SPERMATOGENIC CELLS OF THE PREPUBERAL MOUSE

Isolation and Morphological Characterization

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ABSTRACT

A procedure is described which permits the isolation from the prepuberal mouse testis of highly purified populations of primitive type A spermatogonia, type A spermatogonia, type B spermatogonia, preleptotene primary spermatocytes, leptotene and zygotene primary spermatocytes, pachytene primary spermatocytes, and Sertoli cells. The successful isolation of these prepuberal cell types was accomplished by: (*a*) defining distinctive morphological characteristics of the cells, (*b*) determining the temporal appearance of spermatogenic cells during prepuberal development, (*c*) isolating purified seminiferous cords, after dissociation of the testis with collagenase, (*d*) separating the trypsin-dispersed seminiferous cells by sedimentation velocity at unit gravity, and (*e*) assessing the identity and purity of the isolated cell types by microscopy. The seminiferous epithelium from day 6 animals contains only primitive type A spermatogonia and Sertoli cells. Type A and type B spermatogonia are present by day 8. At day 10, meiotic prophase is initiated, with the germ cells reaching the early and late pachytene stages by days 14 and 18, respectively. Secondary spermatocytes and haploid spermatids appear in increasing numbers between days 18 and 20. Cell separations were attempted throughout this developmental period. The purity and optimum day for the recovery of specific cell types are as follows: day 6, Sertoli cells (purity >99%) and primitive type A spermatogonia (90%); day 8, type A spermatogonia (91%) and type B spermatogonia (76%); day 18, preleptotene spermatocytes (93%), leptotene/zygotene spermatocytes (52%), and pachytene spermatocytes (89%).

Mammalian spermatogenesis is a continuum of cellular differentiation in which three principal phases can be discerned: spermatogonial renewal and proliferation, meiosis, and spermiogenesis. The initial phase of spermatogonial proliferation occurs in the basal compartment of the seminiferous epithelium (7, 9) and consists of a mitotic proliferation of stem cells which form, in sequence, type A spermatogonia, intermediate spermatogonia, and type B spermatogonia (19, 30, 31, 36). The type B spermatogonia divide to form preleptotene primary spermatocytes which undergo a final replication of nuclear DNA before entering meiotic prophase. The second phase, meiosis, occurs while the spermatocytes remain intercalated between cytoplasmic processes of adjacent Sertoli cells on the adluminal side of the intercellular Sertoli junctions. Meiotic prophase,

which includes the leptotene, zygotene, pachytene, and diplotene stages, terminates in the first reduction division with the formation of secondary spermatocytes. The latter cells quickly enter the second reduction division to form the haploid spermatids. Spermiogenesis, the final phase of spermatogenesis, consists of a complex morphological transformation of the haploid germ cell that culminates with the release of late spermatids into the lumen of the seminiferous tubule.

The isolation of homogeneous populations of the respective spermatogenic cells is an essential prerequisite for definitive biochemical studies of germ cell differentiation. The technique which has been applied to the adult testis most successfully for this purpose utilizes differential sedimentation velocity at unit gravity of cells which differ in volume (14, 18, 20, 23, 24, 35). With the recent refinements introduced by Romrell et al. (35), this technique provides purified populations of pachytene primary spermatocytes, round spermatids, and residual bodies, but does not yield germ cells at any step of development preceding the pachytene stage of meiotic prophase. The low frequency of spermatogonia and early primary spermatocytes in the adult seminiferous epithelium does not permit isolation of these particular cell types.

Spermatogenesis is initiated shortly after birth. In consequence, during the prepuberal period the seminiferous epithelium contains only Sertoli cells, spermatogonia, and, with increasing age, primary spermatocytes at progressively more advanced stages of meiotic prophase (3, 10, 27). The feasibility of isolating discrete populations of spermatogonia and primary spermatocytes from prepuberal rats was recently demonstrated by Davis and Schuetz (4). However, their respective populations of germ cells were not sectioned and characterized by light and electron microscopy, and consequently it is difficult to discern the identity and to assess the purity of their cell populations, particularly those at early stages of meiotic prophase.

In this report, a procedure is described which permits the recovery from the prepuberal mouse testis of highly purified populations of primitive type A spermatogonia, type A spermatogonia, type B spermatogonia, preleptotene spermatocytes, leptotene/zygotene spermatocytes, pachytene spermatocytes, and prepuberal Sertoli cells. The identity and purity of the isolated germ cell and Sertoli cell populations have been verified by light and electron microscopy, using as criteria distinctive morphological features which are characteristic of the respective cell types. Identification was aided considerably by a concomitant study of the cells *in situ* within the intact testis and the isolated seminiferous cords. The integrity of the isolated cells has been assessed by Nomarski differential interference microscopy, light and electron microscopy and by the exclusion of trypan blue.

MATERIALS AND METHODS

Materials

Male and female mice CD-1 strain, 70–100 days of age, were purchased from Charles River Breeding Laboratories, Inc. (Wilmington, Mass.). Bovine serum albumin (BSA, fraction V), trypsin (bovine pancreas, type III), and deoxyribonuclease (DNase-I, DN-EP) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Collagenase (CLS) is a product of Worthington Biochemical Corp. (Freehold, N. J.); the preparation of essential amino acids (×100) is supplied by Microbiological Associates (Bethesda, Md.), and Eagle's nonessential amino acids (×100) are obtained from Difco Laboratories (Detroit, Mich.). The glass STA-PUT sedimentation chambers (SP-120 and SP-180) were purchased from Johns Scientific, Toronto, Canada. Nitex filter cloth is a product of Tet/Kressilk, Inc., New York. The enriched Krebs-Ringer bicarbonate medium (EKRB) contained 120.1 mM NaCl, 4.8 mM KCl, 25.2 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4 \cdot 7H_2O$, 1.3 mM $CaCl_2$, 11.1 mM glucose, 1 mM glutamine, 10 ml/liter of essential amino acids, 10 ml/liter of nonessential amino acids, 100 μg/ml streptomycin sulfate, and 60 μg/ml penicillin G (K^+ salt). Immediately before use, the medium was filtered (0.30-μm Millipore filter [Millipore Corp., Boston, Mass.]) and the pH adjusted to 7.3 by a 15–20 min aeration with 5% CO_2 in air. The siliconized glassware and other equipment were sterilized before use.

Preparation of Seminiferous Epithelial Cells

Female mice were naturally mated and observed at 12-h intervals near the end of pregnancy to record the time at which parturition occurred. The day of birth was designated as day 0. Litter size was adjusted to a maximum of ten by removing the appropriate number of female pups. The required number of male pups, 30–80 depending upon the age, were sacrificed on each day from day 6 to 20, inclusive. The testes were excised and decapsulated by making a small incision in the tunica albuginea and gently expressing the contents onto an aseptic surface. The decapsulated testes were kept in EKRB at 22°C until all samples were collected, a period which usually did not exceed 30 min. The method of Romrell et al. (35), with modifications, was used to

isolate the seminiferous cords and to prepare the suspension of spermatogenic cells. The decapsulated testes were placed in 20 ml of EKRB containing 0.5 mg/ml collagenase and incubated under 5% CO_2 in air for 15 min at 33°C in a shaking water bath (Precision Scientific Co., Chicago, Ill.) operated at 120 cycles per min. The dispersed seminiferous cords were isolated by allowing them to sediment in EKRB (3–4 min) and then decanting the supernate. This process was repeated three times to ensure removal of the dispersed interstitial tissue (35). The seminiferous cords were incubated in 20 ml of EKRB containing 0.5 mg/ml trypsin and 1 μg/ml DNase for 15 min at 33°C, using the conditions described above. Most of the cell aggregates which remained after this treatment were sheared gently by repeated pipetting with a Pasteur pipet for 3 min. The dispersed seminiferous cells were then washed twice, resuspended in EKRB containing 0.5% BSA, filtered through Nitex filter cloth (40 mesh), and the cell concentration was determined with a hemocytometer.

