

Development and Restitution of Squamous Metaplasia in the Calf Prostate After a Single Estrogen Treatment

An electron microscopic study

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Received October 15, 1971

The development and restitution of squamous metaplasia in the glandular portion of the calf prostate was studied with the electron microscope at 4 days and 12 weeks following a single intramuscular injection of either of the synthetic estrogenic compounds diethylstilbestrol or hexestrol.

In the control prostates at 8 and 16 weeks, three types of cells were observed: serous, mucous and basal cells. The serous and mucous cells appeared as low columnar cells, showing structures comparable to the prostatic cells in man, dog, rat and mice. However the rough endoplasmic reticulum was less extensive and secretion granules of serous and mucous cells differed in structure. The basal cells had simpler cytoplasm and no secretion granules.

At 4 days after estrogen administration squamous metaplasia had developed as a proliferation of basal cells which transformed into squamous cells with many tonofilaments which pushed the other cell organelles to the periphery of the cells. Some immature secretory cells also showed tonofilaments.

Restitution of the metaplasia into glandular epithelium was found 12 weeks after estrogen administration apparently resulting from lack of estrogen. It appeared to be a very slow process representing a transformation of immature squamous cells into glandular cells. These changes were characterized by a diminishing number of tonofilaments and an increasing Golgi region with development of secretion granules. The strong interdigititation of cell membranes was reduced and typical plications and cell junctions were found again.

In The Netherlands administration of estrogens to fattening calves with the aim to get an increased weight gain, is legally forbidden. Experiments were carried out to investigate whether illegal administration of estrogens to calves could be detected using histological methods (Ruitenberg *et al.*, 1969, 1970; Kroes, 1970). It was found that the prostate displayed squamous metaplasia throughout the entire glandular portion of the organ, which persisted 8-12 weeks after a single parenteral administration of an estrogen (Ruitenberg *et al.*, 1970; Kroes, 1970). At 8 weeks however beginning restitution was observed (Kroes, 1970). The present study was undertaken to investigate with the aid of the electron microscope the development of the metaplasia, and the restitution of the induced metaplasia at a later time.

MATERIAL AND METHODS

Dutch-Frisian male noncastrated prepubertal calves (normally reaching puberty at an age of about 30 weeks) were used in the experiments. They were kept under conditions usual for fattening animals.

Two experiments were undertaken. In one experiment two calves received 80 mg diethylstilbestrol (DES, diethylstilbestroldipropionate 5%, Desexine, Wirtschaftsgenossenschaft Deutscher Tierärzte, Hannover, Western Germany) by intramuscular injection 4 days prior to slaughter at an age of 8.5 weeks to study the development of metaplasia. Two calves of the same age served as controls. Another experiment was carried out with nine calves to study the restitution of the induced metaplasia. Three calves received 80 mg DES by intramuscular injection at an age of 4 weeks; three calves were injected intramuscularly with 80 mg hexestrol (HEX, 2.5%, Animed, Bussum, The Netherlands) at the same age; and another three untreated served as controls. At the age of 16 weeks, when they were still prepubertal, the calves were slaughtered.

For histological study paraplast sections of the prostate were prepared and stained with hematoxylin and eosin (HE) or with periodic acid-Schiff (PAS) method.

Electron microscopy. Prostate tissue was removed as soon as possible after slaughter. Only the distal part of the prostatic body was prepared. A small piece of tissue was placed immediately in a drop of fixative and cut into smaller pieces to obtain good fixation. Immersion fixation was carried out in 2% osmium tetroxide in phosphate buffer, pH 7.3 (Millonig, 1962) for 90 minutes at 4°C. After fixation the tissue blocks were dehydrated in a graded series of ethanol and embedded in epon 812 (Luft, 1961). In order to select the areas for ultrastructural study 1–2 μ sections were cut on a LKB-ultramicrotome, stained with toluidine blue (Trump *et al.*, 1961) or with hematoxylin and eosin (Pool, 1969) and examined with light microscopy. Thin sections obtained with an LKB-ultramicrotome III with glass knives were mounted on copper grids and stained with lead citrate (Reynolds, 1963) or uranyl acetate followed by lead citrate.

These sections were examined with a Philips EM 200 electron microscope at 60 or 80 kV and instrumental magnifications of 550–12,000 \times .

RESULTS

Light microscopy

In the control animals of both experiments (8.5 and 16-weeks-old calves) a single layered epithelium was observed (Fig. 1). In this epithelium two types of cells were present: serous and mucous cells. Most cells had an immature aspect. Furthermore, a few basal cells, lying between the epithelial cells and the basement membrane, were found. Four days after estrogen administration a strong proliferation of basal cells, leading to a squamous metaplasia, was observed throughout the whole glandular portion. These proliferated cells elevated the lining glandular epithelial cells (Fig. 2). In the long-term experiment it was found that the metaplastic changes in the prostatic tissue were most pronounced

