.

5) The morphological and immunocytochemical detection of an outer periacrosomal layer, sandwiched between the plasmalemma and outer periacrosomal layer, a cytoskeletal layer previously not recognized.

6) The observation that there are biochemical similarities between the perforatorium, the inner ventral spur of the postacrosomal sheath and the outer periacrosomal layer.

7) The absence of actin within the perinuclear theca of mature spermatozoa.