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Steroid Hormone Receptor Characterization of Several Histologic Variants of a Rat Prostatic Adenocarcinoma

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Several histologic variants of the transplantable R-3327 prostatic adenocarcinoma carried in male Copenhagen rats have been characterized and the histologic types have been correlated with steroid hormone receptor content. One type is clearly an adenocarcinoma; this tumor is hormonally responsive and contains substantial amounts of both androgen and estrogen receptors. In contrast, another histologic type, a fibrosarcoma, is hormonally nonresponsive and does not contain either receptor. A third histologic variant is classified as a carcinosarcoma and contains histological elements of both adenocarcinoma and fibrosarcoma and is also hormonally responsive. This tumor contains lower receptor levels than the adenocarcinomas but more than the fibrosarcomas. The androgen receptor appears to be identical in the different histologic forms of the tumor; the sedimentation coefficient is 7.8S and the dissociation constant for methyltrienolone is 4×10^{-9} M. Similarly, the estrogen receptor from the different histologic forms of the tumor has a sedimentation coefficient of 8.3S and the dissociation constant for estradiol is 7×10^{-10} M. These findings clearly distinguish the cytosol binding macromolecules from plasma binding proteins, and classify them as steroid hormone receptors. Further, rat serum was devoid of androgen and estrogen binding in the 8S region. Normal prostate tissue from Copenhagen rats contained low levels of an androgen receptor, but no estrogen receptor. It is possible that during growth and/or passage of the R-3327 tumor, the hormonally responsive adenocarcinoma cells do not survive and there is a gradual emergence of the nonresponsive fibrosarcoma. If, as we suspect, the receptors are found in the epithelial cells and not the stromal cells, there clearly should be considerable variation of receptor content in the different intermediary histologic forms of the tumor.

Key words: estrogen receptor, androgen receptor, prostatic adenocarcinoma, estradiol, methyltrienolone, hormonal responsiveness

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Measurement of steroid hormone receptors in prostatic cancer may be useful in predicting response to endocrine manipulation, similar to the application of estrogen receptor determination in breast cancer [1]. As a first step in developing methodology suitable for receptor determination in human prostatic cancer, steroid hormone receptors in an animal model system – the R3327 rat prostatic adenocarcinoma first described by Dunning in 1963 [2] – have been characterized. This tumor arose spontaneously in an aged Copenhagen male rat, line 2331, and has been preserved by subcutaneous transplantation. Recent work by Dunning and her collaborators [3, 4] and by Smolev et al [5, 6] showed that the rat adenocarcinoma, at least from the endocrine status, is quite similar to the human disease. Differences in growth rate of the tumor after transplantation into intact male rats that had previously been castrated indicated that the tumor is androgen-sensitive [3]. The tumor also contains the 5α-reductase enzyme [3] which converts testosterone into 5α-dihydrotestosterone (DHT), a requisite step prior to hormone action. Furthermore, preliminary studies by Dunning and her colleagues [4] showed that protein fractions sedimenting at 3.5S on sucrose density gradient centrifugation, bound either [^{3}H]-5α-dihydrotestosterone or [^{3}H]-17β-estradiol. Smolev et al [5, 6] reported that the R-3327 tumor responds to castration, to antiandrogen (flutamide), and to estrogen (diethylstilbestrol).

The animal tumor, therefore, appears to be a good model system for human prostatic cancer due to the following characteristics: spontaneous origin of the tumor in an aged animal; histologic and histochemical similarity to human prostate cancer; hormonal sensitivity of the tumor (both androgen and estrogen); metastatic capacity; and ease of transplantation, thereby making the tumor accessible for study.

We report here on the characterization of steroid hormone receptors in histologic variants of the hormonally responsive R-3327 tumors and in a rapidly growing line that emerged during transplantation of a cell suspension prepared from the R-3327 tumor. This rapidly growing line is histologically distinct from the established line as well as hormonally nonresponsive, as previously reported [4]. Our efforts to characterize the androgen receptor in the tumor have been aided by the utilization of a synthetic androgen, R1881 (methyltrienolone), which has high affinity for the androgen receptor but low binding capacity for plasma proteins [7]. Preliminary reports of this work have been presented [8–10].

MATERIALS AND METHODS

Materials

17β-Estradiol and diethylstilbestrol (DES) were from Sigma Chemical. [2,4,6,7-^{3}H]-17β-Estradiol ([^{3}H]-estradiol, 91 Ci/mmole) was from New England Nuclear. [6,7-^{3}H]-R5020 (17,21-dimethyl-19-nor-pregna-4,9-diene-3,20-dione, 51 Ci/mmole) and [6,7-^{3}H]-R1881 (methyltrienolone, 17β-hydroxyl-17α-methyl-estra-4,9,11-triene-3-one, 58 Ci/mmole), and radioinert R5020 and R1881, were kindly provided by Dr J. P. Raynaud, Roussel-Uclaf, Romainville, France. Stock solutions of all steroids were made up in absolute ethanol and stored at 0–4°C. Steroids were checked for purity periodically by thin-layer chromatography and were used if chromatographically pure.

Reagent grade Trisma base and dithiothreitol were obtained from Sigma Chemical. Ethylenediaminetetraacetic acid (EDTA) was purchased from J. T. Baker. Ultrapure,

RNAse-free sucrose used for density gradient centrifugation and radioimmunoassay-grade charcoal were purchased from Schwartz-Mann. Dextran T-70 was obtained from Pharmacia Fine Chemicals. All other reagents were of analytical reagent grade.

Tumors and Tissue

The frozen R-3327 tumor and tumor-bearing or normal Copenhagen rats were obtained either from Dr Arthur E. Bogden, Mason Research Institute, Worcester, Massachusetts or from Dr Normal H. Altman, Papanicolau Cancer Research Institute, Miami, Florida. Frozen tumors, after thawing, were transplanted subcutaneously behind the front leg into adult male Copenhagen rats, strain 2331. Tumors became plapable after variable time periods (2–5 months) and were removed from the animals for receptor studies 7–9 months after transplantation. Several tumor-bearing animals were also obtained from Dr David M. Lubaroff, Department of Urology, University of Iowa, Iowa City, Iowa. These tumors were removed from the rats 4–5 months after transplantation. In order to lower endogenous androgen levels, tumor-bearing or normal animals were castrated 16–24 h prior to removal of tumor or of normal prostate, respectively. In other experiments, the R3327 tumor (obtained from the Mason Research Institute) was used to prepare a cell suspension following the method of Lubaroff et al [11]. Cell count was performed and viability was determined by trypan blue exclusion. Cells were diluted to 10^7 viable cells per milliliter and 0.1 ml was injected directly into the prostate or into the uterus in female Copenhagen rats. Three groupings of animals were used: female, intact males, and castrated males. Growth rate, pathologic characteristics, and receptor status were analyzed in these tumors.

