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Purification of Rat Spermatogenic Cells and Preliminary Biochemical Analysis of These Cells

MARVIN L. MEISTRICH,[1,2] JILL LONGTIN,[2] WILLIAM A. BROCK,[2]
SIDNEY R. GRIMES, JR.,[3] and MYLES L. MACE[4]

Department of Experimental Radiotherapy,[2]
The University of Texas System Cancer Center,
M. D. Anderson Hospital and Tumor Institute
Houston, Texas 77030

General Medical Research,[3]
Veterans Administration Hospital,
Shreveport, Louisiana 71130

and

Department of Pathology,[4]
Baylor College of Medicine,
Houston, Texas 77030

ABSTRACT

A method for obtaining highly purified fractions of rat testicular cells is described. Single cell suspensions from adult rat testes were separated by centrifugal elutriation. Fractions enriched in pachytene primary spermatocytes, early spermatids, and cytoplasts detached from late spermatids were obtained. These fractions were further separated by equilibrium density centrifugation on gradients of Percoll. In this manner fractions of 3×10^7 pachytene spermatocytes (98% purity), 1.1×10^8 early spermatids (93% purity), and 1.1×10^8 cytoplasts (98% purity) were obtained within 6 h after sacrificing the rats. The cells appeared to be morphologically intact and to have retained their biochemical integrity.

Analysis of acid-soluble nuclear proteins by polyacrylamide gel electrophoresis showed that histone 4 is synthesized during the pachytene stage, and confirmed that testis-specific histones are also synthesized during this stage. Analysis of a microsomal RNA preparation from purified pachytene spermatocytes and purified early spermatids by sucrose gradients indicated that intact ribosomal RNA (rRNA) can be obtained from purified cells. Both cell types are active in synthesizing presumptive messenger RNA (mRNA) with a wide range of sedimentation values, but no appreciable rRNA synthesis was detected.

INTRODUCTION

Biochemical studies of spermatogenesis require sufficient quantities of purified populations of cells at specific stages of the process. It has been our goal to obtain at least 10^7 cells of specific stages in at least 90% purity. In addition, speed of the preparation and integrity of cells are important criteria.

Methods for purification of germinal cells from rodent testes have included velocity sedimentation or equilibrium density separation (for review see Meistrich, 1977). When a single separation technique was employed, purities have been generally limited to at most 90% (Beckman et al., 1978; Bellve et al., 1977; Chandley et al., 1977; Davis and Schuetz, 1975; Geremia et al., 1977; Go et al., 1971; Grabske et al., 1975; Meistrich et al., 1973; Nakamura et al., 1978; Nyquist et al., 1973; Platz et al., 1975; Romrell et al., 1976). Higher purity can be obtained by taking cells first separated by

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[1] Reprint requests: Dr. M. L. Meistrich, Dept. of Experimental Radiotherapy, The University of Texas System Cancer Center, M. D. Anderson Hospital and Tumor Institute, Houston, TX 77030.

velocity sedimentation and further purifying them by equilibrium density centrifugation. Mouse testis cells had been separated by velocity sedimentation at unit gravity followed by density centrifugation in Renografin gradients; fractions containing 90% round spermatids and 97% late spermatid cytoplasts were obtained (Meistrich and Trostle, 1975). However, the time involved in unit gravity sedimentation, the difficulty in recovering cells from the hypertonic Renografin solutions, and the limited numbers of cells that could be loaded onto the gradients made that method impractical.

Loir and Lanneau (1975, 1977) separated ram spermatids by velocity sedimentation at unit gravity, followed by density centrifugation in gradients of colloidal silica. However, the cytotoxicity and short gelation time of colloidal silica caused problems. Recently, these objections have been overcome by the availability of colloidal silica in the form of Percoll, a preparation specifically designed for cell separation (Pertoft et al., 1977, 1978). Therefore, we reinvestigated the use of density separation of testis cells, using Percoll as the gradient medium.

In this study we have employed the following approach to obtain large numbers of quite homogeneous populations of spermatogenic cells in a relatively short time. Cell suspensions, prepared with trypsin, were first separated by centrifugal elutriation (Grabske et al., 1975). Enriched fractions were then further separated by equilibrium density centrifugation on Percoll gradients. Large numbers of pachytene spermatocytes, early spermatids, and late spermatid cytoplasts were recovered in high purity. The increased purity obtained by this method has permitted further biochemical analysis of the stages of spermatogenesis.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats, weighing 250–300 g, were used in all experiments. In some experiments animals were treated with 400 rads of ^{60}Co gamma radiation 8.7 days prior to sacrifice to reduce the number of spermatogonia and early primary spermatocytes (Dym and Clermont, 1970; Erickson, 1976).

Radioactive Labeling

All radiochemicals were purchased from Schwarz/Mann, Orangeburg, NJ. Spermatogonia and preleptotene spermatocytes were labled by intratesticular injection of 200 μCi (200 μl) per testis of [^{3}H] thymidine (thymidine[methyl-^{3}H]; sp act, 1.9 Ci/mMole), 90 min prior to sacrifice. Proteins were labeled by intratesticular injection of 100 μl per testes of a mixture containing 50 μCi [4,5-^{3}H] L-lysine and 50 μCi of [5-^{3}H] L-arginine 90 min prior to sacrifice. RNA was labeled by injection of 100 μCi (200 μl) per testis of [5-^{3}H] uridine (sp act, 25 Ci/mMole).

