Action and Interaction of Hormones and 3-Methy cholanthrene on the Ventral Prostate Gland of the Rat In Vitro. I. Testosterone and Methylcholanthrene  

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SUMMARY—The effect of testosterone and 3-methylchol anthrene, applied singly and combined, on organ cultures of the ventral prostate gland of the rat, in natural and semidefined medium, was studied. In medium without added hormone the glandular epithelium regressed. The regression was more severe in the natural medium and was accompanied by a marked increase of stroma. Testosterone inhibited the stromal growth and partially maintained the epithelium in the natural medium; in the semidefined medium it fully maintained the epithelium and preserved secretory activity. After more prolonged treatment testosterone induced mild epithelial hyperplasia. 3-Methylcholanthrene caused rapid and extensive hyperplasia of the alveolar epithelium. After transfer to control medium the hyperplasia persisted. Simultaneous administration of testosterone and 3-methylcholanthrene and transfer of explants pretreated with 3-methylcholanthrene to medium containing testosterone resulted in a drastic inhibition of the hyperplasia caused by the carcinogen. It is suggested that the interaction of the two substances may take place at the lysosomal level.—J Nat Cancer Inst 35: 339–348, 1965.

CARCINOMAS of the prostate gland in the rat have been induced by 3-methylcholanthrene in vivo by Dunning et al. (7), Mirand and Staubitz (2), and Allen (3). Allen obtained a higher incidence of tumors in castrated than in intact rats or in castrated rats treated with testosterone propionate. His finding suggested that androgenic hormones slowed down carcinogenesis in the prostate. It is not certain, however, whether this effect was due to a direct interaction of the two compounds or whether it was influenced by the presence of other hormones or general systemic factors. This question could be answered by the use of organ cultures in which both these factors are largely eliminated. Tissues grown in organ culture retain their structural integrity and functional activity for prolonged periods in vitro and are therefore comparable to their parent tissue in vivo. They can be exposed to given quantities of physical or chemical agents.
and the effects of these agents and their modification studied at the tissue or cellular level.

In earlier work the direct effect of 3-methylcholanthrene (MCA) on organ cultures of the ventral prostate gland of the rat was investigated (4), and the carcinogen was found to induce rapid and extensive hyperplasia of the alveolar epithelium. In the present experiments, organ cultures of the ventral prostate gland of the rat were treated with testosterone and MCA to study the interaction of the two compounds.

Since differences in nutrition and traces of hormones in natural media may modify the final result, the action of testosterone alone and the effect of testosterone combined with MCA were studied in cultures grown in natural and semi-defined media.

MATERIALS AND METHODS

Most of the glands were obtained from 8-week-old hooded rats of a laboratory inbred strain and a few from 5- to 6- or 10-week-old animals. The glands were removed under sterile conditions and freed from the surrounding sheath of fat and connective tissue. During dissection they were kept in Tyrode's solution to which 200 units of penicillin and 2 percent horse serum had been added. A knife or scissors was avoided to prevent a regenerative hyperplasia at the cut edges; instead the organ was gently teased apart into its natural subdivisions by means of two pairs of fine forceps.

For cultivation in natural medium, Shaffer's (5) modification of the watchglass technique of Fell and Robison (6) was used. Fragments approximately $2 \times 4 \times 2$ mm were arranged on strips of rayon acetate; 2 of these strips holding 3 to 4 explants each were then placed on a clot contained in a watchglass. The clot consisted of $1 \text{ cm}^3$ of medium composed of cock plasma, horse serum, and dilute chick embryo extract in a proportion of 3:1:1.

For cultivation in a semidefined medium, the rayon strips holding the cultures were supported on metal grids placed in small flat-bottomed dishes of borosilicate glass. The dishes were filled with medium, usually $2.5 \text{ cm}^3$, up to the level of the explants. The medium consisted of Parker's 199 with 5 percent horse serum.

Testosterone B.P. (Organon, London) was dissolved in absolute ethanol, and MCA in acetone. The solutions were suspended in serum and measured quantities incorporated into the clot or added to the fluid medium. The final concentration of testosterone in the medium was $30 \mu g$ per ml and that of MCA $4 \mu g$ per ml of medium. The controls received serum to which corresponding amounts of ethanol, acetone, or both had been added.

