

MOUSE SPERM BASIC NUCLEAR PROTEIN

Electrophoretic Characterization and Fate after Fertilization

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ABSTRACT

Mouse sperm were labeled *in vivo* with [³H]arginine. The sperm were then followed autoradiographically from the time of label incorporation until after fertilization. The label was completely lost from the sperm head after fertilization, during the oocyte's second meiotic division.

That the [³H]arginine was incorporated into a sperm-specific basic protein was demonstrated by fractionating acid extracts of epididymal and ejaculated sperm with polyacrylamide gel electrophoresis. All the histone fractions were resolved in the epididymal extracts, but in addition a band was present that migrated faster than histone F2al and slower than the salmon protamine used as a marker. This new fraction (proposed name: musculine) was also present in ejaculated sperm; it was shown to be the only fraction that was labeled. Musculine therefore represents the end product of a histone transition in mice. It is, however, according to our electrophoretic characterization, not identical to the classical fish protamines. Rather, musculine resembles bovine sperm nuclear protein. Since the loss of this fraction from the sperm head was coincident with the rearrangement of the male genome, before its resumption of transcription, it is suggested that musculine is involved in the control of chromatin that accompanies spermiogenesis and fertilization.

Sperm differentiation is often accompanied by a nuclear protein transition that replaces histones of prespermatid stages with more basic protein(s) (sperm basic nuclear protein, SBNP) which remain complexed to the sperm DNA at least until fertilization (5, 14, 17, 20). Concurrently, nuclear RNA synthesis stops (10, 29, 35) and the spermatid nucleus condenses into its characteristic shape. It has been suggested that the complexing of DNA to these unusually basic proteins, with their unique molecular configurations, permits extensive condensation of the DNA thus repressing transcrip-

tion (31) perhaps by excluding RNA polymerase from the genome. Whether or not this is a general mechanism of genetic repression in sperm is difficult to determine because the transition from histone to protamine is not universal (5, 15, 16, 21, 38). In those cases, however, where chromatin becomes complexed to unusually basic proteins and repressed, a question remains as to how genetic activity may be resumed after fertilization.

Several investigators addressed this question by attempting to find what happened to SBNP after fertilization. They showed, with cytochemical pro-

cedures that are specific for basic protein, that most sperm nuclei stain before fertilization (1-3, 6, 8), but that after fertilization the male pronuclei do not stain (1, 7, 13). Thus it would appear that SBNP may be lost after fertilization. On the other hand, the SBNP may remain complexed to DNA since the staining properties of basic proteins are severely altered by side group modification and complex formation with DNA (34).

These alternative interpretations may be resolved by using radioactively labeled sperm. Such labeled sperm, coupled with autoradiography as a means of monitoring the movements of SBNP, would be less influenced by conformational changes, side group modification, and complex formation. That this approach may be feasible was indicated by Monesi (29, 30) who showed that mouse sperm could be labeled *in vivo* with [³H]arginine and by another report (24) wherein the use of labeled sperm in fertilization experiments was described. Nevertheless, valid conclusions from these kinds of experiments would depend on proof that the label is incorporated into that fraction of basic nuclear protein carried by the sperm into the egg. Under these conditions the loss of label would be *prima facie* evidence for loss of SBNP.

This report is a study in which the specificity of *in vivo* labeling is determined for mouse sperm by extraction and analytical polyacrylamide gel electrophoresis. In addition, the pre- and postfertilization movements of mouse SBNP are monitored by autoradiography from ejaculation to pronuclear fusion. The results we obtained may resolve the alternative interpretations of the cytochemical findings, provide additional properties of mouse SBNP, and help to eventually elucidate SBNP function.

MATERIALS AND METHODS

Animals and Biological Materials

Carworth CF no. 1 strain mice were obtained locally and acclimated for at least 1 wk before use in mating experiments. To insure predictable mating behavior, animals were kept in well ventilated, air-conditioned quarters with an artificial light cycle of 12 h light and 12 h dark. 10-16-wk old animals were used.

Calf thymus glands, obtained shortly after slaughter, were immediately cooled to 0°C and stored at -20°C until processed for basic protein extraction. Salmon and herring sperm protamines (salmine sulfate, grade I and clupeine sulfate, grade III, respectively) were purchased from Sigma Chemical Co., St. Louis, Mo. Pregnant mare's serum gonadotropin (PMS) (Gestyl, Organon

Inc., West Orange, N. J.), was reconstituted with the diluent supplied, then diluted further with 0.15 M NaCl. Human chorionic gonadotropin (HCG) (A.P.L., Ayerst Laboratories, New York) was diluted in a similar manner.

In Vivo Sperm Labeling

Male mice were mated 1 day before isotope administration in order to evacuate their sperm storage organs. Each male was injected intraperitoneally with 50-250 μ Ci L-[4-³H]arginine (sp act = 22 Ci/mmol; Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.). Sites of [³H]arginine incorporation were determined by (a) sacrificing males at intervals after injection or (b) recovering sperm from the uterine contents of their female copulation partners at intervals after injection. Tissues and sperm samples were examined by autoradiography and by biochemical extraction procedures. Each male was mated only once, and duplicates for each interval were run.