Representative sections of all tumors were prepared for histologic study and stained (hematoxylin and eosin) preparations were made. The remaining tissue was stored in liquid nitrogen until receptor studies were performed.

Preparation of Cytosol

Frozen pieces of normal prostate or R-3327 prostate tumor were pulverized in a Microdismembrator (VWR Scientific) at liquid nitrogen temperature. The frozen, pulverized tissue was diluted with two volumes of ice-cold TED buffer (0.01 M Tris-chloride buffer containing 1.5 mM EDTA and 0.5 mM dithiothreitol, pH 7.4) and homogenized at 0°C using a Polytron PT-10 ST tissue homogenizer. The homogenate was centrifuged at 4°C in a Beckman L5-65 ultracentrifuge at 208,000g for 1 h. The supernatant was carefully withdrawn from beneath the lipid layer with a syringe and stripped of endogenous steroids (contributed by blood contamination) by stirring for 10 min at 0°C with the pellet from an equal volume of dextran-coated charcoal (0.05% dextran T-70, 0.5% radioimmunoassay-grade charcoal in TE buffer (0.01M Tris-chloride, 0.5 mM EDTA, pH 7.4). After centrifugation to remove charcoal, the cytosol was stored in an ice bath until use, no more than 1 h later.

Dextran-Coated Charcoal Assay

Cytosol (0.1 ml) was added to 1.5-ml microfuge tubes in which varying quantities of tritiated steroid (total binding) or titrated steroid in the presence of a large molar excess radioinert steroid (nonspecific binding) had been previously dried under nitrogen. Incubations were performed in duplicate for 16–18 h at 0°C. To terminate the reaction, 1.0 ml of a dextran-coated charcoal suspension was added to each of the microfuge tubes and

incubated with periodic agitation for 10 min at 0°C. The tubes were then centrifuged at 0°C in a Beckman Microfuge B. Charcoal efficiency was always better than 99.3%. Aliquots (0.5 ml) were counted in minivials with 5.0 ml of Biofluor (New England Nuclear) in a Beckman LS 3150T scintillation counter at 36% efficiency. Specific binding was calculated from the difference between total binding and nonspecific binding.

Sucrose Density Gradient Centrifugation

Cytosol (0.3 ml) or plasma (0.3 ml) was added to 1.5-ml microfuge tubes containing either tritiated steroid alone or tritiated steroid in the presence of a large molar excess radioinert competitor. Steroids were taken to dryness under nitrogen immediately prior to cytosol addition. The reaction mixture was incubated for 3 h at 0°C. Free steroids were removed by treating the cytosol with the pellet from 1.5 ml of dextran-charcoal for 10 min at 0°C with periodic agitation. Charcoal was removed by centrifugation at 0°C in a Beckman Microfuge B. Aliquots (0.2 ml) of clear supernatant were removed and layered onto 5–40% linear sucrose gradients. Gradients were prepared using TE buffer and formed in 5.0-ml cellulose nitrate tubes using a Buchler gradient former. Gradient tubes were centrifuged at 4°C in a Beckman L5-65 ultracentrifuge at 400,000g for 16 h. Fractions (0.1 ml) were collected from the bottom of each tube into minivials and 5.0 ml of Sucrosolve (Beckman Instruments) was added. Counting efficiency was 36% in the Beckman LS 3150T. Human serum albumin (4.6S) and human γ-globulin (7.1S) were used as marker proteins to estimate sedimentation coefficients according to the method of Martin and Ames [12]. When calculating total binding capacity by sucrose density gradient centrifugation, values for binding inhibited by excess radioinert steroid in both the 8S and 4.5–5S regions were summed.

Serum Binding of Steroids

In view of the possible occurrence of binding components in rat blood that could complicate determinations of steroid hormone receptors in cytosol, tritiated steroid binding by Copenhagen rat serum was investigated. Animals were castrated 16–24 h prior to removal of blood. Free steroids were removed by diluting the serum with four volumes of dextran-charcoal in TED buffer and incubating with intermittent shaking for 10 min at 0°C. After centrifugation, the diluted serum was incubated with tritiated steroid for 3 h at 0°C either with or without 100-fold excess radioinert competitor. The reaction mixture was then treated with dextran-charcoal to remove excess steroid and analyzed by sucrose density gradient centrifugation.

Protein Determination

Protein concentrations of cytosol preparations were determined by the method of Lowry et al [13] using human serum albumin as standard.

RESULTS

Pathologic Characterization and Growth Characteristics of Histologic Forms of R-3327 Prostatic Adenocarcinomas

Histopathologic examination of the transplantable prostatic carcinoma from a number of rats shows that there are several distinct morphologic variants.

One tumor type was found to consist of a very cellular epithelial neoplasm, with the pleomorphic tumor cells forming acinar structures (Fig. 1) which were closely packed and separated by a very thin fibrous stroma. The tumor cells were low cuboidal when arranged around the periphery of the acinar structures. There was piling up of the tumor cells, and they often filled the acini. The nuclei of these tumor cells were quite large and there was a small amount of cytoplasm. Nucleoli were quite prominent and there were moderate numbers of mitotic cells present. These tumor cells were quite different from the normal rat prostate epithelium, which is columnar and has a considerable amount of bright-pink staining cytoplasm. In this morphologic tumor type, there was relatively little secretory material in the lumen of the acini. This variant of the transplantable prostatic tumor is considered a moderately differentiated adenocarcinoma.

The second histologic group of tumors consisted of low cuboidal epithelial cells which formed small to quite large acinar structures containing a pink amorphus secretory material in the lumen (Fig. 2). These tumor cells were usually lined up on a basement membrane in a uniform manner, one or two cell layers thick. The nuclei were smaller and there was slightly more cytoplasmic content than in the first tumor type. The epithelial tumor cell appeared to be more differentiated and there were only occasional mitotic cells. In this tumor type, there was an extensive proliferation of the stromal fibroblastic cells, and the connective tissue component constituted a variable but often a considerable area of the tumor. The fibroblastic cells were occasionally spindle-shaped, but many of them were rounded up or only slightly elongated. The nuclei were large and hyperchromatic,