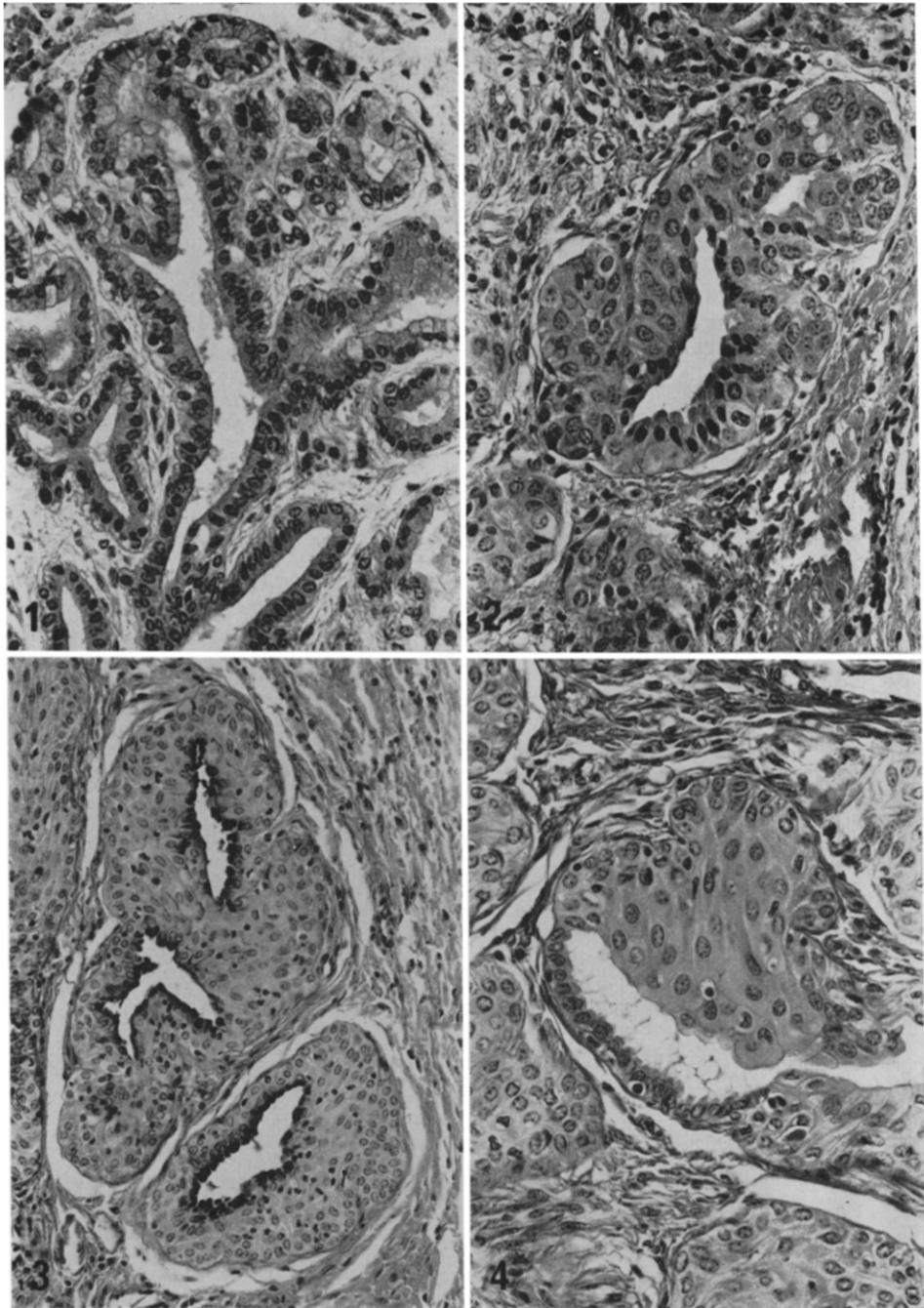


FIG. 1. Prostate, calf, age 16 weeks, control. Tubules lined by columnar epithelium. Mucous (MC), serous (SC) and basal (BC) cells. H-E stain, $\times 210$.

FIG. 2. Prostate, calf, age 8.5 weeks, 4 days after 80 mg DES. Proliferating basal cells, elevating the lining glandular epithelium. H-E stain, $\times 210$.

FIG. 3. Prostate, calf, age 16 weeks, 80 mg DES at an age of 4 weeks. Squamous metaplasia, superficial cellayer of metaplastic epithelium, showing PAS-positive granules. PAS-method, $\times 125$.

FIG. 4. Prostate, calf, age 16 weeks, 80 mg DES at an age of 4 weeks. Squamous metaplasia and "newly formed" or "pre-existent" glandular epithelium. H-E stain, $\times 210$.

in the animals receiving DES (Fig. 3). However, restitution could also be observed. This restitution of the squamous metaplasia was more advanced in the animals receiving HEX. In all estrogen-treated animals, however, squamous metaplasia was noted, but also "newly formed" or "pre-existent" glandular epithelium was observed (Fig. 4).

Electron microscopy

Ultrastructure of normal prostate of control animals. The main difference between the 8-weeks-old and the 16-weeks-old calves was the existence of lower and more immature cells in the single layered lining epithelium of the younger animals. The prostatic secretory cells were cuboidal or low columnar (Fig. 5). The adjacent plasma membranes were lying close together and toward the apical cell regions typical plications and junctional complexes were found (Fig. 6). Junctional complexes always showed a tight junction (zonula occludens), an intermediate junction (zonula adherens) and a desmosome (macula adherens). In addition, desmosomes were distributed irregularly along the lateral surfaces of apposed secretory cells. Toward the base of the cells intercellular spaces were noted, which varied considerably in size (Fig. 5). The apical cell surface displayed microvilli with some variation in size and number, and was coated occasionally with a moderately dense amorphous layer. The membrane at the basal side of the cell was bordered by a moderately dense amorphous and homogeneous layer, about 200–400 Å in width, which was referred to as the basement membrane.

Occasionally basal cells lying between the epithelial cells and the basement membrane were noted (Fig. 8). The periacinar tissue consisted of connective tissue in which fibroblasts and capillaries were lying between collagen and interstitial ground substance.

