Basic Proteins of the Perinuclear Theca of Mammalian Spermatozoa and Spermatids: A Novel Class of Cytoskeletal Elements

Frank J. Longo,*‡ Georg Krohne,* and Werner W. Franke*

*Division of Membrane Biology and Biochemistry, Institute of Cell and Tumor Biology, German Cancer Research Center, Heidelberg, Federal Republic of Germany; and ‡Department of Anatomy, The University of Iowa College of Medicine, Iowa City, Iowa 52242

Abstract. The nuclei of bovine spermatids and spermatozoa are surrounded by dense cytoplasmic webs sandwiched between the nuclear envelope and the acrosome and plasma membrane, respectively, filling most of the cytoplasmic space of the sperm head. This web contains a complex structure, the perinuclear theca, which is characterized by resistance to extractions in nondenaturing detergents and high salt buffers, and can be divided into two major subcomponents, the subacrosomal layer and the postacrosomal calyx. Using calyces isolated from bull and rat spermatozoa we have identified two kinds of basic proteins as major constituents of the thecal structure and have localized them by specific antibodies at the light and electron microscopic level. These are an M_r 60,000 protein, termed calicin, localized almost exclusively to the calyx, and a group of multiple-band polypeptides (MBP; M_r 56,000–74,000), which occur in both the calyx and the subacrosomal layer. The polypeptides of the MBP group are immunologically related to each other, but unrelated, by antibody reactions and peptide maps, to calicin. We show that these basic cytoskeletal proteins are first detectable in the round spermatid stage. As we have not detected any intermediate filament proteins and proteins related to nuclear lamins of somatic cells in sperm heads, we conclude that the perinuclear theca and its constituents, calicin and MBP proteins, are the predominant cytoskeletal elements of the sperm head. Immunologically cross-reacting polypeptides with similar properties have been identified in the heads of rat and human spermatozoa. We speculate that these insoluble basic proteins contribute, during spermiogenesis, to the formation of the perinuclear theca as an architectural element involved in the shape changes and the intimate association of the nucleus with the acrosome and the plasma membrane.

During the past decade it has become increasingly clear that the overall shape of a cell and its internal architecture is dependent not only on membranous elements but also on a group of structures that resist cell lysis and extraction by nondenaturing detergents and buffers of various pH and ionic strength. Such proteinaceous structures are now usually referred to under the collective terms cytoskeleton, as to the cytoplasm, and karyoskeleton, for nuclear architectural elements. Among the cytoskeletal components some of the most prominent structures are the actin-containing microfilaments and the tubulin polymers, i.e., microtubules. The term cytoskeleton also includes certain filamentous webs such as membranous coats in the cell periphery, many of which contain proteins of the spectrin family, the regular clathrin coats of coated pits and vesicles, and the plaques of adhering junctions. Remarkably, some residual cyto- and karyoskeletal components are preserved even after extraction in high salt concentrations, and maintain typical architectural features. Structures of this category include the intermediate-sized filaments (IFs)[1] of the cytoplasm and the biochemically related structure in the cortex of the nucleus, the nuclear lamina. It also includes various kinds of plasma membrane–associated plaques, notably those associated with the desmosome, and, in the nucleus, the subunits of the pore complex.

Several routes of cell differentiation are characterized by spectacular shape changes of both the nucleus and the cytoplasm, one of the most prominent being spermiogenesis in the seminiferous tubules of the mammalian testis (reviewed in references 8 and 23). Contributions of microfilaments and microtubules to shape changes taking place during the development of spermatids and spermatozoa have been discussed, although controversially. In the sperm tail, microtubules are doubtlessly important elements of spermatozoan functions. In addition, various stabilizing elements that are resistant to high salt treatment have also been identified in the sperm tail, including the outer dense fibers and the fibrous sheath (e.g., 3, 8, and 23).

The role of cytoskeletal elements in the formation and maintenance of the nucleus-containing sperm head is even less clear (reviewed in reference 8). In this respect observations that the sperm head also contains structural elements resistant to extractions with detergents and high salt buffers

1. *Abbreviations used in this paper*: IF, intermediate-sized filaments; MBP, multiple-band polypeptides; NEPHGE, nonequilibrium pH gradient gel electrophoresis.

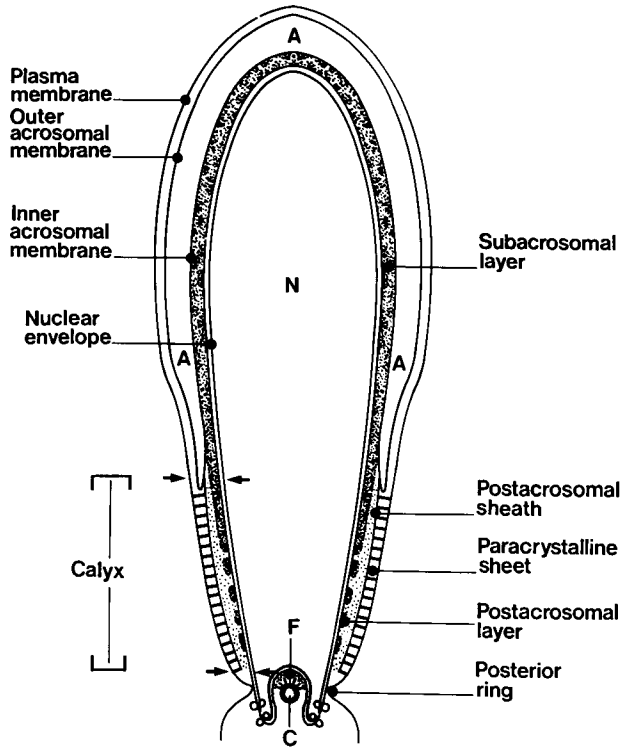


Figure 1. Schematic drawing showing the major components of an idealized mammalian sperm head. The scheme is a modified version of the classic diagram of elements of the primate sperm head (23). *N*, nucleus; *A*, acrosomal content. The membrane types are listed in the upper left; the posterior ring, the centriolar complex (*C*) and the dense layer of the implantation fossa (*F*) are also indicated. The major cytoskeletal elements, as perceived by electron microscopy (indicated on the right hand side), form the perinuclear theca, which can be distinguished, and under certain conditions fragments, into an anterior portion, the subacrosomal layer, filling the subacrosomal space, and a posterior complex, designated here as calyx (*arrows*). The latter is located in the postacrosomal segment of the sperm head (13) and consists of (*a*) the postacrosomal layer intimately applied to the nuclear envelope, (*b*) the postacrosomal sheath, (*c*) the paracrystalline sheet of filamentous ridges (striations; reference 35), and (*d*) some less distinct material in between (*dotted area*). The degree and extent of regularity of the material in the paracrystalline sheet varies between different species and in relation to fixation and isolation conditions. The term postacrosomal sheath (a synonym is postacrosomal dense lamina) is used here in the more restricted definition (23, 52), whereas others have also used it to describe the thin sheath plus the paracrystalline and other material (e.g., 40 and 44). The material of the subacrosomal and postacrosomal layers, plus the interspersed material (*dotted area*), has also been referred to as perinuclear substance in the literature (17). Depending on the type of fixation used the material of the perinuclear theca often appears with a finely filamentous texture (20, 40).

appear to be relevant. Bellvé and O'Brien (8) have attracted attention to the existence of a large, extraction-resistant, extranuclear structural element, the perinuclear theca (Fig. 1). This structure represents a dense layer of variable thickness sandwiched in between the outer nuclear membrane on the one side and the inner acrosomal membrane, and caudally to the posterior ring, the postacrosomal segment of the plasma membrane, on the other side (reviewed in references 17, 23, 40, and 50). The postacrosomal segment of the perinuclear theca is very complex and forms a continuous, rigid, funnel-shaped entity. Since this structural complex can be isolated as a whole we shall specifically refer to it as the calyx. On the basis of ultrastructural and cytochemical observations the calyx complex has been further subdivided into a thin postacrosomal layer that is tightly applied to the nuclear envelope continuous with the subacrosomal layer (perinuclear substance) *sensu* (8, 17), and a thin (~5 nm) layer parallel to the postacrosomal plasma membrane (postacrosomal sheath) that is connected to the inner aspect of the plasma membrane by a paracrystalline sheet of ridges or filaments of 10–14 nm in diameter (Fig. 1). In addition, some indistinct, loosely webbed material is located in between these elements.

Although various procedures for the isolation of detergent- and high ionic strength–resistant structures from mammalian sperm heads have been described, no protein has so far been positively localized to any of these structures. On the other hand, there have been a number of controversial reports on the existence of IF proteins and nuclear lamins in mammalian sperm heads (for details see Discussion). In the course of a study aiming at the clarification of whether mammalian spermatids and spermatozoa possess IFs or nuclear lamina proteins we have found that practically the only cytoskeletal element of the sperm head that is resistant to extractions with detergent and high salt buffers is a scaffold of the perinuclear theca, and we have identified, by the use of antibodies as specific probes, two types of basic proteins as major components of this dense-web structure.

Materials and Methods

Collection and Fractionation of Spermatozoa

Vasa and epididymides were obtained from adult Wistar rats and from bulls slaughtered at a local meat processing firm. Ejaculates containing human spermatozoa were from outdated reference samples obtained from the andrology unit of the Department of Dermatology, Mannheim Medical School, University of Heidelberg, FRG. All procedures were carried out at 4°C unless otherwise indicated and all solutions contained 1 mM phenylmethylsulfonyl fluoride (PMSF). Vasa and epididymides were minced with scissors in PBS and filtered through fine-mesh gauze. Sperm were recovered by centrifugation at 1,000 *g* for 5 min and washed twice in PBS containing 25 mM EDTA (PBS–EDTA) and either stored frozen as a sediment or directly fractionated.

Sperm resuspended in PBS–EDTA (2–3 ml packed sperm in ~30 ml) were fractionated into heads and tails by sonication with a sonifier (output setting of 7, three bursts, each 15 s in duration; Branson Sonic Power Co., Danbury, CT). The suspension of heads and tails was made 50% in sucrose and layered over 75% sucrose (10 mM Tris-HCl, 1 mM EDTA, 2 mM dithiotreitol [DTT], 150 mM NaCl, pH 7.5) and centrifuged at 26,000 *g* for 45 min in a rotor (SW 27; Beckman Instruments, Inc., Fullerton, CA). Tails concentrated as a sharp band at the sucrose interface and heads formed a pellet at the bottom of the tube. Purity of head and tail fractions was assessed with a Zeiss microscope equipped with phase-contrast optics.

Sperm heads were collected and washed once in PBS–EDTA. In this and the following incubations sperm heads were pelleted by centrifugation at 1,000 *g* for 10 min and resuspended with a motor-driven homogenizer. Sperm heads were demembraned by incubation for 10 min with 1.0% Triton X-100 in PBS–EDTA and then treated twice with 2 M NaCl, 5 mM DTT, 10 mM Tris-HCl buffer, (pH 7.4) for a total extraction period of 40 min. After the second extraction, sperm heads were treated with DNase I (100 μg/ml Cooper Biomedical, Wiesbaden, Federal Republic of Germany) in 10 mM Tris-HCl buffer (pH 8.5) containing 2 mM $MgCl_2$ for 30 min at 20°C. The resulting residual structures were extracted a third time for 15 min with 2 M NaCl (same buffer as above), then with DNase (100 μg/ml; 30 min at 20°C), and again with 2 M NaCl (10 min). The final residue was washed two times in 10 mM Tris buffer (pH 7.4) and pelleted. In some ex-

periments 1 instead of 2 M NaCl was used. Tail fractions were also treated in parallel using essentially the same extraction protocol.

To determine the effects of the various treatments used in this protocol on the morphology of spermatozoa and sperm heads, samples were taken after each incubation, washed in PBS, and fixed with 2.5% glutaraldehyde in 50 mM sodium cacodylate buffer (pH 7.2) for 30 min. Samples were washed for 1 h in 50 mM cacodylate buffer, postfixed in 1% OsO_4 for 30 min, dehydrated in ascending concentrations of ethanol, and embedded in Epon (cf. 26). Thin sections, stained with uranyl acetate and lead citrate, were examined with a Siemens 101 electron microscope.

