LIFE HISTORY OF MOUSE SPERM PROTEIN

Intratesticular Stages

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ABSTRACT

A basic protein fraction, migrating as a single band in acetic acid-urea gel, distinct from histones, was isolated from mouse sperm collected from vasa deferentia and caudae epididymides and was used to immunize female rabbits. The presence of antibodies to the mouse sperm protein (MSP) in the rabbit antisera was demonstrated by a cytoimmunofluorescence procedure using the cells of origin of the antigenic protein, the mature mouse sperm. The specificity of the antisera was verified by fluid and gel precipitation tests and by crossed immunoelectrophoresis. The latter procedure demonstrated the presence of two antigen-antibody systems, consonant with earlier reports that the basic chromosomal protein of mouse sperm is heterogeneous.

MSP antigen *in situ* was recognized by the specific antibodies of the rabbit antisera only after the smear of mature sperm was treated with either of two reducing agents: 2-mercaptoethanol or dithiothreitol. However, when the immunofluorescence procedure was applied to untreated smears of mouse testicular cells, spermatids of all stages from 1 to 14–15 were positive, while spermatocytes, stage 16 spermatids and spermatozoa were negative. After treatment of testes smears with reducing agent, only spermatocytes remained negative.

Those observations indicate the following: (*a*) MSP is immunogenic in a heterologous species; (*b*) its antigenic sites are detectable in spermatozoa and spermatids of all stages, but not in primary spermatocytes; (*c*) those antigenic sites become masked at about stage 15 of spermiogenesis and may be unmasked by treatment with a reducing agent. The interpretation is made, therefore, that one or more components of MSP are assembled at the beginning of spermiogenesis and undergo an alteration in the final intratesticular stage of spermatid maturation. That alteration may be presumed to be the formation of disulfide linkages between the cysteine residues.

KEY WORDS sperm · protein · cytoimmunofluorescence · spermiogenesis · sulfhydryl · spermatogenesis

In the maturation phase of mammalian spermatogenesis, two unique sequences of differentiation occur: morphogenesis of the maturing gamete and turnover of the basic chromosomal proteins. Surprisingly, for so conservative a process as spermatogenesis, the products of those sequences display marked species variability.

In all species, the cell arising from the second

meiotic division, the round spermatid, progressively evolves into the intricate species-specific conformation of the spermatozoon. Concurrently, the basic nucleoproteins are altered and the haploid chromosome complement becomes exceedingly compact. Those alterations have been biochemically characterized for a number of species and, in general, include the replacement of somatic-type histones with smaller basic proteins of very high arginine and considerable cysteine content and, subsequently, the formation of disulfide cross-linkages (6). The broad similarities of the various sperm proteins, however, are overlaid by the species-specificity of their amino acid composition (2, 3).

Those two phases of mammalian spermiogenesis, morphologic transformation and turnover of nucleoproteins, and their interrelationships present an interesting system in which to study the molecular bases of an isolated sequence of differentiation as well as mechanisms of spermatogenesis. This series of studies was designed, therefore, to investigate that system, with first attention directed to the life history of the sperm protein of the mouse (MSP).

In previous studies, the temporal relationships between the nuclear changes and the stage of spermatid maturation have been extrapolated from biochemical analyses and on the basis of the anatomical compartment of the male reproductive tract from which the analyzed population of cells was obtained (2, 4, 11, 18). Thus, there is some ambiguity as to the stage at which the specific sperm protein first appears and as to whether the sperm protein itself, its precursor, or other unique histones are present in testicular spermatogenic cells (13, 15, 16, 20). That ambiguity may arise from the fact that there appear to be two classes of nucleoprotein in mature sperm of mammals: that in which the basic protein moiety is homogeneous, e.g., guinea pig (2, 3) and rat (3, 11), and that in which there are two or more closely related but unique proteins, e.g., mouse (2, 3, 7) and man (3, 14).

Since the presence of the disulfide bonds on the sperm protein has been demonstrated in epididymal sperm (4, 18), it has been considered that oxidation of the sulfhydryl groups on the cysteine residues takes place after the sperm leave the testes (1, 4), suggesting that that phase of sperm maturation may be dependent upon the environment of some region of the excurrent duct system. An alternative consideration, however, supported by the observations reported here, is that the progression of spermatid differentiation may not be dependent alone upon a change in the extracellular milieu but is intrinsic in that cell, following a program of commitment initiated by assembly of the sperm nuclear protein.

The materials of these studies were developed for high sensitivity detection in cytologic situ of MSP. This report specifically deals with the demonstration, by immunocytochemical procedures, of the cell type in spermatogenesis in which MSP appears and the stage in spermiogenesis at which the disulfide linkages between its cysteine residues are established.

MATERIALS AND METHODS

Isolation of MSP

MSP was isolated from spermatozoa of vasa deferentia and caudae epididymides by a modification of the procedure originally described by Bellvé et al. (2). The sperm samples were washed three times in 0.075 M NaCl and 0.024 M EDTA (S-EDTA) by resuspension and centrifugation at 2,000 *g* for 5 min. The pellet was suspended in S-EDTA buffer containing 1% of SDS and homogenized (20 stokes, Teflon to glass), and the homogenate was filtered through Nitex cloth, 84 mesh. A pellet was recovered by centrifugation at 200 *g* for 5 min, resuspended in S-EDTA-sodium dodecyl sulfate (SDS) buffer, and the suspension was layered over a cushion of 1.6 M sucrose in S-EDTA-SDS buffer in an SW-27 Beckman rotor tube (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The preparation was centrifuged at 10,000 rpm for 30 min, and a pellet was recovered which contained sperm heads with a low level of contamination with sperm tails. The tails were removed by resuspending the pellet in S-EDTA-SDS and repeating the centrifugation. At this stage of purification, no tails were detectable upon microscope examination. The sperm protein was extracted from the sperm head preparation with 4 M guanidinium chloride (GCl), 50 mM dithiothreitol (DTT) in 0.5 M Tris, pH 8.5, followed by addition of HCl to a final concentration of 0.2 M. The solution was clarified by centrifugation at 15,000 *g* for 30 min, and the sperm protein was desalted by gel filtration on a Sephadex G-25 column (1 × 20 cm), equilibrated, and eluted with 50 mM HCl. An aliquot of each preparation was extensively dialyzed against 9 M urea, 0.9 M acetic acid and 1% 2-mercaptoethanol (BME) before electrophoresis on acetic acid-urea gel (23) (Fig. 1*B*). In each instance, a single band was seen. When similar extraction, dialysis and electrophoresis procedures were carried out on the whole sperm suspension (vas deferens contents), the gel showed a large number of bands including those corresponding to