Preparation of Cell Suspensions

Single cell suspensions were prepared from the testes of six rats by either the EDTA-trypsin method or the trypsin method (Meistrich, 1977; Meistrich and Trostle, 1975). Similar suspensions and separations were obtained with both preparation methods; the trypsin method was preferred because of its simplicity. Testes were chopped with a razor blade. In the trypsin method, they were placed in Dulbecco's phosphate-buffered saline (PBS, Gibco, Grand Island, NY) containing calcium and magnesium. Glucose, purified trypsin (Worthington, Code TRL), and DNase (Sigma, DN-25) were added to final concentrations of 0.1%, 0.1%, and 17 μg/ml, respectively. The samples were incubated with shaking in a gyrotory water bath at 200 cycles/min for 30 min. Fetal calf serum was added to 8% final concentration. The samples were filtered through a coarse (0.3 mm pore size) stainless steel screen, a finer screen (80 μm pore), and then through glass wool to remove most of the spermatozoa and sperm tails. The cells were centrifuged at 500 × g for 15 min and resuspended in 105 ml of PBS containing 2 μg/ml purified DNase (Worthington, Code D), 0.02% soybean trypsin inhibitor, and 5 mM naphthol disulfonic acid, and then cooled to 4°C. Cells were filtered through 25 μm pore size nylon screen (Meistrich, 1977). The EDTA-trypsin and trypsin methods yielded 5×10^8 and 7×10^8 cells per 2 testes, respectively.

Separation by Centrifugal Elutriation

In each experiment, 2.5×10^9 cells were loaded into an elutriator rotor (Beckman Instruments, Palo Alto, CA) with the standard Beckman elutriation chamber. Elutriation conditions, designed to optimize both purity and yield of the cells, were as outlined in Table 1. Other details of the elutriation process were as described previously (Meistrich, 1977).

Percoll Density Gradients

Stock solutions of Percoll (Pharmacia, lot no. 4634), with density of 1.134 g/cm^3, were diluted with water and 1/10 volume of 10X calcium-magnesium-free PBS (Gibco). $MgCl_2$ and DNase (DN-25) were added to concentrations of 1 mM and 100 μg/ml, respectively. DNase was included to minimize cell clumping caused by free DNA. Linear gradients were constructed by using two equal diameter chambers containing the dense and the light Percoll solutions. Gradients of 30 ml in 41 ml cellulose nitrate tubes (Beckman, 302237) were prepared the afternoon prior to use. The following gradients are currently employed: pachytene fraction, 25–37% Percoll; early spermatid fraction, 23–33%; cytoplast fraction, 22–32%. The percentages used must be adjusted slightly for different lot numbers of Percoll with different initial densities.

After elutriation, cells were centrifuged (500 × g, 10 min) and resuspended in PBS containing 20 μg/ml DNase. A 90% Percoll solution was added to bring the concentration of Percoll to 5% below that at the light end of the gradient. Up to 10 ml of this suspension were layered onto each gradient. For the pachytene fraction, one gradient was used for cells from either one or two elutriations; for the early spermatid fraction and for the cytoplast fraction, two gradients were used for cells from either one or two elutriations.

The gradients were placed in adapters made to fit an HB-4 swinging bucket rotor, and spun in a RC5B centrifuge (Dupont-Sorvall, Norwalk, CT) for 10 min at 11,000 × g (10,000 rpm), using a very slow acceleration and deceleration from 0 to 1000 rpm. This centrifugation was sufficient for cells to reach their equilibrium densities.

In preliminary experiments, fractions were collected from the top of the gradient with an Autodensi-flow collector (Buchler Instruments, Fort Lee, NJ). After the separations were characterized, only the desired region was collected by puncturing the side of the centrifuge tube with a greased 25 gauge needle and slowly collecting a predetermined volume with a syringe. The following procedures were used for collecting the cells:

Pachytene fraction. Side of tube was punctured 3.5 cm below center of major band, and 12 ml were withdrawn. All cells from 1.0 to 3.5 cm below level of maximum concentration were collected.

Early spermatid fraction. Side of tube was punctured 0.5 cm below center of major band, and 6 ml were withdrawn. All cells from 0.5 cm below to 0.7 cm above level of maximum concentration were collected.

Cytoplast fraction. Side of tube was punctured 0.2 cm above center of major band, and 6 ml were withdrawn. All cells from 0.2 to 1.4 cm above level of maximum concentration were collected.

Refractive indices of the fractions were determined. The refractive index at 23°C (n) is related to the density at 4°C (ρ, in g/ml) by the following experimentally determined equation:

$$\rho = (6.8340)n - 8.1128$$

Then the remainder of the fractions were diluted fourfold with PBS or PBS containing 0.5% bovine serum albumin and counted in a hemacytometer using phase-contrast optics. Cells were concentrated by centrifugation at 500 × g for 10 min.

Cell Identification

Cells were identified on air-dried smears, fixed in Bouin's, and stained with periodic acid-Schiff and hematoxylin, according to criteria described previously (Meistrich et al., 1973; Meistrich, 1977).

Cells were prepared for electron microscopy by fixation with buffered glutaraldehyde. Rat testes were fixed by perfusion. Samples were subjected to post-fixation osmium tetroxide, routine dehydration, embedding, and staining of thin sections, which were examined on a JEOL 100-C electron microscope.

Radioactivity Determination

Cells labeled with [^{3}H] thymidine were quantitated by filtering the fractions onto glass fiber filters and scintillation counting as described previously (Meistrich, 1972).

Analysis of Histones

Nuclei were prepared by hypotonic lysis in the presence of Triton X-100 and protease inhibitors (Platz et al., 1977), and histones were extracted with 0.25 N HCl. Histones were separated by polyacrylamide gel electrophoresis in an acid 2.5 M urea system (Panyim and Chalkley, 1969). Gels were stained with amido black and scanned at 600 nm to locate protein bands and were sliced and counted to determine the radioactivity profiles (Brock et al., 1980). Slices were counted for 10 min; 5 cpm above background (30

TABLE 1. Elutriation conditions employed for separation of rat testis cells. These settings yield fractions suitable for subsequent separation on Percoll gradients.