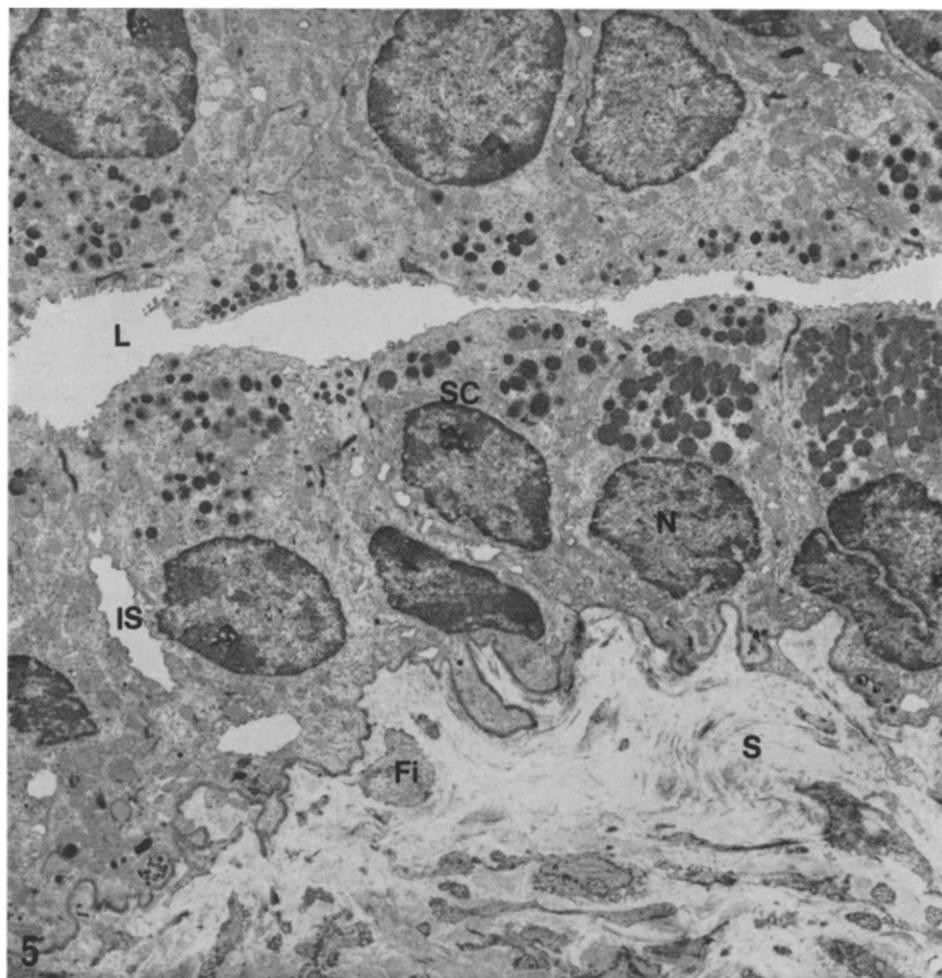
The *serous cells* represented the majority of the epithelial cells. A round to oval nucleus was located near the base of the cells. Cytoplasmic invaginations of the nuclei were occasionally observed.

The nuclear envelope showed pores. Nucleoli were present, and in several nuclei also nuclear bodies were noted.

In the cytoplasm variable amounts of small cisternae of rough-surfaced endoplasmic reticulum (RER) and many vesicles of smooth-surfaced endoplasmic reticulum (SER) were present. A variable number of free ribosomes and some polysomes were found. A prominent supranuclear Golgi apparatus with many microvesicles was observed. Cisternae of the RER often were very close to the "forming face" (Beams and Kessel, 1968) of the Golgi apparatus (Fig. 6). However, no distinct transition of the RER into the Golgi components was noted.

In the apical region of serous cells a moderate to large number of distinct membrane-bound secretion granules containing dense material was found (Fig. 5, 6). Variations in diameter and density of these granules were noted. The granules were lying in a cytoplasmic matrix containing RER cisternae, some SER, free ribosomes and polysomes and well-developed mitochondria with long cristae and a moderately dense matrix.

The secretion granules were most probably formed in the Golgi region. Small



Note: Figs. 5-15 are all electronmicrographs of sections double stained with uranyl acetate and lead citrate, unless stated otherwise. Abbreviations used in these electron micrographs: MC mucous cell, SC serous cell, IS intercellular space, BM basement membrane, CM cell membrane, CJ cell junction (epithelial junctional complex), D desmosome, HD hemidesmosome, PI plication of adjacent cell membranes, Id interdigititation, Mv microvilli, L lumen, N nucleus, NB nuclear bodies, RER rough-surfaced endoplasmic reticulum, R free ribosomes, G. Golgi complex, SG secretion granule, M mitochondrion, DB dense body, T tonofilaments, S stroma, Fi fibroblast.

Fig. 5. Prostate, calf, age 16 weeks, control. Normal single layered glandular epithelium. Approx $\times 3700$.

vesicles pinching off the ends of Golgi lamellae might fuse together to form large vesicles, which would transform into secretion granules. In some cases merocrine secretion or "reversed pinocytosis" (Brandes, 1966) of secretion granules reaching the apical cell membrane was noted (Fig. 7).

The basal area of the cell and its perinuclear region contained moderate amounts of cytoplasmic constituents, mainly RER, SER, free ribosomes and