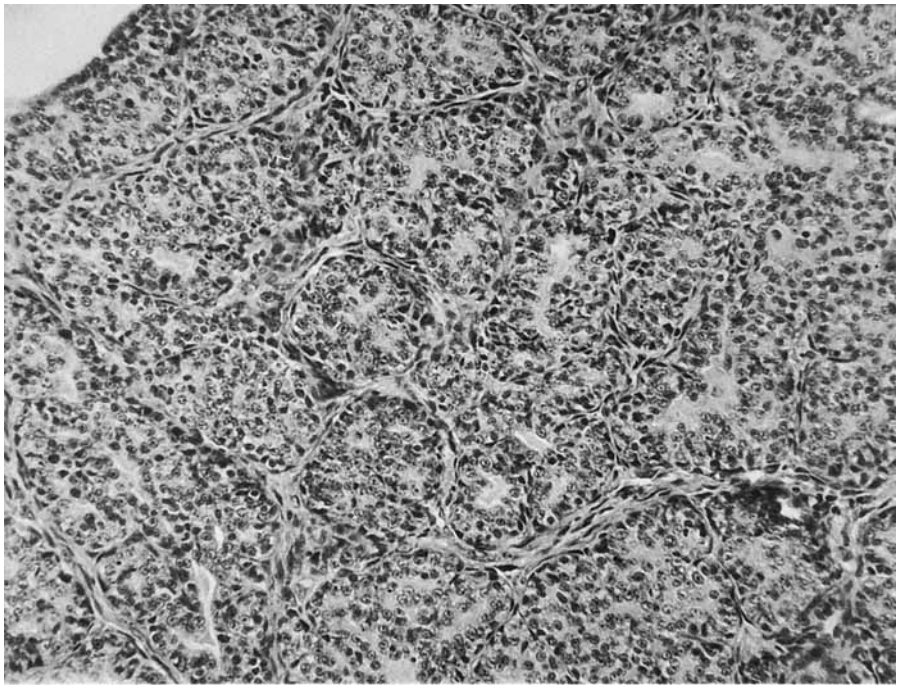

Fig. 1. Typical section of adenocarcinoma, with closely packed differentiated glandular structures. H & E, × 200.

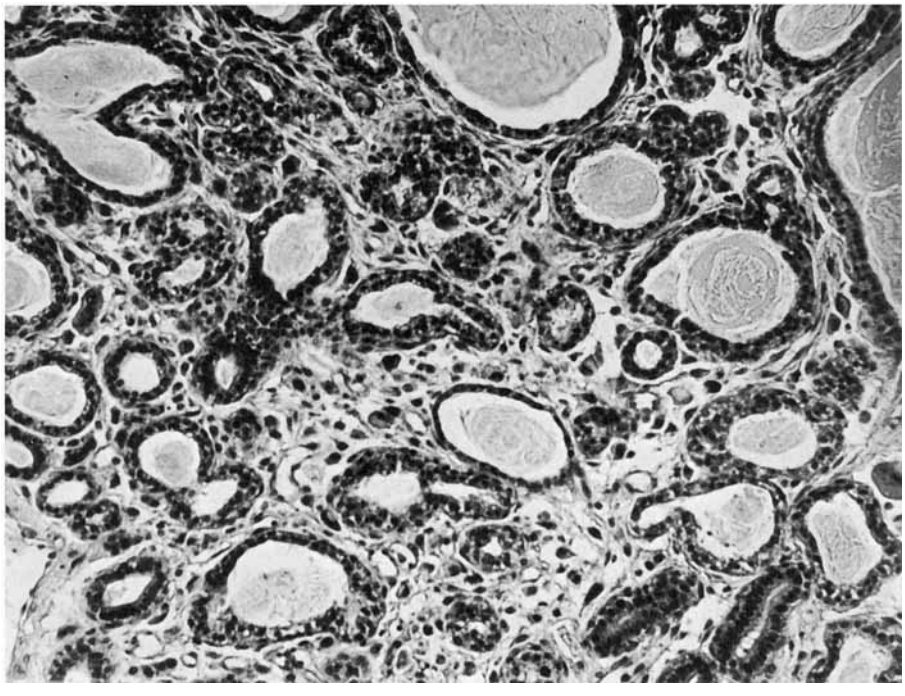

Fig. 2. Typical section of the mixed tumor, showing islands and nests of functional glandular tissue separated by actively proliferating fibrous stroma. This tumor is designated a carcinosarcoma. H & E, × 200.

and mitotic activity in the stromal component was quite marked in some tumors. Occasional binucleated fibroblastic cells were found. This histologic variant was considered to be a carcinosarcoma, due to the mixed nature of the cell types. In metastatic foci, both cell types also proliferated.

The third, and most rapidly growing, tumor type was a pure connective tissue neoplasm, and the tumor cells were densely packed spindle-shaped cells (Fig. 3). The nuclei were pleomorphic and varied from elongated, thin, dark-staining ones to others which were round to ovoid and contained nucleoli and were quite pale-staining. Mitotic activity was quite prominent in this tumor type, and there was a variable amount of pale-staining collagen. The metastatic foci of this tumor were histologically the same as the primary site. This histologic variant was classified as a fibrosarcoma, and the tumor cell type appeared comparable to the stromal cell of the carcinosarcoma. Grossly these tumors were heavily encapsulated, the white capsular layer encasing a reddish-brown tumor mass.

Growth characteristics of the different types of tumors varied, as follows: the adenocarcinoma (which was obtained from Dr Lubaroff) grew subcutaneously in the rats for about 4–5 months. The mixed tumor (which was obtained from Dr Bogdan and Dr Altman) grew slowly over a period of 6–9 months after subcutaneous injection into rats. The fibrosarcoma was observed after preparation of a cell suspension of a tumor obtained from Dr Bogden and direct injection of the cells into the prostate or uterus of Copenhagen rats. This tumor type grew quite rapidly and to a large size within a period of 4–5 weeks. Further, this form of the tumor grew equally as well in female, normal male, or castrated male rats and therefore appeared to be growing independently of the

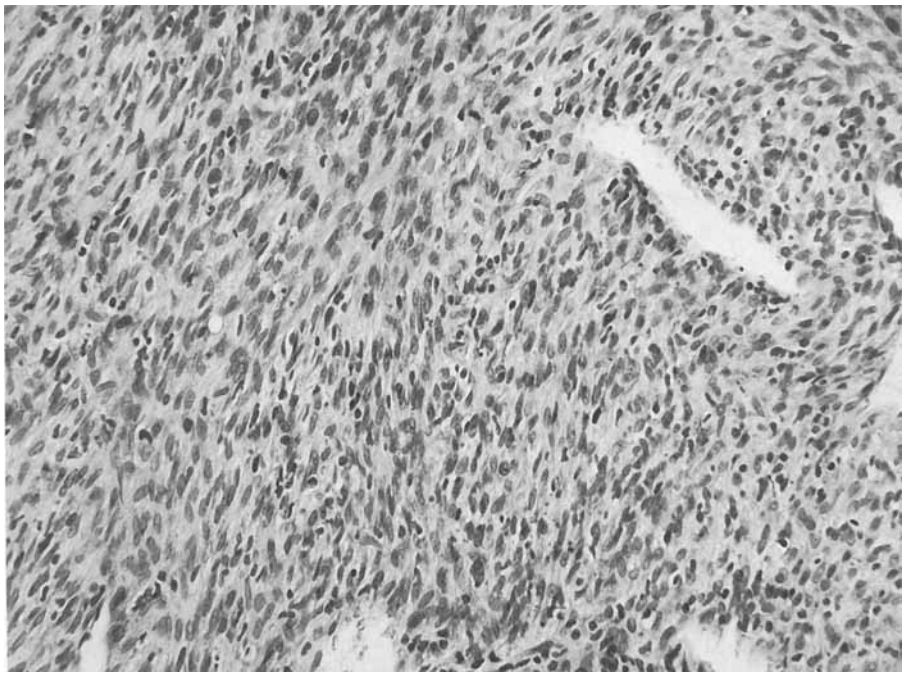

Fig. 3. Typical section of the anaplastic tumor. As can be easily seen, this tissue appears to be indistinguishable from fibrosarcoma. The field is covered with spindle-shaped cells arranged in a storiform manner. H & E, × 200.

endocrine environment. On the other hand, the adenocarcinoma and the mixed tumor have been shown by others to be hormonally sensitive, since they grew better in intact male rats than in castrated male or female rats [3].