Fraction	Rotor speed (rpm)	Flow rate (ml/min)	Volume collected (ml)	Comments and cell purity
1	3000	13.5	180	100 ml loaded at these settings
2	3000	17.9	150	
3	3000	31.3	150	75% early spermatids; 6% late spermatids; 10% cytoplasts
4	2000	23.2	150	
5	2000	40.0	150	80% pachytenes; 9% early spermatids; 3% secondary spermatocytes or cells in meiotic division
W	0	...	100	Wash out chamber
6	3360	10	250	Fraction 1 loaded at these settings
7	3360	22.5	150	76% cytoplasts; 13% late spermatids; 8% early spermatids
W	0	...	100	Wash out chamber

cpm) represents a statistically significant count ($P < 0.05$).

Analysis of RNA Extracted from Rat Testis Microsomes

The microsomal subfraction was isolated from unfractionated rat testis cells or purified cell types essentially as described by Gedamu and Dixon (1976) with 10 μl of diethylpyrocarbonate per liter of each solution to inhibit RNase. The RNA in this fraction consists primarily of ribosomes and polyribosomes (Palade and Siekevitz, 1956). Total RNA was extracted from microsomal preparations and precipitated with 2 volumes of 95% ethanol containing 2% potassium acetate, pH 5.2. The RNA was washed twice with 95% ethanol, dried, and 1 to 5 O.D. (260) units of RNA were dissolved in a 150 μl of a buffer solution (10 mM Tris-HCl, pH 7.6, 30 mM KCl, 1 mM EDTA) containing 0.1 mg/ml of Proteinase K (E. Merck, Darmstadt, Germany). The sample was layered over a linear 7–20% (w/w) gradient of sucrose in the same buffer solution and was centrifuged at 420,000 × g for 2.5 or 3 h in a Beckman SW 65 rotor. Aggregation of RNA was not a problem, presumably because of the low, analytical amounts of RNA loaded onto the gradients and the inclusion of EDTA in the buffer. Gradients were unloaded and monitored using a recording spectrophotometer (Model 2400-2 Gilford Instruments, Oberlin, OH) with a top harvesting gradient fractionating accessory. Approximately 0.1 ml fractions were collected and radioactivity was determined using Ready-Solve HP counting cocktail (Beckman Instruments, Palo Alto, CA). Data were corrected to correspond to 1 O.D. unit of RNA per gradient.

RESULTS

Effect of Irradiation on Depletion of Cells

In an attempt to reduce contamination of the fractions by spermatogonia and early primary spermatocytes, which are highly active in macromolecular synthesis, these cell types were reduced in numbers by prior irradiation (Geremia et al., 1977). The percentages of spermatogonia and early primary spermatocytes were significantly reduced from 1.2% and 1.4% in control rats to 0.2% and 0.6%, respectively, in irradiated rats. Nevertheless, the contamination of purified cell fractions was not significantly reduced by using irradiated rats. Hence, data from irradiated and unirradiated rats were pooled and the irradiation protocol was abandoned.

Separation of Cell Suspensions on Percoll Gradients

Preliminary experiments were performed to determine the separation achieved by equilibrium density centrifugation of unfractionated testis cells. A testis cell suspension was layered on top of a linear 20% to 45% Percoll gradient. After centrifugation, fractions were collected and analyzed (Table 2). Cell recovery averaged 97% in five experiments.

Significant enrichments of spermatid cytoplasmic fragments, round spermatids, elongating spermatids, elongated spermatids, pachytene spermatocytes, residual bodies, and interstitial macrophages were obtained. No significant enrichment of spermatogonia, early primary spermatocytes, cells in meiotic metaphase, secondary spermatocytes, spermatozoa, Leydig cells, or Sertoli cells was observed. Many of the sperm heads, spermatozoa, and Leydig cells were found in the pellet along with all of the erythrocytes.

The distribution of spermatogonia and preleptotene spermatocytes was further examined by measuring radioactivity in cells obtained from rats injected with ^{3}H-TdR. Labeled spermatogonia and preleptotene spermatocytes, prepared with the EDTA-trypsin method, presented an almost equal spread of radioactivity throughout the gradient. When cell suspensions were prepared with trypsin alone, these cells were localized in a specific density region around 1.048 g/cm^3. These results were consistent with the previous observation (Meistrich and Trostle, 1975) that densities of early spermatogenic cells were more heterogeneous when suspensions were prepared with EDTA.

Combination of Density Centrifugation and Elutriation

Based on the above observations, we decided that it would be feasible to purify three cell types by equilibrium density centrifugation of fractions first separated by centrifugal elutriation. The cell types chosen were pachytene spermatocytes, early spermatids (steps 1–11), and cytoplasts from step 10–18 spermatids.

In each experiment cell suspensions were prepared from 10 to 17 rat testes and separated by elutriation. Either one elutriator run was performed or, when greater numbers of cells were required for RNA analysis, two identical elutriation separations were done.

The pachytene fraction was most highly enriched (80%) by elutriation. The contamination in this fraction was primarily by less dense multinucleated round spermatids (Table 1). The early spermatids (steps 1–11 were 75% pure; contamination was primarily by denser elon-

TABLE 2. Percentage cellular composition of fractions of rat testis cells separated by equilibrium density centrifugation in Percoll gradients.[a]

