

Preparation of Epithelial and Stromal Cell Fractions from Immature Rat Prostatic Tissue Using Percoll Gradients

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A method is described for the dispersion, isolation, and partial characterization of epithelial and stromal tissue cells from the rat ventral prostate. Viable epithelial and stromal cells have been prepared from a collagenase and trypsin digest of immature rat ventral prostates. This mixed population of cells was fractionated on two continuous Percoll[®] (a modified colloidal silica) gradients to give an epithelial-enriched fraction and a stromal-enriched fraction (greater than 90% enrichment for each fraction). Cells were viable as demonstrated by exclusion of trypan blue dye and by their ability to metabolize testosterone. Acid phosphatase activity, a marker of androgen action in rat ventral prostate, was found predominantly in the epithelial cell fraction. This cell separation procedure provides a simple, rapid, and reproducible method for the isolation of prostatic epithelial and stromal cells that will be used for studies of androgen-mediated differentiation in rat prostate as it relates to changes in acid phosphatase activity. Separation of these different cell types should also permit investigation of some of their metabolic interactions.

Key words: prostate, androgens, testosterone.

It has been well established that normal prostate tissue and some prostatic tumours are androgen dependent (Huggins and Clark, 1940; Ofner, 1968; Farnsworth, 1970).

Cell and organ culture systems have been developed to aid in the elucidation of the mechanism of androgen action in the prostate gland (Stonington et al, 1975; Stone et al, 1976; Lechner et al, 1978; Rubenstein and Anderson, 1980; Baulieu et al, 1968; Lasnitzki, 1974; Lasnitzki and Mizuno,

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1977). This has allowed investigators to eliminate many of the effects of nonandrogenic hormones and secondary effects of androgens which occur *in vivo* and hence has permitted the study of androgenic effects at the cellular level. In agreement with *in vivo* studies (Bruchovsky and Wilson, 1968a,b), organ culture systems have clearly shown that testosterone is irreversibly reduced to 5 α -dihydrotestosterone (5 α -DHT), which in turn is further metabolized to 5 α -androstane (3 α or 3 β), 17 β -diols (Baulieu et al, 1968). As a principal prostatic metabolite of testosterone, 5 α -DHT is the most important active androgen in the gland (Anderson and Liao, 1968; Bruchovsky and Wilson, 1968a,b).

Using both *in vivo* and *in vitro* techniques, our laboratory has been investigating steroid metabolism as it relates to characteristics of acid phosphatase activity—a marker of androgen action in the rat ventral prostate (Tenniswood et al, 1976). While these studies have provided valuable information, it has become increasingly apparent that we should investigate androgen metabolism and actions in the different cell types of the prostate. There are two major cell fractions in the prostate: (1) epithelial, which is comprised of tall columnar, cuboidal, and basal cells, and (2) stromal cells of mesenchymal origin, which collectively represent the fibroblasts, endothelial cells, and smooth muscle cells. There have been only a few studies that stress the importance of epithelial—mesenchymal cell interactions for

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normal prostatic growth and development (Cunha, 1972a,b, 1973; Lasnitzki and Mizuno, 1980). Franks et al (1970) showed that mechanically separated epithelium from human benign prostatic hyperplasia (BPH) could not be maintained in tissue culture independently of the stroma. This suggested that the epithelium was dependent on factors released by the stroma. Cowan et al (1977) demonstrated that in human BPH, Δ^4 -5 α -reductase activity is located primarily in the stromal cells. Cowan et al (1977) hence speculated that the prostatic stroma may be involved in the supply of active androgens to the epithelium. This work has been extended to show that Δ^4 -5 α -reductase activity is localized in stroma not only in BPH but also in normal and carcinomatous human prostate tissue (Wilkin et al, 1980). Habib et al (1976) have shown that there is little difference in the overall hormonal status of patients with BPH or prostatic carcinoma when compared with normal men, but that hormonal differences occur within the gland. In BPH, the glandular concentration of DHT is dramatically increased in comparison to that in the normal prostate (Siiteri and Wilson, 1970; Habib et al, 1976). This high concentration of DHT has been shown to be a result of elevated Δ^4 -5 α -reductase activity in the stroma (Bruchovsky and Lieskovsky, 1979; Wilkins et al, 1980). In contrast to BPH, Habib et al, (1976) have demonstrated significantly higher levels of testosterone in carcinomatous tissue. They suggest that the accumulation of testosterone is related to the development of carcinoma of the prostate, although the mechanism is unknown. Thus the interaction between these cell types may play an important role in the normal functioning of the prostate.

A variety of techniques have been employed to provide highly purified epithelial cell fractions for study in culture. Most studies have established monolayer cultures of prostatic epithelium from outgrowths of explant cultures (Schroeder et al, 1971; Stonington and Hemmingsen, 1971; Stone et al, 1978). More quantitative studies have involved the use of velocity sedimentation of enzymatically dispersed cells on isokinetic gradients of Ficoll (Dow and Pretlow, 1975; Helms et al, 1975; Rubenstein and Anderson, 1980).