Gel Electrophoresis, Immunoblotting, and Peptide Mapping

Total spermatozoa or pellets of cell fractions were boiled for 5 min in SDS sample buffer (5% SDS, 10 mM sodium phosphate buffer, pH 7.2, 10% 2-mercaptoethanol; cf. 39). For subsequent two-dimensional gel electrophoresis the solubilized proteins were precipitated by addition of 9 vol cold acetone, dried, and either used directly or after further removal of DNA. For DNase treatment the dried pellets were resuspended in 1 ml DNase buffer containing 50 μg DNase I and incubated for 10–15 min at 20°C. The polypeptides were then either collected directly by centrifugation (10,000 *g*, 5 min) or precipitated with 6 vol acetone. The pellets were washed with 90 and 100% acetone and then solubilized in sample buffer (47). In some experiments, nonextracted sperm heads (sediment of 100-μl vol) were boiled directly in 1 ml SDS sample buffer (see above) and precipitated with 6 vol acetone. The pellet was washed with 90 and 100% acetone, dried, and resuspended in 2 ml DNase buffer containing 50 μg DNase I/ml and incubated for 30 min at 20°C. The polypeptides were then collected and processed as described above.

SDS-PAGE was carried out essentially according to Laemmli (39) using variable loads (up to 50 μg/lane); two-dimensional gel electrophoresis using nonequilibrium pH gradient gel electrophoresis (NEPHGE) was as described (47) with minor modifications (2). For immunoblotting, gels were processed as described (12). Proteins transferred to nitrocellulose paper were visualized by staining with Ponceau S in distilled water (Sigma, Munich, FRG). The stained nitrocellulose paper was washed in distilled water and photographed to identify the exact position of proteins. Periodic acid Schiff reaction for glycoprotein was performed as described (21).

Tryptic peptide maps from polypeptide spots after two-dimensional gel electrophoresis were done as described (19).

Antibodies

The following antibodies were used. (*a*) Guinea pig antibodies obtained after immunization with protein eluted from an SDS-PAGE band containing the M_r 60,000 polypeptide, calicin, obtained from the calyx fraction of bull sperm heads (for methods of elution and immunization see reference 11). Antibodies were affinity-purified as described (37) when necessary. (*b*) Guinea pig antibodies prepared similarly but directed against the multiple-band polypeptides (MBP) (M_r 56,000–74,000) were also affinity-purified as mentioned above. (*c*) Guinea pig antibodies specific for lamins that recognize lamins from a wide variety of cell types and different species (for details see reference 11). (*d*) Guinea pig antibodies against cytokeratins and vimentin were as described (cf. references 27–29). (*e*) Monoclonal antibody IFA which reacts with a highly conserved epitope common to all classes of IF proteins (54). (*f*) Monoclonal antibody K_G8.13 reacting with basic (type II) cytokeratins and cytokeratin No. 18 (reference 30; commercially available from Bio-Makor, Rehovot, Israel). (*g*) Monoclonal broad range cytokeratin antibodies AE1 and AE3 (66). (*h*) Monoclonal antibody PKK1 reacting with several cytokeratins (reference 32; commercially available from Labsystems Oy, Helsinki, Finland). (*i*) Monoclonal antibody VIM9 reacting with vimentin (Biochrom, Berlin, FRG). (*j*) Monoclonal antibody PKB8 reacting with lamins A, B, and C (38). Fluorescein- or Texas red–labeled secondary antibodies were from goat (Dianova, Hamburg, FRG).

Light and Electron Microscopic Immunolocalization

Immunofluorescence microscopy on ~5-μm cryostat sections of shock-frozen tissue samples from testes and other tissues (liver, intestine, and heart) of bulls and rats was performed as described (12, 27, 38). For immunolocalization experiments on whole mount preparations, suspensions of washed spermatozoa were placed as droplets on coverslips and gently spread. Cells adhering to the coverslips were then either rinsed briefly in PBS or were first permeabilized by brief incubation in PBS containing 0.5% Triton X-100 and then washed. Thereafter, cells were either directly incubated with antibodies for 30 min or were first immersed in −20°C methanol for 5 min and in −20°C acetone for 2 min before antibody application. All subsequent steps were essentially as described for tissue sections. In all double label immunolocalization experiments both primary antibodies were applied simultaneously (cf. 11).

For electron microscopy of tissue samples, small pieces were fixed for 30 min in 2.5% glutaraldehyde (50 mM sodium cacodylate, 50 mM KCl, 2.5 mM $MgCl_2$, pH 7.2) in the cold (0–4°C), followed by three washes in 50 mM cacodylate buffer and secondary fixation in 2% OsO_4 for 60 min. Further processing was as described (26). For electron microscopic immunolocalization in tissue specimens, cryostat sections were processed as described (71), using colloidal gold–labeled goat antibodies to guinea pig or mouse Ig (~5-nm particle diameter; Janssen, Beerse, Belgium). Alternatively, immunogold labeling was performed on ultrathin sections of Lowicryl K4M–embedded samples as described (58). Samples used for Lowicryl embedding were prefixed for 20 min with 2% formaldehyde (0.1 M sodium phosphate, pH 7.3, 50 mM KCl, 2.5 mM $MgCl_2$). For immunoelectron microscopy of whole spermatozoa one of the two following protocols was used. (*a*) Spermatozoa were resuspended in PBS containing 0.5% Triton and washed in the same buffer. Antibodies were then added (final concentrations ranging from 5–50 μg Ig/ml) and the suspension incubated for 2 h at room temperature. After three washes in PBS to remove unbound Ig, gold-labeled secondary antibodies (see above) were added and incubated overnight. After another three washes in PBS the pelleted material was sequentially fixed with glutaraldehyde and OsO_4 and processed for electron microscopy as described. (*b*) Spermatozoa attached to coverslips were briefly treated with PBS, with or without 0.5% Triton X-100, and then with −20°C methanol and acetone as described for light microscopic preparations. The coverslips were then incubated for 30 min with the primary antibodies (same range of concentrations), washed three times in PBS, incubated with secondary antibodies for 4 h, washed in PBS, fixed, and flat-embedded as described above for immunoelectron microscopic processing of cryostat sections (cf. 11 and 71).

Controls were done in parallel using other primary antibodies unreactive on spermatids and spermatozoa but positive on various other cell types (including Sertoli cells), such as antibodies to vimentin, nuclear lamins (see above and reference 11), and plakoglobin (18), or secondary antibodies alone.

Results

Isolation and Fractionation of the Perinuclear Theca

Nuclei isolated from bull or rat sperm heads with buffers containing 1% Triton X-100 maintained their specific shape and remained delimited by the perinuclear theca, with variable portions of the nuclear envelope persisting (Fig. 2, *a*, *d*, and *e*). Depending on the specific preparative conditions, the perinuclear theca often tended to split, to variable extents, into two sublayers separated by a space, which probably represents an artifact (Fig. 2, *d* and *e*), in a similar pattern as described after cavitation (50) and treatment with low concentrations of Triton X-100 (72). One layer, which was of rather uniform width, undercoated the plasma membrane and included, in the calyx region, the postacrosomal sheath and the material of the paracrystalline layer. The other layer was usually thinner, irregularly patchy in its distribution and conformation, and remained closely associated with the outer margin of the nuclear envelope.

Incubation of such nuclear preparations in high salt buffer containing 2 M NaCl and 5 mM DTT resulted in a pronounced increase in transparency and swelling of the anterior region of the sperm nucleus (Fig. 2, *b* and *c*). This swelling usually disrupted the thin subacrosomal layer in several places (Fig. 2 *c*). Throughout the swelling and extraction of chromatin, however, the cuplike formation surrounding the posterior aspect of the sperm nucleus, i.e., the calyx, maintained its overall size and shape (Fig. 2, *b* and *c*).

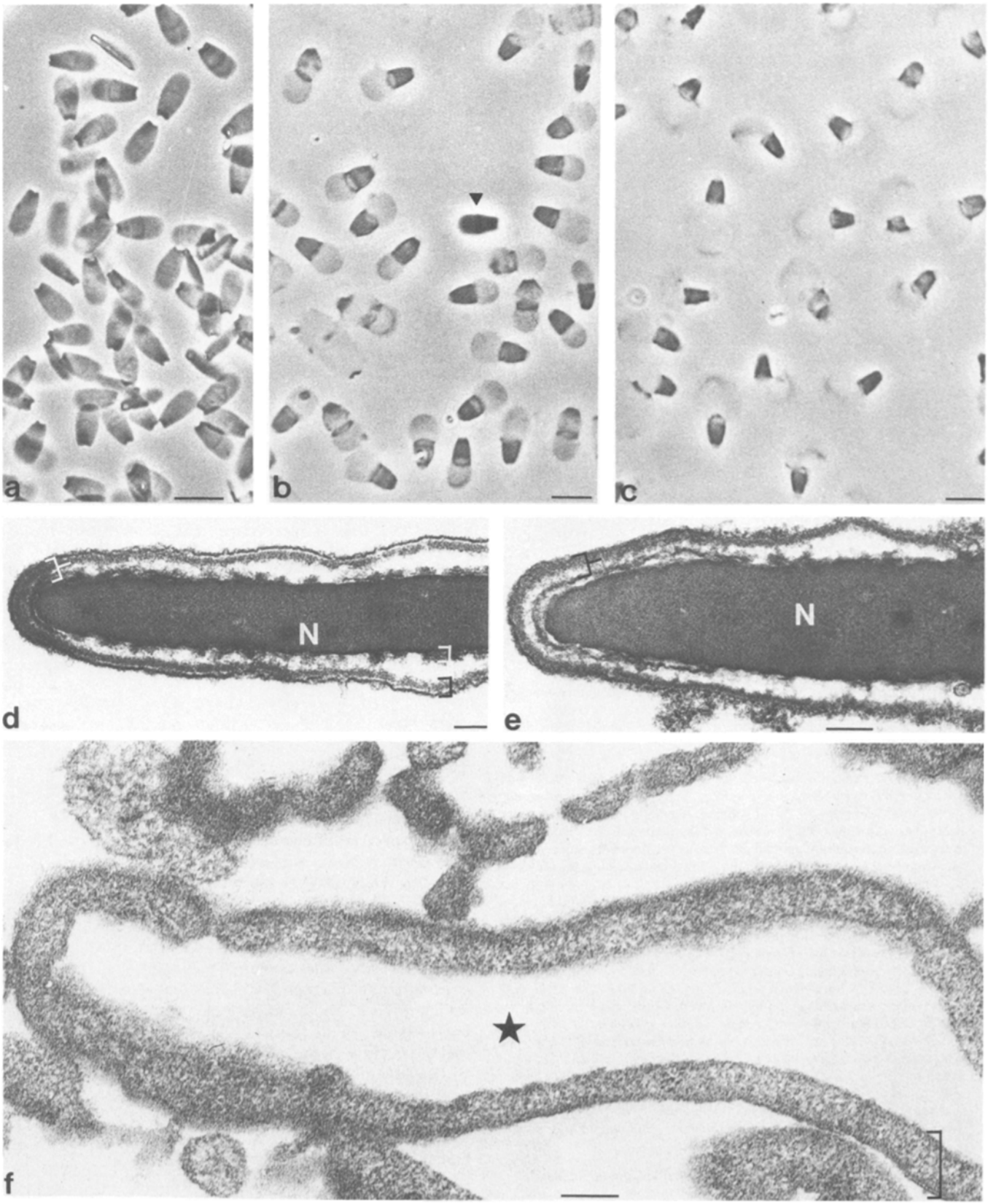


Figure 2. Morphology of extracted and nonextracted bull sperm heads. When isolated bull sperm heads (*a*) were treated with Triton X-100, then incubated in buffer containing 2 M NaCl and 5 mM DTT for 15 (*b*) and 45 (*c*) min, and viewed with phase-contrast optics, large amounts of material were extracted concomitant with chromatin swelling and disruption of the anterior aspect of the sperm head (subacrosomal layer), whereas the posterior part (calyx) by and large retained its original size (the triangle in *b* indicates an intact sperm head of a suspension that was mixed with extracted specimens just before examination). Electron micrographs (*d–f*) of ultrathin sections showing the calyx (fork brackets in *d* and *e*, bracket in *f*) in nonextracted sperm heads (*d*), after incubation with Triton X-100 (*e*), and