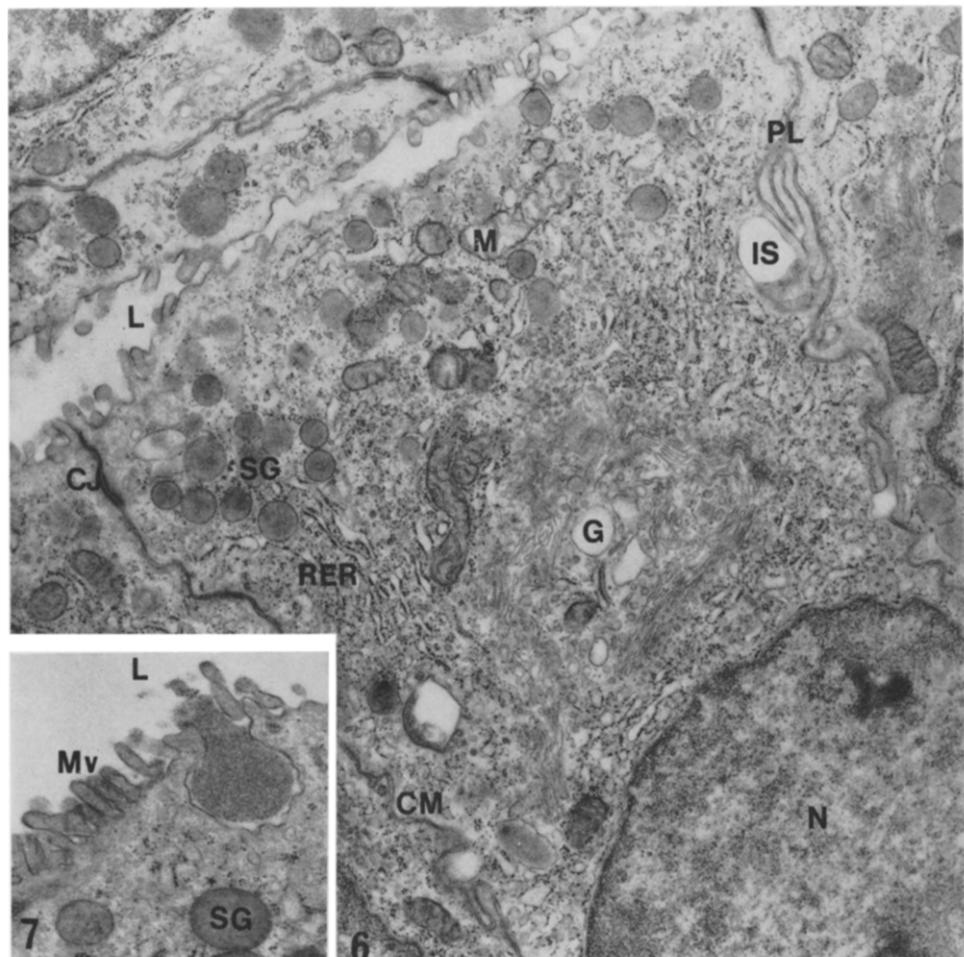


FIG. 6. Prostate, calf, age 16 weeks, control. Apical region of serous cell. Note the presence of RER, SER, Golgi region, secretion granules and mitochondria. Adjacent cell membranes show typical epithelial junctional complex and plications. Microvilli are present at the luminal border. Staining lead citrate, approx $\times 10,500$.

FIG. 7. Prostate, calf, age 16 weeks, control. Meroocrine secretion in serous cell. Approx $\times 21,000$.

mitochondria. Multivesicular bodies and dense lysosome-like bodies were present in several cells.

Serous cells were found both in 8-weeks-old calves and in 16-weeks-old calves. Many of the cells were still immature as they contained less secretion granules and showed a less developed RER and Golgi apparatus than the mature cells.

Both in 8-weeks-old and in 16-weeks-old calves *mucous cells* were found. These cells were characterized by a more dense cytoplasmic matrix and many membrane-bound secretion granules (Fig. 10). The granules showed considerable variation in size. They contained an amorphous material, which was less dense in

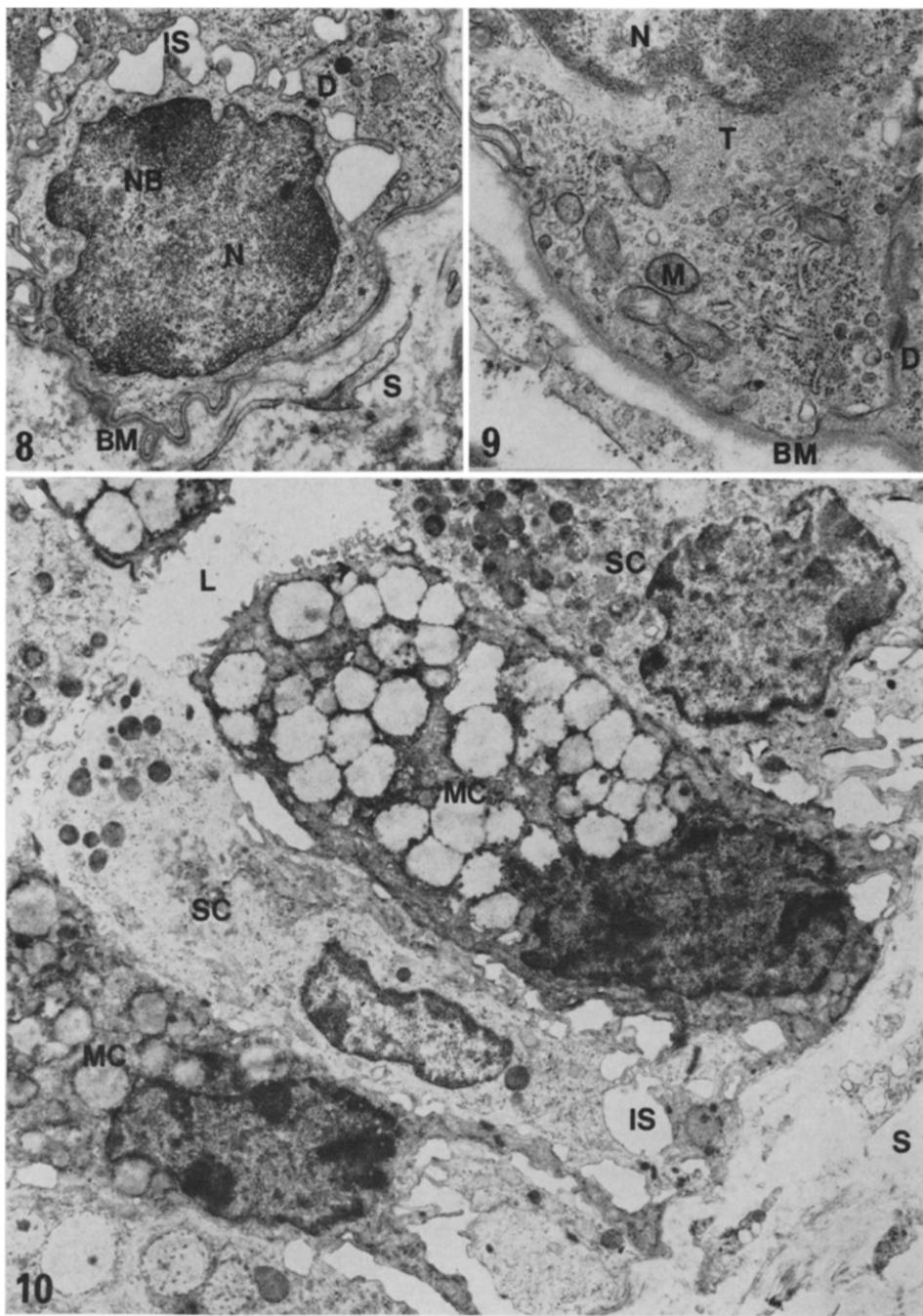


FIG. 8. Prostate, calf, age 16 weeks, control. Basal cell. Note the relatively large nucleus, nuclear body, desmosomes, intercellular spaces and basement membrane. Approx $\times 7800$.