Separation of Seminiferous Epithelial Cells

Cells of the dissociated seminiferous epithelium were separated by sedimentation velocity at unit gravity at 4°C, using a 2–4% BSA gradient in EKRB (35). The cells were bottom-loaded into either the SP-120 or SP-180 chamber in a volume of 10 or 25 ml, respectively, and at a maximum concentration of 10^7/ml. A linear gradient was then generated from either 275 ml of 2% BSA and 275 ml of 4% BSA, or 550 ml of 2% BSA and 550 ml of 4% BSA, depending upon the size of the chamber. The time allowed for sedimentation, including generation of the gradient and unloading of the chamber, was standardized at 4 h. The SP-120 and SP-180 chambers were unloaded from the bottom in 5- and 10-ml fractions, respectively, to ensure that the different cell types were recovered in comparable fraction numbers. The first fraction collected was designated as 1 and the remainder were numbered through to 100. The cells in each fraction were pelleted by centrifugation at 200 *g* for 5 min at 4°C in a Sorvall GLC-2 centrifuge (DuPont Instruments, Sorvall Operations, Newtown, Conn.). The supernate was then aspirated, and the cells were resuspended in 0.5 ml of EKRB containing 0.5% BSA. An aliquot of each fraction was examined by Nomarski differential interference microscopy to assess cellular integrity and, where feasible, to identify the cell types. The mean cell diameter was determined with a calibrated eyepiece micrometer. Fractions containing cells of similar size and morphology were pooled, and an aliquot was taken to determine the percentage of cells which excluded trypan blue. The remaining portion of each sample was prepared for detailed light and electron microscope examination.

The criteria required for the identification of isolated germ cells were obtained from a concurrent morphological study on the postnatal development of the mouse testis. Three mice were sacrificed on each alternative day from day 0 to 20 and the testes excised and prepared for light and electron microscopy. Samples of isolated seminiferous cords, prepared by incubating the testes in collagenase, were also examined.

Preparation of Samples for Microscopy

Whole prepuberal testes were immersed for 60 min in modified Karnovsky's fixative (17) containing 2.5% glutaraldehyde, 2% formaldehyde and 0.05% calcium chloride in 0.1 M cacodylate buffer (pH 7.3). After 15 min, the samples were cut into 1 mm cubes, postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.3) for 2 h, dehydrated in ethanol, and embedded in Epon (21). Isolated seminiferous cords and the respective germ cell populations were initially fixed for 30 min in 2% glutaraldehyde in EKRB (pH 7.3) (35). Secondary fixation was achieved in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.3) for 30 min. The samples were then embedded in low-viscosity Epon (22). 1-μm sections were stained with toluidine blue and examined by light microscopy. Thin sections were stained with uranyl acetate and lead citrate (34) and examined by electron microscopy.

RESULTS

Prepuberal Development of the Seminiferous Epithelium

It was essential to define the morphological characteristics and the temporal appearance of spermatogenic cells in the prepuberal seminiferous epithelium before attempting to separate the cells. These two objectives were accomplished by a light and electron microscope examination of the intact testis at successive intervals during prepuberal development.

The seminiferous epithelium of the newborn mouse testis contains two distinct cell types, gonocytes and Sertoli cells (3). The former are evident in the center of the cords as large round cells, ~20–24 μm in diameter, having a spherical nucleus with dispersed homogeneous chromatin and a central filamentous nucleolus. The cytoplasm contains spherical mitochondria in relatively low numbers.

By 6 days after birth, the germ cells are attached to the basement membrane and have differentiated to form primitive type A spermatogonia (Fig. 1). These cells are similar in appearance to the gonocytes except that they are smaller, ~14–15 μm in diameter, and the nucleus contains scattered flakes of heterochromatin. Furthermore, the nu-

cleoli now have a prominent reticulated nucleolonema, an irregular shape, and occupy an eccentric position in the nucleus. Primitive type A spermatogonia comprise 16% of all cells in the seminiferous epithelium at day 6 (Table I).

In contrast to primitive type A spermatogonia, the more numerous Sertoli cells exhibit frequent irregularities in both nuclear and cytoplasmic contour (Figs. 1 and 2). Scattered areas of heterochromatin occur around the periphery of the nucleus, and the cytoplasm contains many spherical and elongated mitochondria. At this stage in development, the Sertoli cell lacks both the paired nucleolar karyosomes and cytoplasmic lipid inclusions and is considerably smaller in volume than the adult cell.

Type A and type B spermatogonia are present at day 8 after birth (Table I). In comparison to primitive type A spermatogonia, type A spermatogonia have a reduced nuclear and cytoplasmic volume. Nuclear chromatin is again homogeneous, and multiple reticulated nucleoli are seen in close proximity to the nuclear membrane (Figs. 2 and 4). Type B spermatogonia have a diameter of ~8–9 μm and are therefore considerably smaller than type A (Fig. 2). These cells have an increased amount of heterochromatin. A thin rim of condensed chromatin around the nuclear membrane is interspersed with larger areas of more highly condensed chromatin. A single reticulated nucleolus is usually situated near the center of the nucleus.

Primary spermatocytes at the preleptotene and leptotene stages of meiotic prophase are present at day 10 of development (Table I). The preleptotene spermatocytes are the smallest of germ cells with a diameter of only ~7.5–8.2 μm (Fig. 5) and are usually separated from the basement membrane by Sertoli cell processes (28, 29, 40). Localized areas of condensed chromatin occur scattered throughout the nucleus and occasionally in close proximity to the nuclear membrane. The remaining chromatin is dispersed in a homogeneous manner, but is more densely granular than in type B spermatogonia. Characteristically, the preleptotene spermatocyte contains a limited amount of cytoplasm. Leptotene primary spermatocytes are characterized by the appearance of thin axial elements in the nucleus which signify the initiation of chromosome condensation. In other areas, the chromatin of leptotene spermatocytes is homogeneous and granular, with the condensed chromatin seen at the preleptotene stage being compressed against the nuclear membrane (Fig. 3). Fragments of nucleolonema are frequently observed. These cells have a diameter of ~8–10 μm and are therefore similar in size to type B spermatogonia.

Zygotene primary spermatocytes, first observed on day 12 postnatal (Table I), are characterized by the appearance of short segments of synaptonemal complexes (Fig. 4). Condensation of the X and Y chromosomes to form the "sex vesicle" is also initiated at this stage of meiotic prophase. A nucleolus with a reticulated nucleolonema is either tangentially attached to or located in the vicinity of the sex vesicle. These cells have a diameter of ~10–12 μm and are therefore larger than leptotene spermatocytes. The cytoplasm contains increasing amounts of endoplasmic reticulum and piles of narrow cisternae (29). Mitochondria become more elongated with a few dilated cristae.

The transition from zygotene to the pachytene stage of meiotic prophase is gradual (Fig. 5). The autosomes, now paired by the synaptonemal complexes, become increasingly condensed during the prolonged pachytene period. A prominent nucleolar cap later forms over the sex vesicle which becomes closely applied to the nuclear membrane. Increasing numbers of mitochondria with dilated cristae aggregate in clusters as the pachytene stage advances. Both endoplasmic reticulum and areas of thin cisternae also increase substantially during this stage. Primary spermatocytes reach an early pachytene stage by day 14 (Table I), but late pachytene spermatocytes are not observed until days 18–20. During the pachytene stage, cell diameter increases from ~12 μm to a maximum of 18 μm.

The seminiferous cord has acquired a lumen in many areas by day 16. Secondary spermatocytes and round spermatids occur in increasing numbers by day 20, thereby signifying the onset of spermiogenesis.