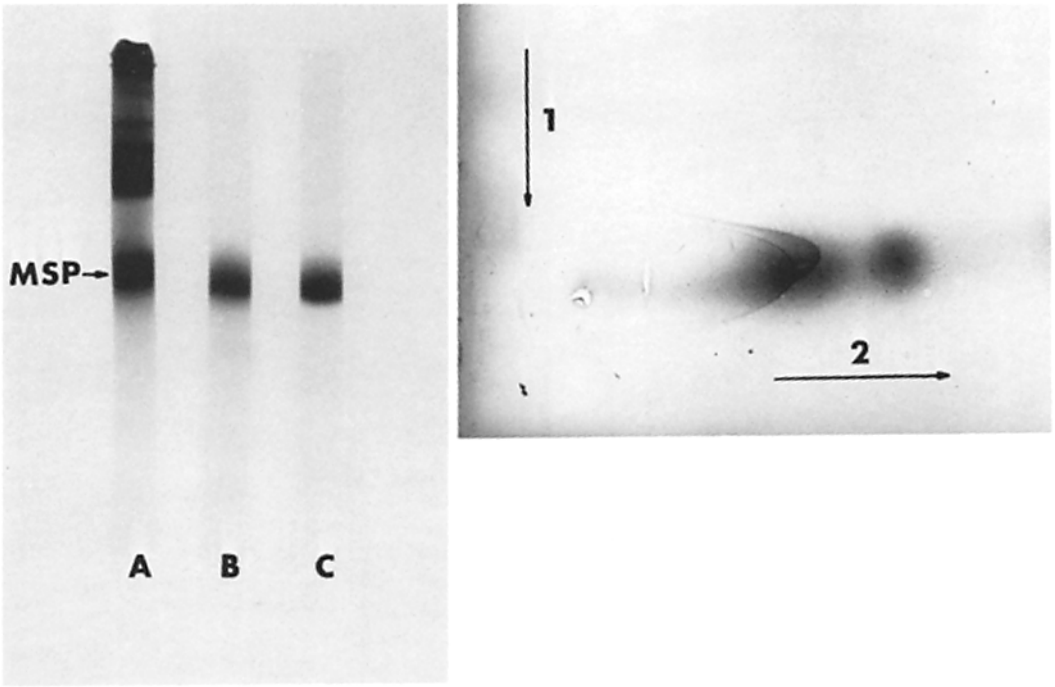


FIGURE 1 Electrophoretic analysis on acetic acid-urea gel of (*A*) contents of vasa deferentia of 5 mice; (*B*) 100 μg of the basic protein fraction isolated from sperm heads (*MSP*); (*C*) 100 μg of S-methylated MSP (see Materials and Methods). Since the gels were electrophoresed at the same time and under the same conditions, the fast moving band at coincident positions is identified as MSP in all three gels. The absence of other bands in *B* and *C* indicate that the fraction isolated as MSP was free of contamination by the other components of the crude suspension displayed in *A*. The slow moving bands in *A* probably represent a miscellany of sperm membrane proteins, histones from somatic cells, etc.

FIGURE 2 Two-dimensional immunoelectrophoresis of S-methylated MSP and anti-MSP antiserum (see Materials and Methods). The direction of electrophoresis in each of the two runs is shown by the arrows. A gel slice (corresponding to the electropherogram of Fig. 1*C*), obtained by electrophoresis of S-methylated MSP in the first dimension, was positioned on a gel containing anti-MSP antiserum and electrophoresed in the second dimension. The two precipitin arcs indicate that two immunologic systems are present, and their positions indicate that the antigenic components of both reside in MSP.

somatic cell histones, and a well separated fast migrating band corresponding to the single band seen on the electropherogram of the purified sperm protein (Fig. 1*A*). The fraction corresponding to that band designated in these studies as MSP was used for the immunization series. Amino acid analysis of MSP demonstrated its correspondence with the fraction characterized as mouse SCP by Bellvé et al. (2) and mouse protamine(s) by Calvin (3) (Table I).

Preparation of Anti-MSP Antiserum

Mature New Zealand white female rabbits were injected intradermally with 0.5 ml of an emulsion of complete Freund's adjuvant containing 0.2 mg of MSP per dose. The injection series consisted of three doses at monthly intervals, three month rest and, finally, two boosters (0.2 mg of MSP) 1 wk apart. The presumptive immune sera were periodically tested for antibody by the indirect immunofluorescence method carried out on mature sperm. Specific antibody was detected 3 wk after the last injection. Quantitation of antibody was not carried out, but visual approximation of intensity of fluorescence indicated that the titer continued to rise for ~6 wk after the first positive testing.

Tests for Specificity of Antisera

Because of the high degree of insolubility of MSP, it was necessary to convert it to its soluble S-methylated derivative to carry out immunochemical tests for antibody specificity. The S-methylated protein was prepared

TABLE I

Amino Acid Composition of Basic Protein(s) Isolated from Mature Mouse Sperm

	SCP (Bellve et al., 1975)	Mouse protamine (Calvin, 1976)	MSP (this report)
Arg	47.0	52.6	48.2
Lys	4.2	5.2	4.0
His	12.3	12.7	12.3
Glu	0	1.0	0
Asp	0	0.5	0
Ala	1.3	1.2	1.2
Gly	4.0	3.5	4.2
Leu	1.8	1.3	1.7
Ile	2.5	1.7	2.3
Val	0	0	0
Ser	12.2	8.1	11.3
Thr	1.5	1.1	1.4
Met	0	0	0
Cys	9.6	10.5	10.0
Pro	0	0	0
Trp	0	ND	ND
Tyr	3.6	2.2	3.4
Phe	0	0	0

Values expressed as mol/100 mol amino acids recovered after acid hydrolysis.

by the method of Heinrikson (12) as modified by Coelingh et al. (5), and its identity with MSP was verified by acetic acid-urea gel electrophoresis (Fig. 1 *C*).

Crossed immunoelectrophoresis (17) was carried out as follows: 5 μg of S-methylated MSP in 1.5% agarose in veronal buffer, pH 9.2, was run in the first dimension for 1 h at 180 V, 50 mA. A 0.75-cm strip of the gel containing the entire pathway of the electrophoresed S-methylated MSP was cut out and placed on the surface of an agarose gel containing 50 μl of anti-MSP antiserum per 0.5 ml of the agarose solution. Electrophoresis was then run in the second dimension (at an angle of 90° to the first dimension) for 18 h at 25 V, 7.5 mA. Similarly, a control test with 50 μl of preimmune serum per 0.5 ml was run. The plates were washed for 48 h in 0.1% Triton X, then stained with amido black (50 ml of ethanol, 25 ml of acetic acid, 25 ml of H_2O, 0.1 g of amido black) (Fig. 2).

The Ouchterlony double diffusion method was carried out in agarose gel (1% in 10 mM borate buffer, pH 7.2). Serial dilutions (1:1 to 1:16) of antiserum, undiluted preimmune serum from the same rabbit, and a sample (15 μg) of S-methylated MSP were placed in the wells. The plates were incubated for 6 h at 37°C, then washed for 3 d in borate buffer with 0.1% Triton X-100 (Fig. 3).

A fluid precipitate test was carried out using 100-3 μg of S-methylated MSP in a volume of 150 μl of 10 mM borate buffer, pH 7.2, plus 200 μl of antiserum or preimmune serum (from the same rabbit). After incubation for 1 h at 37°C, followed by 12 h at 4°C, the precipitate in each tube was collected by 10-min centrifugation at 5,000 *g*, washed three times in borate buffer, dissolved in 0.7 M NaOH, and the OD 280 was plotted (Fig. 4).

Immunocytochemical Procedures

CYTOLOGIC PREPARATIONS: For study of mature spermatozoa, a drop of the contents of a vas deferens, suspended in a few drops of phosphate-buffered saline (PBS), was placed on a slide and allowed to air dry. When fixation was used, the slide was flooded with methanol/acetic acid (3:1), allowed to stand for 10 min, drained, and air dried. For study of the spermatogenic series, the cut and teased surface of a testis was lightly pressed against a slide and the smear was fixed, before drying, with methanol/acetic acid.

"UNMASKING" OF ANTIGENIC SITES ON MSP: The smear was immersed for 3–4 min in a 1:2 dilution (with PBS) of a solution of 4 M GCl, 0.5 M Tris buffer, 1% BME (pH 8.5), then washed in a large volume of PBS followed by distilled water and allowed to air-dry before the test serum was applied. Alternatively, the smear was immersed for 1 h in 3×10^{-3} M DTT in PBS, then washed and dried, as described above.

INDIRECT IMMUNOFLUORESCENCE METHOD: A drop of normal rabbit serum (NRS), i.e., pooled preimmune sera from three of the rabbits used for MSP immunization, or a drop of anti-MSP antiserum was

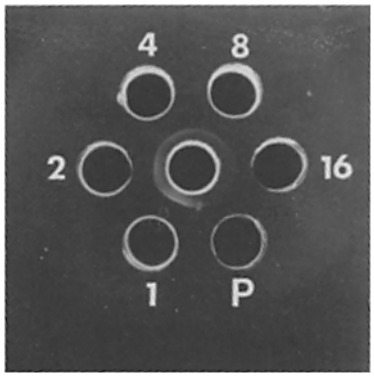


FIGURE 3 Ouchterlony plate of S-methylated MSP tested against serial dilutions of anti-MSP antiserum and preimmune serum from the same rabbit. *1* = 1:1, *2* = 1:2, *4* = 1:4, *8* = 1:8, *16* = 1:16 dilution of antiserum; *p* = undiluted preimmune serum. The center well contained 15 μg of S-methylated MSP. The precipitation band related to the two lowest dilutions is readily apparent, then diminishes in density with increasing dilution. There is no precipitation related to the well with preimmune serum.

