ISOLATION OF PLASMA MEMBRANES FROM PURIFIED MOUSE SPERMATOGENIC CELLS

CLARKE F. MILLETTE, DEBORAH A. O'BRIEN AND CHRISTOPHER T. MOULDING
Department of Anatomy and Laboratory of Human Reproduction and Reproductive Biology, Harvard Medical School, Boston, MA. U.S.A.

SUMMARY

Plasma membranes have been prepared from purified pachytene spermatocytes, round spermatids and residual bodies of the adult mouse testis using procedures modified from other authors. Isolated membranes have been examined using electron microscopy, lectin binding and enzymic assays. Ultrastructural observation reveals smooth unit-membrane vesicles from 0.4-1.7 μm diameter. No contamination by nuclei, mitochondria or lysosomes is detected microscopically. Radiolabelled lectin-binding experiments [125I-RCA, 125I-green pea lectin] indicate that cell surface label cofractionates with material identified morphologically as plasma membrane. Estimates of total recovery of membrane, based upon the lectin data, average 33%. Biochemical analysis of subcellular markers reveal that no detectable DNA and only 1.2% of the total cellular RNA cofractionate with membranes. A variety of enzyme assays suggest little contamination by cytosol enzymes, Golgi material or mitochondria. Assays of 5'-nucleotidase (E.C. 3.1.3.5) indicate that this enzyme is not a major component of developing mouse spermatogenic cell membranes. Instead, Sertoli cells represent the most important source of this enzyme in the adult seminiferous tubule. Polyacrylamide gel analysis of membranes isolated from purified germ cells reveals significant differences in the protein compositions of pachytene spermatocyte and round spermatid membranes. The preparation of highly purified plasma membranes from homogeneous populations of spermatogenic cells should facilitate the biochemical characterization of cell surface antigens specific to developing male germ cells.

INTRODUCTION

The processes regulating the complex pathway of cellular differentiation during mammalian spermatogenesis are generally not well understood at the molecular level. In particular, little detailed information is available concerning the molecular organization of developing spermatogenic cell plasma membranes. Until recently, the inability to obtain highly purified populations of male germ cells at defined stages of spermatogenesis has hindered the analysis of cell surface constituents. Correlative approaches utilizing intact testes at specific periods of development have been used to investigate the appearance of intracellular enzymes in mammalian spermatogenic cells (Blackshaw, 1970; Davis & Langford, 1970). However, these methods are not well suited for the identification of plasma membrane components which are present in low amounts...
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and which often have no known enzymic activity that is easily measured in complex protein mixtures.

Procedures have been developed recently for the isolation of purified cell populations from the mammalian testis (Bellvé et al. 1977a; Bellvé, Millette, Bhatnagar & O'Brien, 1977b; Meistrich, 1977). These techniques provide highly homogeneous cells from most stages of spermatogenesis, including spermatogonia, primary spermatocytes, round spermatids, condensing spermatids and spermatozoa. Using isolated male germ cells, a variety of cell surface antigens specific to spermatogenesis have been identified serologically. A number of these determinants are not expressed until the late pachytene stage of the first meiotic prophase and are then retained by all subsequent germ cells (Millette & Bellvé, 1977; O'Rand & Romrell, 1977; Tung & Fritz, 1978). Some spermatozoan autoantigens are not expressed until the development of haploid spermatids (Tung, Bebe-Han & Evan, 1979). Other surface constituents are partitioned selectively and quantitatively onto the membrane of testicular residual bodies during late spermiogenesis just prior to sperm release (Millette, 1979). The physiological functions of these spermatogenic cell membrane molecules remain unknown.

An important first step in the determination of molecular function is the purification and biochemical characterization of cell surface constituents specific to particular classes of developing male germ cells. This report details the isolation of plasma membrane fractions obtained from purified populations of adult mouse spermatogenic cells including pachytene spermatocytes, round spermatids (steps 1–8) and residual bodies. These preparations are free from significant contamination by intracellular organelles as examined by a variety of biochemical and morphological criteria. The yield of purified plasma membrane is sufficient to allow the analysis of individual cell surface components. Preliminary electrophoretic comparisons of membrane fractions isolated from different stages of spermatogenic cells reveal differences in the membrane composition of pachytene spermatocytes, round spermatids and residual bodies. Therefore, purified plasma membranes from male germ cells should allow the direct biochemical analysis of cell surface markers previously identified serologically.

MATERIALS AND METHODS

Cell preparations

Seminiferous cell suspensions were prepared from adult CD-1 male mice, 60–120 days of age (Charles River Breeding Laboratories, Wilmington, MA.), using collagenase and trypsin according to the procedures of Bellvé and associates (Romrell, Bellvé & Fawcett, 1976; Bellvé et al. 1977a, b). Purified populations of pachytene spermatocytes, round spermatids and residual bodies were isolated by sedimentation at unit gravity (Romrell et al. 1976; Bellvé et al. 1977b). All cells were washed thoroughly in enriched Krebs-Ringer bicarbonate buffer, pH 7.2–7.3 (EKRB; Bellvé et al. 1977a). EKRB used for cell washes also contained 0.5% w/v bovine serum albumin (EKRB-BSA). When necessary, DNase I (≤ 10 μg/ml, Sigma Chemical Co.) was added to the final wash to reduce cell aggregation. Cell viability as determined by the exclusion of trypan blue (0.16% in EKRB) was greater than 95%. Purities of individual spermatogenic cell types averaged 94% for pachytene spermatocytes, 93% for round spermatids and 86% for residual bodies as determined by Nomarski differential-interference microscopy.