In an effort to find a more rapid and simple method to separate epithelial cells from rat ventral prostate suspensions, we have investigated the use of Percoll[®] density gradients. This paper reports our methodology for obtaining epithelial

and stromal enriched cell fractions from immature rat prostates and for determining testosterone metabolism by the individual cell fractions. We have used immature rats because of our long term goal of studying the androgenic control of prostate differentiation, ie the development of the secretory functions of the adult prostate.

Materials and Methods

Animals

Experiments were carried out using immature male Sprague-Dawley rats (21 days old) obtained from Canadian Breeding Farms and Laboratories Ltd. (Montreal, P.Q.).

All animals were housed in an animal room with a controlled environment and a regulated light cycle (12/12 hours light/dark cycle). Rats were fed tap water and Purina Rat Chow *ad libitum*.

Steroids

[1,2-³H]testosterone (59 Ci/mmol), [4-¹⁴C]testosterone (57.5 mCi/mmol), and [4-¹⁴C]DHT (57.5 mCi/mmol) were obtained from New England Nuclear Corp. (Dorval, P.Q.) and were purified prior to use by paper chromatography as described by Bush (1952). [4-¹⁴C]3 α -androstane-17 β -diol (5 α -androstane-3 α ,17 β -diol) was prepared in our laboratory using a 50% rat prostate homogenate in 0.1 M Tris buffer as the source of 3 α -hydroxysteroid dehydrogenase activity for the reduction of [4-¹⁴C]DHT. Unlabelled steroids were obtained from Steraloids Inc. (Wilton, New Hampshire) and Sigma Chemical Company (St. Louis, Missouri). [4-¹⁴C]3 β -androstane-17 β -diol (5 α -androstane-3 β ,17 β -diol) was prepared from [4-¹⁴C]DHT by reduction with NaBH₄ as previously described (Van Doorn et al, 1975). Both ¹⁴C-labeled androstane-17 β -diols were purified by thin layer chromatography (on silica gel G in chloroform:methanol (98:2)) followed by paper chromatography in the Bush B3 system. All labeled steroids were at least 97% pure as determined by thin layer and paper chromatography. [¹⁴C]-recovery standards were prepared in methanol and contained 2000 dpm of the appropriate [¹⁴C] steroid and 25 μ g of cold steroid per 100 μ l.

Chemicals

Sodium chloride, potassium chloride, sodium bicarbonate, sodium dihydrogen phosphate dihydrate, sodium acetate, sodium citrate, and anhydrous dextrose were of reagent grade and were obtained from Canadian Laboratory Supplies (Toronto, Ontario).

Hexane, heptane, benzene, methanol, methylene chloride, and chloroform were obtained from Canadian Laboratory Supplies Ltd. (Toronto, Ontario) and were of spectroquality or reagent grade, in which case they were distilled prior to use.

Trypsin (1:250 "Difco" certified), beef pancreatic DNase I, collagenase-type IV (140 units/mg) and p-

nitrophenyl phosphate were obtained from Sigma Chemical Company (St. Louis, Missouri).

Percoll® was purchased from Pharmacia Fine Chemicals (Montreal, P.Q.). Fetal calf/bovine serum, chicken serum, trypan blue exclusion dye, and Eagle's minimum essential medium with Hank's salts and L-glutamine were purchased from Grant Island Biological Company (New York, New York).

Tissue Disintegration

In each experiment, twelve immature rats were sacrificed by an overdose of ether. The lobes of the ventral prostates were aseptically removed and immediately placed in individual Petri dishes (35 mm) containing 2.0 ml of Moscona's saline (Moscona, 1952) at 4 C. The tissue was cleansed of adhering fat and pooled in a 60 mm Petri dish containing 10 ml Moscona's saline. The tissue was rapidly minced with scissors to 1 to 3 mm³ fragments and then transferred to a 50 ml ground glass stoppered Erlenmeyer flask with 10 ml of 1% chicken serum in Moscona's saline. The tissue was allowed to settle and the supernatant was removed and discarded. The chicken serum wash was repeated three additional times.

The tissue mince was serially digested enzymatically using 10 ml of CTC (0.1% collagenase, 0.1% trypsin, and 1% chicken serum) in Moscona's saline (Kaighn and Prince, 1971). The digestions were performed at 37 C. The supernatant from the first 30-minute digestion was discarded. The cells were harvested in the supernatants from four subsequent digestion periods, each of 20 minutes duration. In the last 5 minutes of each digestion period, 0.25 ml of DNase (0.4% in Moscona's saline) was added. Following each digestion period, the

tissue was allowed to settle and the supernatant was removed and collected. The tissue was then washed once with 10 ml Moscona's saline. The wash was collected and pooled with the supernatants; this cell suspension was placed in an ice bath.