Steroid Hormone Receptor Characterization of Different Histologic Forms of the R-3327 Prostate Tumor: Androgen Receptor

Titration of cytosol from the adenocarcinoma with increasing concentrations of [^{3}H]-R1881 with or without 4 μM radioinert R1881 indicated the presence of a specific binding component that was saturated at 5–15 nM hormone concentration (Fig. 4a) as analyzed by the dextran-charcoal procedure. Scatchard analysis [14] of these data produced a linear plot (Fig. 4b) suggestive of a single class of high-affinity binding sites. The concentration of binding sites was 156 fmoles/mg cytosol protein (the arithmetic mean of two determinations in separate tumors was 170 fmoles/mg). The dissociation constant (K_d) was 1.6×10^{-9}M (the arthmetic mean of two determinations in different tumors was 3.2×10^{-9}M), which agrees with previously reported values for R1881 binding to rat prostate cytosol receptors [7]. In the carcinosarcoma, titration of androgen binding sites revealed that the receptor was saturated at 10–20 nM. Total binding from Scatchard analysis was considerably lower than in the adenocarcinoma, ranging from barely detectable to 50–70 fmoles/mg cytosol protein. The average K_d was 4.3×10^{-9}M (arithmetic mean of two determinations in different tumors). Regardless of the proportion of fibrous stroma, however, Scatchard plots were linear, suggesting a single class of binding sites. The binding proteins in the different histologic forms of the tumor appear to be similar, since the K_d values were almost the same.

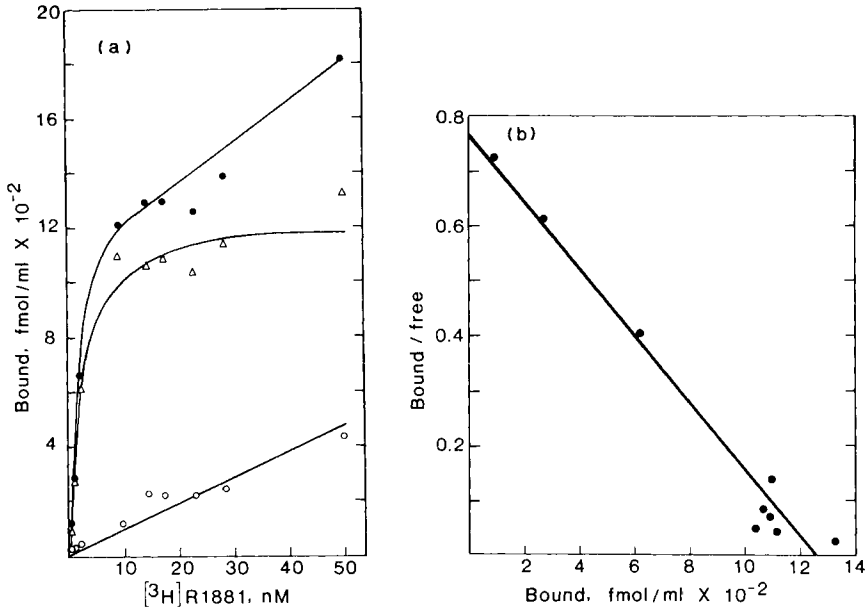

Fig. 4. Titration of androgen-binding sites in R-3327 rat prostatic adenocarcinoma cytosol and Scatchard analysis. a) Binding of 0.25–50 nM [^{3}H]-R1881 in the presence or absence of 4 μM radioinert R1881 was analyzed by the dextran-charcoal procedure following 16 h incubation at 0°C. Cytosol protein concentration was 8.0 mg/ml. Specific binding (△) was determined from the difference between binding in the absence (total binding, •), and binding in the presence (nonspecific binding, ○) of excess radioinert steroid. b) Scatchard analysis of the binding data from Figure 4a after correction for nonspecific binding: $K_d = 1.6 \times 10^{-9}$M; total binding sites = 156 fmoles/mg cytosol protein.

The androgen-insensitive tumor (fibrosarcoma) contained no detectable androgen receptors, as shown by sucrose density gradient centrifugation (Fig. 5). Suppressible binding (binding inhibitable by excess radioinert steroid) was not observed in either the 8S or 4.5–5S regions. There was, however, a binding peak in the 4.5–5S region that was not suppressed by 100-fold molar excess radioinert R1881; this may have been due to contamination of cytosol by a plasma binding component. The central, reddish-brown noncapsular portion of the fibrosarcoma was used for receptor analysis. Tumors grown in either male or female Copenhagen rats gave the same negative results. Our results are in agreement with the preliminary characterization reported by Voigt et al [4].

Molecular characteristics of the cytosol form of the R1881 receptor from the R-3327 prostatic adenocarcinoma were determined by sucrose density gradient centrifugation in low-salt buffer after 3 h incubation with 20 nM [^{3}H]-R1881. Under these conditions binding of [^{3}H]-R1881 was found at 7.8S (and in the 4.5–5S region as well; Fig. 6a). Binding in the 8S region was completely suppressed by incubation with 100-fold molar excess radioinert R1881. Total suppressible binding varied from 150 to 300 fmoles/mg cytosol protein in the two tumors examined. When analyzing [^{3}H]-R1881 binding by sucrose density gradient centrifugation of cytosol from the carcinosarcomas, levels ranging from barely detectable to about 65–70 fmoles/mg cytosol protein (Fig. 6b) were observed. Although the levels of suppressible binding in the carcinosarcomas were considerably lower than in the adenocarcinomas, the sedimentation coefficients of the macromolecular binding components were the same (7.8S), providing additional confirmation of the similarity of the [^{3}H]-R1881 binding protein in the different forms of the tumor as already suggested from Scatchard analysis.