Fraction	4	5	6	7	8	9	10	11	12	13	14	Unseparated suspension
Density (g/cm^3)	1.039	1.040	1.041	1.042	1.044	1.045	1.047	1.048	1.051	1.052	1.053	
Total cells ($\times 10^{-6}$)	2.6	6.4	6.7	7.9	12.3	8.1	4.1	1.3	1.3	1.1	0.5	60.8
Spermatogonia and early primary spermatocytes[b]	2	3	4	1	1	4	3	4	5	3	2	2
Pachytene[c]	0	1	1	1	2	7	10	24	27	21	8	4
Meiotic divisions and secondary spermatocytes	0	0	1	1	1	1	0	0	0	0	0	1
Spermatids												
Steps 1–8	4	8	25	48	48	38	18	12	6	4	2	22
Steps 9–11	1	1	1	2	4	9	11	9	10	11	13	5
Steps 12–15[d]	1	1	1	1	4	15	27	26	29	26	29	14
Sperm heads and sperm	0	1	1	1	0	0	1	1	3	4	6	7
Cytoplasts												
Cytoplasmic fragment[e]	90	83	64	39	30	18	11	5	4	3	4	27
Residual body	1	3	2	5	9	9	18	16	13	21	28	15
Leydig cells	0	0	0	0	0	0	0	1	1	1	1	1
Macrophages[f]	0	0	0	0	0	0	0	1	2	4	7	1
Unknown and degenerating	0	1	1	1	0	0	0	1	1	2	1	1

[a] Cell suspensions prepared by EDTA-trypsin method. Between 800 and 1100 cells per fraction were counted.

[b] Up to and including early pachytene.

[c] Including from midpachytene to diakinesis.

[d] Elongated spermatids with some cytoplasm present but without flagella.

[e] Cytoplast detached from spermatids in steps 10–18; ribonucleoprotein aggregate characteristic of the residual body has not yet formed.

[f] Interstitial macrophages and fibroblasts.

TABLE 3. Two-step purification of rat testicular cells. Numbers of cells and purity of major class of cells. (Mean ± SD of 4 to 8 experiments.)

	Unseparated cell suspension	After elutriation	After density separation
Pachytene fraction			
Total cells	2.5×10^9	$8 (\pm 3) \times 10^7$	$2.6 (\pm 1.2) \times 10^7$
Purity	3 (± 1)%	80 (± 4)%	98 (± 2)%
Number of pachytenes	8×10^7	6×10^7	2.6×10^7
Early spermatid (steps 1–11) fraction			
Total cells	2.5×10^9	$3.2 (\pm 0.6) \times 10^8$	$1.1 (\pm 0.2) \times 10^8$
Purity	26 (± 5)%	75 (± 3)%	93 (± 2)%
Number of early spermatids	6.4×10^8	2.4×10^8	1.1×10^8
Late spermatid cytoplast fraction			
Total cells	2.5×10^9	$8 (\pm 3) \times 10^8$	$1.1 (\pm 0.7) \times 10^8$
Purity	44 (± 4)%	77 (± 3)%	98 (± 2)%
Number of cytoplasts	1.1×10^9	6×10^8	1.1×10^8

gated spermatids, residual bodies, and interstitial cells and by lighter cytoplasmic fragments. The cytoplast fraction consisted primarily of cytoplasmic fragments. Residual bodies (also classified as cytoplasts) were present. Contamination was primarily by dense late spermatids. The separations were highly reproducible and the recovery of the desired cell types was between 38% and 75% (Table 3).

These three fractions were further purified by equilibrium density centrifugation. In initial experiments, the gradients were collected in 3 ml fractions, and the cell counts and purity of the predominant cell types were determined (Fig. 1). Regions of maximum purity of the predominant cell types were evident. From these results the protocol for collection of the gradients by puncturing the sides of the tubes, described in Materials and Methods, was established. Large numbers of cells were obtained in high purity by this procedure (Fig. 2, Table 3). The highest purity (98%) was achieved for the pachytene spermatocytes. The predominant contaminants were 1% round spermatids. In one experiment 99.8% purity was attained; the only contamination was by Sertoli cells (2 cells/1000). Early spermatids (steps 1–11) were obtained in 93% purity. The ultrastructural integrity of these cells has been demonstrated (Fig. 3a). The predominant contaminants were cytoplasmic fragments (4%), which, if nuclear events are to be studied, would not contribute

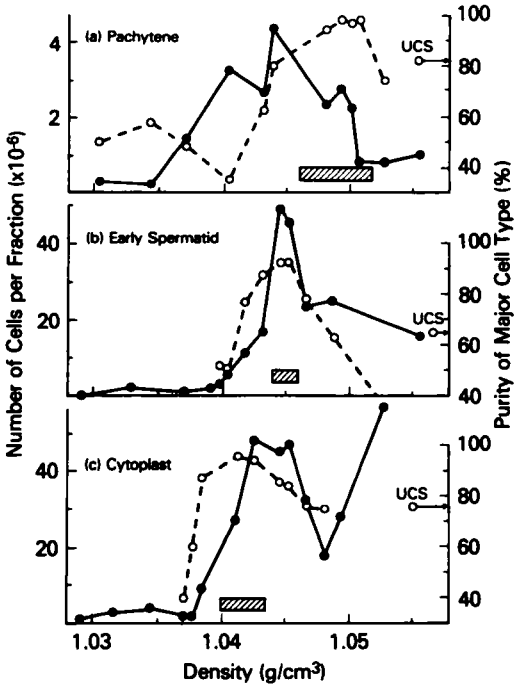


FIG. 1. Distribution of rat testis cells (—) and percentage of predominant class of cells (- - -) after equilibrium density centrifugation in Percoll gradients. Cells were prepared by the EDTA-trypsin method from unirradiated rats (a, b) or from irradiated rats (c). a) Pachytene fraction, 24–36% Percoll gradient; b) early spermatid fraction, 22–32% gradient; c) cytoplast fraction, 22–32%. The regions of the gradients selected for drawing off cells from the side with a syringe are indicated by cross hatching.