Following completion of the enzymatic digestions, the pooled cell suspension was centrifuged at 700 × g for 10 minutes. The resulting supernatant was discarded and the pelleted cells were resuspended in a total volume of 5 ml Moscona's saline. This procedure is summarized in Fig. 1.

Cell Separation

Mixed cell suspensions as obtained above were subjected to continuous isopycnic centrifugation on Percoll gradients (Pertoft et al, 1977; Pertoft and Laurent, 1977). The cells were mixed with the Percoll in Moscona's saline (adjusted to pH 7.4 and 300 m osmol/kg) to give a final Percoll density of 1.075 g/ml. The final cell concentration varied between 0.5 and 1.0 × 10⁸ cells/ml Percoll. Thirty ml polyallomer tubes were used. The tubes were spun at 17,000 rpm (20,000 × g) for 20 minutes at 20 C in a Beckman Ti60 rotor in a Beckman Ultracentrifuge (No. L5-65). Formation of the gradient and separation of the cells occurred simultaneously. The epithelial plus dead cell band at the top of the gradient was pipetted off. The Percoll was washed off the cells with 5 volumes of Moscona's saline. These cells were then spun at 700 × g for 10 minutes and the supernatant was discarded. The cells were resuspended in 2 ml Moscona's saline and reapplied to a second Percoll gradient, with a final density of 1.035 g/ml. This suspension was spun at 17,000 rpm in the Beckman Ti60 rotor for 20 minutes at 20 C. The purified epithelial-enriched band was pipetted

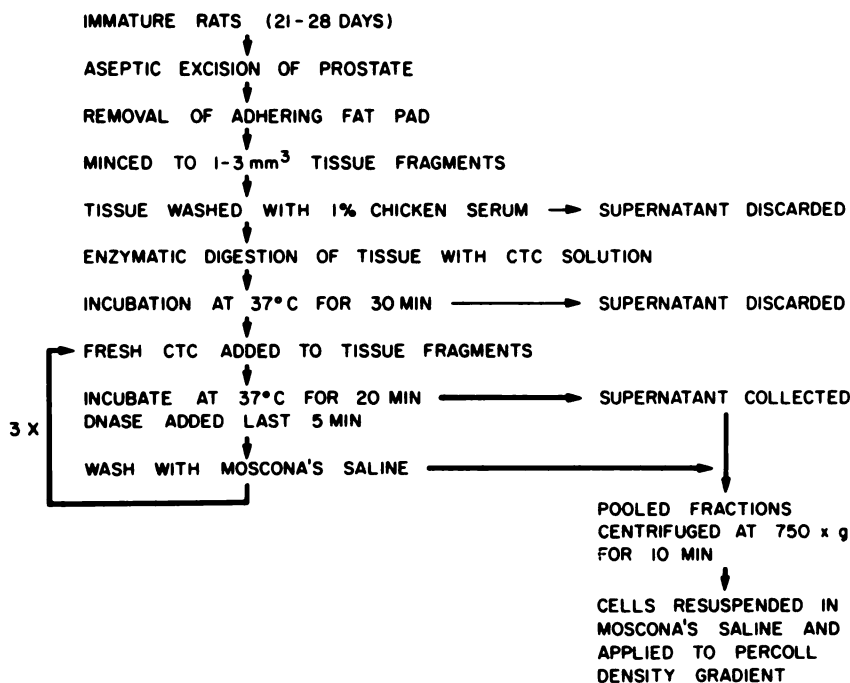


Fig. 1. Flow diagram outlining procedure for disintegration of immature rat prostate tissue.

from the bottom of the gradient and washed several times with Moscona's saline to remove the Percoll; the dead cells remained at the top of the gradient. The stromal-enriched fraction was pipetted from the bottom of the first gradient and washed several times with Moscona's saline.

In a second approach, cells were separated at lower centrifugal forces on preformed gradients. The Percoll gradient (density = 1.075 g/ml) was initially prespun at $20,000 \times g$ for 20 minutes at 20 C, without cells in the gradient medium. The cells were then gently layered on top of the prespun gradient in a small volume of Moscona's saline (2 ml; 7.5×10^6 cells/ml). This preparation was centrifuged in swing-out buckets (rotor 269) at $1000 \times g$ (1455 rpm) for 30 minutes at 20 C in an I.E.C. portable refrigerated centrifuge (Model PR-2). The epithelial plus dead cell and stromal-enriched bands were removed and washed in five volumes of Moscona's saline. The former band was resuspended in 2 ml of Moscona's saline and reapplied to a second prespun gradient of starting density = 1.035 g/ml. The lower centrifugation conditions were repeated as described above. The epithelial-enriched cells sedimented to the bottom, and dead cells remained at the top of the gradient. The epithelial-enriched cell fraction was collected and washed in Moscona's saline as previously described. These procedures are summarized in Fig. 2.