FIG. 9. Prostate, calf, age 16 weeks, control. Basal cell showing a few RER and SER, free ribosomes and mitochondria. Tonofilaments are present in the cytoplasm. Note the basement membrane. Staining lead citrate. Approx $\times 23,000$.

FIG. 10. Prostate, calf, age 16 weeks, control. Mucous and serous cells. Note diversity of secretion granules in the different cells. Approx $\times 6000$.

comparison with the secretion granules of serous cells. Confluence of granules was noted. Because of the numerous secretion granules present in the mature mucous cells, the nuclei were pressed to the base of the cells and were somewhat flattened.

The Golgi apparatus was not very prominent. It consisted of several compressed lamellae, especially in the cells where secretion granules were very numerous. Between the secretion granules some scattered ribosomes, few RER and SER membranes and occasionally mitochondria were found. The perinuclear and the basal parts of the cells contained mainly some RER, SER, free ribosomes and mitochondria.

Immature mucous cells showed round nuclei, less numerous secretion granules and a less flattened Golgi apparatus. Evidence for secretion was not observed in mucous cells.

A number of epithelial cells showed characteristics of serous as well as of mucous cells. Part of the secretion granules were distinct and electron-dense, other larger secretion granules contained less dense material. In addition in the same cases mucous secretion granules were observed with signs of a condensation. It seemed that there were intermediate or transitional forms in secretion granules.

The *basal or reserve cells* were somewhat flattened cells with a round to oval nucleus lying in the centre of the cells (Fig. 8). The long axis of the cells was lying parallel to the basement membrane. In the nuclei nucleoli were present. In some cases nuclear bodies and membrane invaginations were observed.

The cytoplasm of these cells appeared rather undifferentiated (Fig. 9). The majority of the endoplasmic reticulum consisted of SER vesicles. A few short RER membranes were present. Many free ribosomes, sometimes forming polyribosomes, were noted. A rather undifferentiated Golgi apparatus was localized in the apical part of the cell. There was no evidence of secretion.

In most of the basal cells some filaments were found, especially in the basal part of the cells (Fig. 9). These filaments, probably tonofilaments, were often running parallel to the basement membrane. Continuities with desmosomes and hemidesmosomes of the cell membranes were noted. In addition a few mitochondria and dense bodies could be observed.

Changes 4 days after a single estrogen administration. Changes were observed in the basal cells and in the secretory cells. The basal cells increased in size and number thus giving rise to a multilayered epithelium. In the ultrastructural study several stages of development of squamous metaplasia were noted. The first change was the transformation of the basal cell from a relatively undifferentiated cell into a squamous cell (Fig. 11). Many filaments mostly running parallel to the nuclear membrane were observed. The filaments developed primarily in the perinuclear region pushing the other cell organelles to the periphery of the cell. The filaments are believed to be tonofilaments because of their similarity to those found in squamous epithelium (Fawcett, 1966).

A few RER membranes were present and a decreased number of membrane-associated ribosomes was observed. In some cells a slight dilatation of RER cisternae, containing a more dense material, was observed. Ribosomes were lying

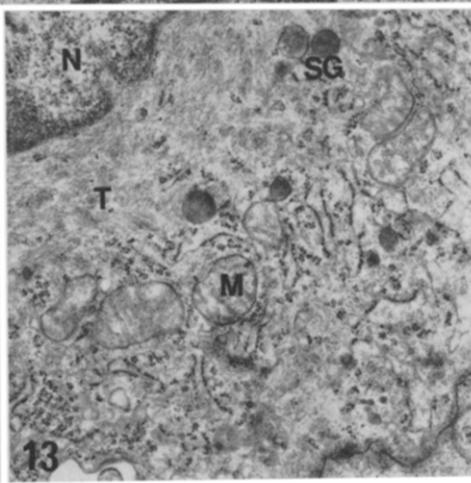
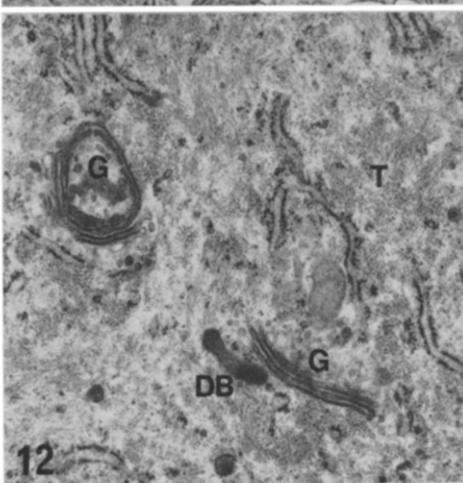
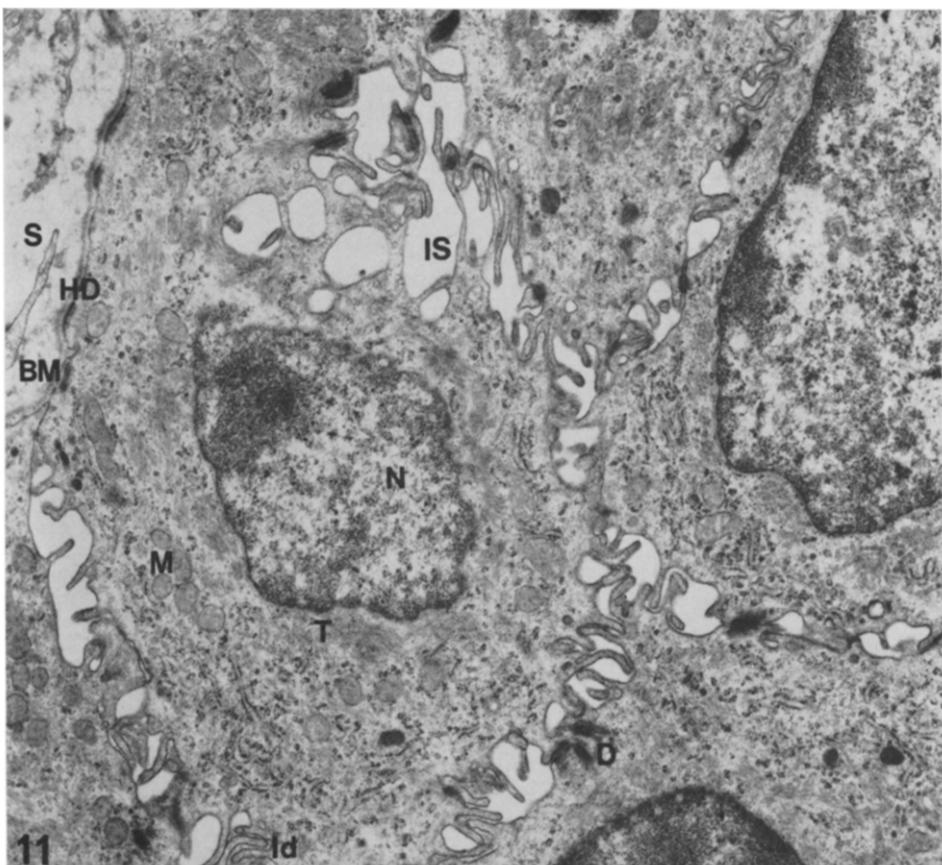