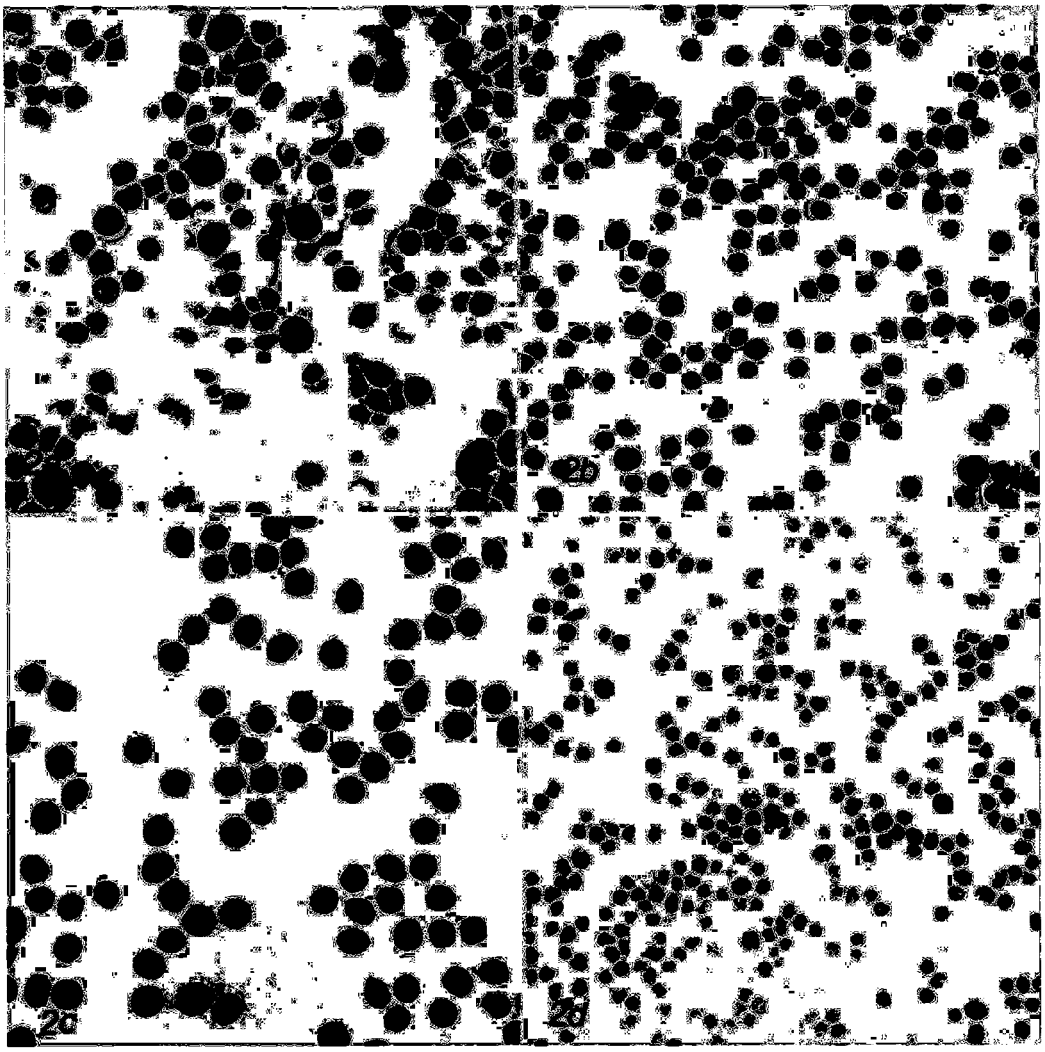


FIG. 2. Photomicrographs of unseparated rat testis cell suspension and fractions separated by elutriation followed by equilibrium density centrifugation. a) Unseparated cell suspension; b) spermatid fraction; c) pachytene fraction; d) cytoplast fraction. X250.

to lowering the purity. Other contaminants were late spermatids (1%), spermatogonia or young primary spermatocytes (1%), and secondary spermatocytes or cells in meiotic division (1%).

The cytoplast fraction was first counted using light microscopy and contained 97% cytoplasts, which did not show development of the residual body, plus 1% residual bodies. Contamination (2%) was by early spermatids.

The origin of the cytoplasts then was determined by ultrastructural analysis and comparison with the cytoplasm of elongated spermatids fixed in situ. The cytoplasm of a step 14 spermatid in situ (Fig. 3c) showed several characteristic features. Smooth endoplasmic reticulum was quite prominent and often formed annulate lamellae. The mitochondria tended to cluster to one side of the cytoplasm and contained more cristae in their interior than did the mitochondria of round spermatids. The late spermatid mitochondria were also easily distinguished from those of Sertoli cells (Fig. 3b) and primary spermatocytes. The cytoplasm of a step 17 spermatid in situ (Fig. 3d) showed several identifying characteristics.

The cytoplasm of late spermatids was more densely stained than that of other cells in the seminiferous tubule. Small membranous vesicles and lipid droplets were common. Features similar to both types of cytoplasts were retained in isolated cytoplasts (Figs. 3e, f). A field of late spermatid cytoplasts is shown in Fig. 3b. For comparison of cell types, a field including a round spermatid and a Sertoli cell cytoplast was chosen. Seven randomly selected fields containing 91 cytoplasts were photographed. Of these cytoplasts, 82 were unequivocally identified as originating from late spermatids and two from Sertoli cells. The remaining seven were most likely from late spermatids, but could not be conclusively identified because of the lack of distinguishing features. Thus, between 88% and 96% of the "cells" in this fraction are cytoplasts of spermatids between steps 10 and 18.

Analysis of Nuclear Protein Biosynthesis

The synthesis of basic nuclear proteins in specific testicular cell types was examined by polyacrylamide gel electrophoresis of histones extracted from the separated cell fractions (Fig. 4b, c). For comparison, the profile of histones extracted from the unseparated cell suspension is also shown (Fig. 4a). The histones appeared to have remained intact during the cell separation procedure.