Mating Procedures and Gamete Collection

Males were caged individually after isotope injection. Females were superovulated according to the method of Fowler and Edwards (18), using 3 IU PMS followed 40-48 h later by 3 IU HCG. The hormone injections were timed to induce ovulation at the beginning of the dark cycle. That ovulation had occurred was confirmed by direct examination of oviducts. Immediately after the HCG injection the female was placed in the male's cage. The next morning females with copulation plugs were sacrificed by cervical dislocation at intervals from the time of HCG injection which was taken as zero time. Ova were dissected out in a short segment of the ampullar oviduct and prepared for autoradiography. Semen was collected by puncturing the uterus with a 100- μ l glass pipet which was drawn out slightly and broken to a sharp orifice. Semen was flushed from this pipet into 2.0 ml of cold 0.15 M NaCl, mixed and washed three times by centrifugation (1500 g, 15 min), and resuspended in the same solution. A final wash in distilled water lysed contaminating somatic cells so that no cell types other than sperm could be seen by phase-contrast microscopy.

Histological and Autoradiographic Methods

Tissue samples (testis, epididymis, oviduct containing ova) were fixed in 2.5% glutaraldehyde in 0.06 M sodium cacodylate adjusted to pH 7.4, imbedded in paraffin, and sectioned at 4 μ m. Ova were serially sectioned. Sperm smears made from fresh uterine contents were air dried and fixed in the same fixative. Slides with sections mounted on them were deparaffinized, rinsed three times in 70% ethanol, and dried. They were then dipped in the nuclear emulsion (Kodak NTB-2 [Eastman Kodak Co., Rochester, N. Y.] diluted with an equal volume of water) and dried in a vertical position. Slides were exposed for 1-6 mo at 4°C in black Bakelite boxes (Union Carbide

Corp., Plastics Div., New York) silica gel. After exposure they were processed in D-19 (5 min), water (20 s), Kodak rapid fixer (2 min, without added hardener), and running water (20 min). These photographic solutions were cooled at 2–4°C in an ice bath before use in order to reduce chemical fog.

Most autoradiographs were stained with hematoxylin through the emulsion after development. Some male tissues, however, were stained according to the alkaline fast green procedure of Alfert and Geschwind (3), modified so that the hot TCA hydrolysis step preceded emulsion coating.

Several controls were included with each set of experimental slides. Background or negative controls consisted of identically prepared tissues which had not been exposed to any exogenous radioisotopes. Oviduct sections containing labeled sperm and the ova fertilized by them required an additional control, namely, similar sections derived from a mating which occurred 2–3 days after the male had received [³H]arginine. These ova were exposed to radioactive seminal plasma containing non-radioactive sperm; thus the radioactivity contributed by the sperm in experimental slides was monitored against a more realistic background level. Positive controls, serving as a check on the sensitivity of our autoradiographic procedure, consisted of a single batch of [³H]thymidine-labeled human chromosome slides of known activity.

Each serial section of each autoradiographed ovum was examined for the presence of sperm, label (grains), and background. These data were recorded for each of the sections and correlated with the developmental stage. Since unfertilized ova are in metaphase of meiosis II, the maternal chromosome configuration was used to index the developmental stages before pronucleus formation. Autoradiographs of sperm smears were analyzed by counting grains over 100 consecutive sperm heads in several different areas of the slides, and averaging the results.

Basic Protein Extraction

Two methods were used. (a) Chromatin was prepared from calf thymus, mouse liver, and testes, according to the method of Marushige and Bonner (27) except that 0.05 M NaHSO₃ was added to the saline-EDTA and Tris buffers in order to inhibit protease activity (4). Basic proteins were extracted with 0.4 N H₂SO₄, then precipitated from the acid extract with 4 vol of acetone (–20°C), washed in acetone by centrifugation, and dried *in vacuo*.

(b) All of the following steps were performed at 0–4°C unless specified. Testes and epididymides were minced and then homogenized at medium speed in a Potter-Elvehjem glass-Teflon homogenizer with 5 vol of 0.075 M NaCl, 0.24 M EDTA, 0.05 M NaHSO₃, and NaOH to pH 8.0 (SEB). Large aggregates were allowed to settle for 1 h and the cells that remained in suspension were washed three times by centrifugation (1,500 g, 10 min)

and resuspended in SEB. The resulting pellets were suspended in 1.0–2.0 ml of 6 M urea, 0.4 M guanidine hydrochloride, 1% 2-mercaptoethanol, 0.05 M NaHSO₃, 0.1 M NaH₂PO₄ and NaOH to pH 7.0 (UGMB) based on the chromatin dissociating solvent of Levy et al. (26) for 1 h at 37°C with continuous mixing. The suspension was briefly sonicated two to three times in order to break up clumps which formed in UGMB. After this treatment sperm heads appeared to lack refractility by phase-contrast examination. An equal volume of 1 N H₂SO₄ was added and the suspension was stirred on ice for 3 h. The suspension was cleared by centrifugation (5,000 g, 10 min) and the supernatant fluid was desalted by pressure dialysis against 10 vol of distilled water concentrated to 0.5 ml in an Amicon model 8MC microultrafiltration cell with a UM 2 filter (Amicon Corp., Lexington, Mass.). Proteins in the retained fluid were precipitated, washed, and dried as in (a) above. The washed pellets of uterine sperm were extracted as in (b) above.