The watchglasses containing the clots and the dishes holding the grids and the fluid medium were enclosed in petri dishes carpeted with damp cotton wool. The petri dishes were stacked in a tightly closed desiccating jar and placed in an incubator; the desiccating jar was perfused with a mixture of 95 percent oxygen and 5 percent CO$_2$ for 40 minutes, at a flow rate of 100 ml per minute after explantation and at each transfer; between transfers it was perfused daily for 20 minutes only. The cultures were incubated at $37.5^\circ C$ and transferred to fresh medium every 3 to 4 days.

In the first series of experiments the explants were exposed to testosterone alone. Both hormone-treated and control explants cultivated in the natural medium were fixed at 10 or 14 days' growth; control and hormone-treated cultures kept in the semidefined medium were fixed at 7 or 14 days' growth.

In the second series the glands were treated with a combination of testosterone and MCA. One group of explants was exposed to both compounds simultaneously and fixed after 11 days' growth in natural or semidefined medium. A second group was grown in natural medium first with MCA only for 11 days and then transferred to natural medium containing testosterone only for 6 more days. This series had two sets of controls kept without testosterone: One was grown without carcinogen and fixed at 11 and 17 days; another was treated with MCA for 11 days, and either fixed after this period or transferred to normal medium for 6 days.

The explants were fixed either in Bouin's or in 3 percent acetic-Zenker solution, embedded in paraffin, and sectioned at 6 $\mu$. The sections were stained by the periodic acid-Schiff reagent (PAS)
after diastase digestion. Approximately 150 cultures were used for the investigation.

RESULTS

The ventral prostate gland consists of tubuli and alveoli lined with cuboidal or columnar secretory epithelium, either straight or slightly folded. The height of the epithelium, the degree of folding, and the secretory activity varied from gland to gland. Many organs showed alveoli in which the epithelium had become necrotic and was shed into the lumen. The stroma between the alveoli was very sparse (fig. 1).

Action of Testosterone

Explants Grown in Natural Medium

Controls.—After 10 days' cultivation the gland had undergone a striking change. The explants had decreased in size, and histological examination of serial sections showed that in many parts of the gland the number of alveoli per unit area was greatly reduced (fig. 2). In all sections the alveolar epithelium had markedly regressed: The folding was lost, the lumen had become narrow, and the cells were much lower owing to a partial loss of cytoplasm (fig. 3). Throughout the explant, the connective tissue had considerably increased and filled the interalveolar spaces with cells and fibers (figs. 2 and 3). After 17 days the changes were similar, but after 14 days there were even fewer alveoli and the epithelium had become flat or atrophic.

Effect of testosterone.—After 10 days' treatment, the explants were larger and contained more alveoli per unit area than the control cultures and the width of their lumen was normal. In the center of the explant the epithelium was straight and flat, but in most peripheral alveoli it was cuboidal or columnar, occasionally folded, and, in some alveoli, small secretory granules could be seen at the free surface. The abnormal stromal growth seen in the cultures grown without hormone was partially inhibited by testosterone (fig. 4). In a few explants the proliferation of the alveolar epithelium was increased, but except in one explant this increase was slight and limited to a few alveoli in which the cells had multiplied to form 2 to 3 rows.

After a 2-week exposure to the hormone, the height and functional activity of the epithelium had been preserved in a greater number of alveoli and the inhibition of abnormal stromal growth maintained, but the incidence and extent of epithelial hyperplasia had become more marked. In nearly half the treated explants hyperplastic alveoli could be recognized in the peripheral areas, and in some of them the cells had increased to 5 to 7 layers which partially occluded the lumen (fig. 5). The hyperplastic epithelium consisted of small, round or columnar cells covered at the lumen by actively secreting elements.

The response to the hormone did not vary significantly with the age of the animals from which the organs had been obtained.

Growth in Semidefined Medium

Controls.—The regressive changes were less severe than in the natural medium and confined mainly to the height and secretory activity of the epithelium. The number of alveoli was similar to that in the gland in vivo. After 1 week's growth the alveoli were slightly dilated, the epithelium was lower, had lost its folding, and secreted less than the organ before explantation. The secretory matter had changed in consistency and staining reaction; it was granular and with PAS stained a pale pink instead of bright red (figs. 6 and 9).