Analysis of RNA Synthesis

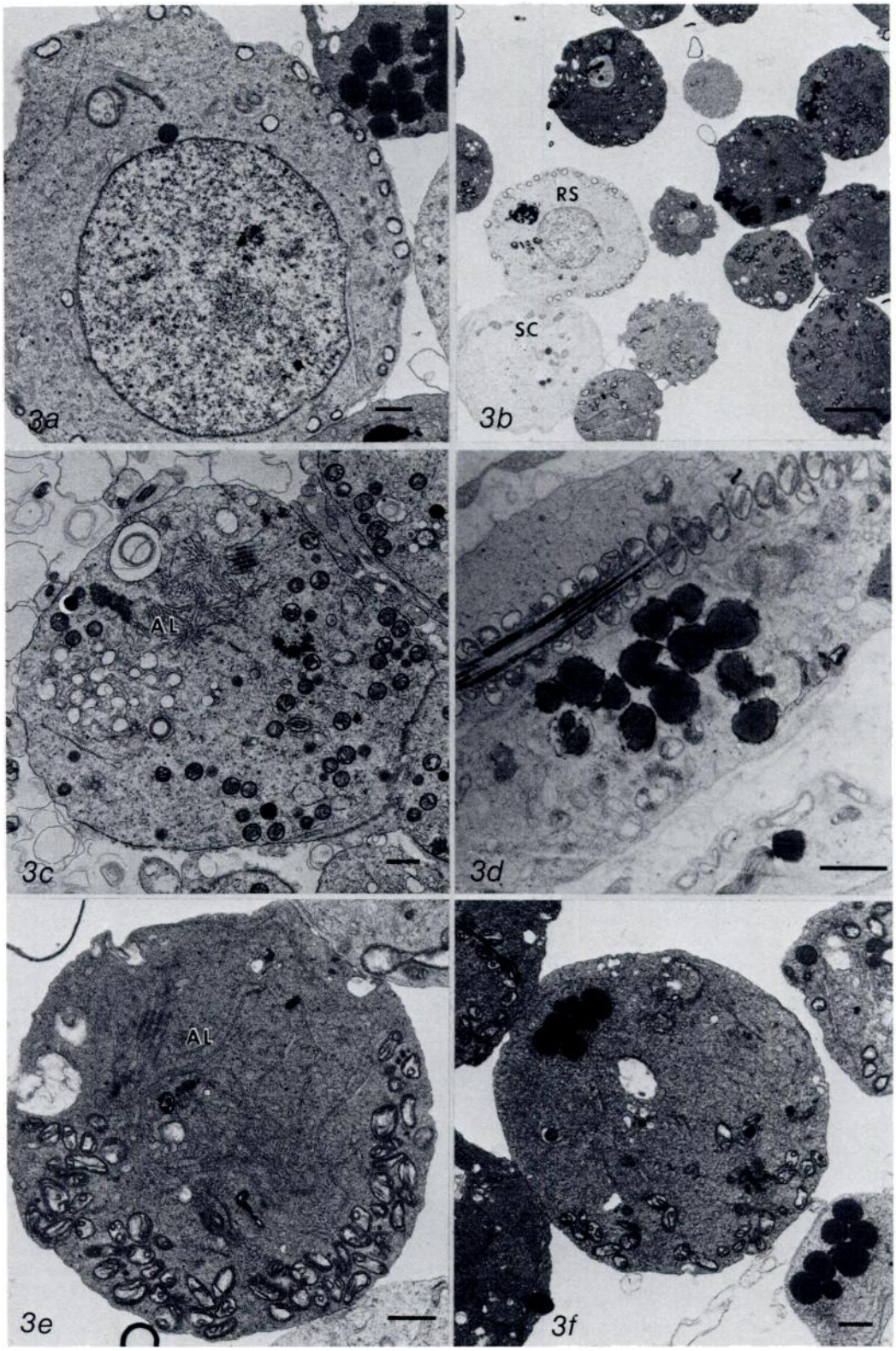
The synthesis of RNA in pachytene spermatocytes and round spermatids was examined by labeling with [^{3}H]uridine and analyzing RNA from the microsomal preparation by sucrose gradient centrifugation 24 h later. The 24 h interval was chosen since the specific activity of the RNA in the microsomal fraction of rat testis nearly reached its maximum value by that time (S. R. Grimes, unpublished results). Such RNA extracted from total testis homogenates (Fig. 5a), purified fractions of pachytene spermatocytes (Fig. 5b), and early spermatids (not shown) shows the peaks at 4S (transfer RNA), 5S, and 18 and 28S (ribosomal RNA), which are characteristic of ribosomes and polyribosomes. This result demonstrated that appreciable RNA degradation was not occurring. The ratio in the area of the 28S peak to that of the 18S peak (Fig. 5a) was unusually low. Although this might indicate some degradation, the peaks characteristic of degraded rRNA were not observed. The reason for the discrepancy in this ratio is not known. The radioactivity profiles of this ribosomal RNA from total testicular homogenates showed major peaks at 18 and 28S (S. R. Grimes, unpublished results). In contrast, the radioactivity profiles of RNA from the purified spermatocytes and spermatids showed a heterogeneous distribution with a modal volume of about 15S, with no evidence for ribosomal RNA synthesis (Fig. 6).

DISCUSSION

The method described for separation of three specific classes of spermatogenic cells will be very useful in biochemical studies of spermatogenesis. The advantages of the method are 1) high purity (93–98%) can be achieved; 2) the time required for separation is reasonably short; 3) large numbers of cells ($\sim 10^8$) containing 600 μg of DNA, 600 μg of basic chromosomal protein, 10 mg of total protein, and 150 μg of microsomal RNA can be obtained in each fraction; and 4) the biochemical and morphological parameters of the cells are unaffected by the separation.