Polyacrylamide Gel Electrophoresis

Acid-urea gels containing either 2.5 M (pH 2.7) or 6.25 M (pH 3.2) urea were prepared according to Panyim and Chalkley (33) without modification. Protein samples were dissolved in a solution of 0.9 N acetic acid, 0.6 M sucrose. After running, the gels were stained with fast green (19), destained, and scanned at 600 nm with a 0.05-mm slit in a Beckman-Gilford spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

Liquid Scintillation Counting

Gels were sliced transversely into 1.5-mm disks which were placed into individual glass scintillation vials. To each vial 0.5 ml of NCS (Nuclear Chicago, Amersham/Searle Corp., Arlington Heights, Ill.) and water (9:1) was added and the disks were digested in capped vials for 8 h at 50°C. After cooling 10 ml of toluene containing 2,5-diphenyloxazole (PPO) (5.8 g/liter) and 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) (0.2 g/liter) were added. The vials were dark adapted for 16 h and counted in the external standard mode. The external standards ratio did not vary significantly among the vials of a single gel; therefore, radioactivity is expressed as counts per minute uncorrected for quenching.

RESULTS

Uptake in Male Urogenital Tissues

Autoradiographs of seminiferous tubules prepared 1 day after [³H]arginine injection showed that elongating spermatids incorporated the label (confirming Monesi, 1964 [29]) but sperm in the ductus epididymis were unlabeled. At 7 days after injection sperm heads in the entire length of the epididymis were labeled although those in the vas deferens were not labeled. Sections which were

hydrolyzed with boiling TCA before emulsion coating showed the same labeling intensity and distribution as the unhydrolyzed sections.

Samples taken from uteri shortly after copulation (Fig. 1) showed that sperm ejaculated 12–14 days after isotope injection were uniformly labeled. Grain counts for individual sperm heads (Fig. 2) showed that 87% of the sperm had 5 grains or more directly over them or touching their borders. The data for Fig. 2 were collected from an autoradiograph which was exposed for 62 days (a period chosen so that most of the sperm would not have too many grains for accurate counting). With longer exposure times (200 days), 94% of the sperm displayed 15 grains or more and none had less than 10 grains. Control autoradiographs displayed less than 0.1 grain/sperm head at both exposure times.

Fate of Sperm Label after Fertilization

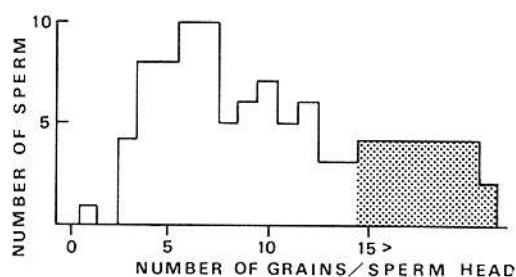
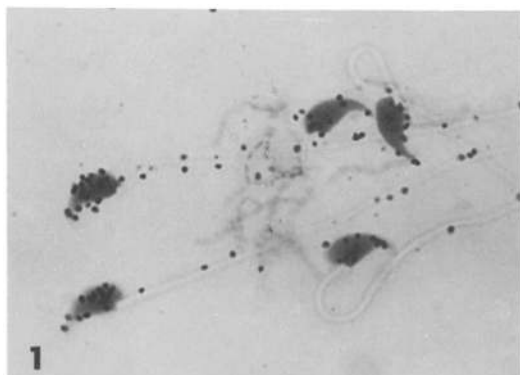
Eight matings were accomplished which yielded labeled sperm in the ampular region of the oviduct. The males had been injected, 12 days before mating, with 250 μ Ci of [3 H]arginine. Four of these matings were not analyzed in detail because the ova were either unfertilized or degenerated. From the remaining four matings, 71 ova were analyzed in detail. The data are summarized in Table I and in Figs. 3–10. The *a* series (Figs. 3–9) are sections of the ova that contained sperm; the *b*

series are sections of the same ova as in *a*, but containing the female chromosomes.

All sperm associated with ova before the start of anaphase II were labeled (Fig. 3 *a* and *b*). Sperm heads remained labeled as anaphase separation commenced (Fig. 4 *a* and *b*), but became progressively less intensely labeled (Fig. 5 *a* and *b*) until, at the end of anaphase, very few grains were detected (Fig. 6 *a* and *b*). After spindle rotation (Fig. 7 *a* and *b*) and polar body II extrusion (Fig. 8 *a* and *b*), only an occasional grain was seen near the sperm head which was developing into the male pronucleus. No labeled pronuclei were observed during the phases of pronuclear maturation (Fig. 9 *a* and *b*) and migration (Fig. 10). In Fig. 10 several labeled sperm were near the pronuclear stage ova, but no unlabeled sperm were observed.

Background levels in control slides were about 1 grain/ovum section and were randomly distributed between ooplasm and pronucleus. Similar patterns were seen in postanaphase and pronuclear experimental sections. After anaphase, no localized accumulations of grains were seen anywhere within the ooplasm, polar bodies, or associated with the oocyte membranes.

Changes occurred in the morphology of the sperm head during anaphase, concomitant with label loss. Its boundaries became less distinct and hematoxylin staining intensity was diminished. These changes have been described ultrastructurally in the mouse (36) where, shortly after sperm-



FIGURES 1 and 2 Labeling pattern of ejaculated sperm. Fig. 1 is a representative autoradiograph of labeled uterine sperm obtained after injection with [3 H]arginine (250 μ Ci) 13 days before mating. This is the first ejaculation since isotope injection. Heads are moderately labeled; midpieces and tails are lightly labeled. Bacteria (out of focus) from the female urogenital tract are unlabeled. Hematoxylin stain, autoradiographic exposure 62 days. \times 1,400. Fig. 2 is a histogram relating the frequency distribution of autoradiographic grains per sperm head. 100 consecutive sperm were scored by counting grains located directly over the sperm head or touching its borders. The stippled area represents 22 sperm which were too heavily labeled to count accurately. These data were derived from the autoradiograph shown in Fig. 1.

