Steroid Metabolism and Steroid Receptors in Dimethylbenz(a)anthracene-induced Rat Mammary Tumors

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ABSTRACT

Mammary tumors were induced in rats by treatment with dimethylbenz(a)anthracene. Cytosol receptors for 17β-estradiol and progesterone were estimated by means of sucrose density gradient centrifugation, and the metabolism of [14C]progesterone, [14C]testosterone, and 17β-[14C]estradiol by minced tumor tissue was studied.

The estradiol receptor (ER) and progesterone receptor (PR) levels of the tumors varied considerably from <5 to 48 fmol/mg protein for ER and to 243 fmol/mg protein for PR. Considering a receptor level lower than 5 fmol/mg protein to be negative, four groups of tumors were found: ER-negative and PR-negative; ER-negative and PR-positive; ER-positive and PR-positive.

In dimethylbenz(a)anthracene-induced tumor tissue, high 5α-reductase and 20α-hydroxysteroid dehydrogenase activities and somewhat lower 3α-hydroxysteroid dehydrogenase and 6α-hydroxylase activities were found. No aromatization was detectable. Steroids, especially estradiol, were also metabolized in a high degree to unextractable metabolites. It was concluded that steroid metabolism of dimethylbenz(a)anthracene-induced rat mammary tumors was not related to the ER and/or PR concentration of tumor tissue.

INTRODUCTION

In 1961, Huggins et al. (18) showed that DMBA2 can induce mammary tumors in rats. The presence of ER and PR in such tumors (7, 8, 21, 34) indicates a possible role of ovarian hormones in the tumor growth process and has led to extensive work on these receptors. Thus, it was found that sedimentation properties and dissociation characteristics of ER and PR complexes of DMBA-induced rat mammary tumors showed the same characteristics as did the receptor complex in human mammary tumors (33) and that some steroid metabolites can enter into competition with estradiol and/or progesterone for these receptors (4, 17, 40, 43, 45). Several studies in which progesterone, testosterone, and dehydroepiandrosterone were used as substrate in incubations of minced tissue or homogenate have indicated the presence of 5α-reductase, 3α-hydroxysteroid dehydrogenase, 20α-hydroxysteroid dehydrogenase, and 7α-hydroxylase in rat and also in human mammary tumors (1, 13, 19, 26, 30, 35–38, 48). Aromatase activity was demonstrated only in human mammary tumors (1–3, 5, 11, 20, 24).

1 To whom requests for reprints should be addressed.
2 The abbreviations used are: DMBA, dimethylbenz(a)anthracene; ER, estradiol receptor; PR, progesterone receptor; [3H]R5020, [17α-methyl-3H]promegestone; R5020, promegestone; TLC, thin-layer chromatography; SDGC, sucrose density gradient centrifugation.

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The aim of the present work was to study progesterone, testosterone, and 17β-estradiol metabolism by substantial amounts of minced tumor tissue from DMBA-treated rats and to examine the relationship between steroid metabolism on the one hand and the presence of ER or PR on the other.

MATERIALS AND METHODS

The following chemical substances were used: toluene, benzene, propylene glycol, petroleum ether (80–100°), dichloromethane, acetone, methanol, n-heptane, cyclohexane, chloroform, sodium molybdate of analytical grade (Merck, Darmstadt, Germany); Sephadex LH 20 (Pharmacia, Uppsala, Sweden); MN-Silica Gel 3/HR (Machery & Nagel, Duren, Germany); silica gel thin-layer plates (20 x 20 mm), 60 F254 (Merck); albumin, catalase, DMBA (Sigma Chemical Co., St. Louis, Mo.); Ritika phosphate-buffered saline (Lumac, Basel, Switzerland); PPO, POPOP, Carboxyl- and Influor (Packard Instrument Co., Downers Grove, Ill.).