Separation of Seminiferous Epithelial Cells by Sedimentation Velocity

It is evident that (*a*) primitive germ cells differentiate in a defined temporal sequence during prepuberal development, (*b*) cell volume decreases progressively with successive divisions of spermatogonia and then increases substantially between the preleptotene and late pachytene stage of meiotic prophase, and (*c*) these cells can be identified by virtue of their distinctive morphological characteristics. This information was applied in

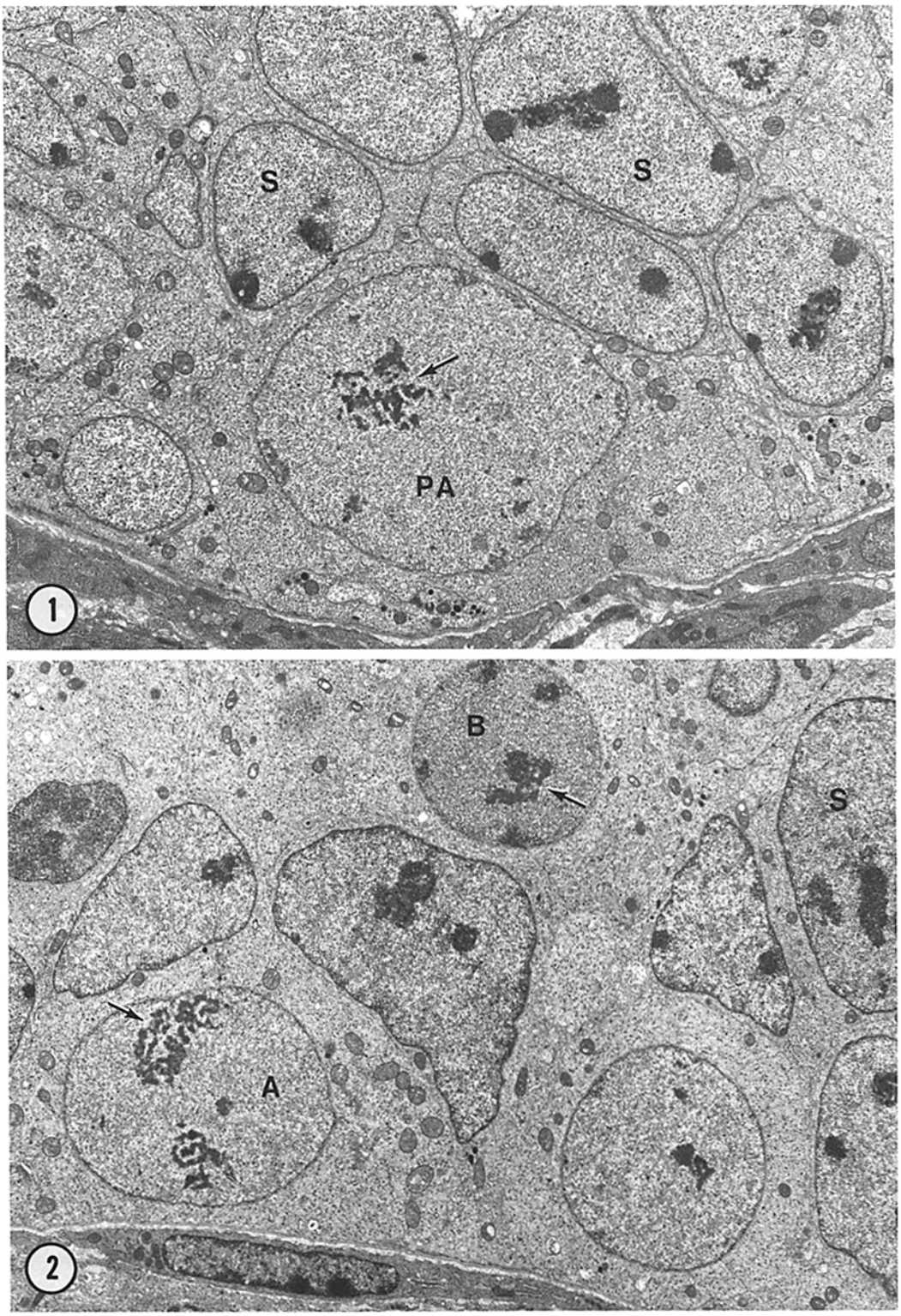


FIGURE 1 Electron micrograph of day 6 mouse seminiferous epithelium showing several Sertoli cells (*S*) and a large primitive type A spermatogonium (*PA*). The latter contains a centrally located nucleolus with reticulated nucleolonema (arrow). × 4,800.

FIGURE 2 Electron micrograph of day 8 mouse seminiferous epithelium showing type A spermatogonium (*A*), type B spermatogonium (*B*), and several Sertoli cells (*S*). Note differences between spermatogonia in the size of mitochondria and position of nucleoli (arrows). × 4,800.

TABLE I

The Temporal Appearance of Spermatogenic Cells in the Prepuberal Mouse Testis

Cell type	Days after birth							
	6	8	10	12	14	16	18	20
Primitive type A spermatogonia	16	–	–	–	–	–	–	–
Type A spermatogonia	–	17	7	7	6	9	3	4
Type B spermatogonia	–	10	11	8	6	8	7	6
Preleptene spermatocytes	–	–	15	11	9	5	10	7
Leptotene spermatocytes	–	–	15	12	13	5	5	9
Zygotene spermatocytes	–	–	–	23	14	7	8	8
Pachytene spermatocytes	–	–	–	–	15	27	36	33
Secondary spermatocytes	–	–	–	–	–	–	1	1
Round spermatids	–	–	–	–	–	–	1	4
Sertoli cells	84	73	52	39	37	39	29	28

Data are expressed as percentage of total cells in the seminiferous epithelium. The cell counts were determined by classifying nuclei present in 50 cross sections of seminiferous cords chosen at random from the testes of three mice sacrificed at each designated age. Degenerating and unidentified cells (2–4%) have not been included in these data.

an effort to isolate and identify discrete populations of spermatogenic cells from the prepuberal seminiferous epithelium.

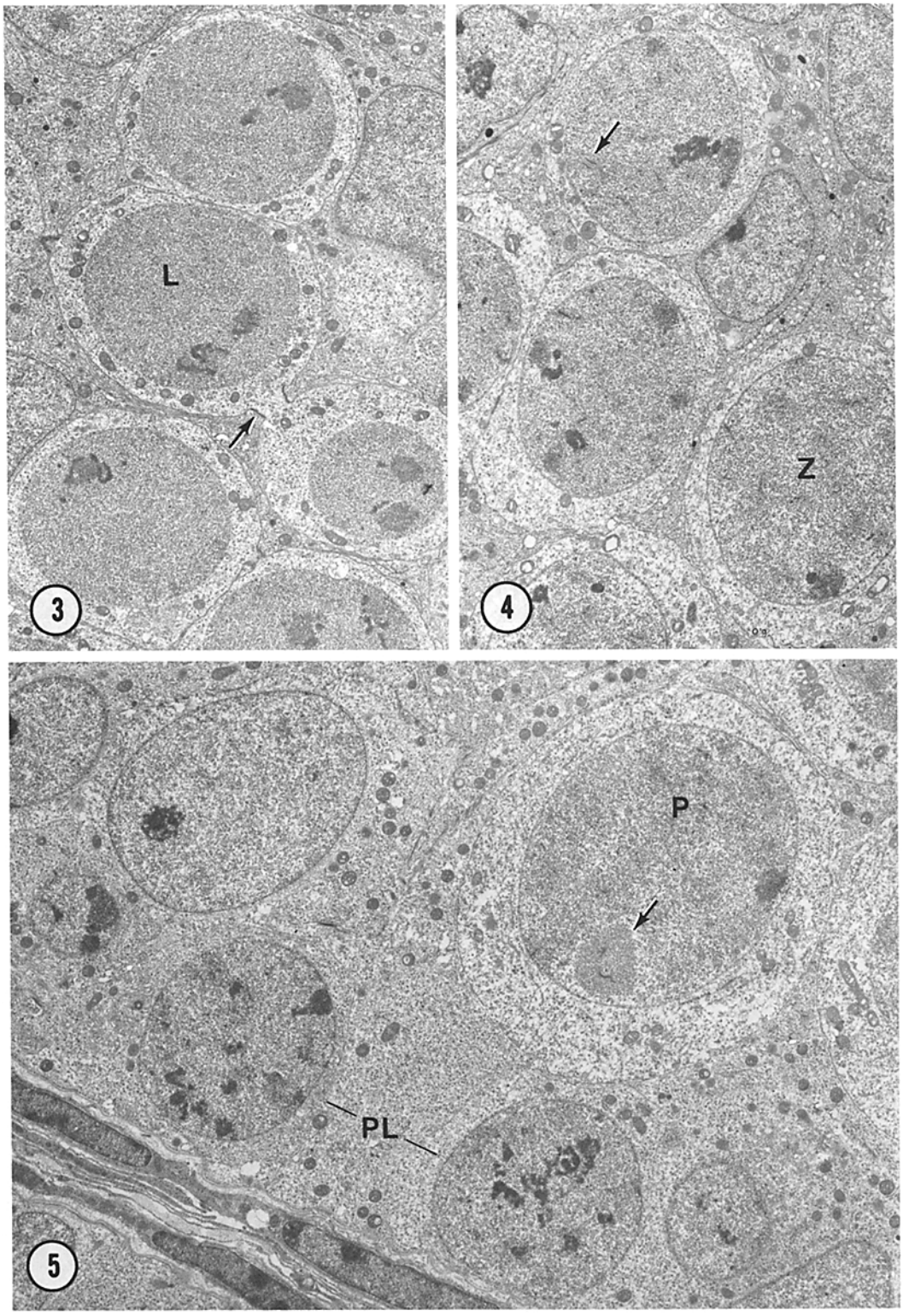
The sequential enzymatic procedure was found to be effective for both the isolation of seminiferous cords and the preparation of cell suspensions from the prepuberal testes. The yield of seminiferous epithelial cells per testis ranges from 7×10^5 at day 6 to 18×10^5 at day 18. Examination of the cells by light and electron microscopy demonstrates that they retain their general morphological characteristics. Furthermore, between 96 and 98% of the cells in the initial suspension and after separation continue to exclude trypan blue. Erythrocytes, lymphocytes, macrophages, myoid cells, and Leydig cells were observed rarely in the isolated cell populations.