FIG. 11. Prostate, calf, age 8.5 weeks, 4 days after the administration of 80 mg DES. Transformed basal cell. Note perinuclear arrangement of tonofilaments, interdigititation of the adjacent cell membranes and increased number of desmosomes. Approx $\times 9700$.

FIG. 12. Prostate, calf, age 8.5 weeks, 4 days after the administration of 80 mg DES. Predominance of tonofilaments partly arranged in bundles. Flattened lamellae and a few microvesicles in reduced Golgi region. Approx $\times 17,000$.

FIG. 13. Prostate, calf, age 8.5 weeks, 4 days after the administration of 80 mg DES. Perinuclear arrangement of tonofilaments. Moderately dilated RER membranes with partial loss of ribosomes. Some secretion granules are present. Approx $\times 21,800$.

scattered between the tonofilaments. SER vesicles, free ribosomes and mitochondria were lying in small accumulations dispersed in the cytoplasm. The Golgi apparatus, normally appearing inactive, showed flattened lamellae and was considerably reduced in size (Fig. 12).

Adjacent cell membranes showed a very extensive interdigititation and many intercellular spaces were observed. The number of desmosomes had increased and they were noted in the intercellular bridges.

The secretory cells covering the proliferated basal cell layers, also showed abnormalities which were mainly similar to those found in the basal cells. These abnormalities varied, however, in the individual cells according to their state of differentiation. The majority of the immature epithelial cells showed tonofilaments which were arranged in bundles mainly in the perinuclear regions, pushing the other cell organelles to the periphery of the cell. The Golgi region was reduced in size. The RER membranes were reduced in number and showed occasionally some dilatation. Between the tonofilaments secretion granules were found (Fig. 13). Their number varied from cell to cell. The typical plications found in normal secretory cells were not observed in all cells. At the apical border microvilli had decreased in number and were atrophic. Signs of early keratinization were not observed in any of the cells mentioned above.

The basement membrane was not altered. In the tubular lumina occasionally desquamated disintegrated secretory cells were noticed. At several places the epithelium was infiltrated by granulocytes and lymphocytes.

Changes 12 weeks after a single estrogen treatment. Metaplastic squamous epithelium was found. The total number of cell layers in the prostatic tubules of HEX-treated animals was less in comparison with the DES-treated animals. In the various sections, however, variation seemed to be dependent on the site where the tubules were localized in the prostate. This was found also in the light microscopical studies.

The squamous epithelium lining the prostatic tubules showed a basal layer of oval cells which were enlarged in size and contained more cytoplasm as compared with the basal cells found in the prostatic tissue of control animals (Fig. 14). The cells showed the same characteristics as were found for the basal squamous cells in the short term study except for the abundance of tonofilaments, the concentration of cytoplasmic organelles in compact regions and the mitochondria which seemed to be smaller and reduced in number. This reduction in number may only be a relative reduction, because of the great increase in size of the cells. No secretion granules were found. Cells showing the same morphological characteristics as described for the basal cells of the prostatic tissue of control animals were never observed.

In the cellular layers covering the basal cells the same pattern was noted. The enlarged cells contained even more tonofilaments. No signs of keratinization were found.

Dependent on the stage of restitution the cells found in the superficial layers showed more or less restoration of secretory function. The stage of restitution was more advanced in HEX-treated animals. The cells changed gradually into cuboidal cells when they were located nearer to the lumen. The cell membranes