Unlabeled testosterone, 5α-dihydrotestosterone, 5α-androsterone, epiandrosterone, 5α-androstanediol-3α, 17β, 5α-androstanediol-3β,17β, 5α-androstenedione, androstenedione, progesterone, 20α-hydroxyprogesterone, 20α-hydroxy-5α-pregnan-3-one, 3α-hydroxy-5α-pregnan-20-one, 5α-pregnen-3α,20α-diol, 5α-pregnen-3α,20α-dione, 17β-estradiol, estrone, and 6α-hydroxy-17β-estradiol were obtained from Makor Chemicals (Jerusalem, Israel); [4,14C]progesterone (58 mCi/mmol), [4,14C]-testosterone (56 mCi/mmol), 17β-[4,14C]estradiol (53.5 mCi/mmol), 17β-[2,4,6,7-3H]estradiol (110 Ci/mmol) were from the Radiochemical Centre (Amersham, England); and [3H]R5020 (87 Ci/mmol) was from New England Nuclear (Boston, Mass.). Diethylstilbestrol was purchased from Koch-Light Laboratories (Coinbrook, England).

Induction of Tumors

Mammary tumors were induced in Sprague-Dawley rats (50 days old) by administration of DMBA (20 mg/ml sesame oil) via a gastric tube, and the tumors were studied 14 to 16 weeks later.

The rats were killed by decapitation, the tumors were excised and freed of fat and the adhering tissues and weighed; 1 g tumor tissue was used for the incubation experiments; the remaining tissue was immediately frozen in liquid nitrogen and used later for ER and PR assay.

Incubation Experiments

Incubation

The tumor tissue (1 g) was minced and transferred to an incubation vessel containing 5 ml Krebs-Ringer buffer solution to which 2 mg NADPH and 50 μl ethanol with 5 μg (1 Ci) of either [4,14C]testosterone, [4,14C]progesterone, or 17β-[4,14C]estradiol were added. Incubations were performed at 37° for 2 hr under a continuous oxygen flow. Incubation was stopped by the addition of 50 ml of acetone/methanol (1:1).

Analytical Procedure

Extraction. Acetone/methanol/water was filtered through Whatman No. 1 filter paper, and the filtrate was reduced to a volume of about 2 ml

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by evaporation under nitrogen. Then, 3 ml of water were added and the metabolites were quantitatively extracted (twice) with 50 ml dichloromethane. The dichloromethane extract was evaporated to dryness and 10 μg of each of the following steroids were added: testosterone, 5α-androstanediol, androstenedione, 5α-androsterone, epipandrosterone, 5α-androstanediol-3α, 17β (testosterone incubations); progesterone, 5α-pregnane-3-20-dione, 20α-hydroxyprogesterone, 20α-hydroxy-5α-pregn-3-one, 3α-hydroxy-5α-pregn-20-one, 5α-pregnane-3α, 20α-diol (progesterone incubations); or 17β-estradiol, estrone, estradiol, 6α-hydroxy-17β-estradiol and 6-keto-17β-estradiol (17β-estradiol incubations).

With this extraction procedure, more than 97% of the initial amount of the steroids was recovered after incubation either in the absence of tumor tissue or in the presence of other (heart, muscle, skin) tissue carcinoma.

Fractionation. The fractionation procedure consisted of Sephadex LH-20 column chromatography, paper chromatography (System A, benzene/n-heptane/methanol/water, 70/30/80/20; System B, petroleum ether/methanol/water, 70/30/80/20; System C, propylene glycol/cyclohexane-benzene, 1/1), and TLC (System D, dichloromethane/ether/ethanol, 95/5/0.5; System E, ethyl acetate/n-hexane/ethanol/acetic acid, 72/13.5/4.5/10; System F, ethyl acetate/n-hexane/ethanol, 80/15/5).

Metabolites produced from either [14C]progesterone, [14C]testosterone, or [14C]androstenedione were first chromatographed on a Sephadex LH-20 column with benzene/dichloromethane/methanol (60/35/5) as elution liquid.