When seminiferous cells of day 6 testes are subjected to sedimentation velocity at unit gravity, three distinct "cell populations" are recovered. Those "cells" having the lowest sedimentation velocity are recovered in fractions 84–92. By Nomarski differential interference microscopy, these cells are 4–6 μm in diameter, smooth in outline, and appear to lack nuclei or cytoplasmic inclusions (Fig. 6). Examination of thin sections reveals that this fraction contains cytoplasts of varying electron density (Fig. 7). Although many of these cytoplasts exhibit concentric whorls of membrane, only a few contain any mitochondria. Other cytoplasts contain numerous small vesicles. Since 98% of the cytoplasts exclude trypan blue, it is assumed that the limiting cytoplasmic membrane is intact. Whether the cytoplasts originate from the cytoplasm of spermatogonia or Sertoli cells is at present unresolved, although the latter origin is more likely.

An extremely homogeneous population of Sertoli cells is obtained on day 6 by pooling fractions 66–76. These cells have a diameter of 7.5–8.2 μm and an irregular outline, and appear almost granular in nature under Nomarski optics (Fig. 8). The latter characteristics make it difficult to discern any nuclear morphology by light microscopy. Electron microscope examination, however, reveals the characteristic irregularities in nuclear configuration and the typical pattern of heterochromatin and dense nucleoli in close association with the nuclear membrane (Fig. 9). The purity of the Sertoli cell population, as determined from both thin and thick sections, is >99% (Table II).

The primitive type A spermatogonia are recovered from the day 6 testis by pooling fractions 36–50 (Fig. 10). These cells have a diameter of 14–16 μm, which is comparable to their size in vivo. They are spherical in outline with large round nuclei which contain flakes of heterochromatin and two to three eccentrically placed nucleoli (Fig. 11). The minimal purity of the primitive type A spermatogonia is 90%, with Sertoli cells constituting the only source of contamination (Table II).

Cell suspensions were prepared from day 8 testes and the separated cells again characterized by light and electron microscopy. Large spherical cells, 12–14 μm in diameter, are recovered in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fractions 40–45 (Fig. 12). They are identified as type A spermatogonia since multiple nucleoli are seen in close proximity to the nuclear membrane, the chromatin is relatively homogenous, and the nuclear/cytoplasmic ratio has increased substantially (Fig. 13). Populations of type A spermatogonia can be prepared so as to exceed 91% purity (Table II). Contaminating cell types include Sertoli cells and type B spermatogonia.

Fractions 64–72 contain type B spermatogonia (Fig. 14). These cells are smaller (8–10 μm in diameter) than type A spermatogonia and contain greater quantities of heterochromatin and small spherical mitochondria (Fig. 15). Many cells are seen in metaphase and late prophase configurations of mitosis. This population of cells may also include intermediate spermatogonia which are difficult to distinguish from type B spermatogonia. On this basis, the population of type B is assessed at 76% purity. The principal source of contamination is Sertoli cells (Table II). Most of the Sertoli cells, however, are recovered in fractions 46–62. The greater sedimentation velocity of the Sertoli cell population on day 8 reflects a 15% increase in cell volume that has occurred since day 6 of postnatal development. Cytoplasts, comparable to those of day 6, are also recovered on day 8 but generally in lower numbers.

Cell separations were also performed over the developmental period of days 9–16, inclusive. Although relatively homogeneous populations of primary spermatocytes can be obtained, the level of purity is inconsistent. The variability is due to contamination of the primary spermatocyte populations by spermatogonia and Sertoli cells. Type A and type B spermatogonia contaminate preparations of zygotene and leptotene spermatocytes, respectively. Furthermore, a proportion of the Sertoli cells loses some cytoplasm during the preparative procedures, and some form aggregates of one or more cells after loading into the sedimentation chamber. The sedimentation velocity of these cells is either less than or greater than that of single intact Sertoli cells. Consequently, they contaminate cell populations in other regions of the gradient. Frequently, two to five preleptotene spermatocytes are seen attached to a single Sertoli cell, thereby forming an aggregate which may also cosediment with pachytene spermatocytes. The recovery of Sertoli cells and spermatogonia, relative to primary spermatocytes, decreases between days 10 and 18. This reduction is due, in part, to the decreasing proportion of Sertoli cells and spermatogonia in the seminiferous epithelium as development progresses (Table I). In addition, however, both of these cell types are apparently lost differentially during the filtration steps.

Attempts were made to isolate primary spermatocytes at successive stages of meiotic prophase from the day 18 seminiferous epithelium. Examination by Nomarski differential interference microscopy reveals cells which are similar in morphology but which differ substantially in size over successive fractions of the gradient. In order to identify which fractions contain cells at the desired stages of meiotic prophase, the following experimental protocol was adopted. Every two successive fractions throughout the gradient were pooled and the mean diameter of cells in each pool was determined by light microscopy using a calibrated eyepiece micrometer. The cells present in each pool were then identified by light and electron microscopy. In subsequent separation attempts, cell diameter was used to select with precision those fractions which could be pooled to yield populations of spermatocytes at specific stages of meiotic prophase. On this basis, populations of preleptotene, leptotene/zygotene, and pachytene primary spermatocytes were recovered.

Preleptotene primary spermatocytes, which

FIGURE 3 Electron micrograph of day 18 mouse seminiferous epithelium showing leptotene primary spermatocytes (*L*). Note homogeneous density of nucleoplasm and relatively pale cytoplasm. Cytoplasmic bridge connects two adjacent cells (arrow). × 4,800.

FIGURE 4 Electron micrograph of day 18 mouse seminiferous epithelium showing zygotene primary spermatocytes (*Z*). Nuclei contain patchy condensed chromatin and short segments of synaptonemal complex (arrow). × 4,800.

FIGURE 5 Section of day 18 mouse seminiferous epithelium depicting preleptotene primary spermatocytes (*PL*) near basal lamina, and pachytene primary spermatocytes (*P*). The latter contains a condensed sex vesicle (arrow) in close proximity to the nuclear membrane. × 4,800.