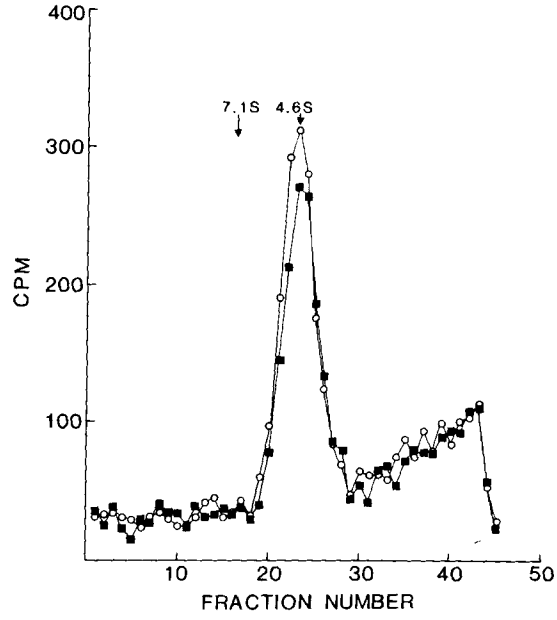

Fig. 5. Sucrose density gradient centrifugation profile of androgen binding in hormonally insensitive fibrosarcoma. Conditions are as described in Materials and Methods; 20 nM [^{3}H]-R1881 binding either alone (○) or with 100-fold molar excess radioinert R1881 (■). The vertical arrows at 7.1S and 4.6S indicate the positions of the marker proteins human γ-globulin and human serum albumin, respectively. Protein concentration 7 mg/ml.

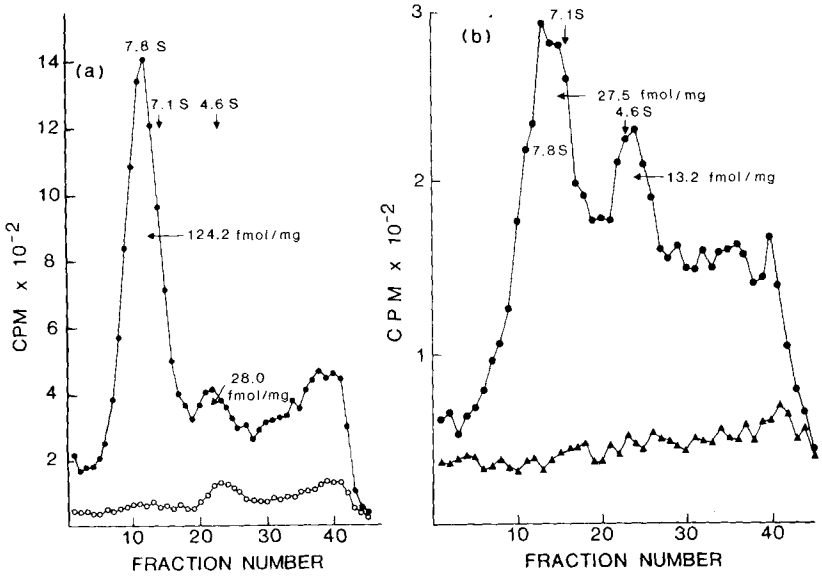

Fig. 6. Sucrose density gradient centrifugation profiles in 0.01 M Tris buffer (see Materials and Methods) of R-3327 prostate tumor cytosol. a) Cytosol from adenocarcinoma incubated with 20 nM [^{3}H]-R1881 alone (●) or with 100-fold molar excess radioinert R1881 (○). Protein concentration 8.8 mg/ml. b) Cytosol from carcinosarcoma incubated with 20 nM [^{3}H]-R1881 alone (●) or with 100-fold molar excess radioinert R1881 (▲). Protein concentration 10 mg/ml. The vertical arrows at 7.1S and 4.6S indicate the positions of the marker proteins human γ-globulin and human serum albumin, respectively.

Estrogen Receptor

Titration of binding sites in cytosol from the R3327 prostatic adenocarcinomas with increasing concentration of [^{3}H]-estradiol in the presence or absence of 2 μM DES indicated that there was a specific binding component that was saturated at 2–6 nM hormone concentration (Fig. 7a). Binding was measured by the dextran-charcoal procedure after 16–18 h incubation at 0°C. Scatchard analysis of these data produced a linear plot suggesting a single class of high-affinity binding sites with a K_d of 5.8×10^{-10}M (the arithmetic mean of two determinations in separate tumors was 6.9×10^{-10}M; Fig. 7b). The number of binding sites was 102 fmoles/mg (the arithmetic mean of two determinations in separate tumors was 186 fmoles/mg). Titration of binding sites in the carcinosarcoma revealed that the receptor was saturated at 2–4 nM hormone concentration. Scatchard analysis again produced a linear plot suggesting a single class of high-affinity binding sites with a K_d of 7.6×10^{-10}M (arithmetic mean of three determinations in different tumors). Although the dissociation constants were essentially the same in the carcinosarcomas and the adenocarcinoma, the number of binding sites in the carcinosarcoma were considerably lower, ranging from barely detectable to 40–60 fmoles/mg cytosol protein.

The fibrosarcoma was completely devoid of [^{3}H]-estradiol binding as measured by sucrose density gradient centrifugation (Fig. 8). There was a binding peak in the 4.5–5S region; however, this binding was not suppressed by 100-fold molar excess radioinert

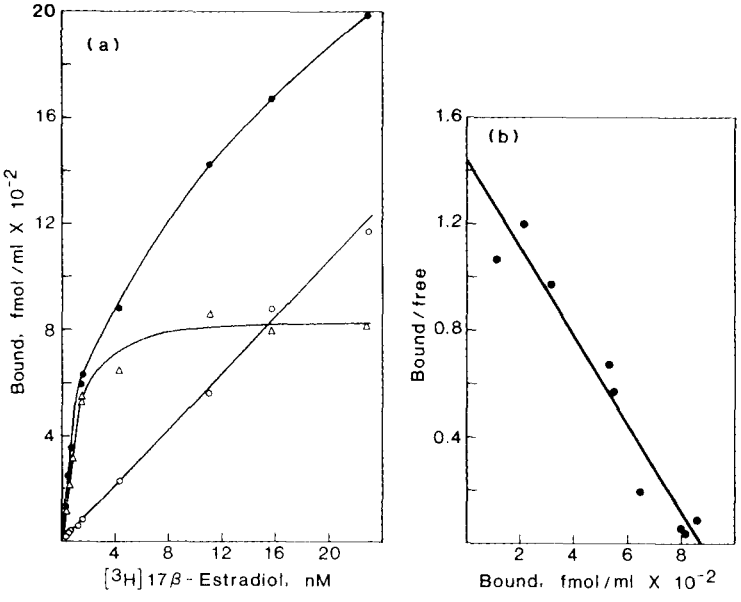

Fig. 7. Titration of estrogen-binding sites in R-3327 rat prostatic adenocarcinoma cytosol and Scatchard analysis. a) Binding of 0.2–23 nM [^{3}H]-estradiol in the presence or absence of 2 μM radioinert diethylstilbestrol was analyzed by the dextran-charcoal procedure following 16 h incubation at 0°C. Cytosol protein concentration was 8.5 mg/ml. Specific binding (△) was determined form the difference between binding in the absence (total binding, •), and binding in the presence (nonspecific binding, ○) of excess radioinert steroid. b) Scatchard analysis of the binding data from Figure 7a after correction for nonspecific binding: $K_d = 5.8 \times 10^{-10}$M; total binding sites = 102 fmoles/mg cytosol protein.