After 1 more week in the same medium the explants underwent a spontaneous recovery (fig. 7). In many alveoli the epithelium had almost regained its original height and new alveoli had formed at the periphery, but the secretory activity had been only partially restored. Thus, only some alveoli contained secretory matter which was homogeneous and stained a brilliant red with PAS.

Effect of testosterone.—After 7 days' treatment the epithelium was higher than in the controls and secreted profusely (figs. 8 and 10); most alveoli were filled with homogeneous PAS-positive matter. This effect could be observed throughout the entire explant, whereas in cultures similarly treated, but grown in natural medium, it was confined to peripheral areas. In most explants, the hormone also induced a mild epithelial hyperplasia in a few alveoli, but not more than 3 rows of cells were formed.
Table 1.—Influence of testosterone on the 3-methylcholanthrene (MCA) effect

<table>
<thead>
<tr>
<th>Medium</th>
<th>Treatment</th>
<th>Total number of cultures</th>
<th>Number of treated cultures showing various grades of hyperplasia*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Natural</td>
<td>MCA only for 11 days</td>
<td>11</td>
<td>2 0 1 4 4</td>
</tr>
<tr>
<td>Natural</td>
<td>MCA and testosterone for 11 days</td>
<td>11</td>
<td>7 0 3 1 0</td>
</tr>
<tr>
<td>Natural</td>
<td>MCA only for 11 days, followed by cultivation in control medium for 6 more days</td>
<td>12</td>
<td>0 0 2 3 7</td>
</tr>
<tr>
<td>Natural</td>
<td>MCA only for 11 days, followed by testosterone for 6 more days</td>
<td>12</td>
<td>0 6 6 0 0</td>
</tr>
<tr>
<td>Semidefined</td>
<td>MCA only for 11 days</td>
<td>14</td>
<td>5 0 9 0 0</td>
</tr>
<tr>
<td>Semidefined</td>
<td>MCA and testosterone for 11 days</td>
<td>8</td>
<td>8 0 0 0 0</td>
</tr>
</tbody>
</table>

*0: No hyperplasia; I: very few alveoli showing mild hyperplasia; II: less than half the number of alveoli showing mild hyperplasia; III: half to two thirds of the number of alveoli showing medium to marked hyperplasia; IV: most of the alveoli showing marked hyperplasia.

Explants exposed to testosterone for 2 weeks were histologically similar to those treated for 1 week. They showed high secretory epithelium, more connective tissue, and a mild hyperplasia of some alveoli (Fig. 11). Since the cultures grown without hormone had partially recovered by this time, there was little difference between them and the experimental cultures except for a greater secretory activity in the treated explants.

Interaction of Testosterone With MCA

Natural Medium

Effect of MCA alone.—The carcinogen stimulated the proliferation of the alveolar epithelium, causing a striking hyperplasia in most treated cultures within 10 to 11 days. In many alveoli many rows of crowded, small, undifferentiated, or columnar cells protruded into the lumen which was partially or completely occluded (Fig. 12). The secretory epithelium continued to function, but in the near occluded alveoli it was shed and replaced by flat nonsecretory cells. Table 1 summarizes the effects of MCA and testosterone on cultures in natural and semidefined media.

In explants maintained 6 more days in medium without carcinogen or hormones, the hyperplastic changes persisted and had become more marked. All cultures showed hyperplastic foci, and in a greater number the hyperplasia was more extensive than in explants exposed to MCA for 11 days only (Fig. 13).

Effect of MCA combined with testosterone.—Simultaneous application of the hormone drastically reduced the incidence and extent of the hyperplasia induced by the carcinogen. After 11 days' treatment with both substances, hyperplasia was present in only 4 of 11 cultures. In 3 explants the peripheral alveoli showed slight hyperplasia and contained 2 to 3 rows of cells; more extensive cell multiplication was seen in only one explant. In the remaining 7 cultures, all alveoli were lined by one layer of cuboidal or columnar secretory epithelium (Fig. 14).