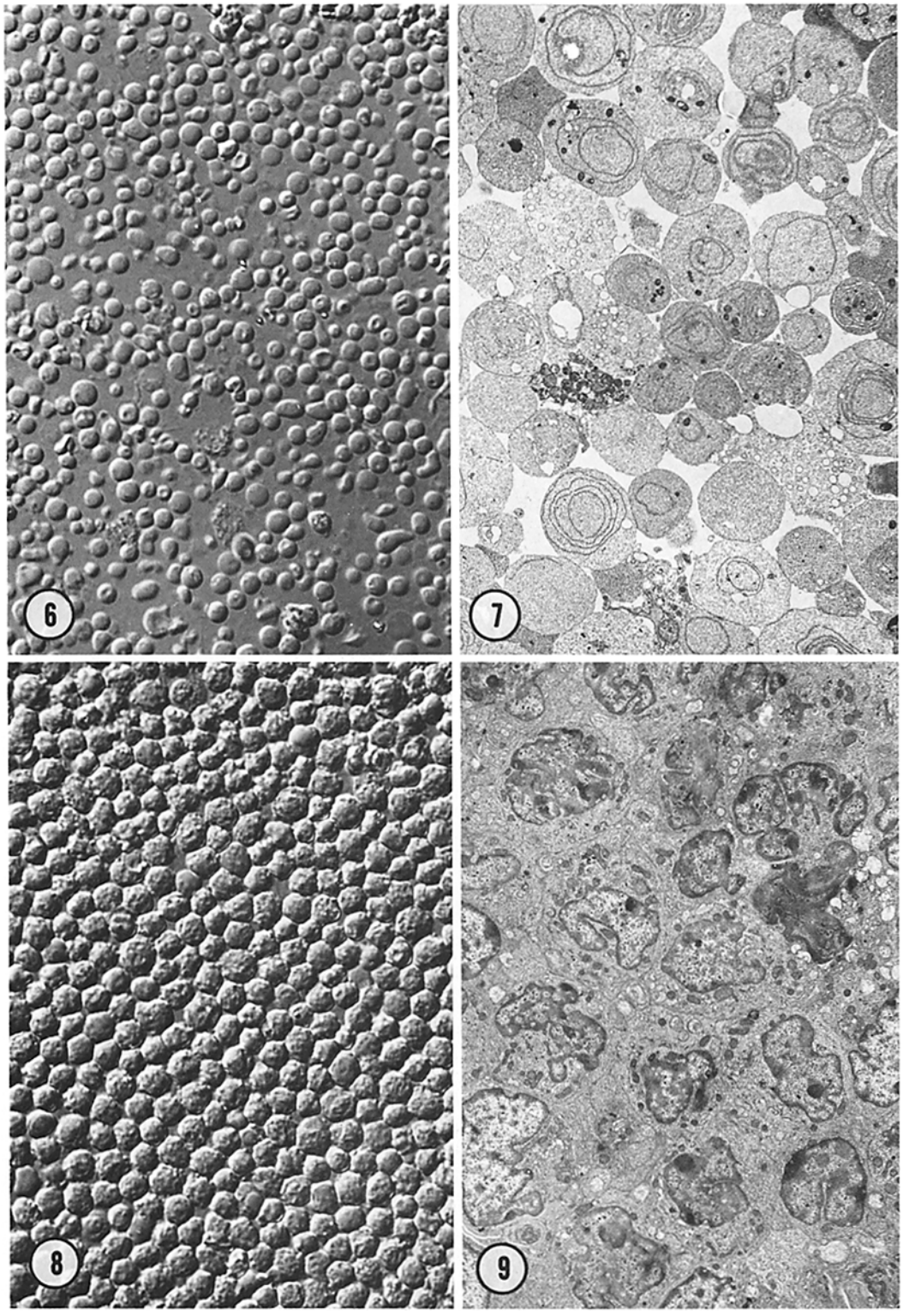
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TABLE II

Purity and Composition of Cell Populations Isolated from the Prepuberal Mouse Seminiferous Epithelium

Cell type	Cell populations						
	PA	A	B	PL	L/Z	P	S
				%			
Primitive type A spermatogonia	**90**	–	–	–	–	–	<1
Type A spermatogonia	–	**91**	6	–	–	<1	–
Type B spermatogonia	–	3	**76**	1	1	–	–
Preleptotene spermatocytes	–	–	–	**93**	7	–	–
Leptotene spermatocytes	–	–	–	3	**31**	–	–
Zygotene spermatocytes	–	–	–	–	**21**	<1	–
Pachytene spermatocytes	–	–	–	–	10	**89**	–
Sertoli cells	10	6	16	3	29	10	**>99**

Data are expressed as percent of total cells recovered in each isolated cell population. Those cells (3–5%) which could not be identified because of necrosis or unfavorable section are not included. The population of type B spermatogonia may contain a small proportion of intermediate spermatogonia which have a greater cell diameter (~10–11 μm) but cannot always be identified with certainty. Cell populations include primitive type A spermatogonia (PA), type A spermatogonia (A), type B spermatogonia (B), preleptotene spermatocytes (PL), pooled leptotene and zygotene spermatocytes (L/Z), pachytene spermatocytes (P), and Sertoli cells (S).

range in diameter from 7.6 to 8.2 μm (mean 7.8 μm), are recovered by pooling fractions 65–72. Except for an occasional Sertoli cell, this cell population appears homogeneous when examined by Nomarski microscopy (Fig. 16). In thin sections, these cells are seen to have regions of condensed heterochromatin throughout the nucleus as well as attached to the nuclear membrane (Fig. 17). Both nuclear and cytoplasmic volumes are smaller than in type B spermatogonia. The purity of the preleptotene spermatocytes is assessed at 93% (Table II).

Leptotene and zygotene primary spermatocytes have cell diameters ranging from ~8.2 to 10 μm and ~10 to 12 μm, respectively. These cells are recovered as a single enriched population by pooling fractions 48–56 (Fig. 18). Leptotene spermatocytes are identified by their larger nuclear volume, homogeneous chromatin, and the occurrence of a few elongated mitochondria in the cytoplasm (Fig. 19). The diffuse threads of axial elements are not always evident in the isolated cells. Zygotene spermatocytes can be discerned by the increase in localized areas of heterochromatin, initiation of the sex vesicle, and short but infrequent segments of synaptonemal complex. This cell population, however, is invariably contaminated with Sertoli cells and less frequently with binucleate preleptotene spermatocytes and early pachytene spermatocytes (Table II and Fig. 19). Due to the contamination by Sertoli cells, separation of leptotene and zygotene spermatocytes, although feasible, was not attempted. Furthermore, it is not possible to isolate homogeneous populations of these cells after day 18 in development, since round spermatids, which occur in increasing numbers (Table I), cosediment with the population of leptotene and zygotene spermatocytes.

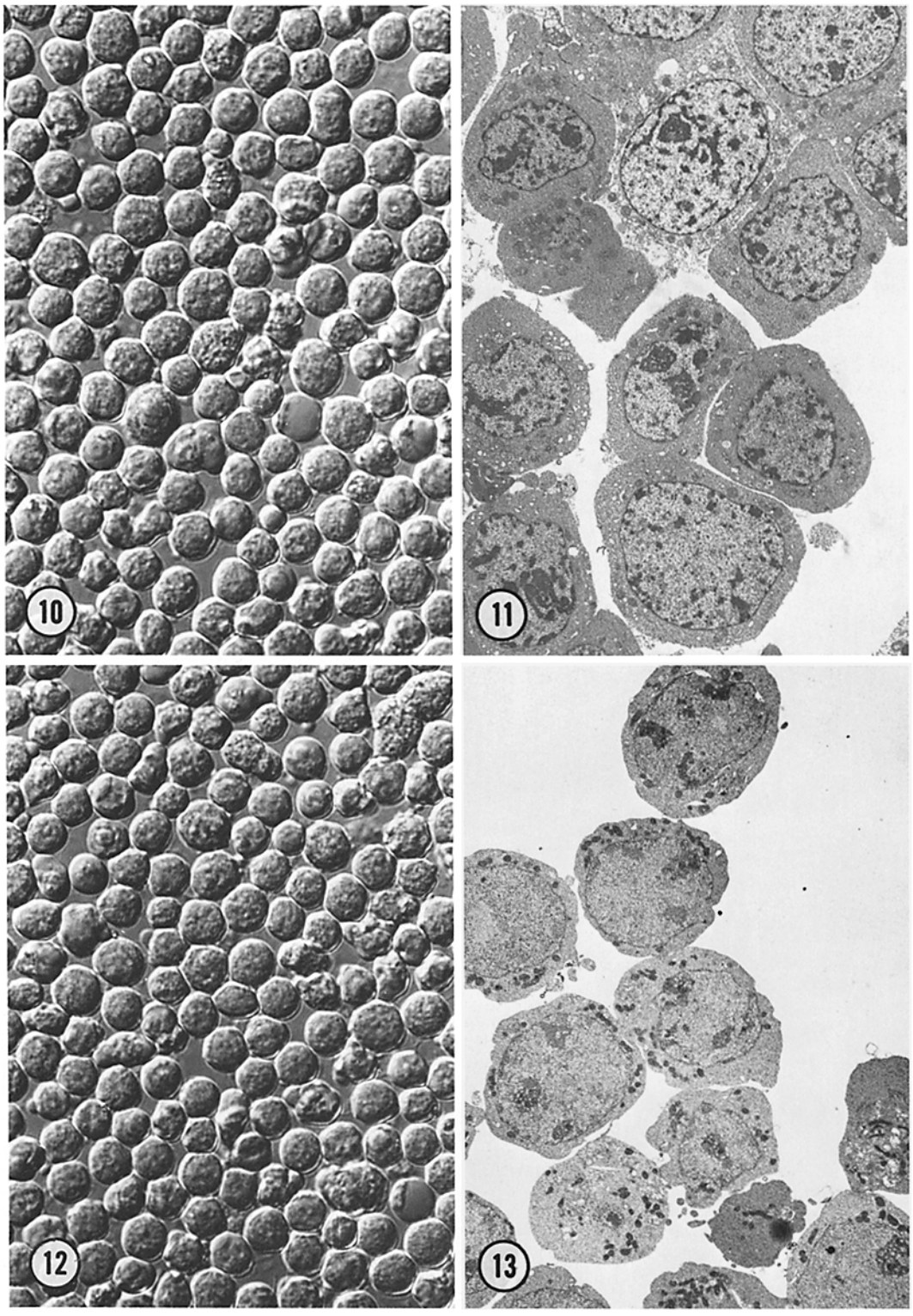
Pachytene primary spermatocytes range from 12 to 18 μm in diameter and can be recovered on