The method employed for preparation of cell suspensions is not as effective at eliminating interstitial cells and symplasts as other techniques (Romrell et al., 1976). Nevertheless, interstitial cells are separated on the basis of

FIG. 3. Electron micrographs of purified cells and cytoplasts, showing comparison with the cytoplasmic regions of elongated spermatids fixed in situ. Bars designate 1 μm except in (b) where the bar represents 5 μm. a) Round spermatid separated by elutriation and Percoll density gradient centrifugation. Note mitochondria without well formed cristae arranged along the periphery of the cell, characteristic of round spermatids. ×5400. b) Purified fraction of late spermatid cytoplasts, illustrating two types of occasional contaminants: a round spermatid (RS) and a Sertoli cell cytoplasmic fragment (SC). The latter is identified by the lightly staining cytoplasm and darkly staining mitochondria with extensive cristae. ×1800. c) Cytoplasmic region of elongated spermatid in Stage XIV tubule showing extensive annulate lamellae (AL) with an aligned array of dense bodies and vacuoles. ×4500. d) Cytoplasmic region of step 17 elongated spermatid in Stage IV or V tubule showing lipid droplets. ×10,000. e) Cytoplast in purified fraction showing many shared characteristics with the Stage XIV above. ×7300. f) Cytoplast in purified fraction showing many shared characteristics with the step 17 shown above. ×4500.



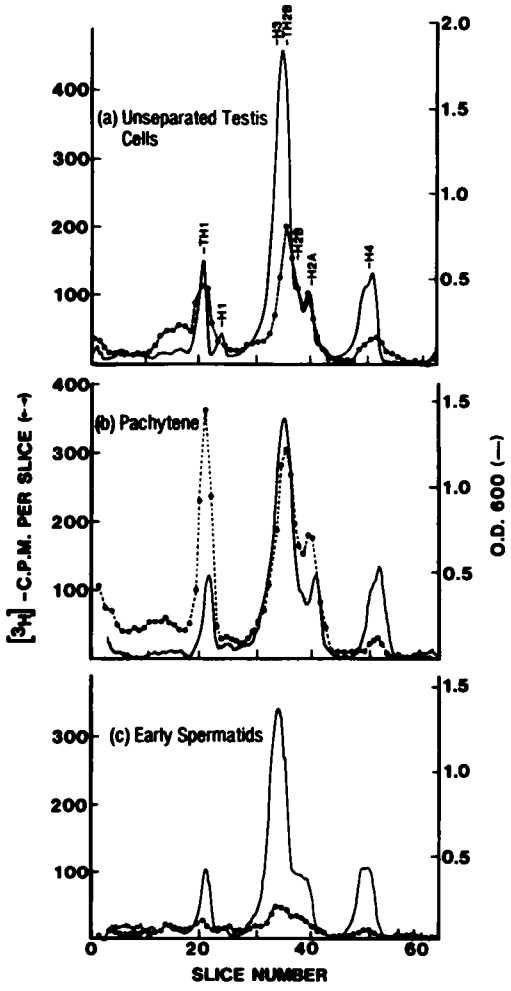


FIG. 4. Densitometric scans and radioactive profiles of rat testicular cell histones separated by polyacrylamide gel electrophoresis on an acid-urea system. Each gel was loaded with 60 μg of protein. a) Histones extracted from a testis cell suspension prepared by the EDTA-trypsin method from irradiated rats; b) histones from a fraction containing 99.8% pachytene spermatocytes; c) histones from a fraction containing 97% early spermatids (based on counts of nuclei).

their increased density, and spermatid symplasts are separated from pachytene cells on the basis of their lower density. Such observations further confirm the utility of two-dimensional cell separation techniques.

The method of separation described here is quite rapid. In practice, 2.5 h are required for the sacrifice of the rats and the complete preparation of the cell suspension. Then only 3.5 h are required for elutriation, concentrating the cells, centrifugation of the Percoll gradients, and collecting the cells.

The separation of pachytene spermatocytes and early spermatids can be applied to the problem of histone and RNA biosynthesis during spermatogenesis. High levels of purity are required in these cases.

In previous studies, we had shown that histone synthesis occurs in pachytene spermatocytes but that little or none occurs in the early spermatids (Brock et al., 1980; Grimes et al., 1975; Grimes et al., 1977). Those results are confirmed in this investigation. Previously (Brock et al., 1980) the low level of H4 synthesis observed in the pachytene fraction was attributed to contaminating cells. However, the high purity obtained in this experiment (99.8%) demonstrates conclusively that low but signifi-

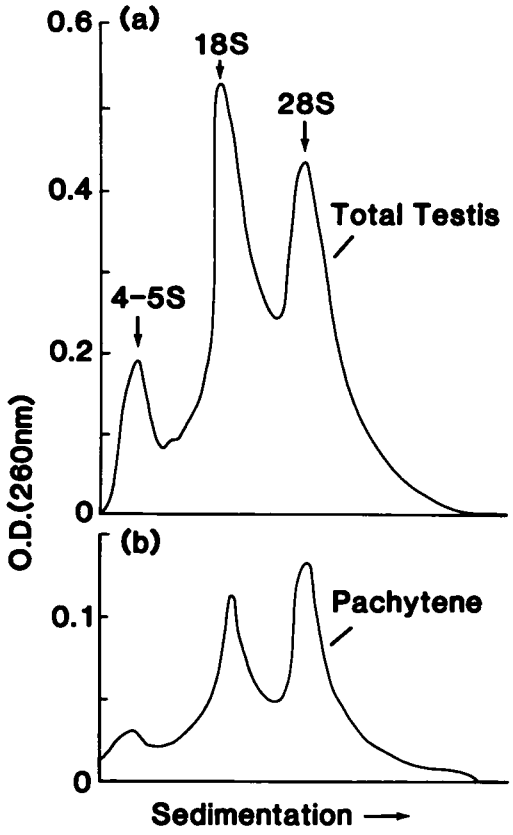


FIG. 5. Optical density profiles of sucrose gradient of RNA from microsomal preparations of a) a testicular homogenate, and b) from purified pachytene spermatocytes. Cells were prepared by the trypsin method from irradiated rats. Gradients were centrifuged for 2.5 h.