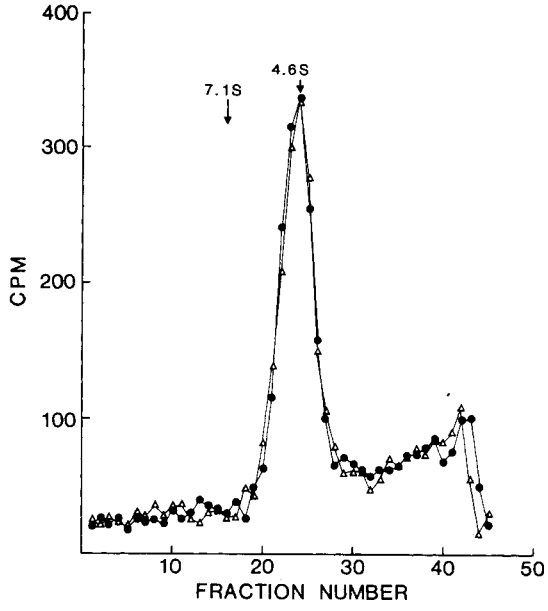

Fig. 8. Sucrose density gradient centrifugation profile of estrogen binding in hormonally insensitive fibrosarcoma. Conditions are as described in Materials and Methods; 6 nM [^{3}H]-estradiol binding either alone (•) or with 100-fold molar excess radioinert estradiol (△). The vertical arrows at 7.1S and 4.6S indicate the positions of γ-globulin and serum albumin, respectively. Protein concentration 7 mg/ml.

estradiol. As with androgen binding in the fibrosarcoma, this may have been caused by contamination of cytosol with a plasma binding component.

Sucrose density gradient centrifugation in low-ionic-strength buffer revealed that the estrogen-binding component in the adenocarcinoma had a sedimentation coefficient of 8.3S; binding was completely suppressed by 100-fold molar excess DES. However, there was also suppressible binding in the 4.5–5S region (Fig. 9A). Total binding varied from 190 to 290 fmoles/mg cytosol protein in the two tumors examined. In the carcinosarcoma, sucrose density gradient centrifugation revealed that there was a considerable range of estrogen receptor levels, varying from barely detectable to 50–70 fmoles/mg cytosol protein (Fig. 9b). The sedimentation coefficient of the estrogen receptor was identical in both tumor types (8.3S). This finding plus the closeness of the K_d values in the different tumors showed that the estrogen receptor is similar in the different histologic forms of the tumor.

Receptor Characterization in the Normal Prostate of Copenhagen Rats

Characteristics of the steroid hormone receptors in normal prostatic tissue from Copenhagen rats were also studied for comparison with those in the R-3327 prostate tumors. Prostates were removed from several normal adult male Copenhagen rats 16–24 h after castration and the total prostates were pooled for receptor studies. Prior to analysis cytosol was stripped of free steroids by dextran-charcoal treatment (see Materials and Methods).

Saturation analysis of estrogen and androgen binding in the normal prostate of Copenhagen rats revealed the presence of a saturable androgen-binding component (Fig. 10), but there was no binding of estrogen (< 2 fmoles/mg cytosol protein). The specific androgen-

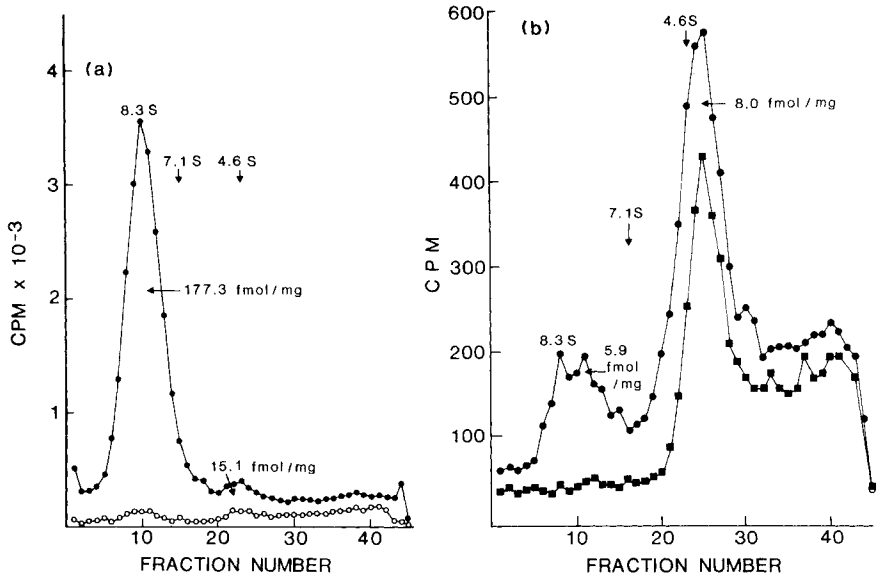

Fig. 9. Sucrose density gradient centrifugation profiles in 0.01 M Tris buffer (see Materials and Methods) of R-3327 prostate tumor cytosol. a) Cytosol from adenocarcinoma incubated with 6 nM [^{3}H]-estradiol alone (•) or with 100-fold molar excess radioinert diethylstilbestrol (○). Protein concentration 8.8 mg/ml. b) Cytosol from carcinosarcoma incubated with 6 nM [^{3}H]-estradiol alone (•) or with a 10,000-fold molar excess of the Parke-Davis antiestrogen CI 628 (▪). Binding profile was identical when 100-fold molar excess diethylstilbestrol was utilized. Protein concentration 14.5 mg/ml. Vertical arrows at 7.1S and 4.6S mark the positions of γ-globulin and serum albumin, respectively.

binding component was saturated at 4–8 nM [^{3}H]-R1881 (Fig. 10a). When analyzed by the method of Scatchard (Fig. 10b), a linear plot was obtained indicating a single class of binding sites. A value of 2.7×10^{-9} M (arithmetic mean of two separate determinations) was obtained for the dissociation constant and the concentration of binding sites was 24 fmoles/mg cytosol protein (arithmetic mean of two determinations).

Sucrose density gradient centrifugation of cytosol from pooled whole prostates from Copenhagen rats revealed no suppressible binding of [^{3}H]-estradiol (Fig. 11a); however, there was macromolecular binding of [^{3}H]-R1881 sedimenting in the 8S region and in the 4.5–5S regions (Fig. 11b). The amount of [^{3}H]-R1881 binding was quite low, ranging from 4–24 fmoles/mg cytosol protein (average of five determinations was 13 fmoles/mg cytosol protein).