FIGURE 6 Cytoplasts isolated from the seminiferous epithelium of the day 6 mouse testis by differential sedimentation velocity. Nomarski photomicrograph. × 850.

FIGURE 7 Electron micrograph of cytoplasts isolated from the day 6 mouse seminiferous epithelium. Note concentric whorls of membrane and absence of nuclei. × 2,800.

FIGURE 8 Sertoli cells isolated from the day 6 mouse seminiferous epithelium by differential sedimentation velocity. Nomarski photomicrograph. × 850.

FIGURE 9 Electron micrograph of isolated prepuberal Sertoli cells. Note irregular configuration of nucleus and cytoplasm, and heterochromatin in association with the nuclear membrane. × 2,800.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this basis in fractions 16–40. These cells are identified by their patchy condensed chromatin and prominent sex vesicle with, at later stages, its associated nucleolar cap. Mitochondria with dilated cristae and the extensive endoplasmic reticulum can also be discerned. The purity of this cell population usually exceeds 89% (Table II).

Unfortunately, it is not possible to obtain valid estimates of cell yield after enzymatic dissociation of the testis. Calculations based on cell number are impractical since serial sections would be required to accurately assess the total population of testicular cells. The problem is complicated further by the removal of interstitial tissue and by a nonselective loss of germ cells from the ends of tubules during the collagenase treatment. Furthermore, estimates of yield based on DNA content are confounded by the differential recovery of cells having a 4C, 2C, and 1C complement of DNA. However, of the total cells present in the final cell suspension, ~65–75% are recovered within the pooled populations of specific cell types. The remaining cells are recovered as heterogeneous populations and are therefore discarded. The approximate number of cells recovered per testis at the appropriate ages is as follows: day 6, 1.6×10^5 cytoplasts, 4.2×10^5 Sertoli cells, and 1.4×10^5 primitive type A spermatogonia; day 8, 1.6×10^5 type A spermatogonia and 1.5×10^5 type B spermatogonia; day 18, 2.1×10^5 preleptotene spermatocytes, 2.6×10^5 leptotene and zygotene spermatocytes, and 7.3×10^5 pachytene spermatocytes.

DISCUSSION

In the adult testis, spermatogenic cells exist in complex cellular associations and are classified into stages of the cycle of the seminiferous epithelium on the basis of steps in spermatid differentiation (19, 30, 31). In this system, identification of the different classes of spermatogonia and early primary spermatocytes is facilitated by their association with spermatids at specific steps of spermiogenesis. Since spermatids are not present in the prepuberal seminiferous epithelium, a precise identification of early germ cells is considerably more difficult (3, 32). This particular problem has been surmounted in the present report by defining distinctive morphological characteristics of the respective seminiferous epithelial cells in the adult and using these criteria to identify the prepuberal cell types. The criteria used are in general concordance with the findings of previous light (3, 19, 30, 31) and electron microscope studies (8, 10–12, 29, 38–40).

The isolation of essentially pure populations of Sertoli cells was fortuitous. Although the prepuberal Sertoli cell lacks the distinctive paired karyosomes of the nucleolus in the adult, the irregular nuclear and cytoplasmic configuration, the peripherally located heterochromatin, and the occurrence of elongated mitochondria make identification of this cell type unambiguous. Previous efforts to isolate Sertoli cells from either the prepuberal (5, 42) or adult rat (41) have involved isolation of seminiferous tubules followed by a period of in vitro culture. During the initial period in culture, many of the contaminating germ cells are phagocytosed by the Sertoli cells. Also, this type of procedure may not entirely remove the peritubular cells which can proliferate and eventually form a fibroblastic contaminant of the Sertoli cell cultures. The present technique for the isolation of Sertoli cells has the distinct advantages of simplicity, brevity, and extreme purity. Furthermore, since the Sertoli cells are isolated during a period of mitotic activity, they may well continue to proliferate in vitro for several days before entering mitotic arrest.

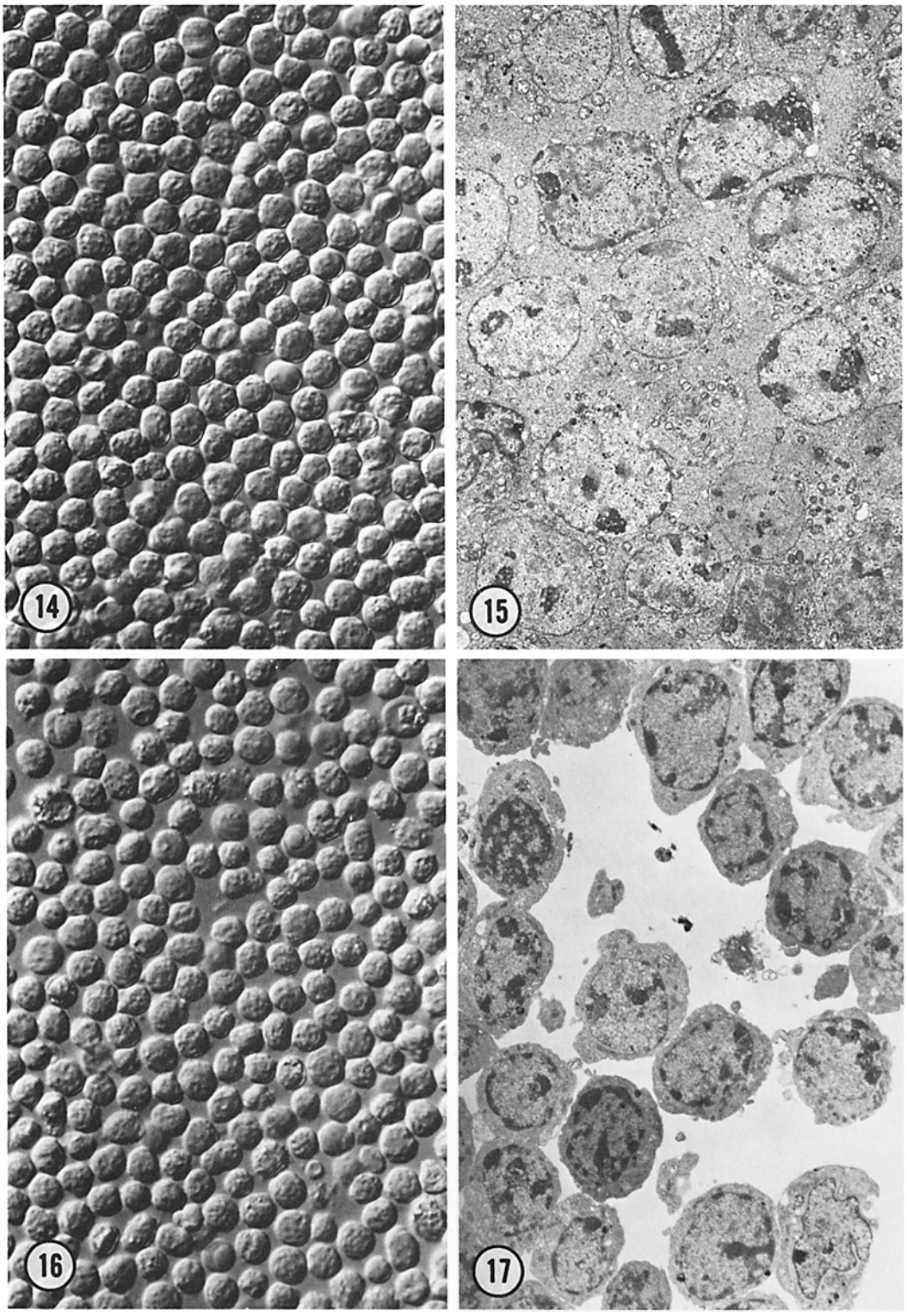
Previous studies have reported that the recovery

FIGURE 10 Primitive type A spermatogonia isolated from the day 6 mouse seminiferous epithelium by differential sedimentation velocity. Nomarski photomicrograph. × 850.