TABLE I
Analysis of Sperm Label after Fertilization

Stage	Number of ova analyzed*	Sperm head or pronucleus	Comments
Unfertilized	2	Labeled	No sperm in contact with ova, but those in vicinity were labeled
Activation	4	Labeled	Sperm within zona contacting the vitelline membrane
Anaphase II	6	Labeled early, unlabeled later	Labeling intensity decreased as anaphase progressed
Pronuclear formation	7	Unlabeled	All accessory sperm were labeled
Pronuclear maturation	4	Unlabeled	All accessory sperm were labeled
Pronuclear apposition	48	Unlabeled	All accessory sperm were labeled

* Total ova analyzed = 71

egg fusion, the sperm chromatin became less dense and diffused into the surrounding ooplasm. Thereafter, it became compact again, forming an elongate structure which transformed into a compact, round pronucleus. These events appear to correspond to the stages in Figs. 5-8.

Electrophoretic Characterization of Sperm Nuclear Proteins

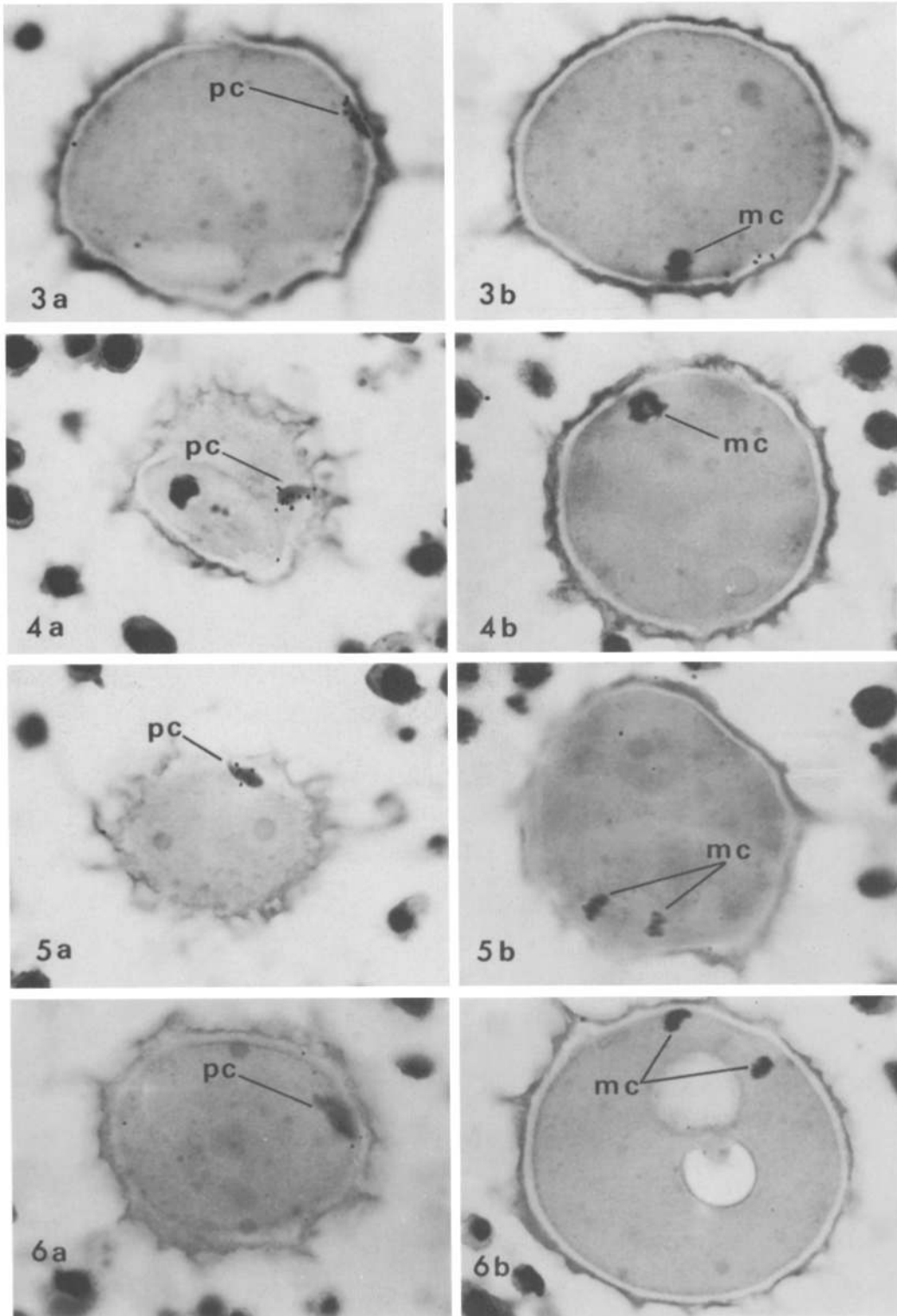
Fig. 11 shows the acid-urea polyacrylamide gel electropherograms (pH 3.2) of extracts of mouse liver, testis, epididymis, and calf thymus histones. The calf thymus and mouse liver extracts (gels *a* and *c*, respectively) were prepared using the chromatin-acid procedure; the mouse testis and epididymis extracts (gels *b* and *d*, respectively) were prepared using the UGMB-acid procedure. The histone bands on all gels are very similar to each other and to calf thymus histones (32). When longer running times were used to amplify differential migration rates, no qualitative differences in the histone patterns could be detected between extracts from any single tissue prepared by either procedure. Their banding pattern was apparently unaffected by the extraction method.