Mouse splenocytes were prepared by excising and mincing the organ followed by gentle
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Homogenization in EKRB. Single cell suspensions were washed three times in EKRB-BSA. Viability was > 95%.

Plasma membrane isolation

The method developed for the purification of mouse seminiferous cell plasma membranes was derived from the techniques of Atkinson & Summers (1971) and Brake, Will & Cook (1978). Gradients were loaded using a procedure similar to that of Monneron & d’Alayer (1978).

Single-cell suspensions of seminiferous cells, purified pachytene spermatocytes, round spermatids or residual bodies were suspended in EKRB-BSA to yield not more than 1.25 x 10^8 cells per tube with an optimal number of 1.25 x 10^8 cells per tube. Cells were pelleted for 5 min at 200 g using a Beckman TJ-6R centrifuge at 4 °C. All traces of supernatant EKRB, including liquid adherent to the tube walls, were removed by absorption with a paper towel. Throughout the membrane isolation steps, the Tris-buffered saline solutions (TBSS) of Brake et al. (1978) were used. TBSS contains 0.16 M NaCl, 3 mM MgCl₂, 5 mM KCl in 10 mM Tris-HCl (pH 7.4 at 4 °C). Cells were first swollen in hypotonic medium by the addition of 0.8 ml homogenizing buffer (TBSS diluted 1/10 with 10 mM Tris-HCl, pH 7.4 at 4 °C). The cell pellets were centrifuged vigorously in the homogenization buffer and swollen for exactly 5 min at 4 °C. Suspensions were then homogenized by 3 strokes in a small glass Dounce homogenizer with Teflon pestle (A.W. Thomas Co., Size O, Cat. no. 3431-Eo4, clearance 0.05-0.1 mm). Immediately thereafter 0.08 ml 10 x TBSS was added to approximate isotonicity and to stabilize the nuclei.

Unbroken cells, nuclei and large aggregates of debris were removed by centrifugation for 30 s at 1000 g using a Fisher microcentrifuge (Model 59). Supernatant was removed carefully and transferred to a glass tube. The pellet was resuspended in 0.8 ml TBSS (1 x) and centrifuged for an additional 10 s at 1000 g in the Fisher centrifuge. The second supernatant was added to the first to yield a final volume of 1.6 ml containing enriched plasma membranes. Supernatants were kept cold at all times.

Plasma membranes were isolated from the supernatant material by centrifugation on discontinuous sucrose density gradients in TBSS. Exactly 1.5 ml of supernatant containing enriched plasma membranes was mixed with 1.5 ml of 80% sucrose w/v to yield 3.0 ml of 40% sucrose containing membranes. Any remaining supernatant was discarded. All the 40% sucrose material (3.0 ml) was layered on top of 3.0 ml of 45% sucrose w/v in TBSS in a cellulose nitrate centrifuge tube (Beckman SW 41). Five millilitres of 30% sucrose (w/v) in TBSS were then layered above the 40% sucrose, followed by 1 x TBSS to fill the tube. Gradients were centrifuged at 125,240 g (32,000 rev/min) for 90 min at 4 °C in a Beckman L2-65B ultracentrifuge equipped with an SW 41 rotor. Fractionated material was collected, diluted ~ 1:10 in TBSS and pelleted at 125,240 g for 30 min.

Lectin assays

Ricinus communis lectin (RCA₁) was prepared according to the method of Nicolson & Blaustein (1972). Green pea lectin was prepared by the homogenization of 1 vol. dried beans, unroasted, in 3 vol. of phosphate buffer saline (PBS; 8.00 g NaCl, 1.15 g Na₂HPO₄, 0.2 g KH₂PO₄, 0.2 g KCl per l, pH 7.4). The extraction mixture was incubated for 1 h at 4 °C. Particulate matter was removed by centrifugation and the supernatant fluid was dialysed overnight against PBS at 4 °C. Purified lectin was obtained by affinity chromatography using Sephadex G-75 in PBS. Lectin bound to the saccharide matrix of Sephadex G-75 was eluted with 0.1 M dextrose in PBS, pH 7.4. Eluted lectin was dialysed against distilled water and lyophilized for storage at 4 °C.

Iodination of lectins was accomplished using chloramine-T according to McConahey & Dixon (1966) as described by Williams & Chase (1967). Specific activities obtained were 7.8 x 10⁶ cpm/mg for RCA₁ and 7.6 x 10⁶ cpm/mg for green pea lectin.