FIGURE 11 Electron micrograph of isolated primitive type A spermatogonia. Note flakes of heterochromatin and eccentrically placed nucleoli. × 2,800.

FIGURE 12 Type A spermatogonia isolated from the day 8 mouse seminiferous epithelium by differential sedimentation velocity. Nomarski photomicrograph. × 850.

FIGURE 13 Electron micrograph of isolated type A spermatogonia. Note smaller nuclear and cytoplasmic volume with nucleoli in close proximity to nuclear membrane. × 2,800.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of Sertoli cells and spermatogonia from the adult testis is substantially lower than expected (24, 35). One suggestion (4, 24) is that these particular cells may be more vulnerable to lysis during the enzymatic dissociation and therefore do not appear in the final cell suspension. This proposal is unlikely, however, since spermatogonia and Sertoli cells are recovered from the seminiferous epithelium on day 6 in normal yields and with viabilities of 96–98%. The current observations support alternative and more plausible explanations.

The occluding Sertoli junctions form during the postnatal period of development (13, 26). In the mouse 6 days after birth, the Sertoli junctions are seen merely as localized networks of intramembranous particles (26). Subsequently, however, the junctional complexes increase in area and length until at day 16 they appear as 40–60 parallel linear arrays of intramembranous particles which course circumferentially around the basal surface of the cell. The appearance at this time of a lumen in many areas of the seminiferous cords attests to the impermeability of the Sertoli junctions. With maturation of these tight junctions, the seminiferous epithelium is effectively divided into two compartments, a basal compartment in which the spermatogonia and perhaps early primary spermatocytes reside and an adluminal compartment in which the more advanced spermatocytes and spermatids are secluded (6). Clearly, the excessive decline in recovery of Sertoli cells and spermatogonia between days 10 and 18 coincides with the development of the Sertoli junctions. In this regard, it is pertinent that the tight junctions which exist between adjacent exocrine cells of the pancreas are not disrupted by digestion with chymotrypsin although dissociation does occur in a medium which either lacks Ca^{++} or contains EDTA (1). On the basis of this evidence, it is proposed that the Sertoli junctions are relatively resistant to tryptic dissociation. Consequently, the Sertoli cells remain as large aggregates in association with fragments of the basal lamina, and, together with spermatogonia entrapped in the basal compartment, are lost during the filtration steps. Thus, the liberation of spermatogonia from the day 20 rat testis in the presence of EDTA, but not after tryptic dissociation (4), may be due to dissociation of the Sertoli junctions. Alternatively, EDTA may cause the release of spermatogonia from the basal lamina although there is no direct evidence to support this contention.

The precise stage in differentiation at which spermatocytes traverse the Sertoli junctions is not as yet clearly delineated (cf. reference 6, 7, 9, 28, and 29). It is feasible, for instance, that preleptotene primary spermatocytes pass into the adluminal compartment and therefore may exist in both compartments of the seminiferous epithelium at different phases of their development. This would account for the high recovery of preleptotene spermatocytes which is observed after enzymatic dissociation of the day 18 mouse seminiferous epithelium. An alternative proposal which could account for the recovery of preleptotene spermatocytes, but not spermatogonia, is not immediately apparent.

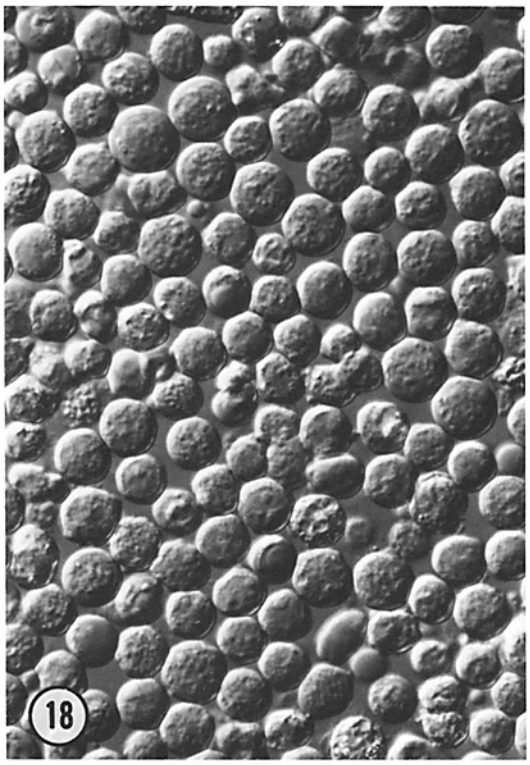
The isolation of seminiferous cells from the prepuberal testis is facilitated primarily by (*a*) the use of animals at specific stages of development, (*b*) the isolation of seminiferous cords before preparing the cell suspension, (*c*) the differential volume of these cells which enables their separation by sedimentation velocity, and (*d*) the identification of isolated cells by virtue of their distinctive morphological features. The sequential enzymatic dissociation permits isolation of intact seminiferous tubules and also ensures removal of the dispersed cellular components of the vascular system, interstitial cells, and virtually all of the peritubular

FIGURE 14 Type B spermatogonia isolated from the day 8 mouse seminiferous epithelium by differential sedimentation velocity. Nomarski photomicrograph. × 850.

FIGURE 15 Electron micrograph of isolated type B spermatogonia. Note decrease in cell size and characteristic pattern of heterochromatin. × 2,800.

FIGURE 16 Preleptotene primary spermatocytes isolated from the day 18 mouse seminiferous epithelium by differential sedimentation velocity. Nomarski photomicrograph. × 850.