Of interest in Fig. 11 is the prominent M_z band in epididymis that does not appear in other gels. M_z migrates approximately twice as fast as the histone F2a1 fraction. This band could only be

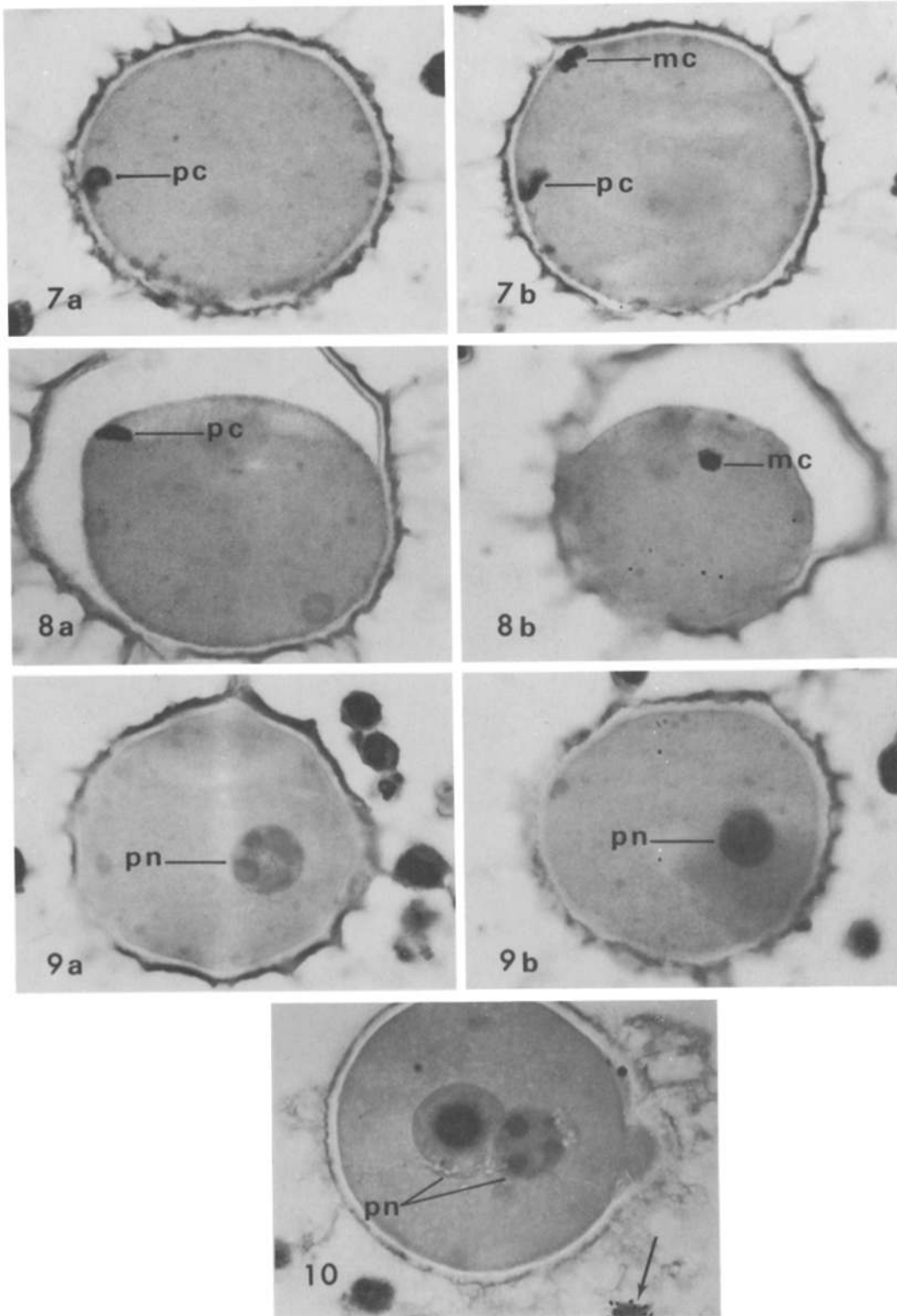
detected in epididymal and uterine sperm extracts using the UGMB-acid procedure, although a very faint band could be detected in testicular extracts when the gels were very heavily loaded. The light testicular band migrated slightly behind M_z but this could be caused by the large volume of sample required to load the gel. Minor bands M_w , M_x , and M_y , slower than M_z , accounted for less than 10% of the nonhistone basic protein, as determined by area measurements.

To investigate M_z further, epididymal and uterine sperm UGMB-acid extracts were run concurrently in longer (110 mm) gels at two different pH's. In addition, protamines from the sperm of salmon (salmine) and herring (clupeine) were placed on separate gels as standards. To prevent closely migrating bands from obscuring each other, relatively light sample loads of uterine sperm extracts were used. The results are shown in Fig. 12. Fraction M_z migrated as a single homogeneous band at either pH. It was not subfractionated at either pH, even though the simultaneously run salmine was further resolved into two subfractions.