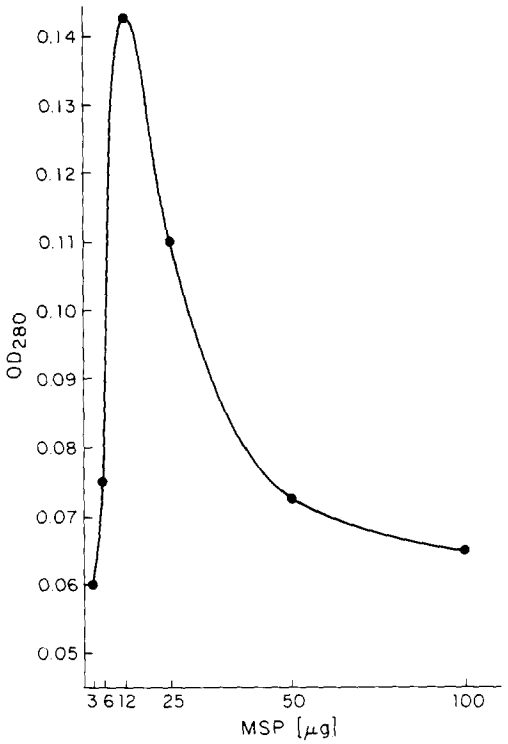


FIGURE 4 Plot of OD 280 of precipitate obtained with increasing amounts (3.0–100 μg) of S-methylated MSP in a constant volume (200 μl) of anti-MSP antiserum. Maximum precipitation was seen with 12 μg, followed by rapid decline with increment in excess antigen.

placed on the dry smear, covered with a cover slip, and the slide incubated in a moist chamber at 25°C for 1 h. The coverslip was removed and the smear was washed in five changes of PBS. The slide was drained, but not allowed to dry, and a drop of a 1:5 dilution of anti-rabbit fluorescein-conjugated goat serum was placed on the smear and covered. A control preparation, using fluorescein-conjugated goat serum but no rabbit serum, was included in each series. After incubation in a moist chamber at 25°C for 1 h, the slide was washed in five changes of PBS, pH 7.1, and mounted in PBS, pH 7.6.

MICROSCOPY AND PHOTOMICROSCOPY: The specimens were examined and photographed in the Zeiss Photomicroscope (Carl Zeiss, Inc., N. Y.). Each field was photographed first, with a tungsten lamp, phase contrast optics, and automatic exposure set for ASA 400 (Kodak tri-x film) and, secondly, with a mercury lamp, fluorescein isothiocyanate (FITC) excitation filter (absorption maximum 500 nM), barrier filter 53, and manually controlled 30-s exposure. For the ×40 objective, the smear was mounted and photographed in PBS. For the ×100 objective, the smear was rapidly dehydrated in ethanol, passed through xylene, and mounted in nonfluorescing immersion oil.

RESULTS

Antigen-Antibody Specificity

The specificity of the MSP antiserum was confirmed in all serological tests (see Materials and Methods). When the S-methylated MSP was tested against the anti-MSP antiserum by the method of two-dimensional immunoelectrophoresis, a single broad band was obtained in the first electrophoresis. Upon electrophoresis in the second dimension, the components of that band interacted with the anti-MSP antiserum to produce two precipitin arcs (Fig. 2). The shape and position of the arcs indicate that the immunologic recognition by the antiserum is that of two antigenic components of MSP which are similar in their migration properties and, consequently, may be presumed to be two closely related molecular species. That observation is consonant with other reports (2, 3) indicating that the basic chromosomal protein of mouse sperm contains two closely related, but unique, proteins. No precipitin lines were obtained when the electrophoresed S-methylated MSP was run into the gel containing preimmune serum from the same rabbit.

The Ouchterlony double diffusion gel precipitation test (25) was carried out utilizing serial dilutions of antiserum and preimmune serum from the same rabbit and, as antigen, S-methylated protein derived from the sample of MSP used in the immunization. Precipitation bands were formed with the 1:1, 1:2, and 1:4 dilutions of antiserum but not with the undiluted preimmune serum (Fig. 3).

The plot of the data of the fluid precipitation test produced a curve with a sharp peak at 12 μg of S-methylated MSP, typical of that produced when a purified soluble antigen is tested against its specific antiserum (25). No precipitate was obtained in any tube of the control test with preimmune serum.

Cytoimmunofluorescence

When either fixed or unfixed, but otherwise untreated, vas deferens sperm were tested by the indirect immunofluorescence procedure, only the acrosomal and tail membranes were stained (Fig. 5). That staining was variable in intensity and was

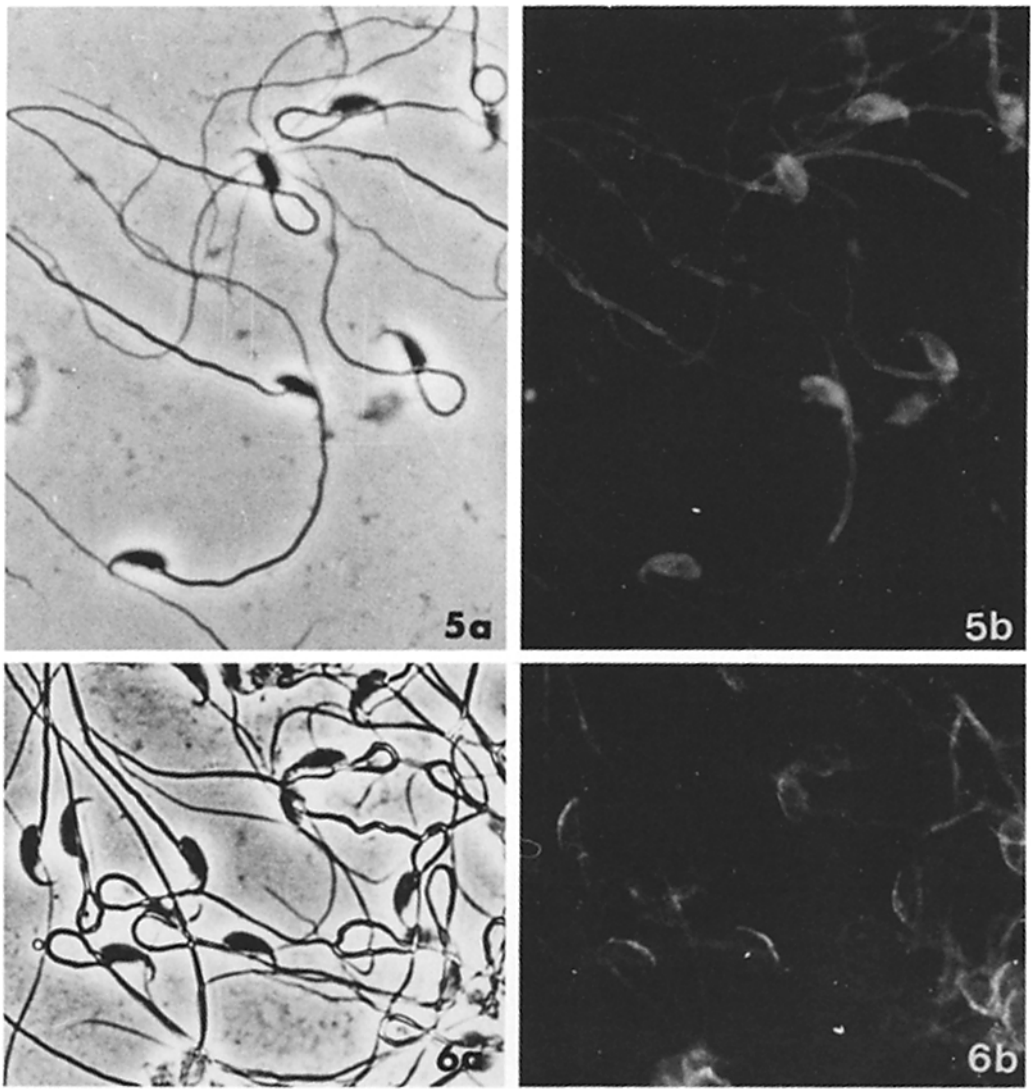


FIGURE 5 Sperm from vas, fixed, no other treatment. Indirect immunofluorescence method with anti-MSP antiserum. Only head and tail membranes are fluorescent. × 800. (For Figs. 5–8, 11–18: (*a*) series are transmitted light, phase contrast micrographs; (*b*) series are fluorescence micrographs of the same fields.)