FIGURE 17 Electron micrograph of isolated preleptotene primary spermatocytes. Note diminished cytoplasmic volume and localized areas of condensed chromatin. × 2,800.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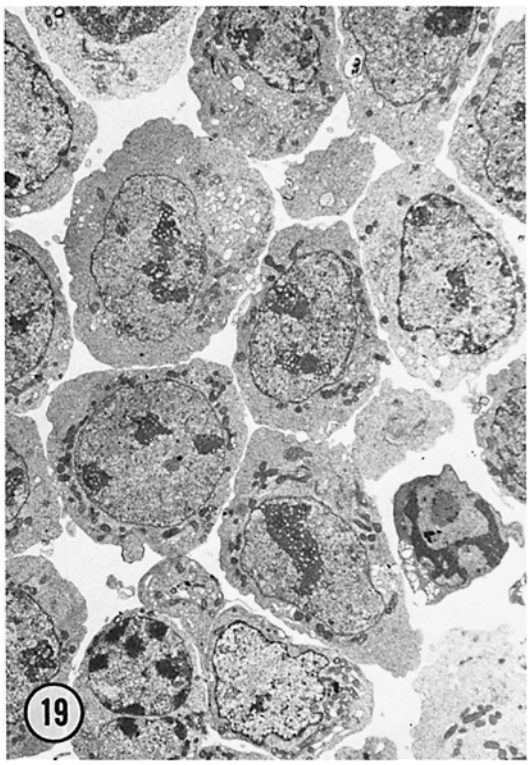
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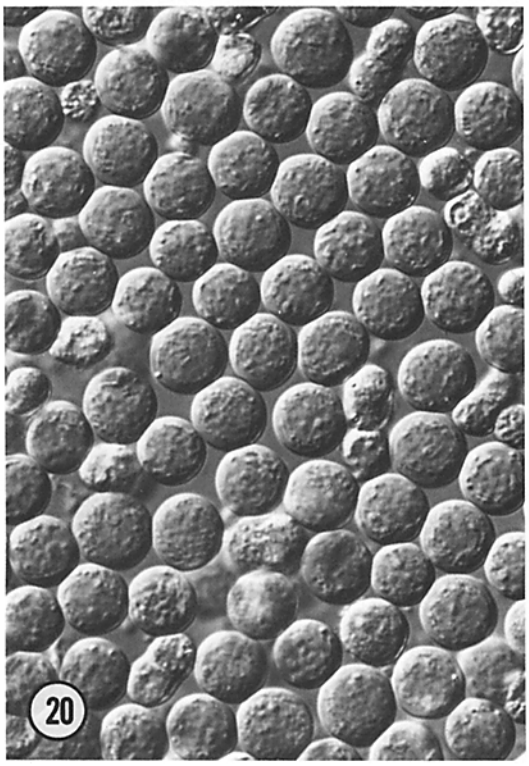
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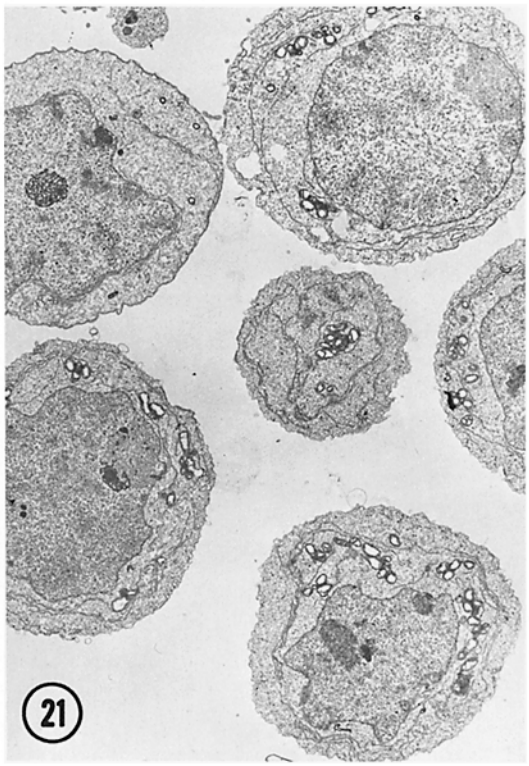
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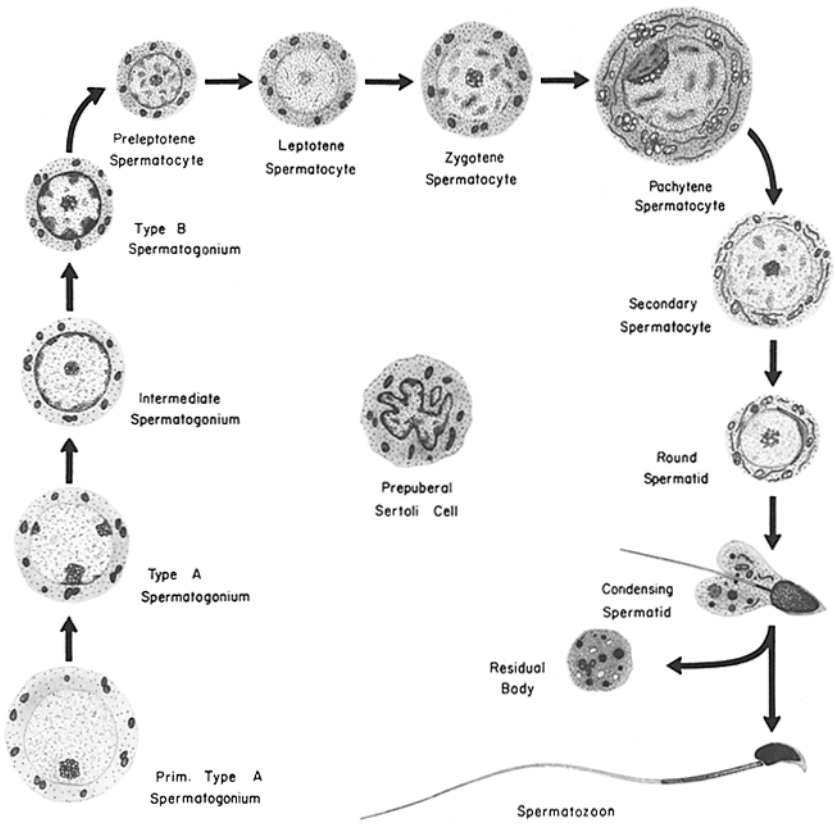

FIGURE 22 Schematic diagram of spermatogenesis in the prepuberal and adult mouse testis showing the relative volumes and characteristic morphology of the respective cell types. This complex process occurs in three phases: the mitotic proliferation of spermatogonia (ascending axis); meiosis with its prolonged meiotic prophase (horizontal axis) and two reduction divisions which yield 2× secondary spermatocytes and then 4× haploid round spermatids; and spermiogenesis (descending axis) which culminates in the formation of spermatozoa. Enriched populations of each cell type, except intermediate spermatogonia and secondary spermatocytes, can now be isolated (see text).

FIGURE 18 Pooled population of leptotene and zygotene primary spermatocytes isolated from the day 18 mouse seminiferous epithelium by differential sedimentation velocity. Contaminating Sertoli cells are slightly refractile and irregular in outline. Nomarski photomicrograph. × 850.

FIGURE 19 Electron micrograph of pooled leptotene and zygotene primary spermatocytes after isolation. Contaminating Sertoli cell (lower center) and a binucleate preleptotene primary spermatocyte (lower left) are also present. × 2,800.

FIGURE 20 Pachytene primary spermatocytes isolated from the day 18 mouse seminiferous epithelium by differential sedimentation velocity. Nomarski photomicrograph. × 850.

FIGURE 21 Electron micrograph of isolated pachytene primary spermatocytes. Note patchy areas of condensed chromatin, sex vesicle, nucleolar cap, and mitochondria with dilated cristae. × 2,800.

cells. The interstitial tissue is thereby effectively eliminated as a source of contamination. Furthermore, the existence of the Sertoli junctions appears to facilitate the isolation of preleptotene spermatocytes and leptotene/zygotene spermatocytes with minimal contamination by type B and type A spermatogonia, respectively. Caution must be taken, however, to prevent contamination of the leptotene/zygotene spermatocytes by round spermatids which first appear in appreciable numbers during days 19 and 20 of development.

By utilizing these physical parameters of the seminiferous epithelium, it proved possible to isolate highly enriched populations of primitive type A spermatogonia, type A spermatogonia, type B spermatogonia, preleptotene spermatocytes, and pachytene spermatocytes. An enriched population of leptotene and zygotene spermatocytes can also be obtained. Furthermore, since the volume of the pachytene spermatocytes ranges from 900 μm^3 to 3,050 μm^3, it may also be feasible to isolate populations of early, mid, and late pachytene spermatocytes. The isolation of spermatogenic cells at these earlier stages of differentiation complements those obtained previously from the adult seminiferous epithelium, namely, late pachytene spermatocytes, round spermatids, and residual bodies (Fig. 22) (35). The extremely short duration of both the diplotene primary spermatocyte and secondary spermatocyte stages does not permit the isolation of these particular cell types. It should be noted that the population of type A spermatogonia may contain, in addition to types A_1 to A_4, a small number of the nonproliferating (Ao) and renewal stem cells (As, Apr, Aal) (2, 16, 32, 33). The isolated seminiferous cells continue to exclude trypan blue (96–98%) and retain their morphological integrity as determined by light and electron microscopy. Moreover, spermatogenic cells isolated from the adult testis under identical conditions show appreciable levels of oxygen consumption (35). The incidence of degenerating prepubberal spermatogenic cells (2–4%) is not greater than expected under the conditions used for their isolation. This suggests that the large number of spermatogenic cells which undergo normal degeneration *in situ* (30, 37) are either quickly phagocytosed by Sertoli cells (15) or else are removed by the enzymatic treatment.

Although further cytological and biochemical characterization is required, the successful isolation of spermatogenic cells representing virtually all major stages of spermatogenesis (Fig. 22) is a major advance towards elucidating the regulatory mechanisms involved in this complex differentiative process. The isolated spermatogenic cells, for instance, have proved an invaluable asset in recent studies on the expression of membrane antigens during mouse spermatogenesis (25).

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