During chromatography of [14C]pregesterone metabolites, 3 fractions (12 to 23, 23 to 28, 39 to 78 ml) were collected: Fraction 1 contained progesterone plus 5α-pregnane-3, 20-dione, Fraction 2 contained 20α-hydroxy-progesterone plus 20α-hydroxy-5α-pregn-3-one plus 3α-hydroxy-5α-pregn-20-one, and Fraction 3 contained 5α-pregnane-3α, 20α-diol. Further separation was obtained by TLC in System D (Fraction 1), by paper chromatography in System B followed by TLC in System D (Fraction 2), and by paper chromatography in System A (Fraction 3).

[14C]Testosterone metabolites were eluted from Sephadex in 4 fractions (10 to 21, 22 to 35, 36 to 75, 76 to 115 ml) and contained androstenedione plus 5α-androstane-3α, 17β-diol (Fraction 1), testosterone plus 5α-dihydrotestosterone plus 5α-androstenedione (Fraction 2), and 5α-androstane-3α (or 3β)-17β-diol (Fraction 3). Estrone and 17β-estradiol, eventually produced from testosterone, were eluted in Fractions 3 and 4, respectively. The metabolites of Fraction 1 were separated by TLC in System D; those of Fraction 2 were separated by paper chromatography in System B followed by TLC in System D; those of Fraction 3 were separated by paper chromatography in System C; the residue of Fraction 4 was chromatographed on paper in System A.

During Sephadex chromatography of [3H]estradiol metabolites, 3 fractions (35 to 80, 81 to 120, 221 to 350 ml) were collected. Fraction 1 contained estrone plus an unidentified metabolite, Fraction 2 contained 17β-estradiol, and Fraction 3 contained 6α-hydroxy-17β-estradiol. Estrone and the unidentified metabolite were separated by TLC in System D; the estradiol fraction was chromatographed on paper in System A, and the 6α-hydroxy-17β-estradiol fraction on a TLC plate in System F.

Radioactive zones on paper chromatograms or TL plates were detected with a Packard radiochromatogram scanner and eluted with ethanol. A portion was analyzed by liquid scintillation counting; the remaining part was used for identification by chromatography and crystallization to constant specific activity in n-heptane/ethanol (9/1), n-heptane/dichloromethane (9/1), n-heptane/chloroform (9/1), or methanol/water (8/2). At each step of the method, a known percentage of sample was analyzed so that correction for experimental losses was possible.

Liquid Scintillation Counting. Liquid scintillation counting was carried out in a Packard liquid scintillation spectrometer. A solution of 300 mg POPOP and 5 g PPO in 1 liter toluene (2% ethyl alcohol) was used as scintillator. The amount of each metabolite recovered was calculated as the percentage of the incubated substrate ([14C]testosterone, [14C]progesterone, or [14C]estradiol).

Preparation of ER and PR

Receptor Studies

Sucrose density gradient profiles of [3H]ER or [3H]PR complexes were characterized by 2 peaks with sedimentation coefficients of 8S and 4S, respectively. Incubations of cytosol with [3H]estradiol (50 nm) or R5020 and a 100 times higher concentration of unlabeled steroid showed complete disappearance of the 8S radioactive peak. However, radioactivity of the 4S region was practically not liberated in the presence of unlabeled estradiol (17β-estradiol or progesterone). The Kd value was calculated by Scatchard analysis from the saturation curves obtained in those conditions.

RESULTS

Receptor Assay

Receptors for 17β-estradiol and progesterone were estimated by SDGC. Cytosol (200 μl) was incubated for 4 hr at 24°C with 50 nm 17β-estradiol (or R5020) in the absence (Tube a) or presence (Tube b) of a 100 times higher concentration of unlabeled steroid or competitor (diethylstilbestrol); the tubes were vortexed every 30 min. Then, 200 μl dextran-coated charcoal suspension (0.05% dextran T-70 and 1% charcoal in 10 ml Tris buffer containing 30% glycerol) were added. The tubes were incubated for a second time at 4°C for 30 min (the tubes were vortexed every 5 min) and finally centrifuged for 10 min at 2000 × g.

Charcoal-treated cytosols (200 μl) from Tubes a and b were each run on a TLC, and the radioactivity in the 8S zone of the tube was corrected for a specific bound radioactivity (Tube b) of this zone. The 8S ER or PR content, expressed in fmol/mg cytosol protein, was calculated from the corrected 8S radioactivity and the specific activity of labeled estradiol or R5020.