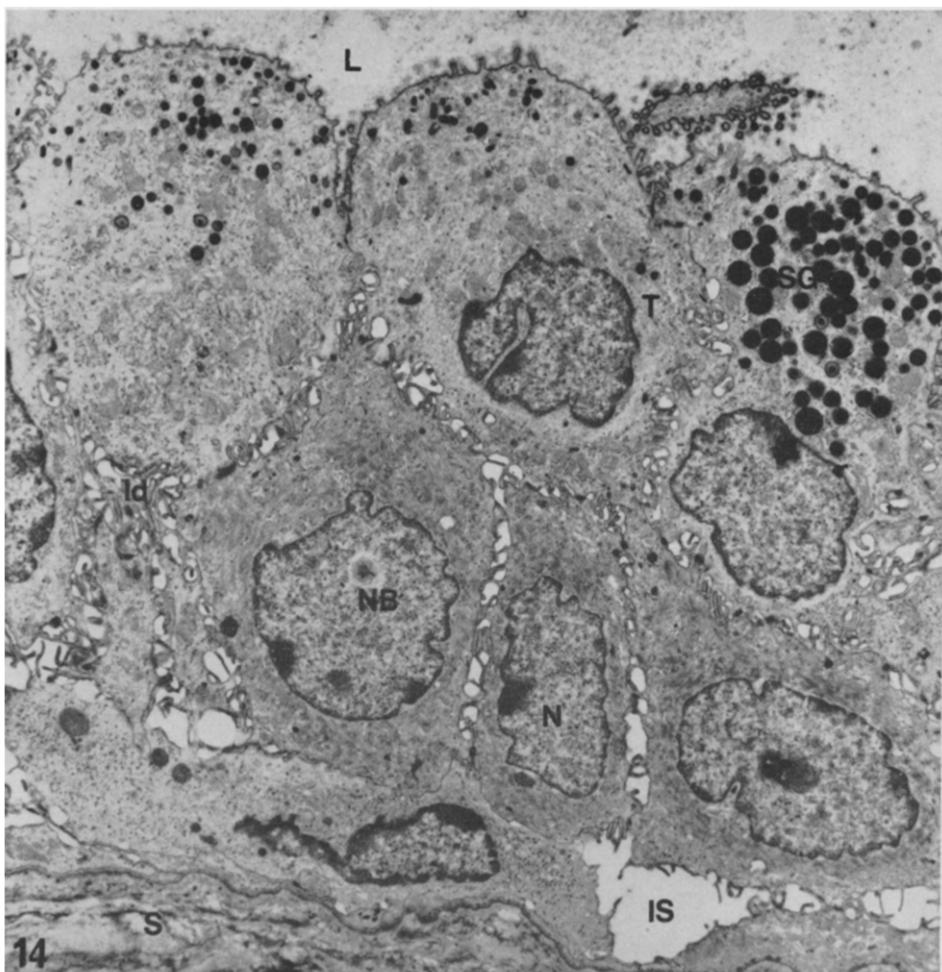


FIG. 14. Prostate, calf, age 16 weeks, 80 mg DES at an age of 4 weeks. Transformation into secretory cells. Note the presence of both secretion granules and tonofilaments in the same cell. Approx $\times 3750$.

showed strong interdigitations which became less pronounced in the cells nearer to the lumen. In a large number of the cells lining the lumen, cell junctions, plications and microvilli were present again. The nuclei showed no changes. Many tonofilaments were present. However, they were most reduced in number in cells in the more superficial layers. An increase in the number of the other organelles was noted. More SER vesicles and RER membranes were present and the Golgi apparatus had increased in size, showing many microvesicles. In the cells located more superficially some secretion granules were observed. The cells lining the lumen showed obvious signs of restoration, especially in the HEX-treated animals. Only a few tonofilaments were present (Fig. 15). A prominent Golgi apparatus was noted and many secretion granules were observed in the apical zone.

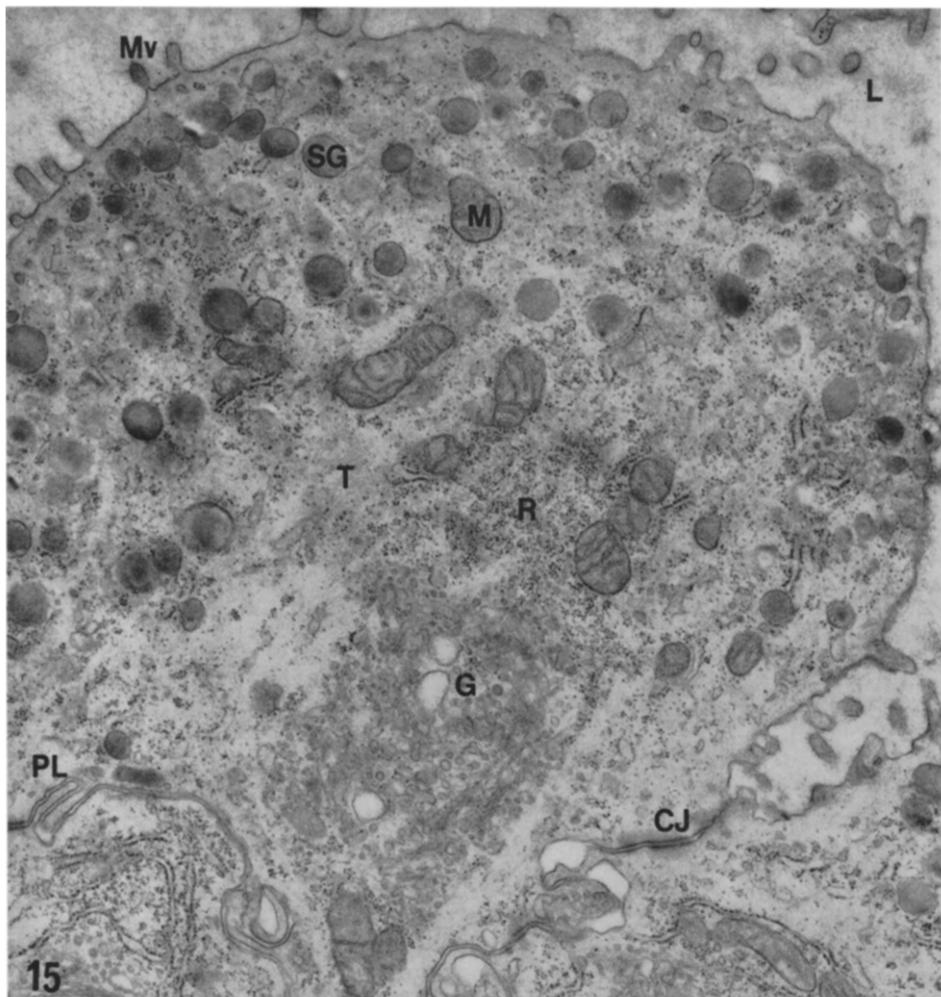


FIG. 15. Prostate, calf, age 16 weeks, 80 mg DES at an age of 4 weeks. Almost complete restitution of epithelial cell. Restoration of secretory activity in prominent Golgi region. Staining lead citrate. Approx $\times 15,000$.