Cell Gradient Characterization

The polyallomer tubes were punctured at the bottom with an 18 gauge needle and 1 ml fractions were col-

lected. The cells in each fraction were counted using an American Optical Spencer hemocytometer and viability was determined by trypan blue exclusion dye (see below). The density was determined using an American Optical refractometer (refractive index of Percoll is directly proportional to density).

The different cell types (epithelial versus stromal) were differentiated on the basis of morphologic size and shape using the standard hematoxylin-eosin procedure. Acid phosphatase activity was measured (see below) and used as a further means of identifying the epithelial cells (Nilsson et al, 1973; Helms et al, 1975; Cowan et al, 1976).

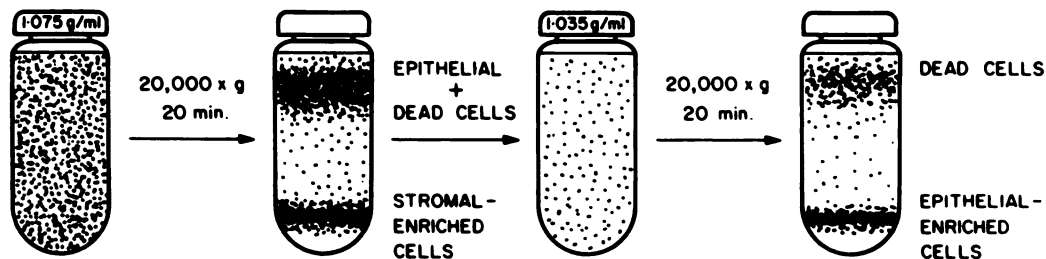
Cell Viability

Cell viability was determined by the trypan blue exclusion dye test. A 0.5 ml aliquot of the cell fraction and 0.5 ml of trypan blue concentration were mixed at room temperature, let stand for 5 to 10 minutes, and then counted in the hemocytometer using a Leitz (Wetzlar) light microscope.

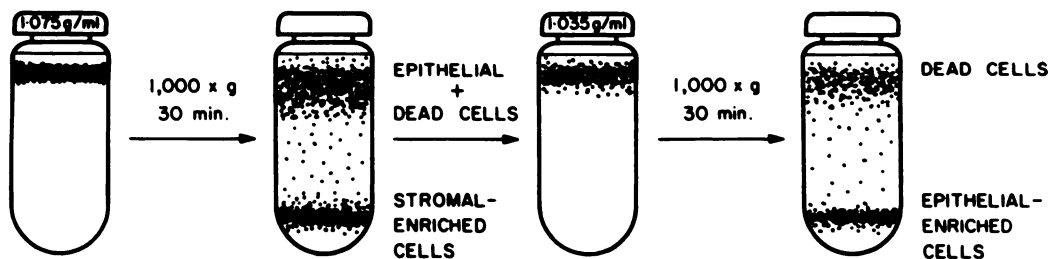
Cell Culture

Short term cell culturing was performed in Eagle's minimum essential medium plus Hanks' salts plus L-glutamine and 10% fetal calf serum. The medium was supplemented with penicillin (100 units/ml) and streptomycin (100 $\mu\text{g}/\text{ml}$). Cells were seeded onto 60 mm plastic culture dishes (Falcon Plastic, Los Angeles) in concentrations of 0.8 to 1.0×10^6 cells/plate/5 ml culture

(A) SELF-GENERATED GRADIENTS *IN SITU*



(B) PREFORMED GRADIENTS



GRADIENTS ARE PRESPUN AT $20,000 \times g$ FOR 20 min. BEFORE CELLS ARE LAYERED ON TOP OF GRADIENT

Fig. 2. Outline of procedures for obtaining epithelial and stromal enriched cell fraction from immature rat prostate using (A) self-generated and (B) preformed gradients of Percoll.

medium. Cells were incubated at 37 C in a humid environment with a 95%:5% air to carbon dioxide ratio.

Slides for microscopic examination were prepared by smearing freshly prepared cell fractions on glass slides. Cells were fixed by an aerosol spray of alcohol—acetic acid (cytospray) and stained by the standard hematoxylin-eosin procedure.

Steroid Metabolism

Steroid metabolism was evaluated using [1,2-³H]testosterone (9.0 nCi/ml; 10⁵ dpm) as substrate (added in 50 μ l methanol) and quantitating [³H]5 α -DHT, [³H]5 α -androstane-3 α ,17 β -diol, and [³H]5 α -androstane-3 β ,17 β -diol in the medium plus cells after 6 hours. Following extraction with methylene chloride, the labeled steroids were separated and isolated using thin layer and paper chromatography as previously described by our laboratory (Van Doorn et al, 1975). Losses were monitored with ¹⁴C-steroids. All radioactive measurements were made using a Beckman LS9000 scintillation counter.

Analytical Methods

Acid phosphatase activity was assayed using a modification of the method of Fishman and Lerner (1953) as described previously (Tenniswood et al, 1976). Protein concentrations were assayed using the Miller (1959) modification of the method of Lowry et al (1951).