In some experiments, 200 μl cytosol were incubated with varying concentrations (from 1.5 to 25 nm) of 17β-[3H]estradiol or [3H]R5020 in the absence or presence of a 100 times higher amount of unlabeled steroid (17β-estradiol or progesterone). The Kd value was calculated by Scatchard analysis from the saturation curves obtained in those conditions.

SEPTEMBER 1983

DMBA Mammary Tumors

Estimation of ER and PR

Preparation of the Cytosol Fraction

The frozen tumor tissue was homogenized to a fine powder with a microdisembranator (Braun, Melsungen, Germany); first, the tissue was cut into small pieces and transferred to a container. The container was cooled in liquid nitrogen and attached to the disemembrator; hammering action was performed for 15 sec (twice). The tissue powder obtained was homogenized in 2 volumes 10 mm Tris buffer containing 10 mm EDTA, 10 mm sodium molybdate (6), and 0.5 mm dithiothreitol. The homogenate was centrifuged in a Beckman ultracentrifuge for 60 min at 105,000 × g. The protein content of the cytosol fraction was estimated according to the method of Lowry et al. (27).

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the receptor-bound tritiated steroid of this region was liberated almost completely during incubation with unlabeled hormone or competitor.

Scatchard analysis of saturation curves of the 8S ER complex obtained by incubating cytosol from ER-positive tumors with increasing concentrations of 17β-[3H]estradiol resulted in a dissociation constant (Kd) of 2.0 × 10^-10 M. In the same manner, a Kd of 6.1 × 10^-10 M was found for the 8S PR complex.

Repeated estimations of 8S receptors at 1-week intervals gave 103, 109, and 116 fmol PR per mg protein for a PR-positive tumor and 36, 42, and 44 fmol ER per mg for an ER-positive tumor. This degree of assay variation indicates that ER as well as PR of the 8S region were estimated with sufficient precision using the SDGC technique.

Tumors from 42 rats were analyzed for ER and PR concentration. The detection limit of the method used was 5 fmol/mg protein. Eleven tumors (26.2%) did not contain ER or PR. The other 31 tumors were receptor positive to either estradiol or progesterone or to both; 20 tumors had ER concentrations ranging from 11 to 48 fmol/mg protein while 23 tumors contained PR levels ranging from 10 to 243 fmol/mg protein. In 12 tumors (28.6%), both ER and PR could be demonstrated; 8 tumors (19%) contained only ER while 11 tumors (26.2%) were positive only for PR. No correlation (r = 0.16; p > 0.4) was found between the levels of ER and PR.

The weight of the tumors varied from 1.76 to 4.10 g. No significant difference (p > 0.6) was found between the mean weight of receptor-negative tumors and that of ER-positive, PR-positive, or ER- and PR-positive tumors.

Steroid Metabolism Studies

Incubations with [4-14C]Testosterone. All incubated tumor tissues converted [14C]testosterone to metabolites characterized by either a carbonyl group at C-17 or a partially or completely reduced A ring (Table 1); these included androstenedione, 5α-androstane-3,17-dione, 5α-dihydrotestosterone, 5α-androsterone, and 5α-androstane-3α (or 3β), 17β-diol. These metabolites were identified by paper chromatography in Systems A, B, and C and by TLC in Systems D, E, and F, after mixing with authentic standards. An important transformation (mean, 16.8%) to nonextractable metabolites was observed in all incubations. Neither 17β-estradiol nor estrone was isolated after incubation with testosterone as substrate.