Radio-iodinated lectins were used as externally applied markers for germ cell plasma membrane. Testes from 6 adult male CD-1 mice were used to prepare seminiferous cell suspensions according to Bellvé et al. (1977b). Cells were washed in EKRB containing 0.5% BSA and divided into 2 equal aliquots of 8.5 x 10⁶ cells each. Cells were pelleted and each aliquot was...
resuspended in 5 ml of EKRB-BSA containing 10 mM sodium azide. Cell suspensions were kept at 4 °C. 1\(^{15}\)I-RCA\(_1\) (200 μg) and 1\(^{15}\)I-green pea lectin (200 μg) were added to the respective cell suspensions which were then incubated with stirring for 20 min at 4 °C. After incubation, unbound lectin was removed by washing the cells 3 times in EKRB-BSA containing 10 mM NaN\(_3\) at 4 °C. Microscopic observation indicated that cells were viable and slightly agglutinated. Cells were pelleted and plasma membranes were prepared as previously described. Gradients were fractionated with an ISCO Model 640 density gradient fractionator. The fraction size was 0.2 ml and 60 tubes/gradient were collected. Radioactive lectin was quantitated by assaying 15 μl of each fraction in 7 ml GammaScint (National Diagnostics, Parsippany, N.J.). Liquid scintillation spectrometry was done on a Beckman LS-255 counter using a window of 0-400 at a gain setting of 0.739.

Biochemical assays

Protein was measured by the method of Lowry, Rosebrough, Farr & Randall (1951). RNA was assayed using the modified orcinol procedure of Volkin & Cohn (1957). DNA was assayed using diphenylamine according to Burton (1968). A variety of enzymes were assayed including the following: 5’-nucleotidase (E.C. 3.1.3.5) according to Heppel & Hilmo (1955); succinic dehydrogenase (E.C. 1.3.99.1) according to Green, Mii & Kohout (1955) as modified by Earl & Korner (1965); thiamine pyrophosphatase according to Yamazaki & Hayashi (1968); acid phosphatase (E.C. 3.1.3.2) according to Igarashi & Hollander (1968); glucose-β-phosphatase (E.C.3.1.3.9) according to Hübscher & West (1965); NADH-diaphorase (E.C. 1.6.99.3) according to Wallach, Hoelzl & Kamat (1966) and lactate dehydrogenase-C\(_4\) (E.C. 1.1.1.27) according to Goldberg & Hawtrey (1967). For experiments designed to assay 5’-nucleotidase in subfractions of mouse testis 5 different samples were prepared, each of which was exposed to identical enzymic treatments. Samples prepared included germ cells, whole testis, total seminiferous cells, collagenase supernatant cells and enriched Sertoli cells. Germ cells were prepared using sequential incubations in 0.5 mg/ml collagenase and trypsin according to Bellvé et al. (1977a). Variations of this procedure were used to obtain the remaining samples. Whole testis material was obtained by pelleting all cells released during each enzyme treatment and by omitting the final filtration through nylon mesh. Total seminiferous cells were prepared by omitting only the filtration step after trypsinization, thereby retaining Sertoli cells. Collagenase supernatant cells were obtained by pelleting all cells remaining after the removal of seminiferous tubules from collagenase incubation mixtures. The supernatant cells, containing an average of 17% Leydig cells with the remainder being germ cells and occasional Sertoli cells, were then treated with trypsin. To prepare an enriched Sertoli cell population, aggregated Sertoli cells were removed by filtration on 80-mesh nylon screen after trypsinization of seminiferous tubules from day-18 animals. Cells recovered from the filters were cultured in vitro for 3 days in a defined medium (Feig & Bellvé, unpublished results). Under these conditions Sertoli cells adhered to the culture dishes allowing the removal of most contaminating germ cells by replacement of the medium. All cell pellets were washed in EKRB-BSA and resuspended in 0.15 M NaCl for homogenization. Aliquots were taken for measurements of both 5’-nucleotidase and protein.

Cytological procedures

Material fractionated on sucrose density gradients was prepared for electron microscopy by diluting samples 10-fold with TBSS and pelleting at 125240 × g in a Beckman SW 41 rotor for 60 min at 4 °C. Fractions were fixed with 2% glutaraldehyde in PBS for 30-60 min and postfixed with 2% OsO\(_4\) in 0.1 m cacodylate buffer, pH 7.4 for 1 h. All fixations were at room temperature. Samples were dehydrated in a series of graded ethanols, infiltrated and embedded in Epon-Araldite. Thin sections were cut on a Porter-Blum MT-2B ultramicrotome and stained with uranyl acetate and lead citrate (Reynolds, 1963). Samples were examined using a Philips EM-200 at 60 kV.

Ultrastructural assays for 5’-nucleotidase were conducted according to the histochemical procedures of Misra, Ladoulis, Estes & Gill (1975).
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Electrophoretic analysis

Polyacrylamide slab gel electrophoresis was conducted according to the procedure of Laemmli (1970), using 20% glycerol and 40 mM dithiothreitol in the sample buffer. Acrylamide concentration of the running gel was 12%. Gels were stained using Coomassie Brilliant Blue R (Sigma Chemical Co.). Destained slab gels were photographed using Ektapan film. Photographic negatives were scanned using an Ortec integrating microdensitometer, Model 4300.

RESULTS

A variety of sucrose density gradients were used to attempt the fractionation of cellular components following hypotonic swelling and gentle homogenization. Initial studies utilizing sucrose density gradients developed by Atkinson & Summers (1971) indicated that satisfactory separation of plasma membrane from intracellular organelles, particularly mitochondria, was not achieved for mouse spermatogenic cells. Accordingly, these gradients were modified using the procedures of Brake et al. (1978) and of Monneron & d'Alayer (1978). The discontinuous gradients used and their appearance after centrifugation are shown in Fig. 1.