FIGURE 6 Sperm from vas, fixed, no other treatment. Indirect immunofluorescence method with NRS. Only membrane fluorescence. × 800.

seen with NRS (Fig. 6) as well as with specific rabbit anti-MSP antiserum (Fig. 5).

Treatment of similar smears with the BME solution caused sperm heads to swell. For methanol-acetic acid fixed smears, it was found that a 3–5-min treatment resulted in a degree of swelling such that the general shape of the sperm head was retained, the acrosome was visible, and at least 50% of the tails remained attached. In such preparations, the sperm heads were brilliantly stained by the indirect immunofluorescence procedure using anti-MSP antiserum (Fig. 7), whereas comparable preparations with NRS were unstained (Fig. 8). The fact that immunologic recognition by MSP antiserum of a component of mouse sperm heads is made only on swollen sperm

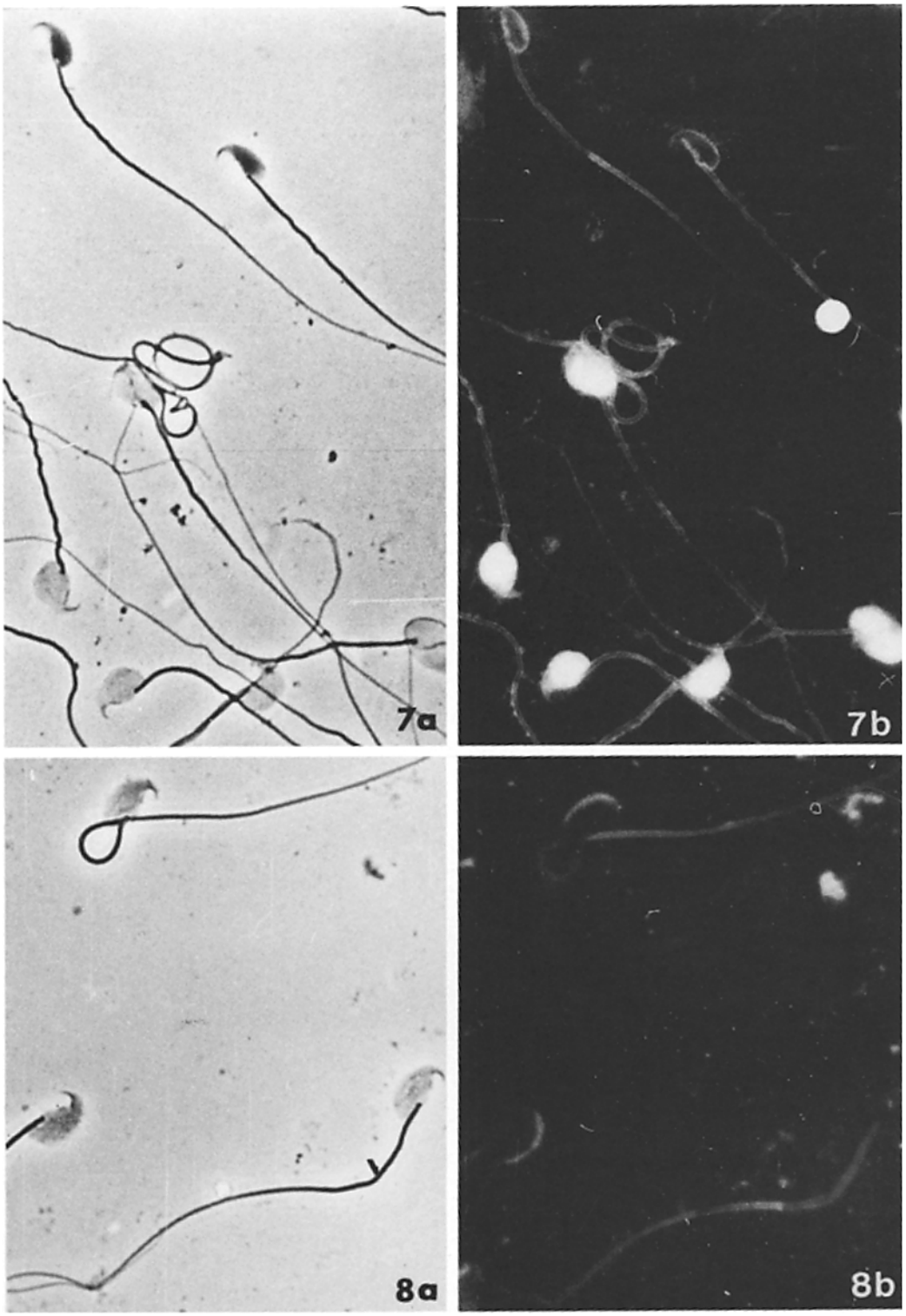


FIGURE 7 Sperm from vas, fixed. The two sperm in the upper portion of the micrograph were untreated, while the other (swollen) sperm were treated with the BME solution (see text). After the indirect immunofluorescence method with anti-MSP antiserum, the treated sperm heads fluoresce but the untreated sperm show only membrane fluorescence. × 800.

FIGURE 8 Sperm from vas, fixed, treated with the BME solution and the indirect immunofluorescence method with NRS. Sperm heads are swollen, but only membranes are fluorescent. × 800.