The extent to which the added testosterone was metabolized varied considerably from tumor to tumor, and 23.1 to 65% unmetabolized [14C]testosterone was recovered. The testosterone which was metabolized showed transformation to androstenedione (1.8 to 8.9%), 5α-androstane-1,5α-diol (1.5 to 10.7%), 5α-androsterone (0.3 to 18.8%), 5α-dihydrotestosterone (main metabolites) (3.9 to 32.2%), 5α-androstane-3α (or 3β), 17β (0.8 to 9.7%), and polar metabolites (10.2 to 24.4%). The mean transformation to 5α-reduced compounds (5α-androstane-3α, 5α-dihydrotestosterone, 5α-androsterone, 5α-androstane-3α (or 3β), 17β) was 34.5%; the conversion to C17-carbonyl compounds (androstenedione, 5α-androstane-3α, 5α-androsterone) was considerably lower (mean, 14.8%). Correlating [14C]testosterone metabolism to the presence of ER or PR, no significant differences (p > 0.2) between ER-negative and PR-positive tumors or between PR-negative and PR-positive tumors were seen. The pattern of testosterone metabolism did not vary with the phase of the estrous cycle of the rats.

Incubations with [4-14C]Progesterone. [14C]Progesterone was transformed by all tumor tissues (Table 2) to metabolites reduced either at 20α (20α-hydroxyprogesterone), 5α (5α-pregnane-3,20-dione), 5α and 20α (20α-hydroxy-5α-pregn-3-one), 3α and 5α (3α-hydroxy-5α-pregn-20-one), or 3α, 5α, and 20α (5α-pregnane-3α, 20α-diol). An important part of the incubated progesterone was also transformed to unextractable metabolites. Considerable variations in metabolism capacity were found, and 13.2 to 62.6% (mean, 33.7%) of the incubated progesterone was not metabolized during the incubation period. The amounts of metabolites isolated also varied considerably: 20α-hydroxy-5α-pregn-3-one, by far the main metabolite, amounted from 18.8 to 52.3% (mean, 32.0% of the incubated progesterone); comparable amounts of 20α-hydroxyprogesterone (mean, 5.7%), 3α-hydroxy-5α-pregn-20-one (mean, 5.8%) and 5α-pregnane-3α, 20α-diol (mean, 5.6%) were formed; transformation to 5α-pregnane-3α, 20α-dione (mean, 1.2%) was low. Conversion to unextractable metabolites varied from 12.4 to 22.8% (mean, 15.9%). Transformation to 5α-reduced metabolites (20α-hydroxy-5α-pregn-3-one, 3α-hydroxy-5α-pregn-20-one, 5α-pregnane-3,20-dione, 5α-pregnane-3,20-diol) (mean, 44.6%) and reduction to 20α-hydroxy metabolites (20α-hydroxyprogesterone, 20α-hydroxy-5α-pregn-3-one, 5α-pregnane-3α, 20α-diol) (mean, 43.4%) were important.

No significant differences (p > 0.3) were found between ER-negative and ER-positive tumors or between PR-negative and PR-positive tumors.

Incubations with 17β-[4-14C]Estradiol. The results of incubations with [4-14C]estradiol varied considerably from 17β-estradiol. An important transformation (mean, 16.8%) to nonextractable metabolites was observed in all incubations. Neither 17β-estradiol nor estrone was isolated after incubation with estrogen as substrate.