Fig. 1. Discontinuous sucrose density gradient used to isolate spermatogenic cell plasma membranes. The stippled region of 40% sucrose indicates that portion of the gradient where enriched membranes were loaded. After centrifugation, material was recovered at each sucrose interface (Bands 1, 2 and 3) and from a small pellet (P). Highly purified plasma membranes were recovered in Band 2.
Three bands of flocculent material were obtained after centrifugation at 125,410 g for 90 min. Band 1 was collected from interface of the TBSS and 30% sucrose layers. This material was usually somewhat diffuse and obviously less in amount than Bands 2 or 3. Bands 2 and 3 were collected from the 30-40% sucrose interface and the 40-45% sucrose interface, respectively. These bands were similar in appearance, although Band 3 was often slightly yellow. Finally, a small pellet (P) was found in most gradients.

**Ultrastructural analysis of gradient fractions**

Electron-microscopic analysis of the fraction isolated from Band 1 revealed small membrane vesicles and amorphous electron-dense material which could not be identified conclusively (Fig. 2A). In contrast, Band 2 was composed predominantly of smooth membrane vesicles of larger size, ranging in diameter from 0.4 to 1.7 μm (Fig. 2B). These vesicles had the appearance of plasma membrane in that no attached ribosomes and few cytoplasmic components were detected. At higher magnification, orthogonal sections revealed unit membrane structure. Most vesicles showed little adherent material trapped inside the membrane. Ultrastructural observation of Band 3 revealed the presence of smooth membrane vesicles, closely resembling those found in Band 2, contaminated with mitochondria and rough endoplasmic reticulum (Fig. 2C). Membrane vesicles found in Band 3 were considerably smaller than those isolated in Band 2.

The morphology of intracellular organelles were not significantly altered by the membrane isolation procedure. Mitochondria concentrated in Band 3 retained the characteristic 'condensed' morphology of the mitochondria seen in situ in developing mouse spermatogenic cells (DeMartino et al. 1979). Pellets (P) obtained from the sucrose gradients contained cell nuclei, many with intact nuclear membranes, a few mitochondria and cellular debris (Fig. 3A). In addition, nuclei derived from developing spermatids had attached acrosomes at various stages of maturation. One such nucleus with a cap-phase acrosome is shown in Fig. 3B. No evidence of acrosomal degradation was detected ultrastructurally. Membrane vesicles were not found in the pellet fractions.

**Lectin binding analysis**

Ultrastructural studies suggested that mouse spermatogenic cell plasma membranes were greatly enriched in Band 2 at the 30-40% sucrose interface. To corroborate this result, intact spermatogenic cells were labelled with either 125I-RCA or 125I-green pea lectin under conditions known to label only cell membrane constituents. Following

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Fig. 2. Ultrastructural examination of material obtained from discontinuous sucrose density gradients. x 9700. (A) Band 1 material consists of small vesicles and amorphous structures. No mitochondria, lysosomes, acrosomes or other intact intracellular organelles are visible. (B) Band 2 material consists almost exclusively of membrane vesicles varying in size from 0.4 to 1.7 μm. (C) Band 3 material is composed of small membrane vesicles contaminated by intact germ cell mitochondria.
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hypotonic swelling and gradient centrifugation the recovery of labelled lectin was assayed by liquid scintillation spectrometry (Fig. 4).

Fig. 4A illustrates the results obtained using $^{125}$I-RCA$_1$. Total recovery of label originally loaded on the gradient was 92.6%, including 13.8% in Band 1, 52.8% in Band 2, and 10.1% in Band 3. No label was detected in the pellet.

Results obtained using $^{125}$I-green pea lectin are shown in Fig. 4B. Recovery of label originally loaded on the gradient was 84.1% with 8.4% recovered in Band 1, 34.0% in Band 2, and 16.3% in Band 3. A significant portion of radio-iodinated material was recovered throughout the 40% sucrose layer, but none was pelleted.

Estimates of the total recovery of plasma membrane, based upon the radio-iodinated lectin data, were 35.4% recovery for cells labelled with RCA$_1$ and 29.7% for cells labelled using green pea agglutinin. Binding of RCA$_1$ was inhibitable to 96% using 0.1 M D-galactose and binding of green pea lectin was inhibitable to 90% using 0.1 M...
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in preliminary experiments, the viability of mouse spermatogenic cells after labelling with radiiodinated lectin was greater than 95% as determined using 0.16% trypan blue in EKRB. Previous data (Millette, unpublished) indicated that FITC-RCA4 and FITC-green pea lectin bound uniformly to mouse spermatogenic cell membranes and were not endocytosed under the conditions employed (4 °C and 10 mM sodium azide). Fig. 4, therefore, demonstrates that plasma membranes of mouse spermatogenic cells were obtained from the 30-40% sucrose interface (Band 2).