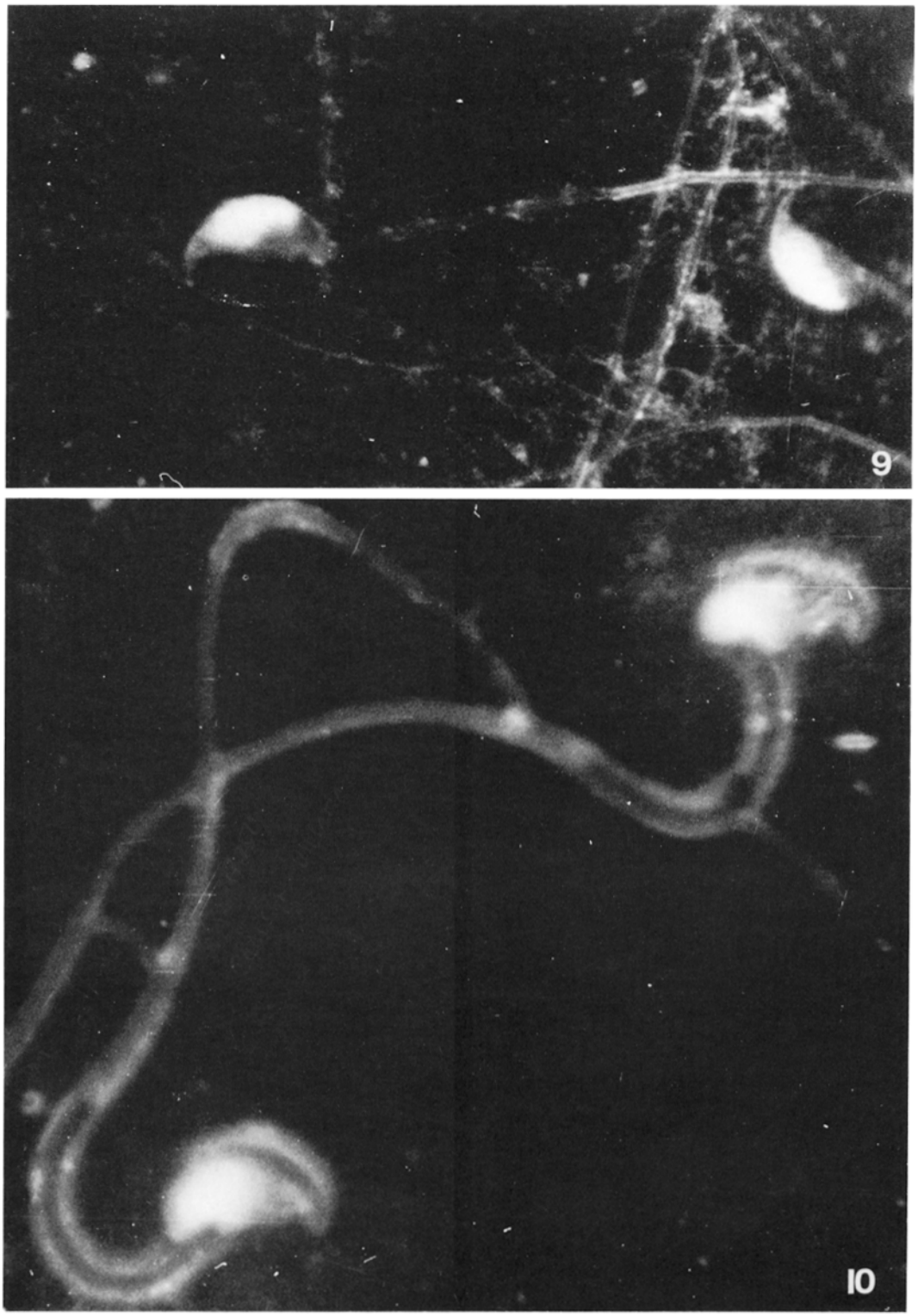


FIGURE 9 Sperm from vas, fixed, treated with the BME solution and the indirect immunofluorescence method with anti-MSP antiserum. Fluorescence is localized to the nucleus. × 2,000.

FIGURE 10 Sperm from vas, unfixed (for better preservation of membrane staining), treated with the BME solution and the indirect immunofluorescence method with anti-MSP antiserum. Nuclear fluorescence is distinct from that of membrane. × 2,000.

is clearly shown in Fig. 7. In that cytologic preparation, a drop of the BME solution was placed on one half of the smear, and the solution was drained and washed off away from the untreated half. The entire smear was then treated with anti-MSP antiserum and fluorescein-conjugated goat anti-rabbit antiserum (see Materials and Methods). Fig. 7 represents the region of interface between the BME treated and untreated portions of the smear. The treated, swollen sperm heads are brilliantly fluorescent while the untreated, unswollen sperm display only the nonspecific membrane staining shown in Fig. 5.

The BME treatment found satisfactory for fixed sperm smears resulted in complete disruption of sperm morphology when applied to unfixed preparations. However, with further dilution of the solution (1:5 with PBS), it was possible to produce moderately swollen sperm. In such preparations, when the immunofluorescence procedure was applied, the sperm heads were stained the same as those of the fixed preparations. Acrosomal and tail membrane staining was more intense with both MSP antiserum and NRS when the methanol/acetic acid fixation was omitted.

The fact tht the staining of the sperm head was a result of recognition of a nuclear component by the specific anti-MSP antiserum could be clearly demonstrated by photomicrographs at higher magnification (Figs. 9 and 10). Comparison of the region of fluorescence in the spermatozoa of Fig. 9 with electron micrographs of mouse sperm (e.g., reference 22) shows that the stained region is exclusively that of the nucleus; the typical morphologies of acrosomal cap and connecting piece are discernible and seen as unstained. The smear preparation represented by Fig. 10 was not fixed and, typical of such preparations, intense membrane staining is displayed. Fig. 10 shows that the staining of the nuclear region is not attributable to the overlying head membranes, but is distinctly and separately that of the sperm nucleus.

Tissue Specificity

Smear preparations of a suspension of bone marrow cells, obtained from mouse femurs, and of liver cells (Fig. 11) with and without BME treatment were negative with both MSP antiserum and NRS.

Species Specificity

Fixed smear preparations were made of a mixed suspension of sperm obtained from mouse, rat (a closely related species), and rabbit (immunization host species). After the BME treatment described above, only the mouse sperm nuclei were stained by MSP antiserum; no sperm nuclei were stained by NRS. Since in such preparations the mouse sperm appeared to be more swollen than the rat or rabbit sperm, separate smear preparations were made of each of the three sperm suspensions and subjected to a series of increasingly longer treatments with the BME solution. Rabbit sperm were found to be less readily swollen or disrupted than mouse sperm, with rat sperm being still more resistant to the reagent. After 8-min treatment with the BME solution, no sperm heads were seen in the mouse sperm preparations, while rabbit

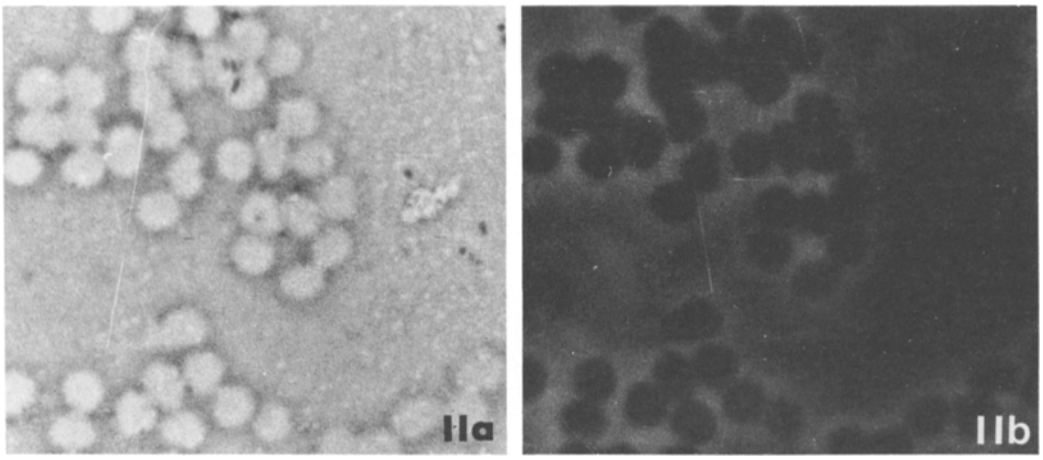


FIGURE 11 Smear preparation of mouse liver cells, fixed, treated by the indirect immunofluorescence method with anti-MSP antiserum. No fluorescence. × 800.

sperm were reasonably swollen with the general shape of the sperm head, connecting piece and tail retained. A similar effect on rat sperm required 12–15-min treatment with the BME solution. The basis for the differential response to the BME is not known, but in each case adequately swollen rabbit and rat sperm preparations showed no nuclear staining with MSP. In both cases, however, there was considerable staining of the membrane, connecting piece, and tail. The latter staining was highly variable, even in the same test preparation, and was seen to a considerable but varied extent in the NRS-treated controls.

To ascertain that the unmasking of the antigenic sites in the mouse sperm nuclei is attributable to the reducing action of the BME rather than to such less specific action as high salt content or high pH, the effect of another reducing agent, DTT, was studied. Previous studies have shown that swelling or decondensation of sperm heads may be induced by treatment with DTT and SDS at pH 9.0 (4) or by DTT and trypsin at pH 7.4 (10).

In this study, it was found that treatment of fixed mouse sperm smears with 3×10^{-3} M DTT in PBS at pH 7.2 for 1 h resulted in swollen sperm heads in which fluorescent staining with anti-MSP antiserum could be demonstrated, while control preparations with NRS were negative.