The extent to which the added estradiol was transformed varied considerably from tumor to tumor, and 23.1 to 65% unmetabolized [14C]estradiol was recovered. The estradiol which was metabolized showed transformation to androstenedione (1.8 to 8.9%), 5α-androstane-1,5α-diol (1.5 to 10.7%), 5α-androsterone (0.3 to 18.8%), 5α-dihydrotestosterone (main metabolites) (3.9 to 32.2%), 5α-androstane-3α (or 3β), 17β (0.8 to 9.7%), and polar metabolites (10.2 to 24.4%). The mean transformation to 5α-reduced compounds (5α-androstane-3α, 5α-dihydrotestosterone, 5α-androsterone, 5α-androstane-3α (or 3β), 17β) was 34.5%; the conversion to C17-carbonyl compounds (androstenedione, 5α-androstane-3α, 5α-androsterone) was considerably lower (mean, 14.8%). Correlating [14C]estradiol metabolism to the presence of ER or PR, no significant differences (p > 0.2) between ER-negative and PR-positive tumors or between PR-negative and PR-positive tumors were seen. The pattern of estradiol metabolism did not vary with the phase of the estrous cycle of the rats.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>11-35 (n = 9)</th>
<th>&lt;5 (n = 11)</th>
<th>10-66 (n = 10)</th>
<th>&lt;5 (n = 10)</th>
</tr>
</thead>
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<tr>
<td>Testosterone</td>
<td>36.7 ± 14.0</td>
<td>41.2 ± 12.3</td>
<td>41.0 ± 15.8</td>
<td>33.9 ± 10.0</td>
</tr>
<tr>
<td>5α-Androstenedione</td>
<td>4.4 ± 2.4</td>
<td>3.4 ± 1.6</td>
<td>4.3 ± 2.4</td>
<td>3.1 ± 1.5</td>
</tr>
<tr>
<td>5α-Dihydrotestosterone</td>
<td>5.8 ± 2.7</td>
<td>4.2 ± 1.8</td>
<td>5.0 ± 2.7</td>
<td>4.4 ± 2.1</td>
</tr>
<tr>
<td>Androsterone</td>
<td>17.9 ± 9.1</td>
<td>19.8 ± 8.4</td>
<td>16.6 ± 8.6</td>
<td>19.4 ± 8.2</td>
</tr>
<tr>
<td>5α-Androstane-3α (or 3β), 17β-diol</td>
<td>5.7 ± 6.2</td>
<td>5.8 ± 5.5</td>
<td>5.6 ± 6.1</td>
<td>6.1 ± 5.5</td>
</tr>
<tr>
<td>5α-Reduced metabolates</td>
<td>34.5 ± 12.5</td>
<td>34.5 ± 12.0</td>
<td>30.7 ± 13.8</td>
<td>38.4 ± 8.8</td>
</tr>
<tr>
<td>C17-Carbonyl metabolites</td>
<td>16.6 ± 10.3</td>
<td>13.4 ± 7.2</td>
<td>14.8 ± 10.1</td>
<td>14.9 ± 7.4</td>
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<tr>
<td>Unextractable metabolites</td>
<td>18.2 ± 3.5</td>
<td>15.7 ± 3.2</td>
<td>18.6 ± 2.8</td>
<td>15.0 ± 3.2</td>
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</tbody>
</table>

* n, number of experiments.
* Mean ± S.D.
bations with 17β-[14C]estradiol as substrate are summarized in Table 3. The data show that about one-half of the added 17β-
[14C]estradiol was metabolized. Only a relatively small percentage of metabolites was extractable with the organic solvent (dichloromethane). Three 17β-estradiol metabolites were isolated: estrone, 6α-hydroxy-17β-estradiol, and an unidentified metabolite. Recovered estrone (mean, 5.8%) was identified by paper chromatography in Systems A and C before and after reduction with NaBH₄. The identity of recovered 6α-hydroxy-17β-estradiol (mean, 3.2%) was indicated by paper chromatography in System C followed by TLC in System F (which permits complete dissociation between the 6α- and 7β-hydroxylated derivatives of estradiol). A considerable conversion (mean, 38.4%) to unextractable 17β-estradiol metabolites was seen.

No significant differences (p > 0.3) were found between ER-negative and ER-positive tumors.

DISCUSSION

SDGC analysis of ER and PR showed a sedimentation peak of bound steroid in the 4S and 8S regions. As indicated under "Results," the 4S radioactivity consisted mainly of aspecifically bound steroid since only a small fraction of labeled steroid was dissociated during incubation with excess unlabelled hormone; on the contrary, triitated steroid bound to the 8S-sedimenting component was liberated almost completely under these conditions. In our study, the data obtained for the 8S component of SDGC were analyzed. This 8S component of sucrose density gradient profiles has been reported to give the most reliable data on receptor concentration (12).

About 65% of the tumors studied has ER and/or PR levels between 10 and 243 fmol/mg protein; the receptor concentration of the remaining tumors were under the limit of detection (5 fmol/mg protein). At least two-thirds of tumor weight were used for preparing cytosol; thus, the receptor levels can be considered as a mean for the whole tumor (15, 44). The relatively low receptor content of the tumors may be due to the fact that receptors were estimated with the SDGC technique. Indeed, lower receptor concentrations have been found using techniques (25) in which receptors were isolated from other interfering proteins than have been found with the dextran-coated charcoal technique. Moreover, the 4S-sedimenting component, which comprises predominantly nonspecifically bound steroid (10), was not taken into account for calculation in our experiments.