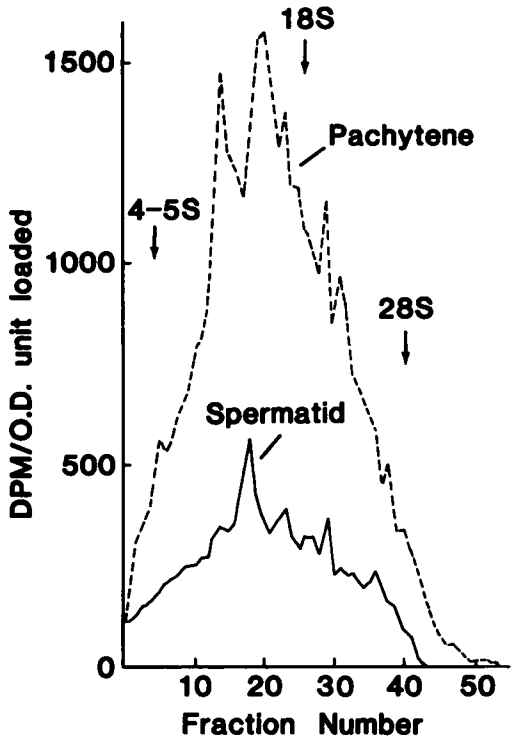


FIG. 6. Radioactivity profiles of sucrose gradients of RNA extracted from microsomal fractions of purified rat testis cells. Cells were prepared by trypsin method from irradiated rats 24 h after intratesticular injection of [^{3}H]uridine. The radioactivity profiles from both purified pachytene spermatocytes (95%) and early spermatids (95%) had modal sedimentation rates of 15S. Gradients were centrifuged for 3 h.

cant H4 synthesis does indeed occur in the pachytene cells and that the newly synthesized histone is bound within the nucleus. This result is surprising because no known variants of H4 exist and no replicative DNA synthesis occurs in these cells. Thus some histone synthesis, not associated with DNA replication or replacement of somatic histones by testis-specific variants, must occur during the meiotic prophase. Further resolution of histone subfractions on Triton-acetic acid-urea or on SDS gels demonstrates that pachytene spermatocytes synthesize H2A variants (Trostle et al., 1981) and one H1 variant (L. Bucci, personal communication).

The present results provide further information on the synthesis of RNA during spermatogenesis. It is generally accepted that most RNA synthesized in rat spermatocytes and spermatids is not ribosomal RNA (rRNA). However, several reports indicate that low levels of rRNA synthesis do occur in these cells. Soderstrom (1976) and Soderstrom and Parvinen (1976) detected newly synthesized rRNA after a 2 h pulse label with [^{3}H]uridine when followed by at least a 14 h chase. They attributed this synthesis to pachytene spermatocytes. We found no evidence for such rRNA synthesis in the present study. There are three possible reasons for this discrepancy. First, the segments of tubules which they studied contained other cell types. Sertoli cells, which are known to be very active in rRNA synthesis (Galdieri and Monesi, 1973), could be responsible for their observations. Second, they performed their incubations in vitro, which could lead to artifacts since no methods for preserving spermatogenic cell viability in vitro exist. Finally, they examined whole cell extracts, as opposed to microsomal preparations in the current experiment. If the rRNA remained in or attached to the nucleus for the 24 h period, we would not have detected its presence. Studies in the mouse (D'Agostino et al., 1978; Geremia et al., 1978) also indicated rRNA synthesis in spermatocytes and spermatids. Polyribosomes extracted from purified pachytene spermatocytes and round spermatids that were incubated with [^{3}H]uridine for 3 or 5 h contained radioactivity in rRNA. Differences with the present results could be due to artifacts arising from the in vitro labeling procedure, species differences, or the lower cell purity in those experiments, although they claim that their preparations are free of spermatogonia, young primary spermatocytes, and somatic cells. Autoradiographic data indicate that labeling over spermatocyte nucleoli, which presumably represents precursors to rRNA, occurs in the rat (Stefanini et al., 1974) and the mouse (Kierszenbaum and Tres, 1978), but the rate of incorporation is low. Our experiments show that these molecules are not found in polyribosomes within 24 h. Our data suggest that no new rRNA is incorporated polyribosomes during the spermatocyte or spermatid stages, but proof must await experiments involving longer chase times, since the rate of rRNA processing may be slow in spermatogenic cells.

The other interesting aspect of RNA synthesis is the presence of newly-synthesized presumptive messenger RNA (mRNA) on polyribosomes in spermatids. This observation suggests the existence of gene expression in haploid cells, a still controversial question (Erick-

son, 1973). Previous studies in the mouse have indicated the synthesis of poly (A)-mRNA in spermatids. D'Agostino et al. (1978) did isolate the polyribosomes for analysis, but used in vitro labeling, which may introduce artifacts. The study of Erickson et al. (1980) used in vivo labeling but examined only whole cell extracts, and the cell purity was not as high as in the current study. In this study we have demonstrated that, after in vivo labeling, newly synthesized RNA is present on polysomes isolated from highly purified round spermatids. Although we did not test for the presence of poly (A) on the RNA, we presume that it is mRNA by its heterogeneous sedimentation rate around 15S and the fact that experiments in the mouse (D'Agostino et al., 1978; Erickson et al., 1980) have shown that 25% of the newly synthesized RNA in spermatids contains poly (A). Thus, these experiments strongly support the occurrence of gene expression in rat spermatids.

The examples presented above demonstrate the application of cell separation by sequential elutriation and density separation steps to studies of spermatogenesis in the rat. Experiments are in progress to apply similar techniques to the separation of spermatogonia and early primary spermatocytes from immature rat testes.

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