Results

Separation of Immature Rat Ventral Prostate Cell Types

The enzymatic method of tissue dissociation by collagenase-trypsin-chicken serum (CTC) provides a ventral prostate cell suspension with 70% viability as indicated by trypan blue exclusion. The tissue was almost completely digested at the end of the fourth digestion period.

Fig. 3(A,B) represents a typical cell distribution profile of enzymatically dispersed prostate cells spun at 20,000 \times g for 20 minutes (self-generated gradient) or at 1000 \times g for 30 minutes (preformed gradients).

Fig. 3A shows that the gradient formation is S-shaped with the greatest resolution at a density of 1.070 to 1.075 g/ml. This can be used as a "buffer" zone to provide maximum distance between the two peaks in order to facilitate pipetting of the cell bands from the gradient. The first peak contained the epithelial-enriched cells. Based on cell morphology determined by light microscopy, the epithelial cells represent 85% of the cells in that peak with a total cell viability of 65 to 70%. The low cell viability is attributed to dead cells

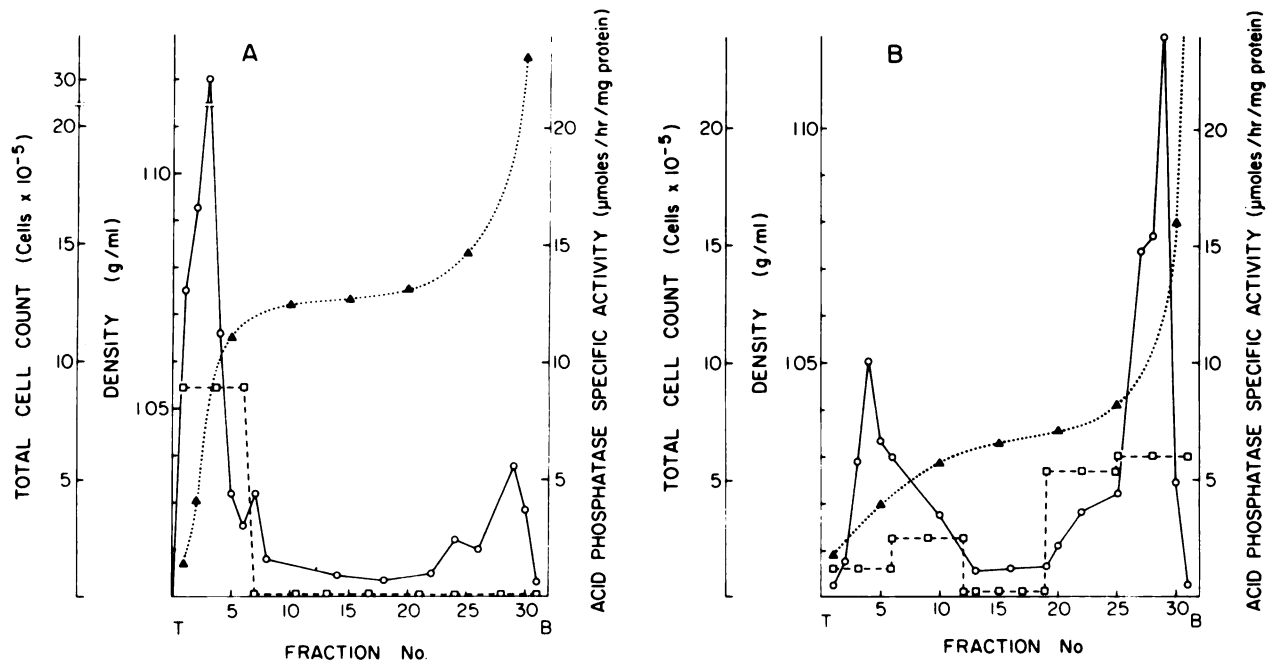


Fig. 3. Cell distribution profiles of enzymatically dispersed immature rat prostate cells for self-generated gradients of Percoll. A. First gradient at starting density 1.075 for separation of epithelial and stromal cells. B. Second gradient at starting density of 1.035 for purification of epithelial cells. $\cdots\blacktriangle\cdots\blacktriangle\cdots$ = gradient density (g/ml); $-\circ-\circ-$ = total cell count ($\times 10^{-5}$); $--\square--\square--$ = acid phosphatase specific activity (μ moles/hour/mg protein).

which sediment at the very top of the gradient and blend in with the viable epithelial cells. In order to remove the dead cells from the epithelial-enriched fraction, the cells were spun on a gradient of much lower density (1.035 g/ml final density; Fig. 3B). The dead cells remained at the top of the gradient and the large viable epithelial cells (85 to 90%) sedimented in a region of 1.050 to 1.065 g/ml density at the bottom of the gradient (Fig. 4A). It should be noted that there were present epithelial cells of different sizes, presumably representing different stages of maturation. The peak at the bottom of the first gradient (Fig. 3A) represents cells of a much smaller size and greater density. There were a few red blood cells present. The majority of the cells, however, (>90%) were larger than red blood cells, and also were nucleated. These cells were designated as being stromal. The shapes of these cells varied from spindle-shaped to small circular cells (Fig. 4B). At the light-microscopic level it is difficult, if not impossible,

to ascertain the exact percentage of fibroblastic cells in relation to other cells that comprise the stromal fraction. Because of the enzymatic procedure, the cell shape is not always characteristic of the *in vivo* situation. However, the cells were 93% nonepithelial and >90% excluded trypan blue.