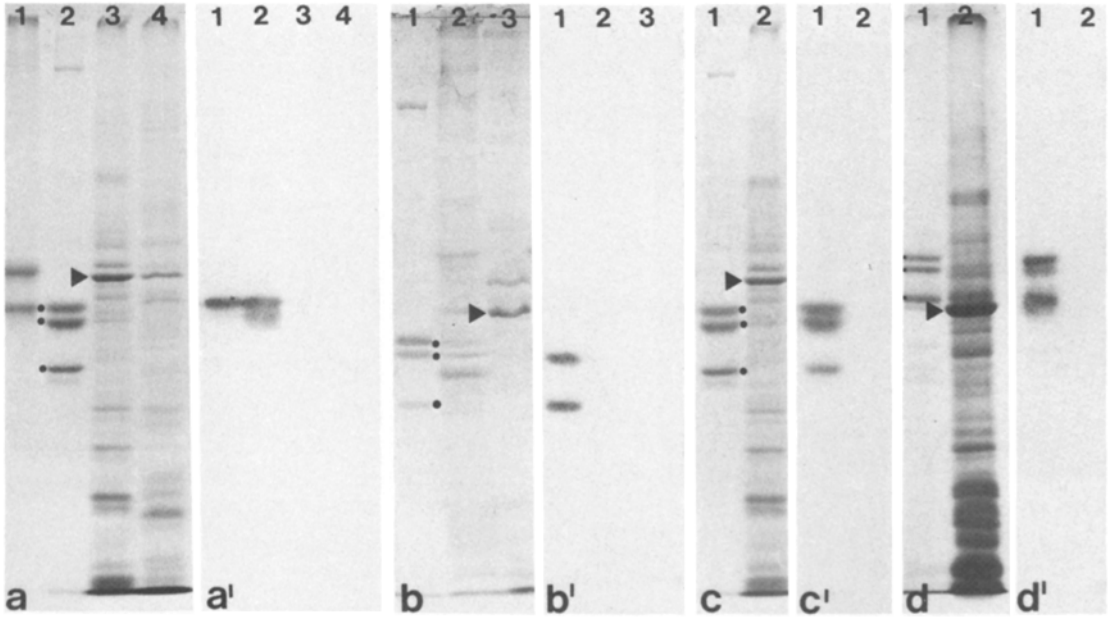


Figure 3. Absence of IF proteins and nuclear lamins in bull and rat sperm as determined by immunoblotting. Coomassie Blue staining after SDS-PAGE (*a–d*) and corresponding autoradiographes of immunoblots (*a'–d'*) reacted with antibodies to vimentin (*a* and *a'*; from guinea pig), cytokeratins (*b* and *b'*; antibody K_G 8.13), monoclonal antibody IFA (*c* and *c'*; reference 54), and nuclear lamins (*d* and *d'*; from guinea pig). (*a*) Lanes *1* and *2*, cytoskeletal preparations of cultured bovine fibroblasts (B1 cells) and kidney epithelial cells (Madin–Darby bovine kidney [MDBK] cells), respectively (cf. 18); lanes *3* and *4*, Triton X-100– and high salt–extracted sperm heads of bull and rat, respectively. Dots denote from top to bottom, vimentin (B1 and MDBK), cytokeratins A (No. 8) and D (human equivalent No. 18; only in MDBK); the arrowheads denote calicin. Antibodies to vimentin show a strong reaction to vimentin derived from B1 and MDBK cells (*a'*, lanes *1* and *2*) but not with any cytoskeletal protein of extracted bull and rat sperm heads (*a'*; lanes *3* and *4*). (*b*) Lane 1, MDBK cytoskeleton; lane *2*, whole bull spermatozoa; lane *3*, Triton X-100– and high salt–extracted bull sperm heads. Antibodies to cytokeratins react exclusively with the two cytokeratins of MDBK cells (*b*, lane *1*) and not with any protein of whole bull sperm or extracted bull sperm heads (*b'*, lanes *2* and *3*). (*c*) Lane *1*, MDBK cytoskeleton; lane *2*, extracted bull sperm heads. Monoclonal antibody IFA reacts exclusively with vimentin and cytokeratins of MDBK cells (*c'*, lane *1*) and not with any polypeptide of extracted bull sperm heads (*c*; lane *2*). (*d*) *Lane 1*, bovine liver nuclear lamins A, B, and C (for preparation see reference 11) are indicated by bars; lane *2*, briefly extracted bull sperm heads. The antibodies recognize exclusively lamins A, B, and C (*d'*, lane *1*) and do not react with polypeptides of extracted bull sperm heads (*d'*, lane *2*).

With this high salt buffer extraction, combined with DNase treatment, rather pure populations of calyces could be isolated, which, for the most part, appeared as somewhat flat (60–90-nm thick), envelope-like elements with finely filamentous, web-like substructures showing occasional fenestrations (Fig. 2*f*). Small segments of membranes were occasionally recognized along the inner and outer margins of the calyx, presumably representing residues of the plasma membrane and nuclear envelope.

Incubation of Triton X-100–treated sperm heads with buffers containing 1 M NaCl did not bring about the extensive extraction of chromatin seen in specimens suspended in 2 M NaCl. Moreover, the perinuclear theca, although swollen, persisted along both the anterior and posterior aspects of the sperm nucleus, so that calyces could not be readily separated. Such preparations, however, also contained more residual material from the nuclear interior, even after DNase treatment. Therefore, preparations made with 2 M NaCl were preferentially used in this study.

Absence of Intermediate Filament Proteins and Nuclear Lamins in Karyo- and Cytoskeletons of Spermatozoa and Spermatids

The presence of known IF proteins and nuclear lamins was examined in immunoblots of proteins from total rat and bull spermatozoa, isolated whole sperm heads, and high salt buffer sperm head residues obtained after extraction with Triton X-100 (isolated calyx preparations), using a panel of highly specific and well characterized antibodies. Cytoskeletal and nuclear lamina preparations were used as positive controls. In all cases from both species the sperm material tested was negative for the presence of vimentin (Fig. 3, *a* and *a'*), a broad range of cytokeratins (one example is shown in Fig. 3 *b* and *b'*), and the highly conserved epitope recognized by antibody IFA (54), which is common to all classes of IF proteins (Fig. 3, *c* and *c'*). These samples were also negative when tested with the cytokeratin antibodies AE1 and AE3 (data not shown). Antibodies recognizing all three

the final residue of sperm heads after extraction with Triton, 2 M NaCl, and DNase (*f*). During the isolation procedure the calyx is often split into two leaflets (small brackets in *d*). In *f*, the area formerly occupied by the sperm nucleus is indicated by the star. *N*, sperm nucleus. Bars: (*a–c*) 10 μm; (*d–f*) 0.1 μm.

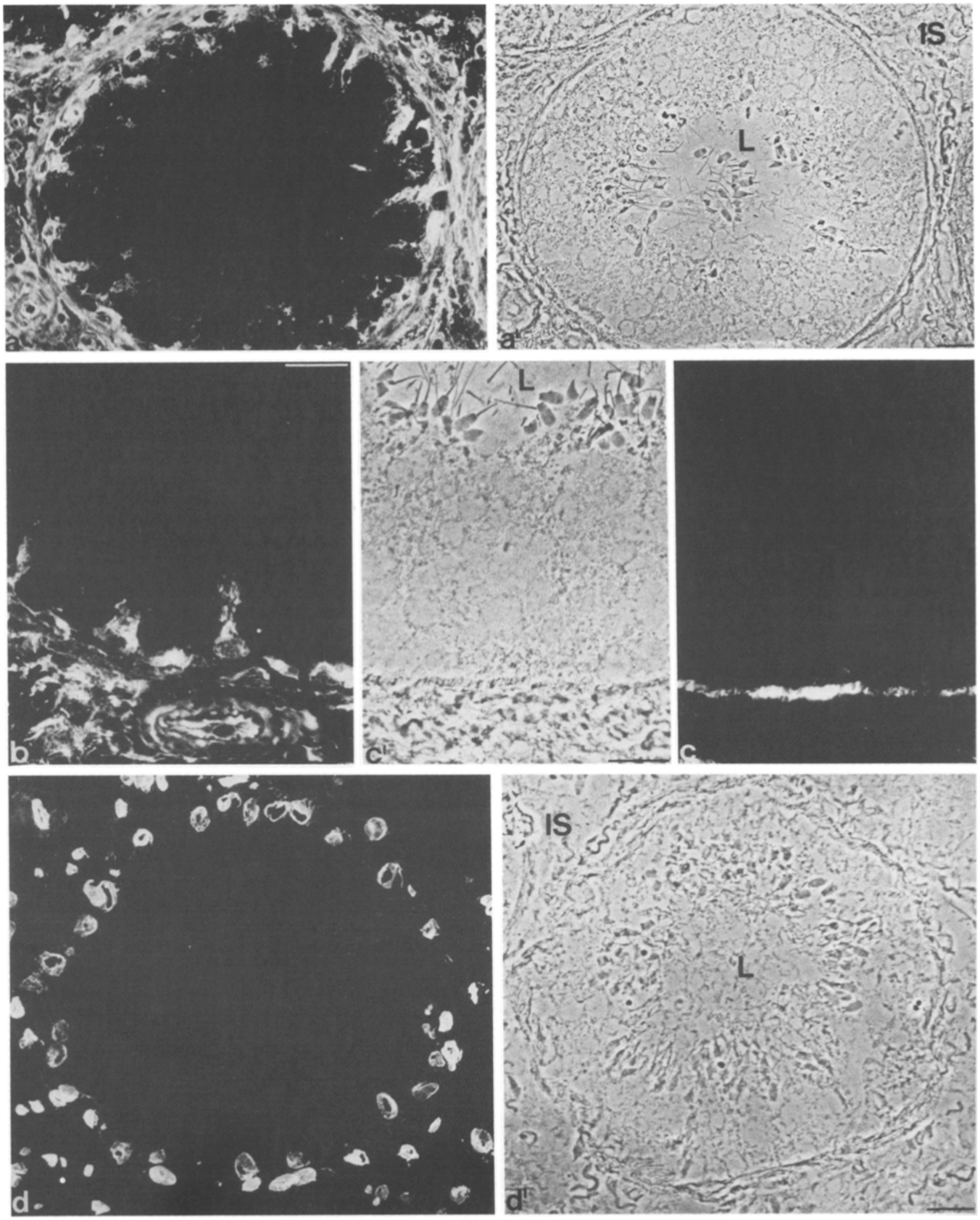


Figure 4. Absence of IF proteins and nuclear lamins in bovine spermatids and spermatozoa as determined by immunofluorescence microscopy. Frozen sections of bull testis reacted with guinea pig antibodies to vimentin (*a*); murine monoclonal antibody IFA to all classes of IF proteins (*b*); guinea pig antibodies to cytokeratins (*c*); and nuclear lamins A, B, and C (*d*). (*a'*, *c'*, and *d'*) Corresponding phase-contrast micrographs demonstrating sperm within the seminiferous tubule lumen (*L*). Antibodies to vimentin (*a*) and antibody IFA (*b*) react strongly with Sertoli and peritubular cells (*IS*, interstitium) but not with sperm cells. Antibodies to cytokeratins (*c*) show no reaction to sperm or spermatogenic cells but (only in the bull) react with cells located along the periphery of the seminiferous tubule. Antibodies to nuclear lamins (*d*) react exclusively with the nuclear periphery of somatic and early spermatogenic cells. Bars, 25 μm.