The yield of purified plasma membrane differed slightly from experiment to experiment, probably dependent upon the efficiency of homogenization. However,

<table>
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<th>Table 1. Biochemical analysis of subcellular fractionation</th>
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<tr>
<td>DNA*</td>
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<tr>
<td>RNA*</td>
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<tr>
<td>5'-Nucleotidase†</td>
</tr>
<tr>
<td>Acid phosphatase†</td>
</tr>
<tr>
<td>Glucose-6-phosphatase†</td>
</tr>
<tr>
<td>Thiamine pyrophosphatase†</td>
</tr>
<tr>
<td>Succinic dehydrogenase§</td>
</tr>
<tr>
<td>Lactate dehydrogenase-C4</td>
</tr>
<tr>
<td>NADH-diaphorase</td>
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<td>ND = non-detectable.</td>
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</table>

Data are expressed as averages of at least duplicate determinations.
* Amounts of DNA and RNA are given in µg/mg protein.
† Specific activities are given in µmol Pi released per mg protein per h.
‡ Specific activity of acid phosphatase is given in mmol p-nitrophenol reduced per mg of protein per h.
§ Specific activity of succinic dehydrogenase is given in µmol indophenol reduced per mg protein per h.
|| Specific activities given as µmol NADH reduced per mg protein per h.

approximately 280 µg of protein were obtained reproducibly from 10⁸ mouse spermatogenic cells. Plasma membranes were also prepared from purified pachytene spermatocytes, round spermatids (steps 1-8) and residual bodies. No significant differences in the recovery of plasma membrane from purified cell types have been noted when compared to mixtures of adult mouse seminiferous cells. The yield of membrane is therefore sufficient to allow the detailed biochemical examination of cell surface constituents from individual classes of spermatogenic cells.

**Enzyme marker analysis**

Table 1 illustrates the distribution of a variety of biochemical markers used to follow the density gradient fractionation of plasma membrane from intracellular organelles and enzymes.

No detectable DNA was found associated with any of the bands isolated from the
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gradients. All 3 bands had small amounts of RNA present, but the RNA associated with Band 2 (35 μg RNA/mg protein) represented only 1.2% of the total cellular RNA when measured in gradient preparations from adult mouse seminiferous cell mixtures. These data indicate that isolated mouse spermatogenic cell plasma membranes (Band 2) are not contaminated with significant amounts of rough endoplasmic reticulum, and corroborate the ultrastructural observations previously described.

Contamination of Band 2 with other cellular constituents was monitored as follows: mitochondria were assayed using succinic dehydrogenase; elements of the Golgi apparatus were detected using thiamine pyrophosphatase; microsomes were assayed using glucose-6-phosphatase and NADH-diaphorase; lysosomes were assayed using acid phosphatase. Lactate dehydrogenase-C₄, the sperm specific isozyme of this enzyme, was used to detect cytosol proteins. It should be noted, however, that some LDH-C₄ may be localized to germ cell mitochondria (Montamat & Blanco, 1976; but see also Wheat, Hintz, Goldberg & Margoliash, 1977).

The results shown in Table 1 indicate that plasma membranes (Band 2 material) were not contaminated by significant amounts of intracellular material from mitochondria or the Golgi apparatus. Measurements of succinic dehydrogenase confirmed electron-microscopic data (Fig. 2) indicating that mitochondria were concentrated in Band 3 material. In addition, little LDH-C₄ was detected in Band 2 suggesting that neither cytosol proteins nor mitochondria contaminated isolated plasma membranes.

Results obtained using glucose-6-phosphatase and NADH-diaphorase suggest that some smooth endoplasmic reticulum may co-fractionate with the plasma membranes in Band 2. Glucose-6-phosphatase is enriched 3-fold in Band 2 when compared to cellular homogenates, while NADH diaphorase is enriched approximately 2-fold in Band 2 and 4-8-fold in Band 3 preparations. Analysis of lysosomal contamination using acid phosphatase indicates some contamination of Band 2 by this enzyme, but amounts are below that detected in cellular homogenates. Intact lysosomes were not detected in Band 2 material examined with the electron microscope.

Thus far, attempts to improve the separation of acid phosphatase and glucose-6-phosphatase activity from Band 2 plasma membrane have been unsuccessful. Experiments included additional centrifugation of cellular homogenates at both 4000 gₑᵥ and 20000 gₑᵥ to remove cytoplasmic organelles and glucose-6-phosphatase activity, respectively, according to the procedure of Misra et al. (1975). Using adult mouse seminiferous cells, these centrifugations resulted in a 5-fold loss of protein from Band 2 without any significant decrease in the specific activity of presumptive lysosomal markers.

Although the radio-iodinated lectin results already described provide biochemical confirmation of plasma membrane fractionation, efforts to identify positive enzyme markers for the plasma membranes of adult mouse spermatogenic cells have been unsuccessful. Results obtained for the enzyme 5'-nucleotidase, a commonly used cell surface marker, indicate that this marker is not localized to the plasma membranes of adult male germ cells of the mouse. In fact, 5'-nucleotidase was enriched in specific activity 4-2-fold in Band 1 material and just slightly enriched in either Bands 2 or 3 (Table 1).
Measurements of other putative enzyme markers of plasma membranes included Na\(^+\), K\(^+\)-dependent ATPase (E.C. 6.6.1.3) and L-leucyl-\(\beta\)-naphthylamidase (E.C. 3.4.1.1). Results obtained indicated low specific activities for these enzymes and were not reproducible. Alkaline phosphatase (E.C. 3.1.3.2) and \(\alpha\)-glycerophosphate dehydrogenase (E.C. 1.1.1.8) were also assayed. No conclusive evidence was obtained indicating that these markers were localized to particular components of adult mouse spermatogenic cells.