Acid phosphatase was primarily associated with the epithelial enriched fractions, although some acid phosphatase activity was associated with the dead cell fraction. This can be attributed to active acid phosphatase (AP) trapped with dead cells, as well as to viable epithelial cells trapped in a gel-like matrix (liberated deoxyribonucleoprotein) that has been found to be associated with dead cells (Norrby et al, 1966). The latter authors have noted that exposure of the digested tissue to DNase for short periods of time greatly reduces the formation of gels which is caused by liberated DNA. Hence DNase was used in our procedure.

The overall yield of viable cells was 40 to 50% of the original cell suspension. Of this yield, ap-

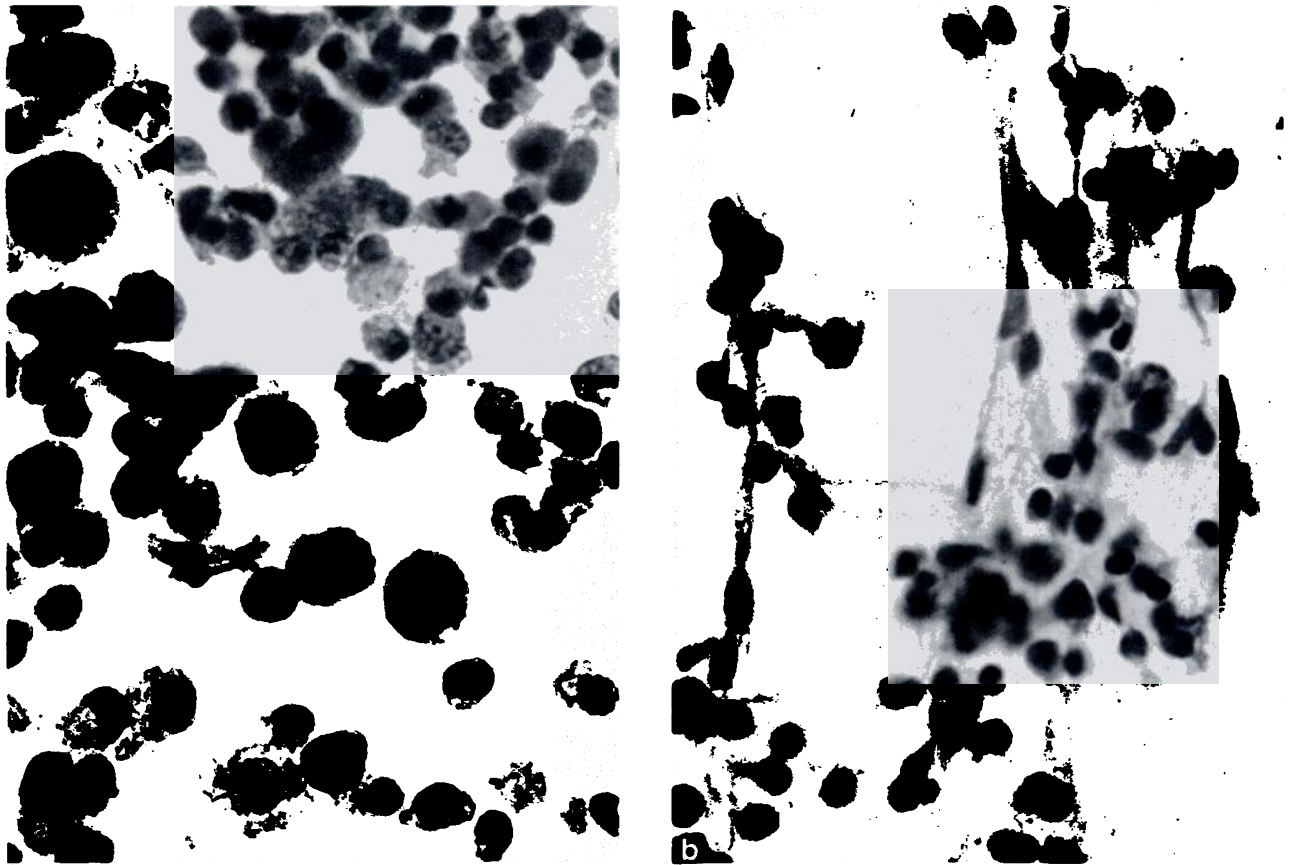


Fig. 4. Microscopic pictures of cells stained with hematoxylin-eosin. A. Epithelial cell enriched fraction. B. Stromal cell enriched fraction ($\times 512$).

proximately 65 to 70% were epithelial cells, with the remainder being stromal.