Spermatogenic Series

The appearance of MSP in spermatogenic cells was determined by applying the indirect immunofluorescence method to smears of testicular cells. Since no satisfactory combination of chromatic staining (e.g., Giemsa's) and the fluorescence procedure could be devised, identification of the cell type was made on transmitted-light, phase-contrast micrographs. With that system, primary spermatocytes in the various stages of prophase could be identified by size or by their occurrence as syncytia (9), while spermatids at the successive stages of spermiogenesis could be identified by reference to the classical diagram of Oakberg (21). Secondary spermatocytes and spermatogonia could not be separately distinguished with certainty, but all cells which were considered to be either spermatogonia or (nondividing) secondary spermatocytes as well as those positively identified as primary spermatocytes were negative when stained with the fluorescence procedure using anti-MSP antiserum (Figs. 12 and 17). In the same or similar fields, those cells identified as round spermatids either free or in syncytial structures were positively stained with MSP antiserum, without treatment with either the BME or the DTT solution (Figs. 12 and 13). Also, more differentiated spermatids of all stages up to about stage 15 were positively stained with MSP in the absence of prior treatment with either of those two reagents, while, in the same fields, spermatids of about stage 15 and spermatozoa were unstained (Figs. 13–17). When similar testicular smear preparations were treated with the BME solution before anti-MSP antiserum and fluorescein-conjugated anti-rabbit goat serum, all spermatids and spermatozoa were stained, while those that could be tentatively identified as spermatocytes were unstained (Fig. 18). For all preparations, NRS and goat anti-rabbit antiserum controls were negative. Fig. 19 is a summary of the information obtained in this study: designation of the cell type in spermatogenesis and stages of spermiogenesis in the mouse in which the presence of MSP was revealed by recognition of its antigenic sites by its specific antiserum and the stage at which treatment with a reducing agent was necessary to make available or unmask those sites.

DISCUSSION

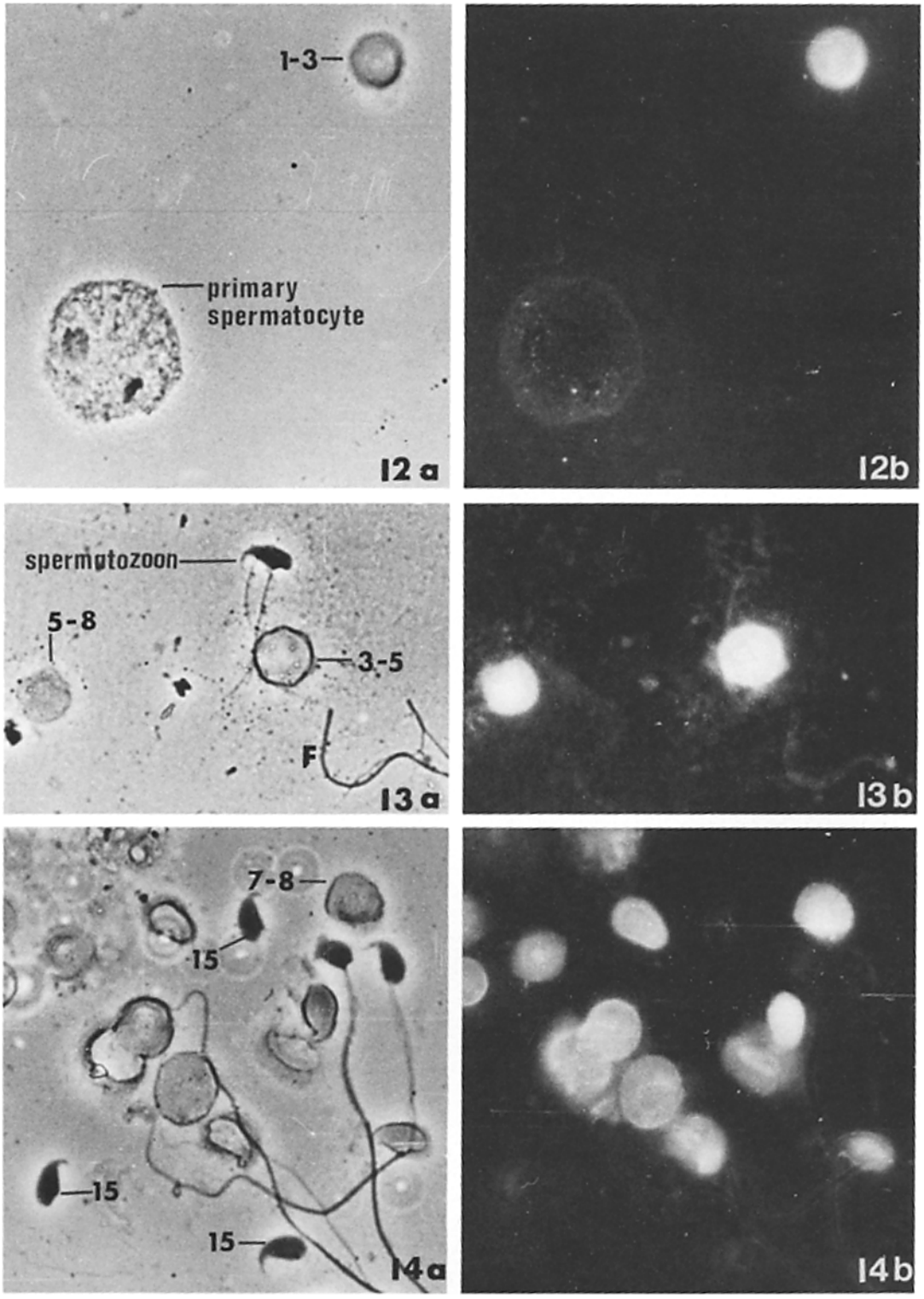
There is reasonable evidence, for all mammalian species studied thus far: rat (3, 11), rabbit (2, 3),

FIGURES 12–17 Smear preparations of testicular cells, fixed, treated by indirect immunofluorescence method with anti-MSP anti-serum. × 800. Numbers on micrographs and in legends refer to stages of spermiogenesis as defined by Oakberg (21).

FIGURE 12 Round spermatid (*1–3*), fluorescent. Primary spermatocyte, nonfluorescent.

FIGURE 13 Spermatids (*3–5*), (*5–8*), fluorescent. Spermatozoon, nonfluorescent.

FIGURE 14 Spermatid (*7–8*) fluorescent, while (*15*) is nonfluorescent. (Many spermatids seen here and in other figures, showing varying intensities of fluorescence, are swollen or otherwise abnormal in appearance. These probably represent atretic cells that have failed at different stages of development.)


1-3
primary spermatocyte
12 a
12b
spermatozoon
5-8
3-5
F
13 a
13 b
7-8
15
15
15
14 a
14 b