Transfer of MCA-treated cultures to medium containing testosterone for 6 more days diminished the hyperplastic changes to an even greater degree than after simultaneous administration of the two compounds. Thus, in all cultures only a few peripheral alveoli showed mild hyperplasia (Fig. 15), while the rest were lined by one layer of cuboidal or columnar secretory cells.

Semidefined Medium

Effect of MCA.—The carcinogen was much less effective than in the natural medium and produced only mild changes in 9 of the 14 cultures. In these explants the epithelial cells in a few alveoli had multiplied, usually to form not more than 2 to 3 layers which were flat and had ceased to secrete.

Effect of MCA and testosterone.—Hyperplasia was completely absent in all explants treated with the two substances, and the tissue consisted of alveoli lined with one row of cuboidal, strongly secreting epithelium.
DISCUSSION

The results showed that the appearance of the prostate gland varied in different culture media, but in both media the organ underwent regressive changes. In the almost hormone-free semidefined medium only the height of the epithelium and its secretory activity were affected. In natural medium the epithelial atrophy was more severe and associated with an abnormal increase of stromal growth. This suggests that lack of testosterone was responsible only for the involution of the epithelium, and that the growth of the connective tissue may be promoted by the presence of other hormones or substances in the natural medium.

It is interesting that in the semidefined medium epithelial height and functional activity were partially restored after more prolonged cultivation. Delost (7) described a spontaneous recovery of the ventral prostate of the field vole 1 month after castration, but he showed that this was due to adrenal hormones. The recovery in vitro is more difficult to interpret. In earlier work Lasnitzki and Lucy (8) found that the arginase activity was greatly increased in organ cultures of the ventral prostate gland of the mouse as compared with the fresh tissue, and it is possible that changes in other enzyme systems may take place in the rat prostate during cultivation, which make the organ independent of testicular hormones.

In the natural, semisolid medium, testosterone inhibited stromal growth and restored the epithelium at the periphery of the tissue, but in the semidefined fluid medium it maintained the epithelium throughout the entire explant. This more pronounced effect is probably due to the greater ease of diffusion of nutrients and testosterone from the fluid medium into the explant and to the absence of hormones that may antagonize the action of the androgen.

Testosterone not only preserved the normal differentiation of the alveolar epithelium but also promoted its proliferation and induced hyperplasia after more prolonged cultivation. The growth promotion by testosterone is not specific for the rat prostate but was also observed in organ cultures of mouse prostate glands (9). It is interesting that the hyperplastic changes are greater in natural than in semidefined medium; it is possible that the hormone is more stable in the protein-rich clot and/or that only the latter can provide the materials necessary for rapid cell multiplication.

The growth promotion by testosterone suggested that the hormone might potentiate the action of the carcinogen. Instead, the combination of hormone and carcinogen drastically inhibited the hyperplastic changes normally seen after the administration of the carcinogen alone. Both after simultaneous application of the two compounds and in cultures pretreated with MCA and transferred to medium containing testosterone, the incidence of hyperplasia was considerably reduced. This response to the hormone was slightly more marked in explants pretreated with MCA.

The inhibition by testosterone of the carcinogenic action of MCA seen in these experiments agrees well with the in vivo results obtained by Allen (3) on the ventral prostate of the rat and with those of Horning (10) on the ventral prostate of the mouse, both exposed to MCA. The authors observed that the presence of androgenic hormones during carcinogenesis diminished the incidence of tumors. This similarity between the in vivo and the in vitro results suggests a direct interaction of hormone and carcinogen at the cellular level.

Horning (10) noticed that in implants of mouse prostate impregnated with MCA the neoplastic changes began in cells in the nonsecretory phase. He postulated that such cells are more susceptible to the carcinogen and that the inhibition of carcinogenesis was due to the maintenance of the secretory activity by androgens. In the rat prostate in culture, however, the secretory process does not diminish cell multiplication, and secretory activity is preserved even during advanced stages of hyperplasia.

It has been shown that carcinogenic hydrocarbons are bound in appreciable amounts to cytoplasmic key proteins (11, 12). Testosterone may prevent the binding by competing for sites on the protein molecule or, if introduced after MCA treatment, may break the protein-carcinogen bond.