In Fig. 12 the uterine sperm gels (*d* and *h*) show that mature spermatozoa do not contain any proteins that migrate with histone mobility. Rather, they show the single prominent band, M_z , in a position that is intermediate between the



FIGURES 3-10 Autoradiographs of eggs fertilized by labeled sperm. The stages are shown in chronological order from the time of sperm-egg contact until just before pronuclear syngamy. The sperm head has silver grains over it until the stage shown in Fig. 6; thereafter, no grains are present over either pronucleus. In Figs. 3-9 the sperm head or paternal pronucleus is shown in panel *a* while the maternal chromatin of the same egg is shown in panel *b*. Fig. 10 is an ovum with both pronuclei apposed before syngamy. A labeled sperm (arrow) is outside the ovum. Hematoxylin stain, autoradiographic exposure 138 days. *mc*, maternal chromatin; *pc*, paternal chromatin or sperm head; *pn*, pronucleus. $\times 960$.



FIGURES 7-10 Legend on preceding page.

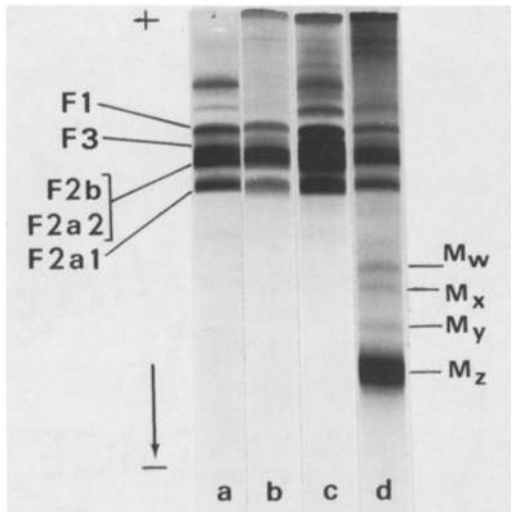


FIGURE 11 Electrophoretic patterns of histones and acid-soluble proteins at pH 3.2. (a) calf thymus, (b) mouse testis, (c) mouse liver, (d) mouse epididymis. Extraction methods are given in text. Electrophoresis was performed at 160 V, 1 mA/gel for 120 min. Gels measured 5 mm diam \times 90 mm. Fast green stain. F1, F3, F2b, F2a2, and F2a1 are standard histone designations (15). M_w , M_x , M_y , and M_z designate mouse sperm fractions.

fastest histone fraction and the protamines, salmine (gels *b* and *f*) and clupeine (gels *c* and *g*). On the other hand, the extracts of epididymal sperm (gels *a* and *e*) do contain typical histone bands in addition to the M_z fraction. These histones, however, probably result from contamination by somatic nuclei that were found in the epididymal sperm samples after microscope inspection.

Specificity of *In Vivo* Labeling

Fig. 13 shows the optical density and radioactivity profiles of two representative epididymis extract gels. The extracts were prepared at 1 and 8 days after [3 H]arginine injection. At 1 day (Fig. 13 *a*) the histones are moderately radioactive while M_z is not. At 8 days, however, when the epididymis autoradiographs showed the ductus to be packed with labeled sperm, fraction M_z is radioactive (Fig. 13 *b*) and the histones show no more activity than they did at 1 day. These data support the conclusion that *in vivo* administration of [3 H]arginine is incorporated into fraction M_z of mouse SBNP.

DISCUSSION

We have demonstrated that mouse sperm, labeled *in vivo* with [3 H]arginine, lose all their radioactiv-

ity after fertilization. The isotope is incorporated into the sperm basic nuclear proteins during spermiogenesis and is lost during the relatively short interval corresponding in time to the anaphase II interval of the activated ovum. The radioactivity does not remain confined to the male pronucleus or to any particular area of the ooplasm; rather, it must be diluted by the ooplasm, or perhaps even leaves the egg. These results can explain the inability of basic protein dyes to stain the male pronucleus after fertilization in the mouse (2) and in other species (7, 13).

In a previous work on the behavior of labeled sperm after fertilization (24), male mice that had been mated three times between isotope ([14 C]arginine) injection and the mating which provided ova for autoradiography were used. This resulted in such a dilution of labeled sperm that only 68% of

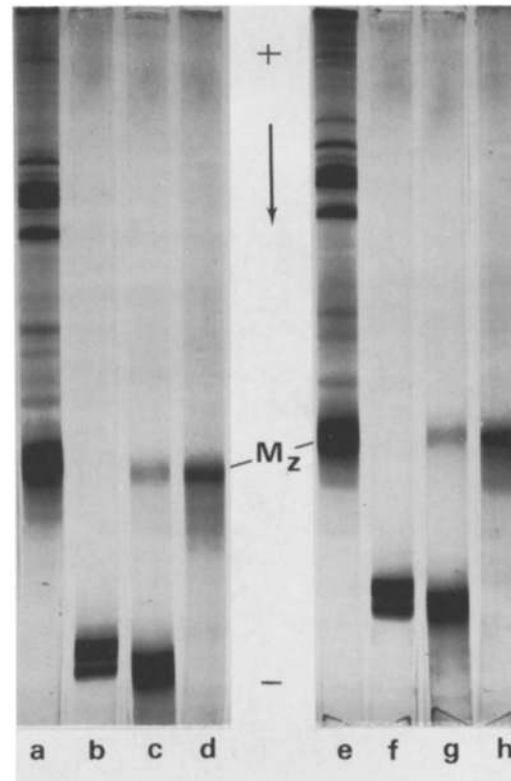


FIGURE 12 Electrophoretic patterns of histones, protamines, and acid-soluble proteins at pH 2.7 and 3.2 (a and e) mouse epididymis, (b and f) salmine, (c and g) clupeine, (d and h) mouse purified uterine sperm. Gels *a-d* are pH 2.7; *e-h* are pH 3.2. Electrophoresis was performed at 170 V, 1 mA/gel for 180 min. Gels measured 5 mm diam \times 110 mm. Fast green stain. M_z designates the mouse sperm fraction.