The effects of collagenase and trypsin treatment on the expression of putative cell surface enzymes was examined to test the possibility that proteolytic cleavage of 5'-nucleotidase had occurred during cell preparation. First, CD-1 mouse splenocytes were prepared and incubated in 0.5 mg/ml solutions of each enzyme under the same conditions employed for seminiferous cells. Other splenocytes were left untreated.

Table 2. Activity of 5'-nucleotidase

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Enzymic sp.act. *</th>
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<tbody>
<tr>
<td>Splenocytes, enzyme-treated</td>
<td>1.14</td>
</tr>
<tr>
<td>Splenocytes, untreated</td>
<td>1.02</td>
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<tr>
<td>Whole testis</td>
<td>1.32</td>
</tr>
<tr>
<td>Seminiferous cells</td>
<td>2.84</td>
</tr>
<tr>
<td>Collagenase supernatant cells†</td>
<td>2.44</td>
</tr>
<tr>
<td>Germ cells</td>
<td>0.80</td>
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<tr>
<td>Enriched Sertoli cells†</td>
<td>7.28</td>
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</table>

All testicular cell samples and the indicated spleen cell population were treated with both 0.5 mg/ml collagenase and trypsin as described in Materials and methods.

* Specific activity equals \(\mu\)M PO\(_4\)\(^{3-}\) released/h/mg protein.
† Cell population contained 17% Leydig cells.
‡ Cell population contained 82% Sertoli cells.

Spleen cells were used since mouse lymphocytes are known to have high levels of 5'-nucleotidase on their surface (Misra et al. 1975). After homogenization, samples were assayed for protein and enzyme activity. As shown in Table 2, enzyme treatment had little detectable effect on the specific activity of 5'-nucleotidase. These data suggested that proteolytic removal of plasma membrane 5'-nucleotidase was not responsible for the relative lack of this enzyme in preparations of surface membranes from mouse seminiferous cells.

It was, therefore, necessary to test the possibility that 5'-nucleotidase was not a prominent cell surface marker for adult mouse spermatogenic cells. This would explain the lack of cofractionation of the enzyme and membrane vesicles (Band 2), but would be seemingly inconsistent with previous studies using 5'-nucleotidase as a cell surface marker in total testicular homogenates (Xuma & Turkington, 1972; Abou-Issa & Reichart, 1976). To examine briefly the distribution of 5'-nucleotidase between different testicular cell types, experiments were conducted on various fractions of adult mouse testis as indicated in Table 2. All samples were exposed to both collagenase and trypsin. The data show that adult mouse germ cells had the lowest
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specific activity for 5′-nucleotidase of any testicular fraction assayed. In contrast, the fraction composed of enriched Sertoli cells had 9-fold the specific activity of the germ cells and 5·5-fold the specific activity of total testis. Although these studies did not exclude the possibility that the in vitro culture of Sertoli cells in some way stimulated 5′-nucleotidase activity, the comparison between seminiferous tubules containing Sertoli cells (2.84 μM PO₄³⁻/h/mg protein) and isolated germ cells containing <0·5% Sertoli cells (0·80 μM PO₄³⁻/h/mg) also suggested that Sertoli cells were the predominant source of 5′-nucleotidase in the seminiferous tubule.

Confirmation of these data was obtained using ultrastructural histochemical techniques designed to localize the subcellular distribution of 5′-nucleotidase (Misra et al. 1975). Results of these investigations indicated that adult mouse germ cells did not express significant amounts of 5′-nucleotidase on their cell surfaces. Primary pachytene spermatocytes, round spermatids and residual bodies were all negative in the histochemical assay. Mouse splenocyte controls, however, were positive, indicating the validity of the procedure. Occasional Sertoli cells encountered in germ cell preparations showed extensive deposition of lead phosphate on the plasma membranes corroborating the biochemical data in Table 2.

Electrophoretic analysis

Polyacrylamide gel analyses of purified populations of intact pachytene spermatocytes, round spermatids and residual bodies reveal numerous polypeptides ranging in molecular weight from ~10 K to >100 K (Fig. 5A). The overall profile of total cellular proteins is similar for each cell population, but both quantitative and qualitative differences in particular polypeptides can be detected, especially in those portions of the gels containing proteins <30 K molecular weight. Residual bodies, for example, show major changes in the relative abundance of low molecular weight proteins. Fig. 5A has been labelled to indicate some of these components. At least one protein band (1) is greatly increased in intact residual bodies, while other proteins are virtually absent (2) or significantly decreased in amount (3, 4). Quantitative alteration in proteins obtained from intact pachytene spermatocytes, round spermatids and residual bodies have been confirmed by microdensitometric analysis. However, more detailed descriptions of the total protein complements of purified mouse spermatogenic cells must await 2-dimensional electrophoresis, due to the complexity of the polypeptide patterns detected on single-dimensional gels.

One-dimensional gel analyses of purified plasma membranes obtained from isolated pachytene spermatocytes, round spermatids, or residual bodies are illustrated in Fig. 5B. Microdensitometer tracings of these gels are shown in Fig. 6. Purified plasma membranes contain many fewer polypeptides than do isolated whole cells (compare Fig. 5A with B). In particular, plasma membranes exhibit few major protein bands of molecular weight <45 K when compared with intact cells.