Serum Binding of Steroid Hormones

Since prostate tissue (normal and malignant) obtained for the receptor studies is vascularized, there is significant contamination of cytosol by plasma proteins. To eliminate the possibility that binding observed in cytosol from the R-3327 prostatic adenocarcinoma was caused by binding components in blood, steroid binding in serum from Copenhagen rats was investigated. Sucrose density centrifugation revealed that there were low levels of [^{3}H]-estradiol-binding (Fig. 12a) and [^{3}H]-R1881-binding (Fig. 12b) macromolecules sedimenting in the 4.5–5S region but not in the 8S region. Further, this binding was not suppressed by excess radioinert competitor, indicating that it was not receptor-like in nature.

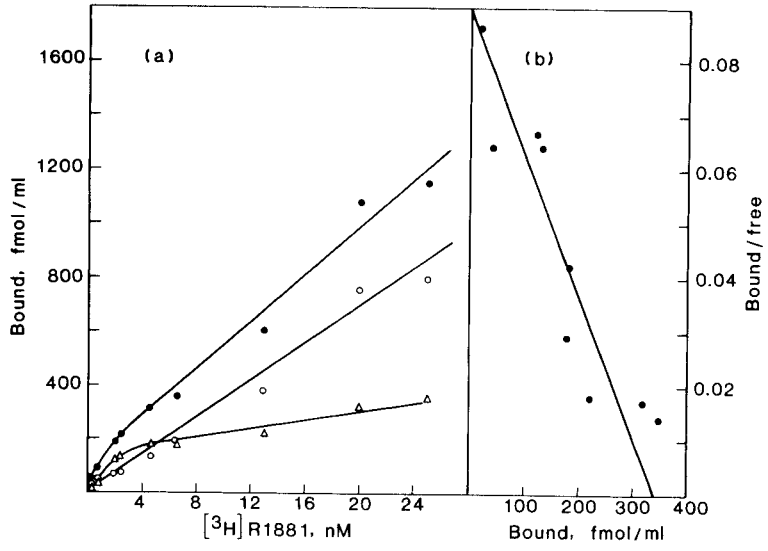

Fig. 10. Saturation analysis of [^{3}H]-R1881 binding in cytosol from normal prostate tissue of Copenhagen rats. Prostates were removed from several normal rats 16–24 h after castration and the whole prostates were pooled and frozen in liquid nitrogen. Prior to analysis cytosol was stripped of free steroids by dextran-charcoal treatment. a) Binding of 0.3–25 nM [^{3}H]-R1881 in the presence or absence of 4 μM radioinert R1881 was analyzed by the dextran-charcoal procedure after 16 h incubations at 0°C. Cytosol protein concentration 11 mg/ml. Specific binding (△) was determined from the difference between binding in the absence (total binding, •) and binding in the presence (nonspecific binding, ○) of 100-fold molar excess radioinert R1881. b) Scatchard analysis of the binding data from Figure 10a after correction for nonspecific binding: $K_d = 3.8 \times 10^{-10}$M; total binding sites = 31 fmoles/mg cytosol protein.

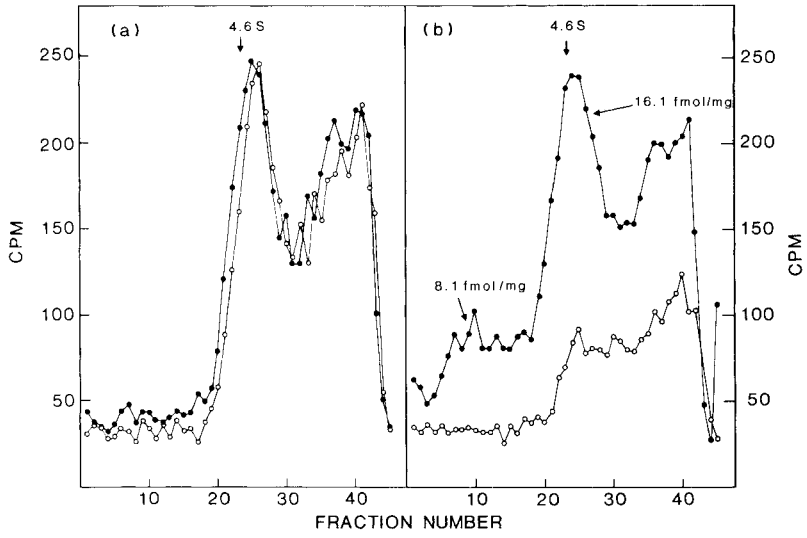

Fig. 11. Sucrose density gradient entrifugation profiles in 0.01 M Tris buffer (see Materials and Methods) of Copenhagen rat prostate cytosol. a) incubation with 6 nM [^{3}H]-estradiol alone (•) or with 100-fold molar excess radioinert diethylstilbestrol (○); b) incubation with 20 nM [^{3}H]-R1881 alone (•) or with 100-fold molar excess radioinert R1881 (○). Protein concentration 5.3 mg/ml. The vertical arrow at 4.6S marks the position of serum albumin.

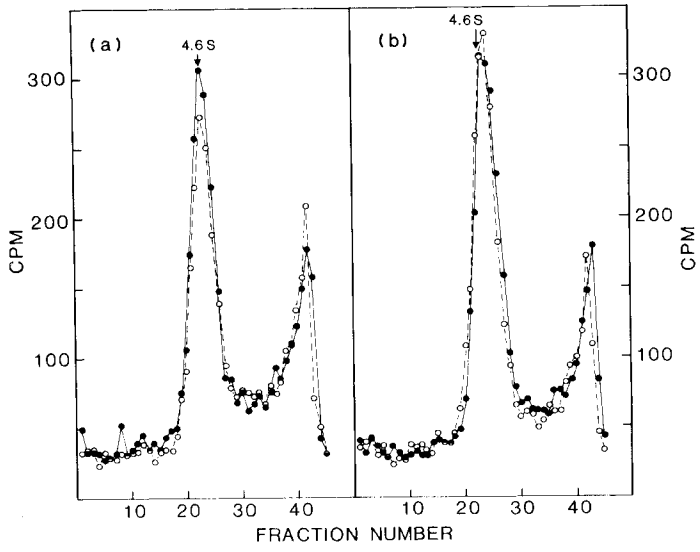

Fig. 12. Sucrose density gradient centrifugation profile in 0.01 M Tris buffer (see Materials and Methods) of Copenhagen rat serum that had been previously treated with dextran-charcoal to remove endogenous steroids. a) Incubation with 6 nM [^{3}H]-estradiol alone (•) or with 100-fold molar excess radioinert diethylstilbestrol (○). b) Incubation with 20 nM [^{3}H]-R1181 alone (•) or with 100-fold molar excess radioinert R1881 (○). Protein concentration 5.6 mg/ml. The vertical arrow at 4.6S marks the position of serum albumin.