No correlation was seen between ER and PR concentration (r = 0.19; p > 0.6) of the tumors. This is not in agreement with the observations of Koenders et al. (22) and Pichon and Milgrom (42), who found a significant correlation between ER and PR levels in DMBA-induced rat tumors and also in human mammary tumors. The data from Pichon and Milgrom show that PR-positive human breast tumors usually contain receptors for estrogen and thus support the hypothesis of Horwitz and McGuire (16) that PR presence may be a marker of estrogen action. This seems not to be the case for DMBA-induced rat tumors since a considerable number of the PR-positive tumors did not contain ER. The lower ER content found in these PR-positive tumors could, however, eventually be due to the presence in the particular animals of higher levels of circulating estradiol or other compounds with ER binding affinity. We have no data in favor of or against such possibilities.

The tumors in the present study were all analyzed 15 weeks after treatment with DMBA; their weights varied from 1.76 to 4.10 g. No statistical differences (p > 0.6) were seen between the mean weight of tumors with undetectable ER and PR levels
and that of ER and/or PR-positive tumors. This may be an indirect indication that other factors besides receptors to estradiol and progesterone are involved in the regulation of DMBA tumor growth. Recent studies reported that prolactin and insulin may play an important role (14, 23, 31, 39, 46, 49, 50). There is even evidence that the growth of some tumors can be supported by prolactin alone (31), while other reports showed that tumor growth is controlled by the synergistic action of estrogen and prolactin (23). On the other hand, the possibility also remains that very low undetectable ER levels may be sufficient for maintaining tumor growth. In this context, it can be noted that a binding capacity of 1 fmol estradiol per mg cytosolic protein corresponds to a significant number (about 100) of binding sites per individual cell.

The results of our study on steroid metabolism indicate the presence of several enzyme systems in DMBA-induced mammary tumors, including 5α-reductase, 20α-hydroxysteroid dehydrogenase, 3α-hydroxysteroid dehydrogenase, and 17α-hydroxysteroid dehydrogenase. They confirm the observations of other investigators who demonstrated the presence of NADPH-linked enzymes in DMBA-induced tumors (35, 37, 38).

All tumors of this study metabolized testosterone to 5α-dihydrotestosterone to a high degree; other 5α-reduced metabolites (5α-androstane-3β, 5α-androstane-3α, 5α-androstane-3β) were produced to a lower extent. The physiological significance of the great 5α-reduction capacity of DMBA-induced tumors and more particularly the important transformation of testosterone to 5α-dihydrotestosterone are not clear. Estrogen-dependent augmentation of cytosolic PR content of human breast cancer was reported to be inhibited by 5α-dihydrotestosterone (29). Dihydrotestosterone can also inhibit the aromatization process of human breast cancer cells (51) and may as such regulate the aromatase activity in the tumor cell (24). Some studies (10, 43, 47, 52) also reported that androgens such as 5α-dihydrotestosterone and 5α-androstanediol not only bind to androgen receptors but also have a specific, although low, affinity to ER. There are also indications (52) that the androgen-translocated nuclear ER is active at specific sites which are normally occupied by the ER complexes. Binding of 5α-dihydrotestosterone to ER, however, would become important only for high concentrations of the compound. Blood contains dehydroepiandrosterone and intratumor conversion into testosterone has been demonstrated (1); further 5α-reduction may lead to sufficiently high 5α-dihydrotestosterone levels.