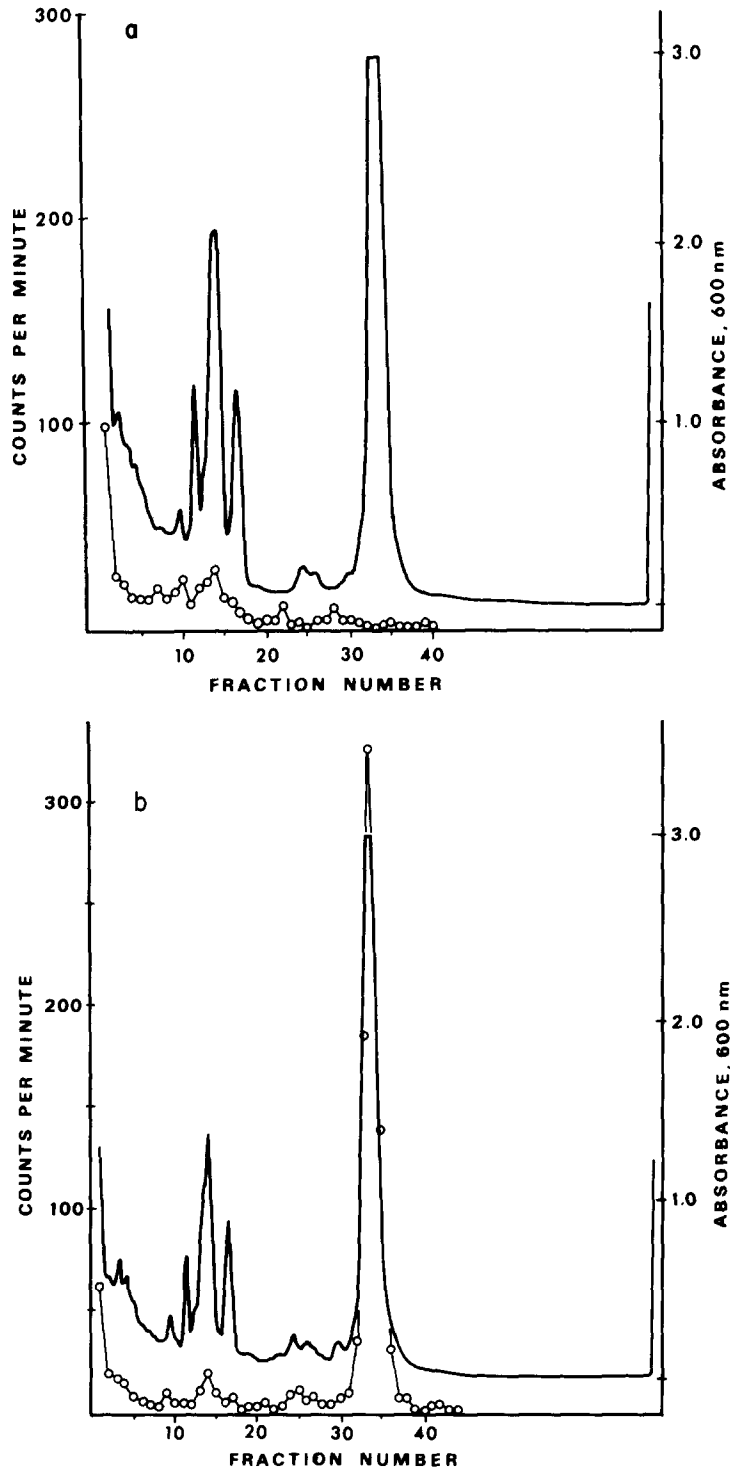


FIGURE 13 Polyacrylamide gel electrophoretic separation of [^3H]arginine-labeled basic proteins from mouse epididymal homogenates. Extract preparation and electrophoretic conditions are identical to those given in Fig. 12 for gel *a*. Fig. 13 *a* shows the optical density and radioactivity profiles of an extract prepared 1 day after isotope injection. Fig. 13 *b* shows similar data for an extract prepared 8 days after isotope injection. Note that the major peak, M_z , is labeled at 8 days but is not labeled at 1 day. On the other hand, the histone peaks show comparable radioactivity in both gels. Fractions 10–20 contain histone bands; fractions 31–37 contain band M_z . (—), optical density; (O—O), radioactivity.

the sperm in the fertilizing ejaculate were labeled. Since only 10 ova were analyzed, a probability existed that the ova autoradiographed were fertilized by unlabeled sperm. Our results are in agreement with Kopečný's (24) data, but they are less equivocal. As described earlier, the present data resulted from an experimental protocol which maximized the percentage of labeled sperm in the fertilizing ejaculate so that no unlabeled sperm were detected. 71 ova spanning the interval between sperm-egg contact and pronuclear fusion were examined in detail. The absence of labeled male pronuclei in 58 eggs after anaphase II would not be a very likely result of fertilization by a fortuitously unlabeled sperm.