Comparisons of surface membranes obtained from purified spermatogenic cells reveal that most major polypeptides are shared by pachytene spermatocytes, round spermatids and residual bodies. However, significant differences can be detected. At this level of resolution, pachytene spermatocyte membranes differ from either round
Fig. 5. Polyacrylamide gel electrophoresis of intact mouse spermatogenic cells and purified spermatogenic cell plasma membranes.

(A) Intact spermatogenic cells. P = purified pachytene spermatocytes, RS = purified round spermatids, RB = purified residual bodies. Positions of proteins used as mol. wt standards are indicated at the left. Representative differences in the polypeptide compositions of the various cell populations are shown to the right.
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Fig. 6. Microdensitometric analysis of isolated plasma membranes from purified populations of mouse spermatogenic cells. Labels are as indicated for Fig. 6A.

Relative amounts of protein 1 are increased in residual bodies. Protein 2 decreases during spermatogenesis and is absent from residual bodies. Proteins 3 and 4 are found in approximately equivalent amounts in both pachytene spermatocytes and round spermatids, but are decreased significantly in residual bodies.

(a) Purified plasma membranes from pachytene spermatocytes (P), round spermatids (RS) and residual bodies (RB). Positions of proteins used as mol. wt standards are shown at the left. P1 indicates material that does not migrate effectively in 12% acrylamide. This material is enriched in membranes from pachytene spermatocytes. RS1 and RS2 indicate polypeptides which are enriched in the membranes of round spermatids and residual bodies.
spermatid or residual body membranes in at least 3 regions of the gels. The micro-
densitometer scans in Fig. 6 have been labelled to indicate these regions (RSi, RS2, P1). The relative amounts of proteins RS1 and RS2 are reduced in pachytene sperma-
tocytes. Both proteins are prominent constituents of membranes isolated from round spermatids and residual bodies. In contrast, pachytene spermatocyte membranes exhibit a prominent amount of protein (P1) too large for effective migration in 12% acrylamide. Plasma membranes from round spermatids or residual bodies exhibit similar electrophoretic profiles and both have only low levels of P1 material.

DISCUSSION

This report describes a method for the purification of plasma membranes from isolated homogeneous populations of mouse spermatogenic cells. Previous investi-
gators have obtained crude preparations of plasma membranes from whole mammalian testicular homogenates (Abou-Issa & Reichart, 1976; Cheng, 1975; Davies, Lawrence & Lynch, 1978; O'Shaughnessy & Brown, 1978; Pacuska, Osborne, Brady & Fishman, 1978), but few morphological or biochemical studies have been conducted to assay the relative purity of isolated fractions. Abou-Issa & Reichart (1976) used a variety of enzyme markers and ultrastructural observations as criteria of membrane enrichment. Presumptive enzyme markers for plasma membranes included both 5'-nucleotidase and adenylate cyclase which are now known to be poorly suited for the analysis of spermatogenic cell surfaces (Neer, 1978; Millette, vide supra). O'Shaughnessy & Brown (1978) performed electron-microscopic analysis of their membrane prepara-
tions but presented none of these data. Although some investigators have used density gradient fractionation techniques (Abou-Issa & Reichart, 1976; O'Shaughnessy & Brown, 1978), previous testicular membrane preparations have usually been obtained as 100000-g pellets from total cellular homogenates. As a result, these samples were most probably contaminated by intracellular organelles. Such preparations are useful for studies of hormone binding, but not for the biochemical examination of individual plasma membrane constituents from particular classes of testicular cells.

The greatest difficulty in the isolation of testicular plasma membranes lies in the extreme cellular complexity of the testis. Only Abou-Issa & Reichart (1976) first isolated seminiferous tubules, thereby eliminating the testicular interstitium consisting of Leydig cells, lymphoid cells, erythrocytes and up to 17% macrophages (Dym & Raj, 1977). The seminiferous tubule itself consists of both non-germinal elements, the Sertoli cells, and the various classes of germ cells including spermatogonia, spermatocytes, spermatids and spermatozoa. Detailed analysis of plasma membrane molecules from particular classes of spermatogenic cells therefore requires the prior isolation of homogeneous cell populations. Experiments utilizing whole testicular homogenates or seminiferous tubules almost certainly yield preparations containing a significant proportion of Sertoli cell membranes. Recent stereological analysis of the monkey testis indicates that Sertoli cells occupy from 24 to 31% of the total volume of the seminiferous epithelium, depending upon the stage of the semi-
niferous cycle (Cavicchia & Dym, 1977). In addition, since Sertoli cells completely
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surround all developing spermatogenic cells, except for the basal side of early spermatogonia and the lateral aspects of developing sperm flagella, the relative contribution of Sertoli cell membrane compared to germ cell membrane must approximate 50%. The predominance of Sertoli cell membrane is also reflected in the enzyme marker studies of Abou-Issa & Reichart (1976) who detected 7- to 13-fold increases in the specific activities of 5'-nucleotidase, adenylate cyclase and ATPase in their membrane fractions. Furthermore, studies of FSH-binding to membrane fractions from intact testis suggest strongly that Sertoli cell membranes comprise a major portion of the assayed material since Sertoli cells, but not germ cells, are targets for follicle stimulating hormone (Steinberger et al. 1979).