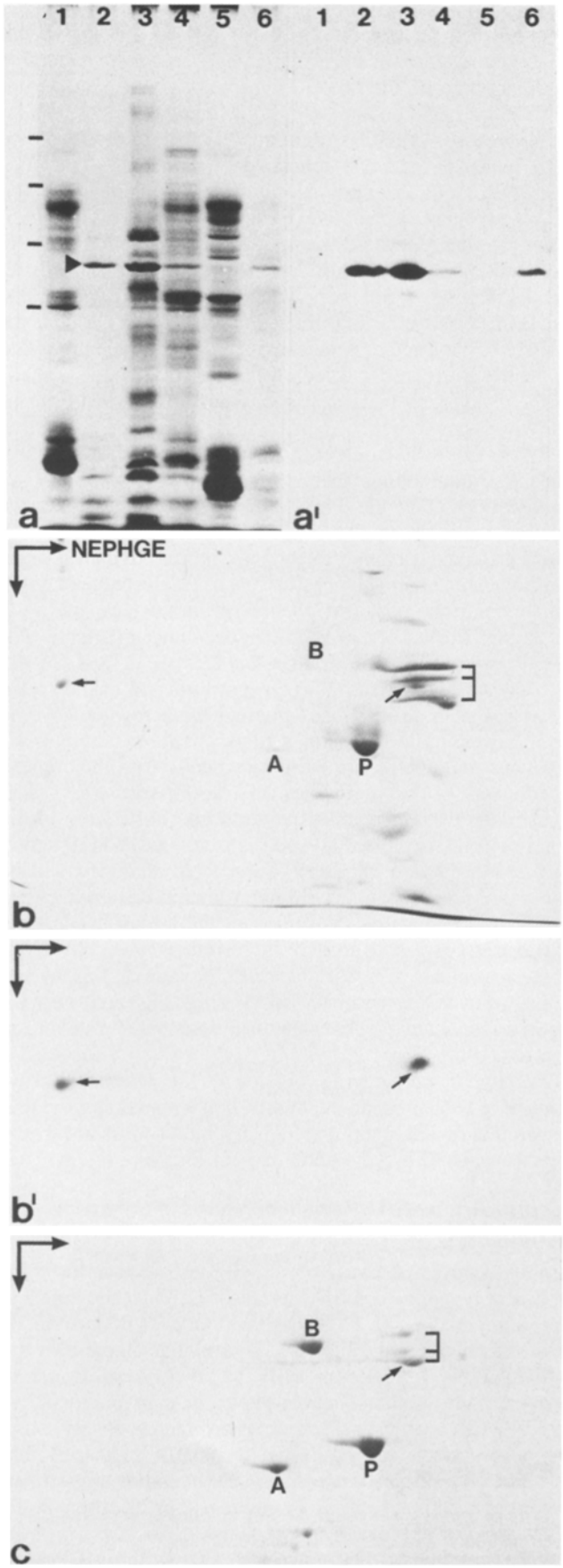


Figure 5. Enrichment of basic proteins of the perinuclear theca in rat and bull sperm head fractions. (*a*) Coomassie Blue staining of proteins separated by SDS-PAGE and (*a'*) corresponding autoradiograph of a parallel immunoblot reacted with affinity-purified antibodies against the M_r 60,000 polypeptide, calicin. Lanes *1–4* contain proteins from bull sperm; lanes *5* and *6* contain proteins from rat sperm. Lanes *1* and *5*, sperm tails; lanes *2* and *6*, fractions of calyx-enriched perinuclear theca material obtained after extraction of sperm heads in buffers containing Triton X-100, 2 M NaCl, and DNase I; lane *3*, whole sperm heads; lane *4*, whole sperm. Bars on the left margin of *a* indicate the position of reference proteins: β-galactosidase (M_r 116,000), phosphorylase a (M_r 94,000), BSA (M_r 68,000), and skeletal muscle α-actin (M_r 42,000). The arrowhead designates calicin found in both bull and rat sperm heads to be enriched during extractions. (*b* and *c*) Coomassie Blue–stained two-dimensional polyacrylamide gel electrophoresis of polypeptides from whole bull sperm heads (*b*) and fractions of perinuclear thecal material (*c*) obtained after extraction as in *a*, lane *2*. First dimension, NEPHGE; second dimension, SDS-PAGE. The left arrow points to the M_r 60,000 protein (calicin) that has not entered the first dimension gel and the right arrow denotes the calicin migrated into the gel. The bracket indicates a triplet of MBP (M_r 74,000–56,000) that are abundant in isolated sperm heads and can be partially extracted between 1 and 2 M NaCl (*c*). Reference proteins (acidic proteins are to the left) used for coelectrophoresis are: BSA (*B*), actin (*A*), and yeast phosphoglycerokinase (*P*). (*b'*) Autoradiograph of an immunoblot corresponding to the gel shown in *b* and reacted with the same affinity-purified calicin antibodies used in *a'*. The antibodies react exclusively with calicin (*a'* and *b'*).

mammalian lamins (A, B, and C) also failed to react with any polypeptide of sperm heads and subfractions made therefrom in bull (e.g., see Fig. 3, *d* and *d'*) and rat (not shown).

These results were confirmed by immunofluorescence microscopy with frozen sections from testicular tissue of both bull (Fig. 4) and rat (data not shown). With antibodies to vimentin, Sertoli cells as well as cells of the interstitium and the wall of the seminiferous tubule were positive, whereas spermatozoa and spermatogenic cells were negative (Fig. 4 *a*). Identical results were obtained with the monoclonal antibody IFA to the highly conserved epitope of IF proteins (Fig. 4 *b*). A broad range of antibodies to cytokeratins were negative for all cell types examined in rat testis, i.e., both somatic and spermatogenic cells (data not shown). In bull testis sperm, spermatogenic cells, Sertoli cells, and components of the interstitium were negative (Fig. 4, *c* and *c'*). In this species, however, cells that, remarkably, were positive for both cytokeratins and desmin (probably myoid cells) were present within the wall of the seminiferous tubules (Fig. 4 *c*).

Frozen sections of testes incubated with antibodies to nuclear lamins demonstrated an intense staining along the periphery of all somatic cell nuclei (Fig. 4, *d* and *d'*). Weak staining was apparent in nuclei of early spermatogenic cells (data not shown), whereas spermatids and spermatozoa were completely negative.

Identification of an M_r 60,000 Polypeptide as a Major Component Specific to the Calyx

Gel electrophoretic analyses of proteins from bull and rat whole sperm and isolated sperm heads showed an enrichment of several polypeptide bands in the M_r range from 56,000 to 74,000. Isolated calyces revealed a prominent M_r 60,000 polypeptide (Fig. 5, *a* and *c*) that showed a pink-reddish metachromatic staining with Coomassie Blue (Fig. 5 *a*, lanes *2* and *6*). In addition, between M_r 56,000 and

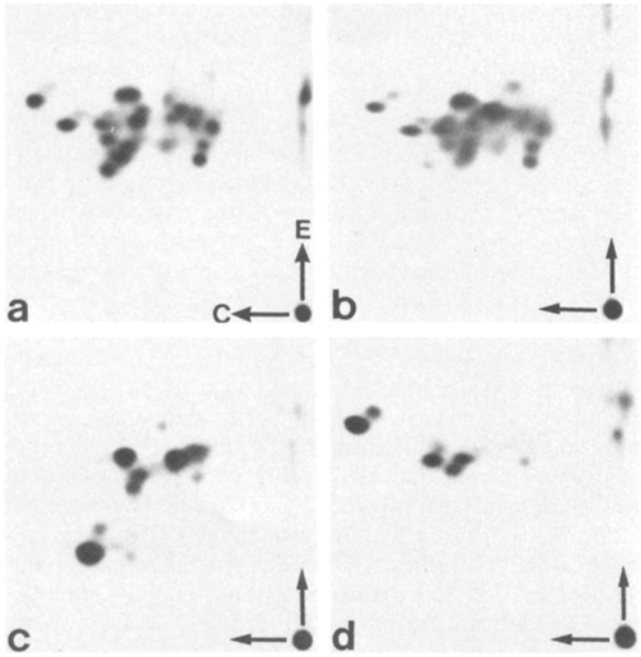


Figure 6. Comparison of ^{125}I-labeled tryptic peptides of basic cytoskeletal proteins of the nuclear theca from bull and rat spermatozoa. (*a*) M_r 60,000 polypeptide (calicin) of bull obtained from a preparation as shown in Fig. 5 *c*. (*b*) Same protein (calicin) from rat (see Fig. 5 *a*, lane *6*). (*c*) M_r 74,000 polypeptide of the MBP group from bull (for preparation see Fig. 5, *b* and *c*, bracket). (*d*) M_r 56,000 polypeptide of the MBP group from bull. *E*, first dimension by electrophoresis; *C*, second dimension by chromatography.

74,000 a group of MBP were present as major components in isolated sperm heads and in sperm heads extracted with 1 M NaCl (data not shown), but were less prominent in calyx preparations obtained with 2 M NaCl (Fig. 5 *a*). In both calyx preparations and isolated sperm heads, the MBP showed, upon staining with Coomassie Blue, a strong scattering effect to obliquely incident light, as is characteristic of several very basic proteins (cf. 34). Both the M_r 60,000 polypeptide and the MBP were not stained with the periodic acid Schiff reaction, indicating that they are not considerably glycosylated. Fractions of rat and bull sperm tails did not reveal the M_r 60,000 protein nor the MBP (Fig. 5 *a*, lanes *1* and *5*).

Because the M_r 60,000 band represented the predominant polypeptide of isolated calyces it was cut out from preparative gels (fraction is shown in Fig. 5 *a*, lane 2) and the protein was eluted and used to generate antibodies. The reaction of affinity-purified antibodies to the M_r 60,000 polypeptide, when examined in immunoblot assays (Fig. 5 *a'*), indicated the specificity of the antibodies used, the presence of the same antigen in both rat and bull calyces, the enrichment of the antigen in isolated sperm heads and in extracted calyx fractions, and its absence in sperm tails.

Two-dimensional gel electrophoresis of calyx preparation and nonextracted sperm head proteins showed that both the M_r 60,000 and the MBP are very basic proteins. During NEPHGE the M_r 60,000 polypeptide migrated exceptionally fast (Fig. 5, *b* and *c*), indicative of an isoelectric point >pH 8 (given the limitations of current isoelectric focusing techniques the exact isoelectric point could not be determined). The migration of the MBP was more variable. Depending on the gel conditions, they moved as a group, which, at the completion of electrophoresis, was either located in conjunction with the M_r 60,000 protein or had moved to an even more basic position (Fig. 5, *b* and *c*).

The reaction of antibodies affinity-purified to the M_r 60,000 bull sperm polypeptide separated by NEPHGE confirmed our SDS-PAGE observations and showed that only one polypeptide species was reactive (Fig. 5, *b* and *b'*). This also indicated that the M_r 60,000 does not form an extended series of polypeptide modifications. Tryptic peptide map analysis revealed that the M_r 60,000 polypeptides of bull (Fig. 6 *a*) and rat (Fig. 6 *b*) are closely related to each other and that the MBP (Fig. 6, *c* and *d*) represent a group of polypeptides that is clearly different from the M_r 60,000 polypeptide. Details of the interspecies relationships of the corresponding proteins in bull, rat, and human sperm will be presented elsewhere.

When the localization of the M_r 60,000 protein was examined by immunofluorescence microscopy of whole mount preparations of bovine (Fig. 7), human, and rat spermatozoa (data not shown), the location of this protein was seen with special clarity. In bovine and human spermatozoa the antigen was localized practically exclusively to the perinuclear calyx surrounding the posterior portion of the nucleus, revealing its funnel-shaped organization. In the morphologically different rodent sperm head (for references see 4, 7, 8, 23, 40, 44, and 48) the acrosome-free ventral portion of the sperm head was positively stained, whereas the acrosome-covered part was essentially unstained (data not shown).

Under immunofluorescence microscopy with the affinity-purified M_r 60,000 antibodies on cryostat sections of various frozen tissues, positive reaction was noted only in the seminiferous tubules of all species examined (Fig. 9 shows bull testis; data on the other two species examined will be presented elsewhere). The immunostaining was exclusive to the posterior, i.e., calyx, region of the sperm heads. The earliest stages found positive were round to slightly elongated spermatids (Fig. 8 *a*), in which the staining was, by and large, restricted to a subacrosomal ring (Fig. 8 *b*) near to, but not identical with, the equatorial segment (for definition see references 8 and 23).

Because of the specific location of the M_r 60,000 polypeptide in the calyx and the finding that it constitutes the major protein of detergent- and high salt buffer–resistant sperm heads, we shall use the name calicin for this protein.

Identification of MBP as Components of the Entire Perinuclear Theca

In most analyses the very basic MBP appeared in the form of four polypeptide components (Fig. 5, *a* and *b* and Fig. 10). M_r values of 56,000, 58,000, 63,000, and 74,000 could be resolved (Fig. 5 *b* and Fig. 10). The relative amounts of the individual bands within the MBP, however, varied from one experiment to another. Characteristically, these polypeptides appear at the same position on two-dimensional gel electrophoresis using the NEPHGE technique, indicating that they are very similar in electrical charge and/or migrated in a common complex. They migrated either close to, faster (Fig. 5 *b*), or slower (Fig. 9 *a*) than calicin.