Androgen Metabolism Studies

In order to determine in a preliminary way if there were cellular compartmentalization of androgen metabolism, both cell fractions were incubated with ^3H -testosterone. Fig. 5 is representative of the results obtained with cells separated by high speed, self-generated gradients. From the results it can be seen that in cells from the normal immature prostate, both cell types can metabolize testosterone. The epithelial cells metabolize testosterone to a greater extent than do stromal cells. In contrast, however, cells separated by low speed, preformed gradients show a fourfold increase in overall metabolism, with the two cell fractions demonstrating apparent similar abilities to metabolize testosterone to its 5α -metabolites (Fig. 6). In experiments with cells obtained by both types of gradients, 90 to 100% of the starting tritium was accounted for after the 6-hour incuba-

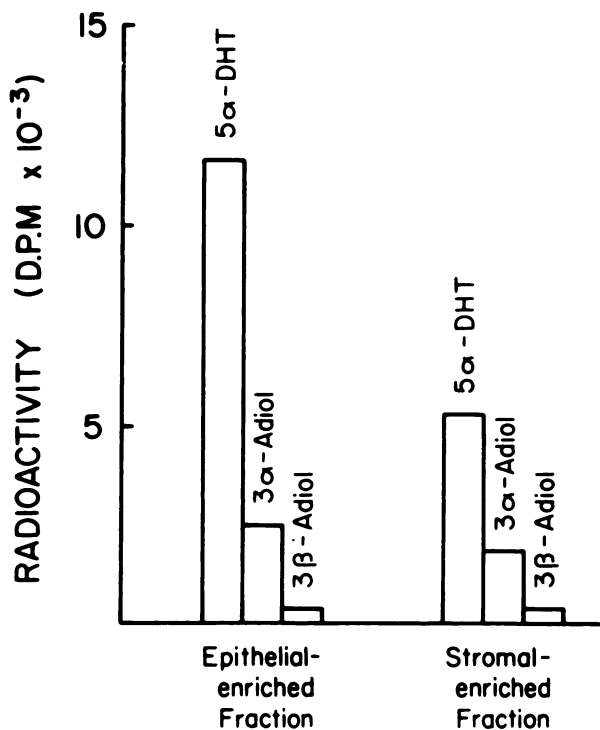


Fig. 5. Amounts of ^3H -labeled 5α -dihydrotestosterone (5α -DHT), 5α -androstane- $3\alpha,17\beta$ -diol (3α -Adiol), and 5α -androstane- $3\beta,17\beta$ -diol (3β -Adiol) formed from ^3H -testosterone over a 6-hour period by epithelial and stromal enriched fractions obtained by self-generated Percoll gradients.

tion. Metabolites other than those identified accounted for 1 to 4% of the tritium. For the cells obtained by self-generated gradients, 60 to 80% of the starting ^3H -testosterone remained at the end of the 6-hour culture. For cells obtained from preformed gradients, 40 to 50% of the starting substrate was recovered unaltered after the incubation.

Discussion

Methods for disaggregating tissues to obtain isolated cells for primary culture have been reviewed by Waymouth (1974). We have adopted a procedure for enzymatic digestion of rat ventral prostate using a progressive digestion with a CTC