In all animals studied restitution was noted. In some tubules of both DES- and HEX-treated animals normal secretory epithelium was observed again. These secretory cells were present between or covered the restorating cells and partly lined the tubules. Morphologically they were similar to the secretory cells found in the control animals. Also in the long-term experiments the metaplastic epithelium was infiltrated at several places by granulocytes and lymphocytes.

DISCUSSION

The ultrastructure of prostatic cells has been studied in different species (Groth and Brandes, 1960; Harkin, 1961; Kanai, 1961; Seaman and Winell, 1962; Brandes and Groth, 1963; Mao and Angrist, 1964; Rowblatt and Franks,

1964; Fisher and Jeffrie, 1965; Szirmai and Van der Linde, 1965; Brandes, 1966). The ultrastructure of the epithelial cells of the calf prostate can be compared to a great deal with the prototype cell described by Brandes (1966). The cell membranes showed cell junctions as found in glandular epithelium (Fawcett, 1966). The typical plications found in the lateral cell membranes were also observed in the prostate of the dog (Seaman and Winell, 1962) and it was suggested that they may serve as an anchoring device.

In the light microscopical studies a distinction could be made between serous and mucous cells. Immature or degranulated mucous cells or transitional forms may be discerned by special stains (Kroes, 1970). In the ultrastructural study, transitional or intermediate cells were found. This is not unique, since the same was noticed (Sasano *et al.*, 1969) in the chief cells of the human gastric mucosa. A strict separation between types of secretory cells seems to be to our opinion a concept of the past.

All epithelial cells of the calf prostate showed a Golgi apparatus, which was especially well-developed in serous cells. Meroerine secretion in serous cells or aprocrine secretion in mucous cells is not frequently found in the epithelial cells. The major reason may be that the calves are immature at this age. Meroerine and apocrine secretion were also described for prostatic epithelial tissue of man and rats (Brandes, 1966). The total number of RER membranes found in the secretory cells was less in comparison with that found in other animals (Brandes, 1966). This may be due to the immature state of the animals. To give a definite answer to this problem, however, the prostate of the adult bull needs to be studied in the future.

The third cell type observed in the calf prostate was the basal cell. The ultrastructure of the basal cells of the calf prostate is very similar to the structure found in man by Mao and Angrist (1964). According to these authors the basal cells can be considered as precursors of the epithelial secretory cells.

Ultrastructural studies concerning metaplastic changes are scarce. In ultrastructural studies of estrogen-induced squamous metaplasia of the columnar epithelium of the mouse uterus (Wadell, 1965; Nilsson and Wadell, 1966) only the ultrastructure of the metaplastic squamous cells is described. Other ultrastructural studies concerned metaplasia in mouse urinary bladder induced by implantation of paraffin pellets containing methylcholanthrene (Ghidoni and Gueft, 1961; Ghidoni and Campbell, 1969) or dimethylbenzanthracene-induced squamous metaplasia in rat tracheal epithelium cultured *in vitro* (Dirksen and Crocker, 1968). The ultrastructural pattern of the metaplastic basal cells described in those studies (Ghidoni and Gueft, 1961; Dirksen and Crocker, 1968; Ghidoni and Campbell, 1969) was similar to that found in the squamous basal cells in the present studies. In the literature mentioned above no information about restitution was found.

In the short-term study some of the basal cells and a considerable number of the secretory cells showed dilated RER cisternae. This distention may be only temporary since normal cisternae were also noticed. It is of interest that in ventral prostate of rats, a short time after estrogen administration also a distention of RER cisternae was observed (Harkin, 1961). The decrease in number of RER mem-

branes and the increase in number of ribosomes found especially in the secretory cells after estrogen administration has been described (Groth and Brandes, 1960).

The basal cells were transformed into squamous cells which proliferated and elevated the secretory epithelium. It appeared that these undifferentiated cells transformed into squamous cells when the estrogen reaches the cells. Using radioactive estrogens it was shown that in the rat the estrogen is incorporated and probably utilized in target organs such as prostate (Mangan *et al.*, 1967; Uhnjem, 1969) and uterus (Eisenfeld and Axelrod, 1966; Jensen and Jacobson, 1962; Bengtsson *et al.*, 1963; Ullberg and Bengtsson, 1963; Gorski *et al.*, 1965; Inman *et al.*, 1965; Stumpf, 1968). The direct action of estrogen on prostatic epithelium was shown in *in vitro* experiments (Lasnitzki, 1958; 1963). When the estrogen has reached the cells, cellular metabolism will be altered in some unknown way and transformation into another cell type is started. In our studies this transformation into another cell type is also found in a number of immature secretory cells. The state of differentiation of the secretory cells at the moment of estrogen application, determines the extension of the squamous transformation of that cell. Fully differentiated cells did not show squamous transformation.

In the long-term experiment a restoration of the squamous epithelium into a secretory epithelium was found. Evidence for the restitution was found in the decreased number of tonofilaments, the increased number of RER membranes, the enlargement of the Golgi apparatus and the reappearance of secretion granules.

It appeared that the restitution to a normal situation, in which basal cells develop to secretory cells, was a slow process. This transformation may be induced by a lack of estrogen. It seems justified to assume that the estrogens administered are bound and probably utilized in the prostatic epithelial cells (Mangan *et al.*, 1967; Uhnjem, 1969). It seems likely that when estrogens do not circulate any longer, the amount of estrogen incorporated in the basal cells will decrease by every cellular division. At a certain time the daughter cells will no longer possess enough estrogen to prolong their transformation into a squamous cell. The inhibition of the differentiation into glandular epithelium is removed and differentiation into glandular epithelium starts again.

In both DES- and HEX-treated animals a restitution in the prostatic tissue was found. From the ultrastructural studies it can be concluded that restitution was not yet finished. In order to evaluate the restitution process it would be interesting to study the effect after a long interval of lower parenteral dosages of estrogens.

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