When affinity-purified antibodies against the MBP were examined by immunoblotting, all bands of this group reacted (Fig. 9 *a'* presents an example of a NEPHGE separation).

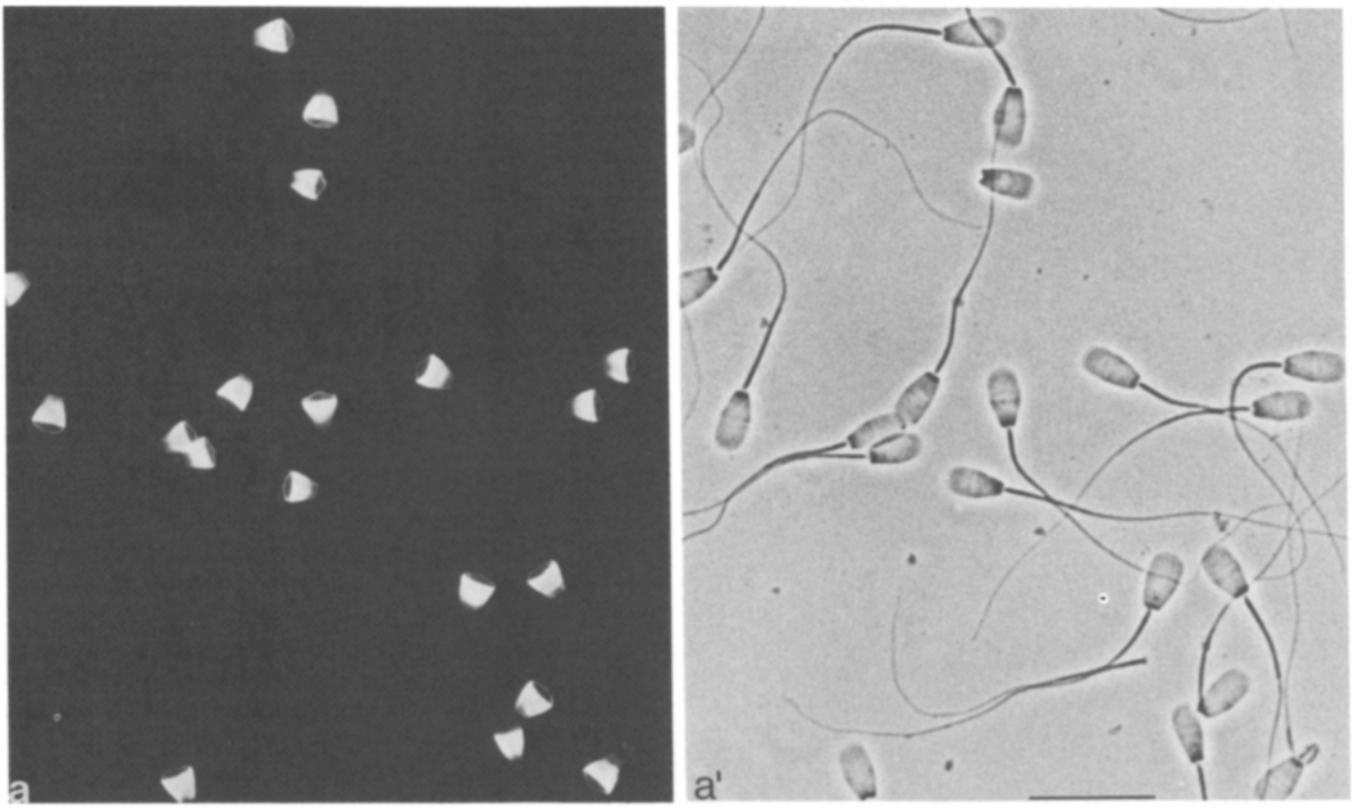


Figure 7. Immunofluorescence localization of M_r 60,000 cytoskeletal polypeptide (calicin) in whole spermatozoa of bull. Spread preparation of epididymal bull sperm incubated with affinity-purified antibodies to calicin (*a*, epifluorescence; *a'*, phase-contrast optics). The antibodies localize exclusively to the posterior aspect of the sperm head and, in the three-dimensional perspective afforded by this preparation, demonstrate that the protein is located in a funnel-shaped calyx surrounding the posterior margin of the nucleus. Bar, 20 μm.

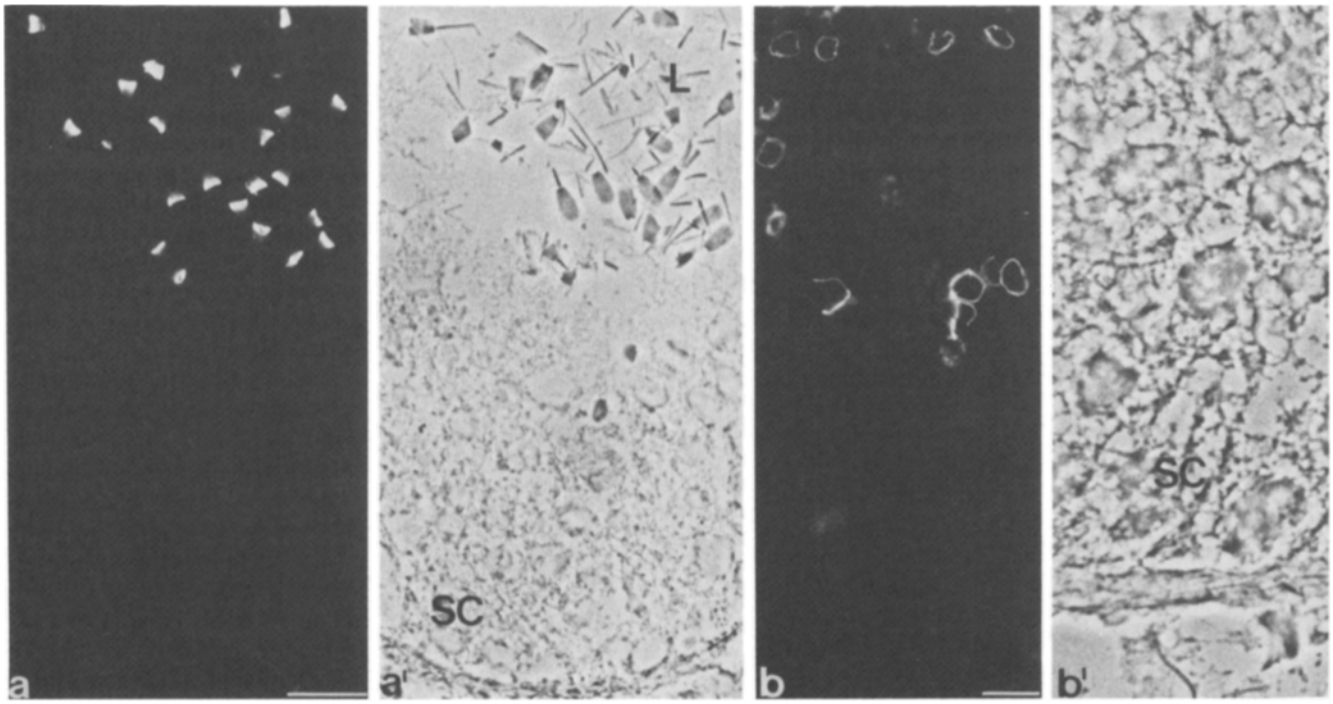


Figure 8. Immunofluorescence microscopy of calicin (M_r 60,000) in bull testis. (*a* and *b*) Frozen sections through bull seminiferous tubules incubated with affinity-purified antibodies to calicin showing (*a* and *b*, epifluorescence optics) areas with mature sperm (*a*) and spermatids (*b*). (*a'* and *b'*) Corresponding phase-contrast micrographs showing luminal spermatozoa (*L*) and spermatogenic cells (*SC*). Luminated spermatozoa (*a*) are stained in the entire posterior aspect corresponding to the calyx. Antibody staining is first detectable in round to slightly elongated spermatids (*b*) in a ringlike fashion. Bars, 20 μm.

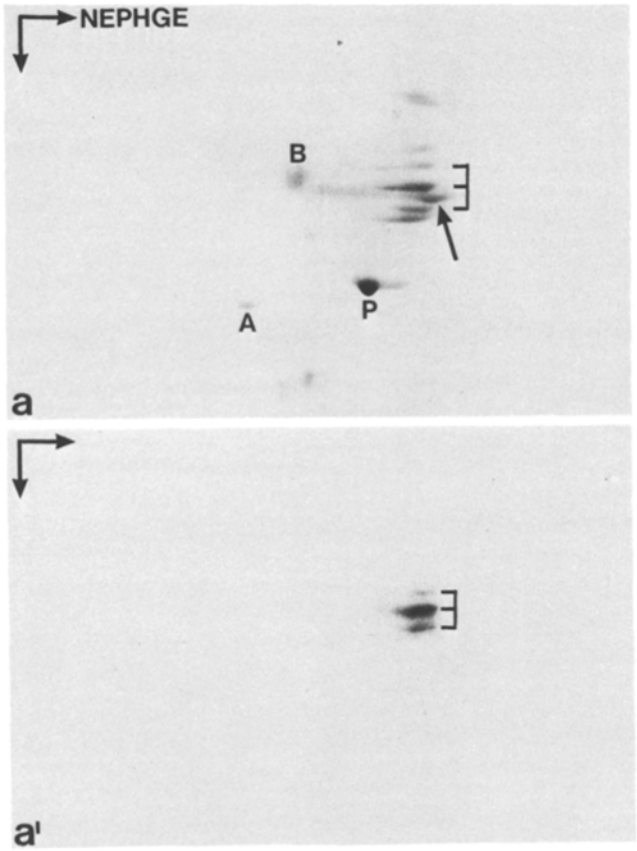


Figure 9. Immunoblot with antibodies to the MBP group. (*a*) Coomassie Blue staining of residual proteins of Triton X-100– and 1 M NaCl-extracted fractions of perinuclear thecal material from bull sperm heads, separated by two-dimensional gel electrophoresis (as in Fig. 5, *b* and *c*), and corresponding autoradiography of immunoblot (*a'*) reacted with affinity-purified antibodies against the upper polypeptide of the MBP (M_r 74,000). The arrow indicates the position of calicin. The brackets denote the position of the triplet polypeptide of the MBP that react with the purified antibody. Internal reference proteins as in Fig. 5, *b* and *c*.

Affinity-purified antibodies prepared by elution from one of the bands using the nitrocellulose strip purification method (37) reacted with the other three bands, thus indicating immunological cross-reactivity. Analysis of the individual polypeptide spots by tryptic peptide mapping (Fig. 6, *c* and *d*) resulted in peptide patterns different from each other and from the pattern obtained from calicin. Based on these results, as well as the different metachromatic appearances after Coomassie Blue staining, we conclude that the MBP are different from calicin and are a group of polypeptides that are immunologically related to each other but exhibit significant peptide map differences that make it unlikely that they form a series of proteolytic degradation products.

When frozen sections of testis and whole mount preparations of spermatozoa were examined by immunofluorescence microscopy during affinity-purified antibodies specific for the basic MBP, an exclusive reaction on spermatozoa (Fig. 10, *a–c*) and spermatids (Fig. 10 *d*) was found. Unlike the calicin reaction, however, the MBP antigens were detected within the entire perinuclear theca, i.e., in the postacrosomal calyx as well as in the subacrosomal layer (Fig. 11, *b* and *c*). In spermatids the MBP immunoreactivity appeared to cover an area smaller than in spermatozoa.