One possible error in the interpretation of the autoradiographs should not be overlooked. Tritium's weak beta particles may only penetrate 1–2 μm through tissue. Therefore the beta emissions from labeled sperm on the bottom of a 4- μm section would not reach the emulsion on top of the section. This shielding effect by the ooplasm could account for the absence of grains over sperm inside ova, and the loss of label we described would then be an artifact. We point out, however, that ova were serially sectioned. The fully developed male pronucleus (Figs. 9–10) appeared in five or six consecutive sections; the developing male pronucleus (Figs. 6–8) was present in at least two consecutive sections. Thus, one cut male pronuclear surface had to be in direct contact with the emulsion. Nevertheless, the combined number of grains over all sections never exceeded the background level (2 grains, total) over nonradioactive controls. The male pronuclei were therefore both unlabeled and unshielded. In the earlier stages of activation and anaphase II initiation (Figs. 4 and 5) the sperm were labeled. Shielding may not have been completely effective because the sperm were included in very superficial sections of the ova. In Fig. 3 *a* the sperm in the perivitelline space is also labeled. Here, again, any possible shielding may have been minimized by the relative lack of dense structure in the clear perivitelline space. We conclude therefore that the complete loss of label cannot be the result of shielding, and that the results we describe are not artifacts. Rather, they represent a phase in the normal sequence of sperm postfertilization behavior. This conclusion is consistent with the results from earlier cytochemical experiments (2, 7, 16) and the autoradiographic study of Kopečný (24).

Extraction of labeled epididymal sperm and

autoradiographic examination of sperm in the various regions of the male urogenital tract at 1 and 8 days after [^3H]arginine injection showed that a unique fraction, M_z , became labeled at the same time as the sperm heads. Although some of the radioactivity in sperm heads may result from a specific incorporation, the data indicate that the majority is probably incorporated into nuclear protein. It appears unlikely that the uterine sperm-specific M_z is a breakdown product of histones since we determined that the extraction procedures have no deleterious effects on histones. Furthermore, the specific radioactivity of histones at 8 days was too low to account for the radioactivity present in M_z .

Fraction M_z is detected in relatively large amounts in extracts of both mature uterine sperm and homogenates of whole epididymides, but it is just barely detected in testicular extracts using the UGMB-acid procedure. Histones of the typical somatic type are not detected in purified uterine sperm using the same procedure. The histones of epididymides could come from three sources, exclusive of the spermatozoa contained in that organ; (*a*) nuclei of the epithelial cells lining the duct, (*b*) nuclei of leukocytes which are often concentrated in nodules in the organ, and (*c*) the "sphere chromatophile" which may contain histones that are displaced from spermatid nuclei as the new SBNP occupies basic protein-binding sites (37). Although this evidence does not constitute final proof, it strongly suggests that no histones are present in the nuclei of epididymal and ejaculated spermatozoa. If this were the case, then a histone-to-protamine transition, analogous to the occurring in salmonoid fish (14), occurs in the mouse. It would appear therefore that M_z is the discrete fraction that replaces histones during spermiogenesis.

Rodent SBNP's were also obtained by Lam and Bruce (fraction MP'; reference 25) and Kistler et al. (fraction S_1 ; reference 22). These fractions were similar to M_z and bovine SBNP (12): they resisted simple acid extraction; they required disulfide bond reduction (9), hydrogen bond disruption, and high salt concentrations for solubilization. Furthermore, they incorporated arginine during spermiogenesis, and their electrophoretic mobilities were between those of histone F2a1 and protamine. Coelingh et al. (11) found several cysteines in the sequence of bovine SBNP; cysteine was also present in the sequence of S_1 (23). The extraction requirements for MP' (25) and M_z suggested that

these SBNP's also contained cysteine. These similarities may broadly define a family of similar proteins, but more information on their primary structures than those data currently available is needed to assess the extent of their relatedness.

On the other hand, there are minor differences in the SBNP electrophoretic mobilities, and certain inconsistencies have appeared in testes-specific basic protein (TP) fractions. Lam and Bruce (25) presented evidence that MP' from sperm was equivalent to a more readily extractable fraction, MP, from testes. Kistler et al. (22, 23) also extracted a testes-specific basic protein which was clearly unrelated to their S₁ from sperm and to MP. Now we report that only a small amount of M₂ is extracted from testes. These inconsistencies are difficult to explain at present, but perhaps are to be expected since different extraction and electrophoretic procedures were used on different rodents: TP was obtained from rat testes; MP and M₂ were from two different strains of mice. These inconsistencies define a need for the adoption of uniform methods for the isolation and characterization of testes-specific basic nucleoprotein. However, for the purposes of this communication, we have demonstrated that the labeled protein that is lost after fertilization is a sperm-specific nuclear protein and not an adventitiously bound or other accessory protein. In keeping with the established practice of naming sperm-specific basic proteins after their genus or species origin, we suggest *musculine* as a name for fraction M₂ from the sperm of *Mus musculus*.

Our data may have some bearing on hypotheses for the function of SBNP, but only indirectly. M₂ is acquired during spermiogenesis when DNA is being packaged into a uniquely condensed form for transmission by the sperm. When packaging is completed, the sperm DNA does not transcribe RNA. On the other hand, M₂ is lost after fertilization as the sperm DNA is unpacked before its replication and resumption of transcription. Thus, the behavior of M₂ (its acquisition and loss) is correlated with the regulation of the form and function of mouse DNA both in the sperm and after fertilization. It is therefore conceivable that M₂ plays a role in the management of the mouse male genome during its genetic transmission and reactivation.

This work was submitted to the Graduate Division, Wayne State University in partial fulfillment of the requirements for the degree of doctor of philosophy. A

preliminary report of the findings was presented at the 13th Annual Meeting of the American Society for Cell Biology, Miami Beach, Fla. November, 1973.

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