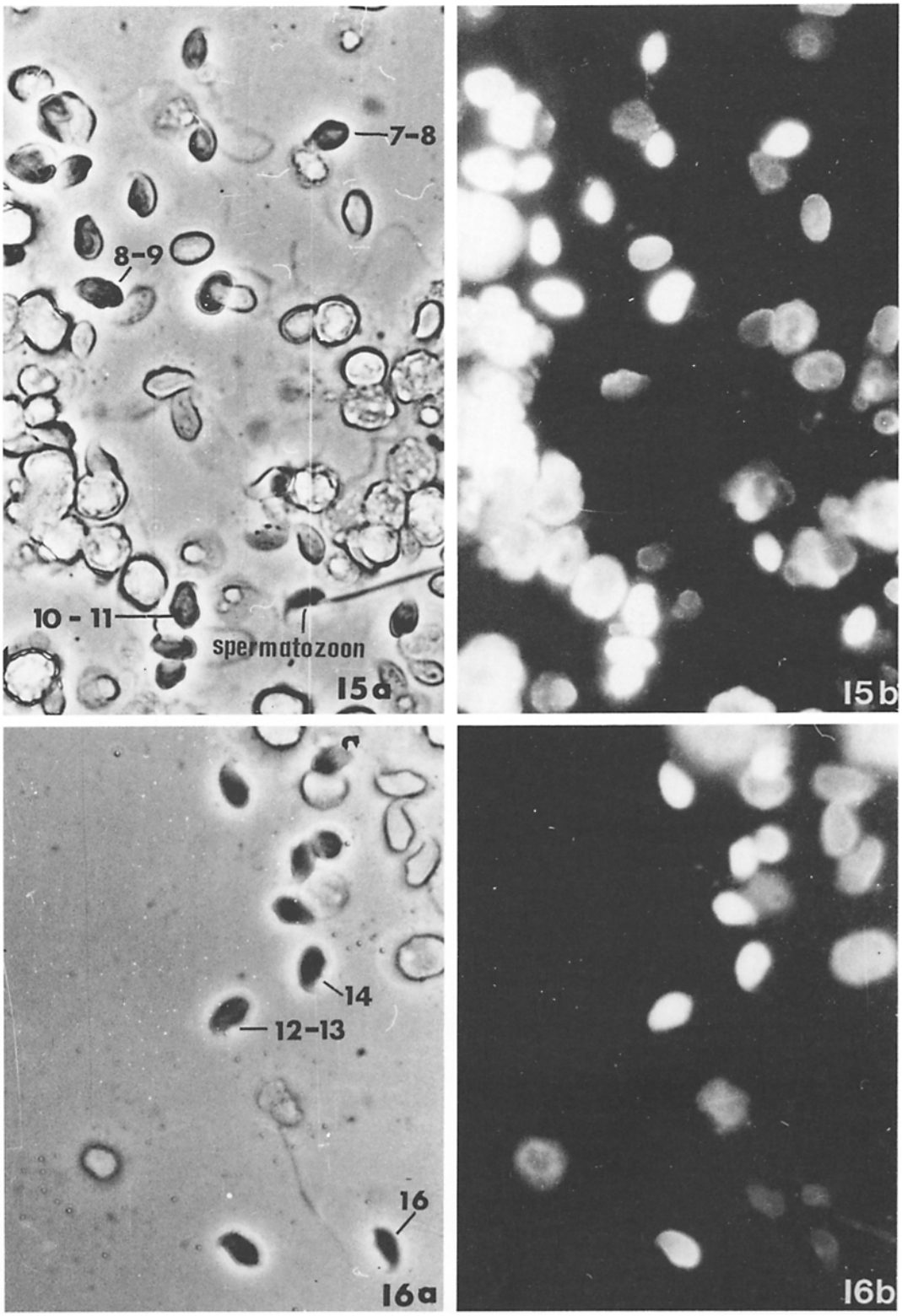


FIGURE 15 Syncytial mass of round spermatids, spermatids (*7–8*), (*8–9*), (*10*) are fluorescent while the spermatozoon is nonfluorescent.

FIGURE 16 Spermatids (*12–13*), (*14*), others are fluorescent. Spermatid (*16*) is nonfluorescent.

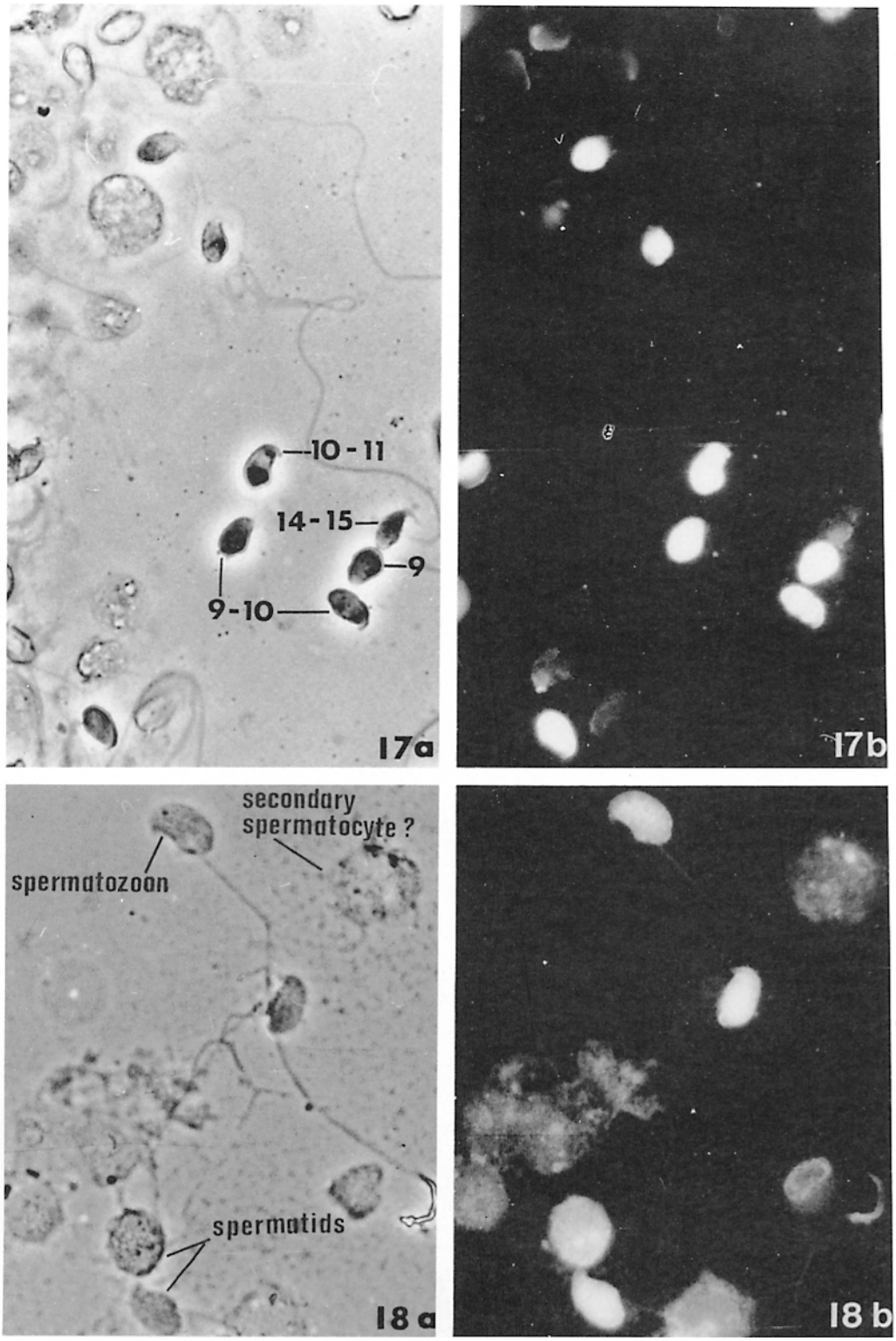


FIGURE 17 Spermatids (*9–10*), (*10–11*), others are fluorescent. Note: spermatid (*14–15*), showing small region of low fluorescence, is closely proximate to spermatid (*9*) showing brilliant fluorescence.

FIGURE 18 Testicular smear, fixed, treated with BME solution and indirect immunofluorescence method with anti-MSP antiserum. The swollen spermatozoa and spermatids are fluorescent, while the cell identified as a spermatocyte (secondary?) is nonfluorescent.