Electron Microscopic Localization of Calicin and the MBP

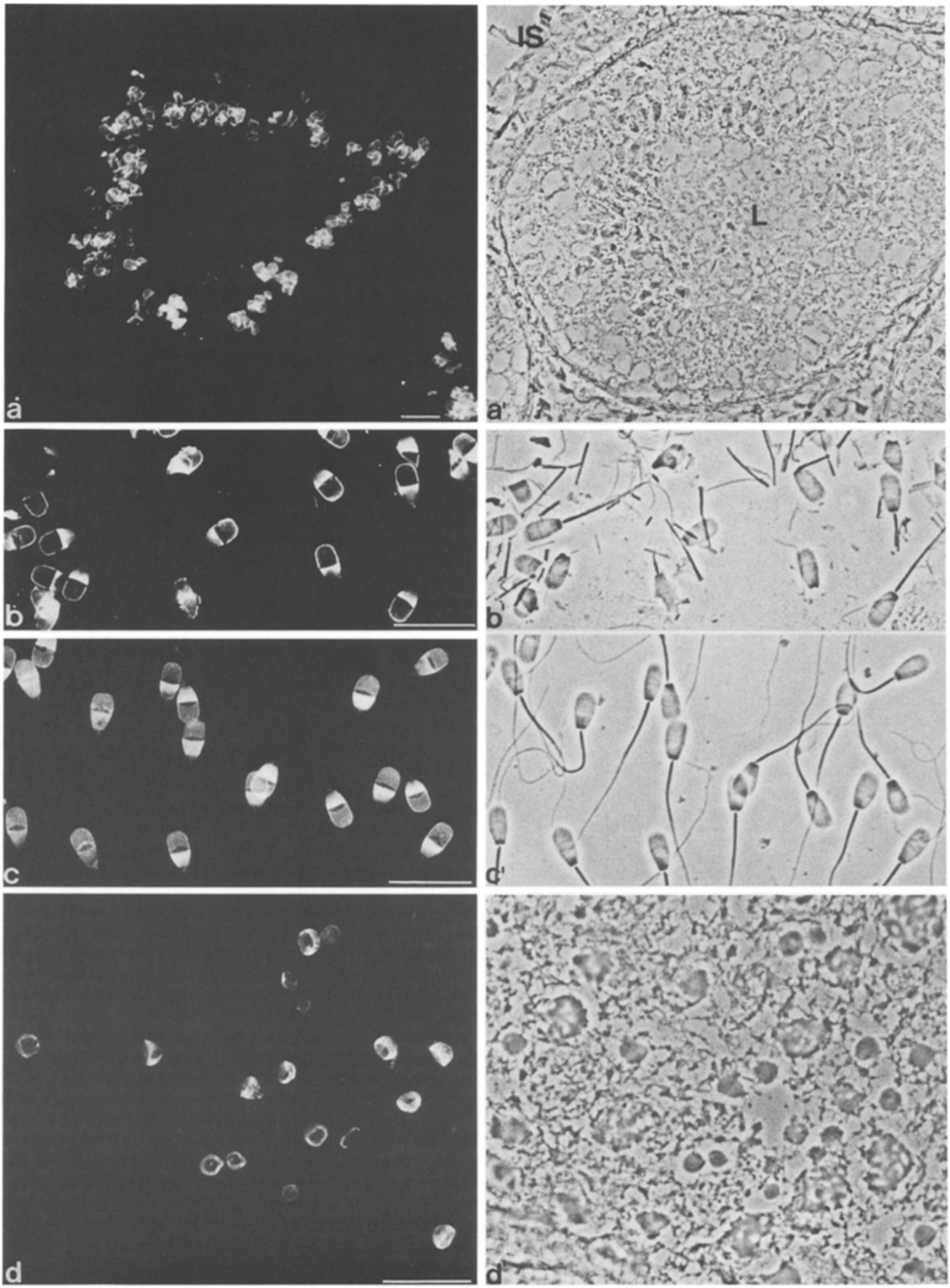
The electron microscopic appearance of the major components of the perinuclear theca is shown in Fig. 11, *a* and *b*. The subacrosomal layer was mostly composed of a web of fibrillogranular material without distinct substructures. The postacrosomal calyx was more complex (see also Fig. 1), revealing a similar fibrillogranular web immediately covering the nuclear envelope (postacrosomal layer) and the ~5-nm thick, electron-dense postacrosomal sheath connected, in some regions, to the plasma membrane by the paracrystalline sheet formed by parallel filamentous ridges of 10–14 nm in diameter and a center to center spacing of ~16 nm.

Immunoelectron microscopy, using colloidal gold–coupled secondary antibodies on frozen tissue sections of testicular tissue as well as permeabilized (suspended or spread) spermatozoa, confirmed our light microscopic observations of intense calicin labeling in the calyx (Fig. 11, *e* and *f*). MBP were also concentrated in the calyx, notably in spermatids fixed in situ (Fig. 11 *c*), but were also detected in the subacrosomal layer (Fig. 11 *d*). Within the calyx most of the immunogold label seen after incubation of specimens with either antibodies to calicin (Fig. 11 *f*) or MBP (Fig. 11 *d*) was associated with the material of the postacrosomal layer on the outer nuclear membrane, where it appeared to be rather homogeneously distributed. At present we are unable to decide with which of the major substructures of the postacrosomal layer of the perinuclear theca the calicin and the MBP label is associated. A more detailed morphological and immunoelectron microscopic study of the components of the perinuclear theca in spermatozoa and during spermiogenesis will be presented elsewhere (Paranko, J., F. Longo, G. Krohne, and W. W. Franke, manuscript in preparation).

Discussion

The most important conclusions from our study are (*a*) that the major cytoskeletal element of the heads of bovine spermatozoa is the perinuclear theca, a densely woven web connecting the nuclear envelope with the acrosome and postacrosomal plasma membrane, respectively, and (*b*) that this cytoskeletal structure contains a class of basic proteins un-

Figure 10. Localization of the MBP by immunofluorescence microscopy in bull spermatozoa and spermatids. (*a–d*) Epifluorescence; (*a'–d'*) corresponding phase-contrast optics. Frozen sections through bull testis showing seminiferous tubules with luminal spermatozoa (*L*) in a survey (*a*), and at a higher magnification (*b*) after reaction with affinity-purified antibodies to the MBP. (*c*) Spread preparation of whole epididymal bull spermatozoa incubated with the same antibodies. The antibodies localize to the entire perimeter of the sperm nucleus and show a more intense local reaction in the calyx (*b* and *c*). (*d* and *d'*) Frozen section of testis showing earlier stages of spermiogenesis. The MBP are first detectable in round to slightly elongated spermatids. Bars, 20 μm.


a
a'
IS
L
b
b'
c
c'
d
d'

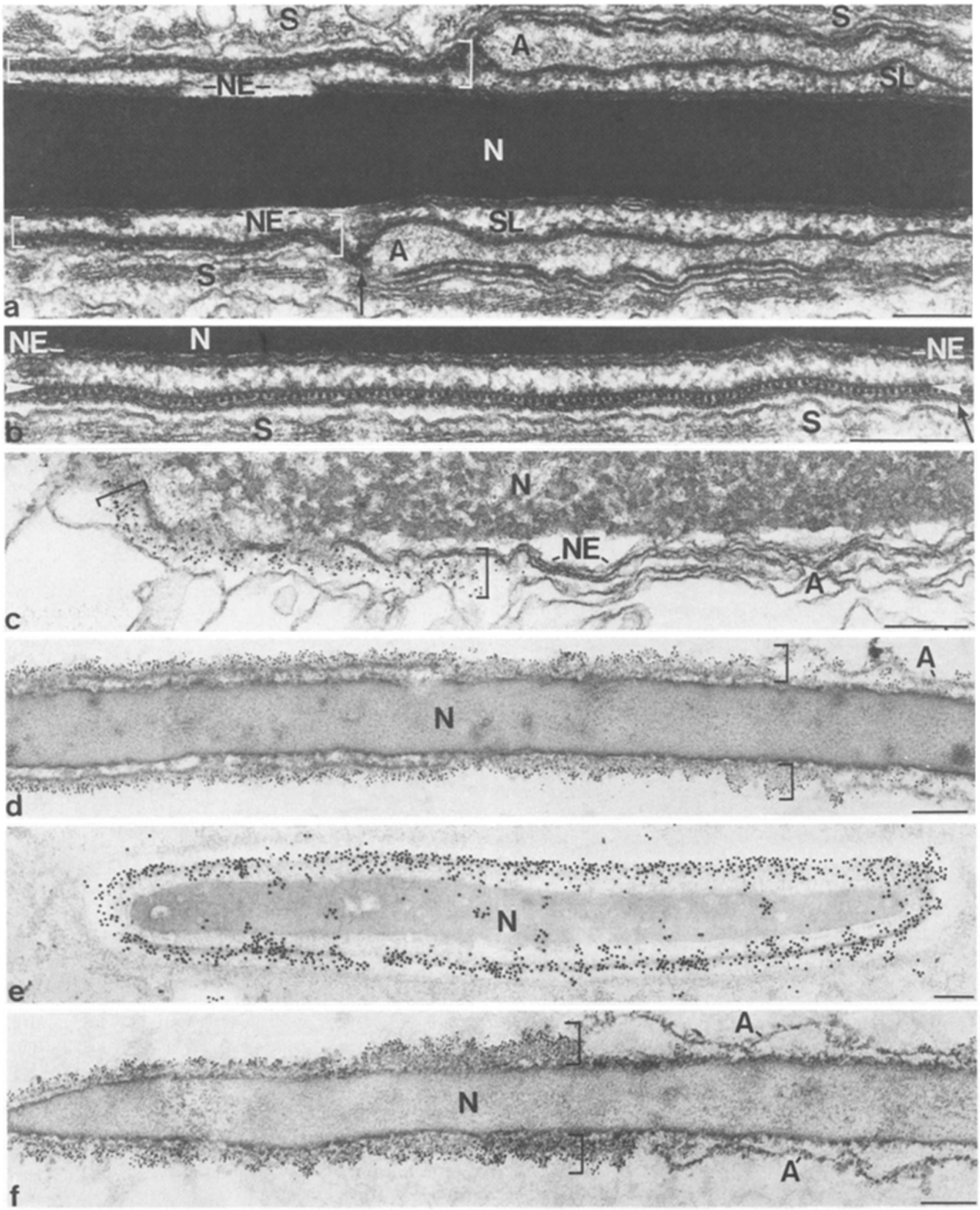


Figure 11. Morphology of perinuclear theca in bull sperm heads and immunolocalization of the M_r 60,000 protein (calicin) and MBP at the electron microscopic level. (*a*, *b*) Electron micrographs of ultrathin sections through in situ fixed testicular sperm (*a* and *b*) showing details of perinuclear structures at the transitional region from the anterior to the posterior part (*a*) and of the posterior region (calyx) of the sperm head (*b*). *S*, Sertoli cell cortex with microfilament bundles; *A*, acrosome; *SL*, subacrosomal layer; *N*, nucleus; *NE*, nuclear envelope. The arrow in *a* denotes the junction between the subacrosomal layer and the calyx region. The arrow in *b* denotes the plasma

related to all cyto- and karyoskeletal proteins described so far. We have also presented evidence to show that the insoluble perinuclear theca is a compositionally complex structure comprising common proteins such as the MBP, as well as at least one protein specific for the calyx. Cytoskeletal proteins similar to calicin and the group of MBP also occur in sperm of rat and man, suggesting that they may be widespread, if not ubiquitous, in mammalian sperm, in agreement with electron microscopic descriptions of perinuclear theca structures in a broad range of species (e.g., 13, 17, 23, 24, 40, 44, 50, 52).

Previously, several authors have noted that on extraction of sperm heads with detergents and high salt buffers under reducing conditions a residual nuclear structure is obtained (8, 9, 53, 57, 65); most authors have ascribed this stability of a residual nuclear cortex to the preservation of intranuclear matrix and/or a nuclear lamina as described for a variety of somatic cells. However, our present finding that the perinuclear theca is the only major structural complex of the spermatozoon resistant to treatments with nondenaturing detergents and a broad range of salt concentrations supports the notion of Bellvé and colleagues (8, 9, 45). These authors have reported that the perinuclear layer that withstands extractions in buffers containing 1% SDS and high ionic strength as well as the removal of nuclear DNA and protamins (190 mM divalent cations) is derived from a structure external to the nuclear envelope and does not contain the nuclear lamins characteristic of somatic cell nuclei (8, 33). The extraction protocols used by these authors were less rigorous than our extractions in 2 M NaCl-containing buffer and, like our extractions in 1 M NaCl, left the perinuclear theca as a more or less continuous layer around the entire nucleus, thus not allowing the separation of calyces.