Progestin Binding in the R-3327 Tumors

Cytosol from R3327 prostatic adenocarcinoma was also analyzed for progestin binding. Using [^{3}H]-R5020 (a synthetic progestin), sucrose density gradient centrifugation of cytosol from three separate specimens (one from an adenocarcinoma and two from carcinosarcomas) indicated the complete absence of suppressible binding in the 8S region and little or no suppressible binding in the 4.5–5S region. Identical aliquots of the same cytosols incubated with [^{3}H]-R1881 showed significant suppressible binding in the 8S region (50–125 fmoles/mg cytosol protein).

DISCUSSION

The availability of an appropriate animal tumor model for prostate cancer would be a valuable asset for research aimed at studying the human disease state. In this report we have characterized the steroid hormone receptors found in the R3327 transplantable prostatic adenocarcinoma carried in male Copenhagen rats and first described by Dunning [2]. Recent reports [3–6] have shown that this rat tumor is hormonally sensitive and responds to endocrine ablation. Further studies by Dunning and her collaborators [4] indicated that binding components for both androgen and estrogen observed in the R-3327 tumor were absent in R-3327A, an androgen-insensitive line derived from R-3327.

Our work has confirmed the histologic variations and extended the steroid binding studies. We have described several histologic variants derived from the R-3327 rat prostate tumor: one type is clearly an adenocarcinoma, whereas another group of tumors contain a mixture of epithelial and stromal elements and are called carcinosarcomas; and yet another type is a connective tissue neoplasm, classified as a fibrosarcoma. In general, both

the adenocarcinoma and carcinosarcoma contain androgen- and estrogen-binding proteins that satisfy the physical-chemical and kinetic criteria for classification as steroid hormone receptors. First, the steroid hormones were bound with high affinity. By Scatchard analysis the specific receptors have dissociation constants (K_d) in close agreement with those reported for comparable steroid hormone receptors in other tissues and cells. Second, steroid binding was saturable and of low capacity. Third, the specific receptors sediment consistently at approximately 8S as determined by sucrose density gradient centrifugation in low salt concentration. The similarity both in K_d values for R1881 binding and in sedimentation properties of the androgen receptor in the different histologic forms of the tumor suggest that a single receptor species is present regardless of the tumor type. These findings also apply to the estrogen receptor. The sedimentation properties also clearly distinguish the specific receptors from albumin, sex hormone-binding globulin, and other steroid-binding components in plasma which sediment in the 4S region. Finally, steroid competition studies (not reported) indicate that the androgen and estrogen receptors are distinct and unique entities.

Two problems that have been encountered in androgen receptor analysis in clinical prostate material have been eliminated in our studies with the rat model system. One of these, the interference by plasma binding proteins with receptor determination, has been eliminated by use of the synthetic steroid R1881, which Raynaud and co-workers have shown does not bind to sex hormone-binding globulin [7, 15]. Others [15, 16] have shown that in rat serum there is no sex hormone-binding globulin, so this is not a problem in our studies in any event. Sex hormone-binding globulin contamination in human prostate tissue remains a significant complication and the potential for use of R1881 to eliminate this problem is of considerable importance. However, one problem that was encountered by investigators [15, 17–19] during studies on the binding of R1881 to receptors in cytosol prepared from human benign prostatic hypertrophy tissue was the affinity of the synthetic hormone for the progestin receptor. This is not a complication in our studies with the R-3327 tumor, since there is no progestin receptor present.

The second problem in androgen receptor determination in prostate tissue is the presence of endogenous steroids which block available receptor sites. This problem has been mentioned by others working with human prostate tissue [16, 20, 21]. In our studies, however, endogenous androgens do not appear to be a significant problem. First, animals are castrated 16–24 h prior to removal of tumors to lower the endogenous steroid levels. Second, the hormonally responsive adenocarcinomas and carcinosarcomas, which grow slowly, almost always contain both the androgen and estrogen receptors, the receptor levels in the tumors apparently being dependent not on the endogenous steroid levels, but on the epithelial content of the tumor (the more epithelial the tumor and the less fibrous stroma, the higher the steroid hormone receptor levels). The complete absence of suppressible binding in the hormonally insensitive and rapidly growing fibrosarcoma is, therefore, attributed to the absence of the receptors (both estrogen and androgen), rather than to excessively high androgen levels in this tumor. In contrast, the low levels of androgen receptor and complete absence of estrogen receptor in the normal rat prostate remain unexplained.

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REFERENCES

1. McGuire WL, Carbone PP, Sears ME, Escher GC: In McGuire WL, Carbone PP, Vollmer EP (eds): "Estrogen Receptors in Human Breast Cancer." New York: Raven Press, 1975, pp 1–7.
2. Dunning WF: Nat Cancer Inst Monogr 12:351, 1963.
3. Voigt W, Dunning WF: Cancer Res 34:1447, 1974.
4. Voigt W, Feldman M, Dunning WF: Cancer Res 35:1840, 1975.
5. Smolev JK, Heston WDW, Scott WW, Coffey DS: Cancer Treat Rep 61:273, 1977.
6. Smolev JK, Coffey DS, Scott WW: J Urol 118:216, 1977.
7. Bonne C, Raynaud JP: Steroids 26:227, 1975.
8. Markland FS, Lee L: Fed Proc (Abstracts) 36:913, 1977.
9. Markland FS, Lee L: Proc Am Assoc Cancer Res (Abstracts) 18:151, 1977.
10. Markland FS, Chopp RT, Cosgrove MD, Howard EB: J Supramol Struct (Suppl) 2:129, 1978.
11. Lubaroff DM, Canfield L, Feldbush TL, Bonney WW: J Nat Cancer Inst 58:1677, 1977.
12. Martin RG, Ames BN: J Biol Chem 236:1372, 1961.
13. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: J Biol Chem 193:265, 1951.
14. Scatchard G: Ann NY Acad Sci 51:660, 1949.
15. Asselin J, Labrie F, Gourdeau Y, Bonne C, Raynaud JP: Steroids 28:449, 1976.
16. Mobbs BG, Johnson IE, Connolly JG: J Steroid Biochem 6:453, 1975.
17. Dubé JY, Chapdelaine P, Tremblay RR, Bonne C, Raynaud JP: Hormone Res 7:341, 1976.
18. Cowan RA, Cowan SK, Grant JK: J Endocrinol 74:281, 1977.
19. Menon M, Tananis CE, Hicks LL, Hawkins EF, McLoughlin MG, Walsh PC: J Clin Invest 61:150, 1978.
20. Mainwaring WIP: In Menon JKM, Reel JR (eds): "Steroid Hormone Action and Cancer." Current Topics in Molecular Endocrinology, vol 4. New York: Plenum, 1976, pp 152–171.
21. Menon M, Tananis CE, McLoughlin MG, Lippman ME, Walsh PC: J Urol 117:309, 1977.