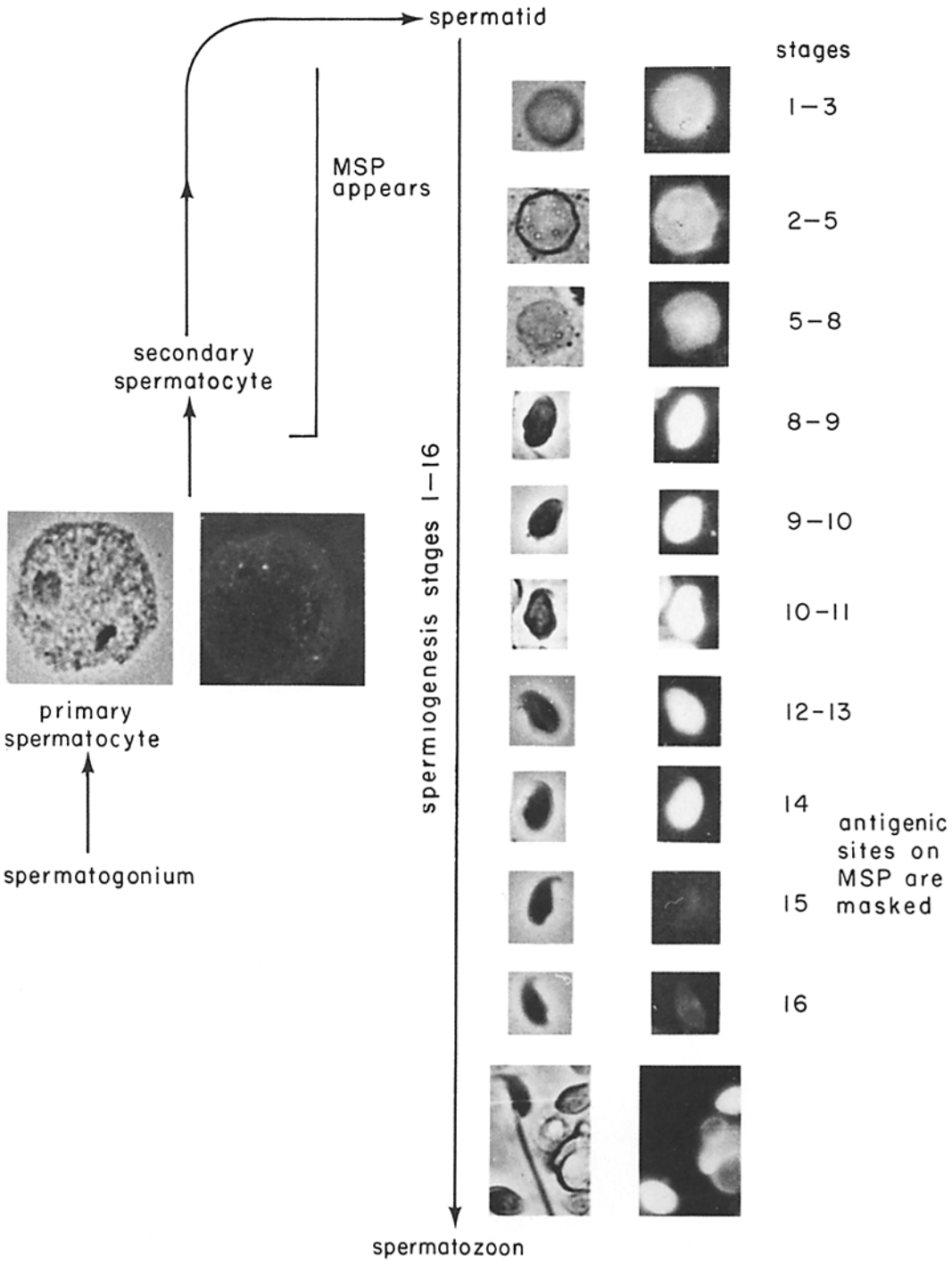


FIGURE 19 Summary of data presented by Figs. 12–18, showing the phase in spermatogenesis at which MSP appears and the stage in spermiogenesis when its structure is altered so that its antigenic sites are masked. Each cell has been selected from one of the micrographs of Figs. 12–17, where each may be seen in the context of the field.

guinea pig (2, 3), human (2, 3, 14), mouse (1, 2, 3), that spermatogenesis includes a turnover of the basic chromosomal proteins. The dynamics of the displacement of somatic-type histones and the assembly of the sperm-unique nucleoprotein are not understood, and there is some question whether certain unique basic proteins are transiently associated with the chromatin in the early stages of spermatogenesis (13, 15, 24). It is well accepted (1, 2, 3, 7), however, that the nucleus of the mature sperm contains none of the complement of the conventional "somatic type" histones detectable by electrophoretic analysis, and that at least 99% of its protein content is composed of one or more arginine-rich basic proteins with a high content of cysteine residues (1).

Biochemical analyses have indicated that while the sperm nuclei of some species, e.g., rat (3, 11) and guinea pig (2, 3), contain only one basic protein, those of human (3, 14) and mouse (2, 3) contain two or more. Critical electrophoretic analysis of the heterogeneity of the mouse sperm protein fraction has demonstrated two very close major bands and trace amounts of components of greater electrophoretic mobility (3), with the latter possibly being attributable to proteolysis of the sperm protein (19).

It has also been shown that, after aminoethylation, the mouse sperm chromosomal protein may be resolved into two electrophoretically distinct bands (2). The similarity in amino acid content (Table I) of MSP and the nuclear proteins of each of the cited studies (2, 3) suggests that MSP, as well, includes two unique proteins. The data of the two-dimensional immunoelectrophoretogram (Fig. 2), demonstrating that the antiserum contains specific antibodies to two antigenically distinct components of MSP, is consistent with that suggestion.

Although there is ample evidence for the heterogeneity of MSP, it has not been possible, thus far, to isolate the separate fractions. Therefore, the entire fraction represented by the single band on the acetic acid-urea gel (Fig. 1 *B*) was used as the immunogen in this study. The immunofluorescence test, carried out on mature sperm, demonstrates that one or both of the antibodies of the anti-MSP rabbit antiserum recognize and react with one or both components of mouse sperm basic protein *in situ*. The cytoimmunofluorescence data also show that some antigenic component of MSP is present in the cells of the earliest identifiable stages of spermiogenesis (Figs. 12, 13, and 15). It cannot yet be said with certainty that both of the components of the basic chromosomal protein of mature mouse sperm appear at the same time in spermiogenesis and, thus, whether the immunofluorescence of the round spermatids represents one or both of the unique antigenic proteins demonstrated in the immunoelectrophoretogram (Fig. 2).

Autologous immunogenicity of human sperm protein(s) was previously inferred by demonstration of immunologic reaction between sera of vasectomized men and ejaculated sperm (14). That reaction was seen only in sperm that were swollen as a result of either abnormality in gametogenesis or experimental treatment of the sperm suspension.

In this study as well, the immunologic reaction between the anti-MSP rabbit serum and mature mouse sperm was demonstrable only on sperm made to swell by either of two different reagent solutions. Each solution contained an agent known to reduce disulfide linkages, BME in one and DTT in the other. Since the DTT solution was of low molarity in PBS at pH 7.2, neither the swelling effect nor the unmasking of the antigenic sites could be attributed to high salt content or high pH. The swelling, unmasking, and disulfide reduction, therefore, appear to be related, and all, in addition, may be related to decondensation of the sperm chromatin. It has been suggested that the swelling of sperm heads induced by treatment with DTT and proteases simulates the swelling and concomitant chromatin decondensation of sperm heads in the egg cytoplasm at fertilization (10). The observations of this study further suggest that the swelling and unmasking of antigenic sites are the morphologic indicia of a reversal of the final steps of spermatid maturation. The progression displayed in Fig. 19 demonstrates that the volume of the entire sperm head as well as that of the nucleus decreases as maturation progresses and that the masking of antigenic sites takes place in the final stages of spermiogenesis.

It has been considered that the completion of chromatin condensation, presumably effected by disulfide bonding between cysteine residues of the sperm protein(s), takes place in the epididymis (4, 18). The data reported here indicate that the antigenic sites of MSP that are detectable by the anti-MSP antiserum become masked at about stage 15 of spermiogenesis (21) and, thus, before the excursion of the spermatozoa from the testis. The masking of those sites may result directly

from the change in conformation of the basic protein induced by the cysteine-to-cysteine linkages or by a change in orientation of the nucleoprotein complex which, in turn, may result from formation of the disulfide bridges. It has been shown (8) that the Feulgen stainability of the DNA in ram spermatozoa is reduced by ~50%, with most of the reduction taking place before release of the spermatozoa from the seminiferous tubules, and that, after treatment with DTT, the Feulgen haploid equivalence is restored. The latter observation suggests that a loss in DNA reactivity is a result of inaccessibility of the Feulgen reactive groups as a consequence of the oxidation of the sulfhydryl moieties of the DNA associated protein.

Over 50 slides of testicular cell suspension treated by the indirect immunofluorescence method were examined. In no instance was fluorescence exhibited by a spermatid of stages 15–16 or by a spermatozoon (Figs. 13–17), while, in the same fields, spermatids of earlier stages were just as consistently positive by that test. When, however, such slide preparations were treated with BME before the immunofluorescence procedure, cells that could be clearly identified as spermatozoa, since their tails were attached, were brilliantly stained as were all spermatids, while spermatocytes showed no staining (Fig. 18). Although the swelling obliterated the indicia by which spermatids are staged, it seems fair to conclude that all spermatids of those stages in which the antigenic sites are masked responded as did the spermatozoon of Fig. 18. Those observations also allow the interpretation that the masking of the antigenic sites on MSP results when the chromatin condensation resulting from establishment of disulfide bonds occurs and, further, that that event takes place in the testis.

Thus, it may be concluded that a component or all of MSP is detectable in the nuclei of spermatids at all stages and that alterations in the protein structure occur in the terminal stages of spermiogenesis. The final phase of the life cycle of MSP, its sojourn in the egg cytoplasm, is under study in these laboratories.

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