The discovery of a novel class of basic proteins as the major components of the perinuclear theca as a whole and the calyx specifically provides biochemical support for the morphologically derived concept of the perinuclear theca as a distinct structural entity (8, 17, 40). Clearly, both kinds of thecal proteins, calicin and MBP, are different from all known cytoskeletal proteins, notably IF proteins and nuclear lamins. We cannot explain why these abundant proteins have not been detected earlier in fractions of the similarly extracted preparations of Pruslin and Rodman (53) and in fractions of basic proteins recovered in nuclear residual structures from mouse sperm heads (45, 56). Perhaps interspecies differences also contribute to the differences of composition reported in the literature. On the other hand, the basic character of the major perinuclear theca proteins provides an explanation for previous conclusions, based on cytochemical staining reactions with phosphotungstic acid, that the dense perinuclear material is composed mainly of nonglycosylated, lysine-rich proteins (17, 31, 44), and probably also explains its known argyrophilia (36).

The localization of the abundant MBP in the entire perinuclear theca suggests that they represent a major component of the perinuclear substance, a finely filamentous web common to both the subacrosomal and postacrosomal layers (Fig. 1). Actin has also been localized by some authors to the filamentous web of the subacrosomal layer, including the apicalmost subacrosomal pit, the perforatorium of some rodents and birds (4, 14–16, 25, 26, 59, 64, 68–70). However, as actin is extracted in high salt buffers, we consider it unlikely that this protein makes a quantitatively important contribution to the cytoskeletal theca. Moreover, a cystein-rich polypeptide of M_r 13,000 has been described in fractions of perforatorium material from rat spermatozoa (49), but it does not occur in the subacrosomal material of other species lacking a perforatorium (15). A basic M_r 15,000 murine sperm protein has also been identified in the perinuclear theca but this protein is also extractable in high salt concentrations (57). Acid phosphatase activity has also been reported to occur in the subacrosomal layer (13).

In contrast to the anterior portion of the perinuclear theca, the calyx is characterized by a greater stability and complexity, both in morphological terms and in its protein complement. We have shown that calicin is a major protein of this structural complex, in addition to the MBP and probably other proteins. The topological specificity of the distribution of calicin is remarkable. Its immunofluorescence microscopic distribution correlates with that of a surface-exposed membrane polypeptide of a very similar M_r value, the posterior head protein described in guinea pig spermatozoa (43). It will be important to determine the molecular basis for such domain restrictions within the plasma membrane, restrictions which are also indicated by the higher frequency of membrane-intercalated particles as seen by freeze-cleavage in this area (reviewed in reference 23), and to identify possible cytoskeletal components involved in the establishment and maintenance of such constraints. While our immunoelectron microscopic findings have identified the presence of calicin in patches of material of the postacrosomal layer covering the nucleus, at present we cannot exclude that this protein also contributes to the postacrosomal sheath and the filamentous ridges of the paracrystalline sheet, as accessibility of these tightly packed structures to the gold-coupled antibodies used might have been insufficient.

We have taken great care to examine the presence of structures and proteins similar to those known from somatic cells, notably IF proteins and lamins. The results are negative, and, because of the perplexingly controversial literature, shall be discussed in some detail.

(*a*) IFs have never been reported to occur in spermatids

membrane. The calyx material is demarcated by brackets in *a* and subcomponents of the calyx are shown in *b*: postacrosomal sheet (*arrowhead*); paracrystalline sheet, regularly spaced filamentous ridges seen in cross sections between arrow and arrowhead; postacrosomal layer, dense material on surface of nuclear envelope (*NE*). (*c–f*) Electron microscopic immunogold localization of MBP (*c* and *d*) and calicin (*e* and *f*) using the preembedding procedure on frozen sections of testis (*c*) and isolated spermatozoa (*d* and *f*) and the postembedding procedure on ultrathin sections of Lowicryl-embedded testis (*e*) with gold-conjugated secondary antibodies. The MBP antibodies react intensely with perinuclear theca material of the calyx (indicated by brackets in *c–f*) and less intensely with material of the subacrosomal layer (*d*); *A*, residues of inner acrosomal membrane; the very thin subacrosomal layer probably has not been accessible to the antibodies in the preembedding procedure (*c*). In contrast, the reaction of the calicin antibodies appears to be largely restricted to the thecal material of the calyx (*f*); *A*, as above; *e*, sperm cross-section at the level of the calyx. In the intense labeling shown in *e* the occasional clusters of gold particles in the nucleus are not significant, compared with control preparations. Bars: (*a–f*) 0.2 μm.

and spermatozoa in any of the numerous electron microscopic studies with these cells. The 10–14-nm filaments or ridges of the paracrystalline sheet of the calyx and the 14–15-nm filaments described in the subacrosomal space of certain spermatid stages (e.g., 59) differ in their structural details from IFs and are not decorated with antibodies to IF proteins (see also reference 50). The 11–13-nm filaments located between the acrosome and the plasma membrane in certain regions of hamster sperm heads (48) also look different from IFs. Our finding of a lack of reactivity of antibody IFA (54), which reacts with all classes of IF proteins, including their homologues in several invertebrates (6), also speaks against the presence of IFs and IF proteins. (*b*) Cytokeratin IFs have been identified in truly epithelial components of the testis as the *rete testis* (1, 42) and in the myoid cells of the bull testis (this study), but not in spermatogenic and Sertoli cells of the seminiferous tubules of mature testes of various species (1, 27, 50, 51, 55, 61, 67–69; for certain pathological situations see reference 42). Reports of the occurrence of certain antigens believed to cross-react with cytokeratins in the acrosomal region (46) or the tail (5) of ejaculated spermatozoa are based on the use of antibodies of unknown specificity and have not identified the keratinous nature of the testicular antigen. (*c*) Our present study and most reports in the literature agree that in mature testis vimentin IFs are expressed in myoid and Sertoli cells but are absent from spermatids and spermatozoa (27, 51, 55, 67). The significance of reports that certain vimentin antibodies react with the equatorial segment of ejaculated human spermatozoa (46, 68) or in the postacrosomal sheath (5) remains unclear, and the minuscule amounts of antigen reported might be due to the notorious contaminations of ejaculates by nonspermiogenic material. Negative results were also obtained when testicular tissue was examined by the same group (69). In summary, from the weight of previously published experimental evidence and that presented here we conclude that IF proteins are either not expressed during spermiogenesis or are present in amounts too low to be detected.

Our findings by immunoblotting, immunocytochemistry, and peptide mapping that proteins immunologically and biochemically similar to nuclear lamins of somatic cells are not expressed, or are present only in undetectable amounts, in spermiogenic cells of bull and rat are in agreement with several previous reports of an absence, in avian and mammalian spermatids and spermatozoa, of a nuclear lamina structure and of karyoskeletal proteins identical with, or closely related to, lamins of somatic cells (8, 22, 33, 38, 63). Only sparse and spotty immunofluorescence with lamin antibodies on murine sperm has been reported by Schatten et al. (60), who concluded that lamins are vastly reduced during spermiogenesis. In contrast, Pruslin and Rodman (53) and Maul et al. (41) have reported the presence of lamin-related proteins in mouse sperm heads, as judged from immunofluorescence staining and gel electrophoretic positions, but without positive identification of the individual polypeptides localized. At present this discrepancy cannot be satisfactorily resolved. We do not exclude the possibility that low amounts of cell type–specific lamin(s), which have epitopes in common with somatic lamins recognized by the antibodies used by some authors (41, 53) but not by the antibodies used by us and others, may be expressed during spermiogenesis. Indeed, a spermatogenesis-specific nuclear lamin has been described in *Xenopus laevis* by Benavente and Krohne (10).

Therefore, we conclude that the largest contribution to the densely webbed residual structures of sperm heads is made by the perinuclear theca and, hence, the basic cytoskeletal proteins described in this paper. It is tempting to speculate that both kinds of proteins, calicin and MBP, are involved in spermiogenesis in that they contribute to the reduction of the cytoplasm in the forming sperm head and to the compression of the residual cytoplasm into the densely woven web, which practically represents the only vestigial cytoplasm left above the posterior ring. They may also contribute to the nuclear–acrosome association and provide some sort of intermembranous cement (cf. 8 and 17). In discussing possible functions of these proteins it should also be remembered that it is the posterior aspect of the sperm head, including the calyx region, which makes contact and fuses with the egg plasma membrane (e.g., 7, 62, and 73), so that possible contributions of thecal proteins to the fertilization process should also be taken into consideration.

We gratefully acknowledge the essential technical assistance of Christine Grund, Cäcilie Kuhn, and Stefanie Winter and the careful typewriting of Friederike Schmitt. We also thank Dr. Ricardo Benavente (Heidelberg) for valuable discussions.

This work was performed during Dr. Longo's sabbatical year at the German Cancer Research Center and has been supported in part by the German Research Foundation (Deutsche Forschungsgemeinschaft, DFG.) Dr. Krohne is a grant fellow of the Heisenberg fund of the DFG (Kr 758/3-1; 758/2-1).

Received for publication 24 March 1987, and in revised form 11 May 1987.

References

1. Achtstaetter, T., R. Moll, B. Moore, and W. W. Franke. 1985. Cytokeratin polypeptide patterns of different epithelia of the human male urogenital tract: immunofluorescence and gel electrophoretic studies. *J. Histochem. Cytochem.* 33:415–426.
2. Achtstaetter, T., M. Hatzfeld, R. A. Quinlan, D. C. Parmelee, and W. W. Franke. 1986. Separation of cytokeratin polypeptides by gel electrophoretic and chromatographic techniques and their identification by immunoblotting. *Methods Enzymol.* 134:355–371.
3. Baccetti, B., V. Pallini, and A. G. Burrini. 1976. The accessory fibers of the sperm tail. III. High-sulfur and low-sulfur components in mammals and cephalopods. *J. Ultrastruct. Res.* 57:289–308.
4. Baccetti, B., E. Bigliardi, and A. G. Burrini. 1980. The morphogenesis of vertebrate performatorium. *J. Ultrastruct. Res.* 71:272–287.
5. Baccetti, B., E. Bigliardi, A. G. Burrini, G. Gabbiani, B. Jockusch, and P. Leoncini. 1984. Microfilaments and intermediate sized filaments in sperm tail. *J. Submicrosc. Cytol.* 16:79–84.
6. Bartnik, E., M. Osborn, and K. Weber. 1985. IFs in non-neuronal cells of invertebrates: isolation and biochemical characterization of keratin-like intermediate filaments from the esophageal epithelium of the mollusc *Helix pomatia*. *J. Cell Biol.* 101:427–440.
7. Bedford, J. M., H. D. M. Moore, and L. E. Franklin. 1979. Significance of the equatorial segment of the acrosome of the spermatozoon in eutherian mammals. *Exp. Cell Res.* 119:119–126.
8. Bellvé, A. R., and D. A. O'Brien. 1983. The mammalian spermatozoon: structure and temporal assembly. *In* Mechanism and Control of Animal Fertilization. J. F. Hartmann, editor. Academic Press, Inc., New York. 55–137.
9. Bellvé, A. R., E. Anderson, and L. Hanley-Bowdoin. 1975. Synthesis and amino acid composition of basic proteins in mammalian sperm nuclei. *Dev. Biol.* 47:349–365.
10. Benavente, R., and G. Krohne. 1985. Change of karyoskeleton during spermatogenesis of *Xenopus*: expression of lamin L_{IV}, a nuclear lamina protein specific for the male germ line. *Proc. Natl. Acad. Sci. USA.* 82: 6176–6180.
11. Benavente, R., and G. Krohne. 1986. Involvement of nuclear lamins in postmitotic reorganization of chromatin as demonstrated by microinjection of lamin antibodies. *J. Cell Biol.* 103:1847–1854.
12. Benavente, R., G. Krohne, and W. W. Franke. 1985. Cell type-specific expression of nuclear lamina proteins during development of *Xenopus laevis*. *Cell.* 41:177–190.