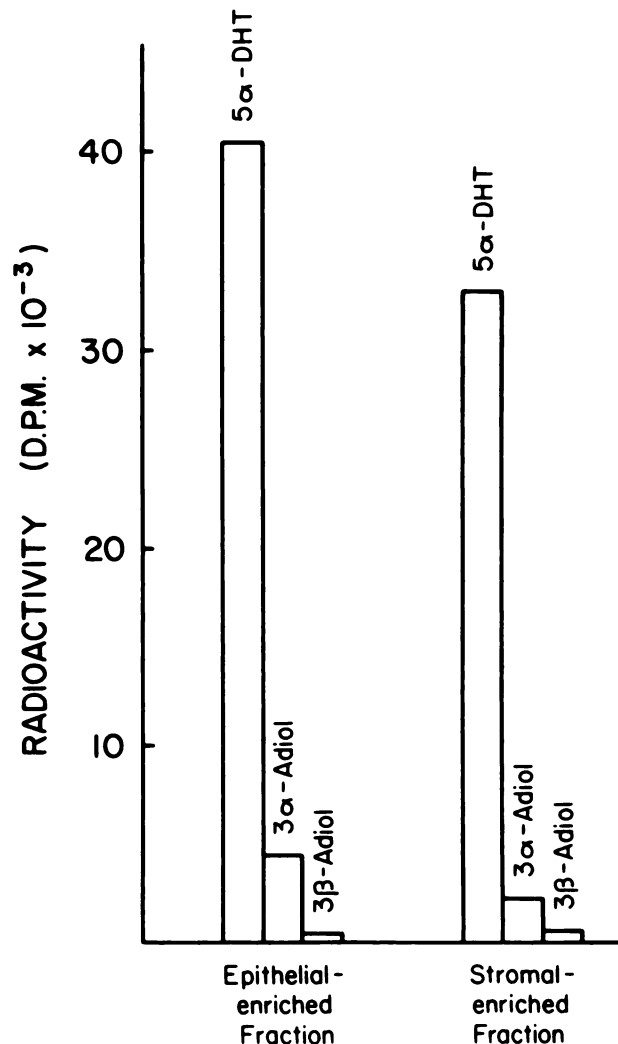


Fig. 6. ^3H -testosterone metabolites formed by cells obtained by preformed Percoll gradients. See legend for Fig. 5 for details.

mixture that has been successfully used by Kaighn and Prince (1971) and Coon (1968) for cell digestion of human and rat liver, respectively. This enzymatic method provides almost complete dissociation of the tissue into individual cells, thereby facilitating cell separation by centrifugation. Collagenase used alone with rat prostate tissue results in a greater frequency of cell clumps (aggregates) which hamper efficient cell separation (Rubenstein and Anderson, 1980).

The use of Percoll as a density gradient medium for cell separation is felt to be superior to isopycnic sedimentation with Ficoll (Helms et al, 1975; Dow and Pretlow, 1975). The gradients of the latter were very shallow and highly viscous, requiring up to 90 minutes to spin. The resultant distribution of cells showed banding within a very narrow range and very little, if any, separation. On the other hand, Helms et al (1975), Dow and Pretlow (1975), and Rubenstein and Anderson (1980) have found isokinetic sedimentation in Ficoll a more successful technique for purification of prostatic epithelial cells. This technique, however, is highly dependent upon the extent of prostatic dissociation, the linearity and viscosity of the gradient, and the speed and time of centrifugation (Helms et al, 1975; Rubenstein and Anderson, 1980). Also, the preparation of the Ficoll gradient is more laborious than that of Percoll, as the latter requires only a simple centrifugation step.

Stonington and Hemmingsen (1971) used a "rolling explant" technique to obtain monolayers of purified epithelial cells. This technique involved rolling the explants in culture dishes until the epithelial cells migrated to the surface of the explants. The enveloped explants were allowed to settle in the culture dish and grow out onto the dish. This technique, however, is long (in terms of days) and tedious and only a few explants gave good epithelial outgrowth.

Stone et al (1976) used collagenase to remove stromal elements and then allowed the glands to settle before pipetting off the supernatant and hence the bulk of fibroblasts. However, it is questionable as to how efficient this technique is in removing the fibroblasts from the "purified" epithelial glandular elements.

The successful use of Percoll in separating different cell types was initially demonstrated by Pertoft et al (1977). He has shown that primary calf testicular cells are able to survive banding in Percoll at $20,000 \times g$ and retain their ability to grow *in vitro*. However, our results show that, although

the prostate cells appear to survive banding at $20,000 \times g$ and demonstrative viability by trypan blue exclusion, their ability to metabolize testosterone is markedly reduced as compared with cells spun at lower gravitational forces ($1000 \times g$). Thus we feel that preformed gradients centrifuged at lower gravitational force are better able to maintain cellular integrity than self-generated gradients spun at high gravitational forces.

The metabolism studies show that at lower centrifugal forces the two cell types have similar abilities to metabolize testosterone to 5α -dihydrotestosterone and 3α - and 3β -androstanediols. Rubenstein and Anderson (1980) have shown qualitatively similar, although quantitatively different, patterns of testosterone metabolism in their epithelial and nonepithelial cell fractions. Thus, unlike the normal, neoplastic or cancerous adult human prostate in which the majority of the Δ^4 - 5α -reductase is in the stroma (Wilkin et al, 1980), the immature rat ventral prostate appears to have similar abilities to metabolize testosterone in the epithelial and stromal enriched fractions. Further studies on the kinetics of androgen metabolism by the two cell types are required to establish this with certainty. Assay results for Δ^4 - 5α -reductase and 3α -hydroxysteroid dehydrogenase activities in the two cell types support this conclusion (unpublished results). Also in support of this conclusion are the findings (unpublished results) that similar metabolic patterns exist for cells after they have been in culture for seven days either in the absence or presence of testosterone. Hence, there is some evidence that both cell types remain viable for significant periods of time.

We believe that CTC enzymatic digestion and Percoll isopycnic centrifugation provides a very simple, rapid, and quantitative means of separating prostate cells. At present our laboratory is investigating methods of maintaining normal prostatic epithelial cells in culture for longer periods of time in order to look at the effects of androgens on acid phosphatase activity. Although epithelial cell attachment is low, numerous colonies of epithelial cells are formed within one week (unpublished observations). The few stromal cells that are present do not appear to proliferate in our modified medium.

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