In the current study plasma membranes were prepared to allow the molecular analysis of germ cell surface differentiation during mouse spermatogenesis. Accordingly, a population of adult male germ cells was first isolated in order to remove interstitial tissue as well as Sertoli cells. Electron microscopy indicates that smooth membrane vesicles are concentrated in Band 2 at the interface of 30 and 40% sucrose. In contrast to results obtained with HeLa cells (Atkinson & Summers, 1971), hypotonic lysis of mouse germ cells does not yield a high percentage of large cell ghosts. Instead, smaller membrane vesicles form. The reason for this discrepancy is not known, but alterations in the time of swelling or the degree of homogenization did not increase the yield of membrane ghosts. Ultrastructural studies suggest that plasma membranes are not significantly contaminated by intracellular organelles. Enzymic assays demonstrate little contamination of Band 2 by mitochondria, Golgi or cytosol proteins, although some smooth endoplasmic reticulum and lysosomal material may cofractionate with plasma membranes. Based upon ultrastructural examinations, neither nuclear nor acrosomal membranes seem likely to contaminate plasma membranes and no rough endoplasmic reticulum is detected in these preparations. The virtual absence of ribosomes and DNA was confirmed by biochemical assays. Only 1.2% of the total cellular RNA cofractionated with plasma membranes. These data are in accord with recent studies showing that only 3-0% of the total cellular RNA is obtained in isolated HeLa S3 plasma membranes (Costantino-Ceccarini, Novikoff, Atkinson & Novikoff, 1978).

Biochemical identification of germ cell plasma membrane was achieved using radio labelled lectins. Other workers have used lectin binding as a satisfactory measure of membrane purification from a variety of cell types (Barchi, Bonilla & Wong, 1977; Cohen, Kalish, Jacobsen & Branton, 1977). The present work demonstrates that both RCA1 and green pea lectin cofractionated almost exclusively with Band 2 material identified morphologically as membranes. Although other studies indicate that many spermatogenic cells do not, in fact, exhibit a high degree of plasma membrane mobility (Millette, 1976; Tung et al. 1979; Millette, 1979); lectin experiments were conducted in the cold and in the presence of sodium azide to minimize the possible redistribution of membrane bound ligand. It is unlikely, therefore, that labelled lectin had access to intracellular membranes. No indication of endocytosis was detected in earlier experiments using fluorescein-labelled lectins bound to mouse spermatogenic cells (Millette, unpublished results).
Positive enzymic markers for adult mouse spermatogenic cell surfaces were not detected in these studies. Marker enzymes for somatic cell plasma membranes include adenylate cyclase and 5'-nucleotidase. Adenylate cyclase, however, is not a good marker for mammalian germ cells. Braun & Dods (1975) and also Neer (1978) have demonstrated that 2 distinct adenylate cyclases exist in the mature rat testis. One enzyme is similar to somatic cell adenylate cyclase and is localized mainly to the membranes of Leydig cells and Sertoli cells. In contrast, the second testicular adenylate cyclase is localized predominantly in the cytoplasm of developing spermatogenic cells (Neer, 1978) and is not a useful plasma membrane marker. Similarly, 5'-nucleotidase is a plasma membrane marker for many, but not all, eukaryotic cells (Misra et al. 1975; Brake et al. 1978). Results presented here suggest that this enzyme is not prominent on adult seminiferous germ cells, at least in the mouse. There is ample precedent for the relative lack of 5'-nucleotidase on plasma membranes. Sun & Reinach (1977) and also Harshman & Conlin (1978) have reported that mouse 3T3 and SV-3T3 cells have very low or negligible levels of 5'-nucleotidase associated with isolated plasma membranes. In addition, rat fat cells, mouse L-929 fibroblasts and Chinese hamster cells have no detectable 5'-nucleotidase on purified cell surface membranes (McKeel & Jarrett, 1970; Trams & Lauter, 1974; Peterson & Biedler, 1978).

Current evidence suggests that extensive alterations occur in the molecular organization of male germ cell membranes during spermatogenesis. Such alterations may have important regulatory functions. A variety of cell surface antigens have been previously defined using serological procedures. Some plasma membrane antigens first appear during pachynema of the first meiotic prophase (Millette & Bellvé, 1977; O'Rand & Romrell, 1977; Tung & Fritz, 1978). Tung et al. (1979) have recently reported that other cell surface antigens found on guinea-pig spermatozoa first appear on germ cell membranes during early spermiogenesis. In addition, some spermatogenic cell surface constituents do not remain on the surface of mature spermatozoa, but are instead sequestered quantitatively to the plasma membrane of the residual body (Millette, 1979; see also Tung & Fritz, 1978). No spermatogenic cell surface antigens have been characterized biochemically, however. The availability of purified plasma membranes prepared from homogeneous classes of developing male germ cells should allow the biochemical identification of antigenic components using labelled antibodies for the in situ localization of particular proteins in polyacrylamide gels (Burridge, 1976; Adair, Jurvich & Goodenough, 1978; West, McMahon & Molday, 1978). These studies, in turn, should aid the analysis of germ cell membrane differentiation during spermatogenesis.

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