13. Bernstein, M. H., and R. J. Teichman. 1972. Regional differentiation in the heads of spermatozoa of rabbit, man and bull. *Am. J. Anat.* 133: 165–178.
14. Camatini, M., G. Anelli, and A. Casala. 1986. Identification of actin in boar spermatids and spermatozoa by immunoelectron microscopy. *Eur. J. Cell Biol.* 42:311–318.
15. Campanella, C., G. Gabbiani, B. Baccetti, A. G. Burrini, and V. Pallini. 1979. Actin and myosin in the vertebrate acrosomal region. *J. Submicrosc. Cytol.* 11:53–71.
16. Clarke, G. N., F. M. Clarke, and S. Wilson. 1982. Actin in human spermatozoa. *Biol. Reprod.* 26:319–327.
17. Courtens, J. L., M. Courot, and J. E. Fléchon. 1976. The perinuclear substance of boar, bull, ram and rabbit spermatozoa. *J. Ultrastruct. Res.* 57:54–64.
18. Cowin, P., H.-P. Kapprell, W. W. Franke, J. Tamkun, and R. O. Hynes. 1986. Plakoglobin: a protein common to different kinds of intercellular adhering junctions. *Cell.* 46:1063–1073.
19. Elder, J. H., R. A. Pickett, J. Hampton, and R. A. Lerner. 1977. Radioiodination of proteins in single polyacrylamide slices. *J. Biol. Chem.* 25:6510–6515.
20. Escalier, D. 1984. The cytoplasmic matrix of the human spermatozoon: cross-filaments link the various cell components. *Biol. Cell.* 51:347–364.
21. Fairbanks, G. T., T. L. Steck, and D. F. H. Wallach. 1971. Electrophoretic analysis of major polypeptides of the human erythrocyte membrane. *Biochemistry.* 10:2606–2617.
22. Fawcett, D. W. 1966. On the occurrence of a fibrous lamina on the inner aspect of the nuclear envelope in certain cells of vertebrates. *Am. J. Anat.* 119:129–146.
23. Fawcett, D. W. 1975. The mammalian spermatozoon. *Dev. Biol.* 44: 394–436.
24. Fawcett, D. W., and S. Ito. 1965. The fine structure of bat spermatozoa. *Am. J. Anat.* 116:567–610.
25. Flaherty, S. P., W. G. Breed, and V. Sarafis. 1983. Localization of actin in the sperm head of the plains mouse, *Pseudomys australis. J. Exp. Zool.* 225:497–500.
26. Franke, W. W., C. Grund, A. Fink, K. Weber, B. M. Jockusch, H. Zentgraf, and M. Osborn. 1978. Location of actin in the microfilament bundles associated with the junctional specialisations between Sertoli cells and spermatids. *Biol. Cell.* 31:7–14.
27. Franke, W. W., C. Grund, and E. Schmid. 1979. Intermediate sized filaments present in Sertoli cells are of the vimentin type. *Eur. J. Cell Biol.* 19:269–275.
28. Franke, W. W., E. Schmid, S. Winter, M. Osborn, and K. Weber. 1979. Widespread occurrence of intermediate-sized filaments of vimentin-type in cultured cells from diverse vertebrates. *Exp. Cell Res.* 123:633–654.
29. Franke, W. W., D. L. Schiller, R. Moll, S. Winter, E. Schmid, I. Engelbrecht, and H. Denk. 1981. Diversity of cytokeratins. Differentiation specific expression of cytokeratin polypeptides in epithelial cells and tissues. *J. Mol. Biol.* 153:933–959.
30. Gigi, O., B. Geiger, Z. Eshhar, R. Moll, E. Schmid, S. Winter, D. L. Schiller, and W. W. Franke. 1982. Detection of a cytokeratin determinant common to diverse epithelial cells by a broadly cross-reacting monoclonal antibody. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:1429–1437.
31. Gordon, M. 1969. Localization of the "apical body" in guinea pig and human spermatozoa with phosphotungstic acid. *J. Reprod. Fertil.* 19: 367–369.
32. Holthoefer, H., A. Miettinen, R. Paasivuo, V.-P. Lehto, E. Linder, O. Alfthan, and I. Virtanen. 1983. Cellular origin and differentiation of renal carcinomas. *Lab. Invest.* 49:317–326.
33. Ierardi, L. A., S. B. Moss, and A. R. Bellvé. 1983. Synaptonemal complexes are integral components of the isolated mouse spermatocyte nuclear matrix. *J. Cell Biol.* 96:1717–1726.
34. Kleinschmidt, J. A., and W. W. Franke. 1982. Soluble acidic complexes containing histones H3 and H4 in nuclei of *Xenopus laevis* oocytes. *Cell.* 29:799–809.
35. Koehler, J. K. 1973. Studies on the structure of the postnuclear sheath of water buffalo spermatozoa. *J. Ultrastruct. Res.* 44:355–368.
36. Krimer, D. B., and P. Esponda. 1978. Preferential staining of the postacrosomal lamina of mouse spermatids. *Mikroskopie.* 34:55–59.
37. Krohne, G., R. Stick, J. A. Kleinschmidt, R. Moll, W. W. Franke, and P. Hausen. 1982. Immunological localization of a major karyoskeletal protein in nucleoli of oocytes and somatic cells of *Xenopus laevis. J. Cell Biol.* 94:749–754.
38. Krohne, G., E. Debus, M. Osborn, K. Weber, and W. W. Franke. 1984. A monoclonal antibody against nuclear lamina proteins reveals cell type-specificity in *Xenopus laevis. Exp. Cell Res.* 150:47–59.
39. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
40. Lalli, M., and Y. Clermont. 1981. Structural changes of the head components of the rat spermatid during late spermiogenesis. *Am. J. Anat.* 160:419–434.
41. Maul, G. G., B. T. French, and K. B. Bechtol. 1986. Identification and redistribution of lamins during nuclear differentiation in mouse spermatogenesis. *Dev. Biol.* 115:68–77.
42. Miettinen, M., I. Virtanen, and A. Talerman. 1985. Intermediate filament proteins in human testis and testicular germ-cell tumors. *Am. J. Pathol.* 120:402–410.
43. Myles, D. G., P. Primakoff, and A. R. Bellvé. 1981. Surface domains of the guinea pig sperm defined with monoclonal antibodies. *Cell.* 23: 433–439.
44. Nicander, L., and A. Bane. 1966. Fine structure of the sperm head in some mammals, with particular reference to the acrosome and the subacrosomal substance. *Z. Zellforsch. Mikrosk. Anat.* 72:496–515.
45. O'Brien, D. A., and A. R. Bellvé. 1980. Protein constituents of the mouse spermatozoon. *Dev. Biol.* 75:386–404.
46. Ochs, D., D. P. Wolf, and R. L. Ochs. 1986. Intermediate filament proteins in human sperm heads. *Exp. Cell Res.* 167:495–504.
47. O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell. 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell.* 12:1133–1142.
48. Olson, G. E., and V. P. Winfrey. 1985. Substructure of a cytoskeletal complex associated with the hamster sperm acrosome. *J. Ultrastruct. Res.* 92:167–179.
49. Olson, G. E., D. W. Hamilton, and D. W. Fawcett. 1976. Isolation and characterization of the perforatorium of rat spermatozoa. *J. Reprod. Fertil.* 47:293–397.
50. Olson, G. E., T. D. Noland, V. P. Winfrey, and D. L. Garbers. 1983. Substructure of the postacrosomal sheath of bovine spermatozoa. *J. Ultrastruct. Res.* 85:204–218.
51. Paranko, J., M. Kallajoki, L. J. Pelliniemi, V.-P. Lehto, and I. Virtanen. 1986. Transient coexpression of cytokeratin and vimentin in differentiating rat Sertoli cells. *Dev. Biol.* 117:35–44.
52. Pedersen, H. 1972. The postacrosomal region of the spermatozoa of man and *Macaca arctoides. J. Ultrastruct. Res.* 40:366–377.
53. Pruslin, F. H., and T. C. Rodman. 1983. Proteins of de-membranated, protamine-depleted mouse sperm. *Exp. Cell Res.* 144:115–126.
54. Pruss, R. M., R. Mirsky, M. C. Ruff, R. Thorpe, A. J. Dowding, and B. H. Anderton. 1981. All classes of intermediate filaments share a common antigenic determinant defined by a monoclonal antibody. *Cell.* 27:419–428.
55. Ramaekers, F., A. Huysmans, O. Moesker, A. Kant, P. Jap, C. Herman, and P. Vooijs. 1983. Monoclonal antibody to keratin filaments, specific for glandular epithelia and their tumors. *Lab. Invest.* 49:353–361.
56. Rodman, T. C., F. H. Pruslin, H. P. Hoffmann, and V. G. Allfrey. 1981. Turnover of basic chromosomal proteins in fertilized eggs: a cytoimmunochemical study of events *in vivo. J. Cell Biol.* 90:351–361.
57. Rodman, T. C., F. H. Pruslin, N. Chiorazzi, M. A. Michelis, and R. Winston. 1983. p15, a nucleus-associated protein of human sperm: identification of four variants and their occurrence in normal and abnormal seminal cells. *Gamete Res.* 8:129–147.
58. Roth, J. M., M. Bendayan, E. Carlemalm, W. Villinger, and M. Garavito. 1981. Enhancement of structural preservation and immunocytochemical staining in low temperature embedded pancreatic tissue. *J. Histochem. Cytochem.* 29:663–671.
59. Russel, L. D., J. E. Weber, and A. W. Vogl. 1986. Characterization of filaments within the subacrosomal space of rat spermatids during spermiogenesis. *Tissue & Cell.* 18:887–898.
60. Schatten, G., G. G. Maul, H. Schatten, N. Chaly, C. Simerly, R. Balczon, and D. L. Brown. 1985. Nuclear lamins and peripheral nuclear antigens during fertilization and embryogenesis in mice and sea urchins. *Proc. Natl. Acad. Sci. USA.* 82:4727–4731.
61. Schlegel, R., S. Banks-Schlegel, and G. S. Pinkus. 1980. Immunohistochemical localization of keratin in normal human tissues. *Lab. Invest.* 42: 91–96.
62. Stefanini, M., C. Oura, and L. Zamboni. 1969. Ultrastructure of fertilization in the mouse. 2. Penetration of sperm into the ovum. *J. Submicrosc. Cytol.* 1:1–23.
63. Stick, R., and H. Schwarz. 1982. The disappearance of the nuclear lamina during spermatogenesis: an electron microscopic and immunofluorescence study. *Cell Differ.* 11:235–243.
64. Tamblyn, T. M. 1980. Identification of actin in boar epididymal spermatozoa. *Biol. Reprod.* 22:727–734.
65. Tsanev, R., and Z. Avramova. 1981. Nonprotamine nucleoprotein ultrastructures in mature ram sperm nuclei. *Eur. J. Cell Biol.* 24:139–145.
66. Tseng, S. C. G., M. J. Jarvinen, W. G. Nelson, J.-W. Huang, J. Woodcock-Mitchell, and T.-T. Sun. 1982. Correlation of specific keratins with different types of epithelial differentiation: monoclonal antibody studies. *Cell.* 30:361–372.
67. Van Vorstenbosch, C. J. A. H. V., B. Colenbrander, C. J. G. Wensing, F. C. S. Ramaekers, and G. P. Vooijs. 1984. Cytoplasmic filaments in fetal and neonatal pig testis. *Eur. J. Cell Biol.* 34:292–299.
68. Virtanen, I., R. A. Badley, R. Paasivuo, and V.-P. Lehto. 1984. Distinct cytoskeletal domains related in sperm cells. *J. Cell Biol.* 99:1083–1091.
69. Virtanen, I., M. Kallajoki, O. Närvänen, J. Paranko, L.-E. Thornell, M. Miettinen, and V.-P. Lehto. 1986. Peritubular myoid cells of human and rat testis are smooth muscle cells that contain desmin-type intermediate

filaments. *Anat. Rec.* 215:10–20.
70. Welch, J. E., and M. C. O'Rand. 1985. Identification and distribution of actin in spermatogenic cells and spermatozoa of the rabbit. *Dev. Biol.* 109:411–417.
71. Wiedenmann, B., and W. W. Franke. 1985. Identification and localization of synaptophysin, an integral membrane glycoprotein of M_r 38,000 characteristic of presynaptic vesicles. *Cell.* 41:1017–1028.
72. Wooding, F. B. P. 1973. The effect of Triton X-100 on the ultrastructure of ejaculated bovine sperm. *J. Ultrastruct. Res.* 42:502–516.
73. Yanagimachi, R., and Y. D. Noda. 1970. Electron microscope studies of sperm incorporation into the golden hamster egg. *Am. J. Anat.* 128: 429–462.