The results of the progesterone incubations demonstrate that tumor tissues also contain 20α-hydroxysteroid dehydrogenase and 3α-hydroxysteroid dehydrogenase. Our results, however, are difficult to compare with the data on progesterone metabolism collected by Lloyd (26), since relative transformation values to individual metabolites were not given by this author. In any case, 3α-hydroxysteroid dehydrogenase activity in the tumors used in our study was found to be much lower than that in the DMBA-induced tumors studied by Mori et al. (38). A considerable 20α-hydroxysteroid dehydrogenase activity, almost as important as the 5α-reductase activity, was present in our tumors as was the case in those analyzed by Mori et al. Otherwise, in our experiments, progesterone was mainly converted to 20α-hydroxy-5α-pregnene-3-one; in the study of Mori et al., 20α-hydroxy-4-pregnen-3-one and 5α-pregnenediol were the main progesterone metabolites. These differences may be due to the fact that these authors incubated homogenates instead of teased tissue.

The physiological role of 20α-hydroxylation with respect to tumor growth is not known. The metabolite 20α-hydroxy-4-pregnen-3-one, a more potent progestational steroid than progesterone, may perhaps play a functional role in feedback mechanisms. All metabolites, however, have a low affinity to the PR (42) with the exception of 5α-pregnen-3,20-dione, the production of which is very low.

Transformation of 17β-estradiol by DMBA-induced rat tumors gave a low yield of extractable estrogens. Estrone formation was low, and no significant differences between ER-positive and ER-negative tumors were observed. Thus, 17β-hydroxysteroid dehydrogenase activity of ER-negative tumors was not higher than that of ER-positive tumors in contrast to what was found for human mammary tumors by Wilking et al. (51). The tumors, on the other hand, possess very active enzyme systems able to convert steroids, especially 17β-estradiol, to highly polar compounds not extractable with organic solvents commonly used for steroid extraction. Small percentages of these unextractable metabolites (0.9 to 1.7% of progesterone incubations; 1.6 to 3.2% of testosterone incubations; 2.2 to 6.7% of estradiol incubations) were liberated during hydrolysis with Sarc Helix pomatia extract, indicating that only a small part of the unextractable compounds consisted of steroid conjugates. The greatest part of these metabolites may be polyhydroxylated steroids or steroid-glutathione thioethers, the production of which is mainly based on hydroxylation reactions. The fact that 6α-hydroxylated 17β-estradiol could be isolated from all incubations with 17β-estradiol proves that mammary tumors contain hydroxylase enzymes. Transformation of 17β-estradiol to high polar hydrophilic or glutathione-bound estrogens may be important since these metabolites are biologically inactive.

Neither desmolase nor aromatase enzyme activity could be demonstrated in the DMBA tumors. Our data confirm the findings that the aromatization capacity of rat mammary tumors is very low or undetectable (9, 35). Contradictory information is present in the literature as to the ability of human mammary tumors to aromatize androgens; in some studies, no estrogen formation could be demonstrated (9, 35), while in others aromatization capacity was established (1, 2, 5, 11, 24, 28). Whether these estrogens were synthesized by the tumor cells themselves or by the important mass of adipose tissue that usually occurs in human tumors, as opposed to rat mammary tumors, is under discussion. Opposite results from incubation experiments of cell lines were reported. MacIndoe (28) demonstrated a low but substantial conversion of testosterone to 17β-estradiol; no estrogen formation was observed by d'Agata et al. (9) under similar conditions. The fact that after a 2-h-incubation period about 60% of incubated 17β-estradiol was recovered as estrone plus estradiol (Table 3) indicates that the failure to observe estrogen formation from testosterone in our incubations with DMBA-induced rat tumors was not due to subsequent rapid conversion of the estrogens.

From our experiments, it can be concluded that DMBA-induced rat tumors contain the following steroid-metabolizing enzymes: 5α-reductase, 20α-hydroxysteroid dehydrogenase, 3α-hydroxysteroid dehydrogenase, and 6α-hydroxylase. The tumors are also able to convert steroids to highly polar unextractable metabolites. Aromatization enzyme activity, however, could not be demonstrated. Steroid metabolism of DMBA-induced tumors.
was also not related, quantitatively or qualitatively, to ER or PR content.

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REFERENCES


Steroid Metabolism and Steroid Receptors in Dimethylbenz(a)anthracene-induced Rat Mammary Tumors

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