Another attractive theory might be based on recent results by Allison and Mallucci (13) who demonstrated by fluorescent microscopy the presence of carcinogenic hydrocarbons in the lysosomes of cell cultures treated with doses similar to those
used in the present experiments. Lysosomes are cytoplasmic particles, enclosed by a lipoprotein membrane, containing enzymes, in particular, acid hydrolases (14). Allison and Mallucci suggest that the carcinogens may release enzymes from the lysosomes that initiate carcinogenesis by disturbing the control of cell division.

Testosterone affects lysosomal activity in a variety of tissues, including rat liver (15) and the Müllerian ducts of chick embryos in vivo and in culture (16), and it is tempting to postulate that the hormone interacts with MCA at the lysosomal level. In this connection it is of interest that excess vitamin A, which has been shown to alter the permeability of lysosomal membranes (17), also inhibited the effect of MCA on the mouse prostate in organ culture (18).

The possibility that the steroids antagonize the MCA effect in the prostate gland by modifying the lysosomal activity of the tissue is now being investigated.

REFERENCES


All sections are stained by the periodic acid-Schiff technique after diastase digestion.

**Figure 1.**—Section through a ventral prostate gland of 8-week-old rat, showing alveoli lined with cuboidal or columnar secretory cells, and very sparse stroma. *Note desquamation of epithelium in one alveolus (black line).* × 150

**Figure 2.**—Section through gland from 8-week-old rat, cultivated for 10 days in natural medium, showing reduction in number of alveoli and increased connective tissue. × 150

**Figure 3.**—Section through gland from 8-week-old rat, after 10 days in natural medium, showing alveoli with narrow lumen lined with low cells and a striking increase of stroma. × 150

**Figure 4.**—Section through gland from 8-week-old rat, cultivated for 10 days in natural medium to which testosterone had been added. Alveolar lumens are much wider than in the controls, the lining epithelium higher, and there is less increase of connective tissue. × 150
FIGURE 5.—Section through gland from 8-week-old rat, cultivated for 2 weeks in natural medium with testosterone, showing epithelial hyperplasia of peripheral alveoli. × 70

FIGURE 6.—Section through gland from 6-week-old rat, kept for 1 week in semidefined medium, showing wide alveoli lined with flat or cuboidal epithelium. × 150

FIGURE 7.—Section through an explant from gland of 6-week-old rat, kept for 2 weeks in semidefined medium. Epithelium is higher than after 1 week (fig. 6) and new alveoli have been formed at the periphery. × 150

FIGURE 8.—Section through an explant from gland of 6-week-old rat, kept for 1 week in semidefined medium to which testosterone had been added. Note high epithelium, intense secretory activity, and mild hyperplasia of central alveoli. × 150
**Figure 9.**—Epithelium from gland of 6-week-old rat, kept for 1 week in semidefined medium. Higher magnification shows low epithelium. $\times$ 900

**Figure 10.**—Alveolar epithelium from gland of 6-week-old rat, kept for 1 week in semidefined medium with testosterone, showing high columnar cells in contrast to the control (fig. 9). $\times$ 900

**Figure 11.**—Section through an explant from gland of 6-week-old rat, grown for 2 weeks in semidefined medium with testosterone, showing alveoli lined with columnar epithelium at periphery and others with slight epithelial hyperplasia in center. $\times$ 150

**Figure 12.**—Section through a ventral prostate gland from 8-week-old rat, grown for 11 days in natural medium with 3-methylcholanthrene, showing extensive epithelial hyperplasia with near occlusion of alveolar lumina. $\times$ 150
Figure 13.—Section through gland from 8-week-old rat, cultivated for 11 days in natural medium with 3-methylcholanthrene and transferred to control medium for 6 days. Note persistence of hyperplasia. × 150

Figure 14.—Section through gland from 8-week-old rat, cultivated in natural medium for 11 days with testosterone and 3-methylcholanthrene. Note absence of hyperplasia in contrast to figure 12. Alveoli are lined with one row of cuboidal or columnar cells. × 150

Figure 15.—Section through gland from 8-week-old rat, cultivated in natural medium for 11 days with 3-methylcholanthrene and transferred to medium containing testosterone. Note marked reduction of hyperplasia as compared with figure 13. × 150