Binding Characteristics of a Major Protein in Rat Ventral Prostate Cytosol That Interacts with Estramustine, a Nitrogen Mustard Derivative of 17β-Estradiol

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ABSTRACT

The tissue distribution of [³H]estramustine, the dephosphorylated metabolite of estramustine phosphate (Estracyt), in the male rat was compared to that of [³H]estradiol 30 min and 2 hr following i.p. administration. In contrast to estradiol, estramustine was found to be efficiently concentrated in the ventral prostate gland by a soluble protein. The binding characteristics of this protein were studied in vitro using cytosol preparations of the gland. With a dextran-coated charcoal technique, the protein was found to bind estramustine with a broad pH optimum between pH 7 and pH 8.5, with an apparent Kₐ of 10 to 30 nm, and with a binding capacity of about 5 nmol/mg cytosol protein. The estramustine/protein complex was not retained by DNA-cellulose. None of the natural steroids tested inhibited the binding of 10 nm [³H]estramustine by more than 35% (progesterone), even when added in 4500-fold excess. The protein was found to bind estramustine with a broad pH optimum between pH 7 and pH 8.5, with an apparent Kₐ of 10 to 30 nm, and with a binding capacity of about 5 nmol/mg cytosol protein. The estramustine/protein complex was not retained by DNA-cellulose. None of the natural steroids tested inhibited the binding of 10 nm [³H]estramustine by more than 35% (progesterone), even when added in 4500-fold excess. The presence of a nitrogen mustard moiety at position 3 of the steroid was necessary for high-affinity binding to the protein. The protein was calculated to constitute about 20% of the total cytosol protein content.

INTRODUCTION

Estramustine phosphate [Estracyt; 1,3,5(10)-estratriene-3,17β-diol-3-N-bis(2-chloroethyl)carbamate 17β-dihydrogen phosphate (NSC 89199)] was introduced in 1966 as a theraeutic agent in the treatment of prostatic carcinoma. It has been used mainly in patients with advanced carcinoma of the prostate who do not respond to conventional endocrine therapy. Clinical experience shows that objective and/or subjective remissions are obtained in 20 to 50% of these cases, including regression of original tumor and soft tissue and skeletal metastases, as well as relief of pain (for references, see Ref. 18). The mechanism of action of estramustine phosphate is complex. Studies in humans and animals have shown that the drug decreases testosterone and gonadotropin levels in serum; causes atrophy of the testes and accessory sex organs; reduces the uptake of zinc by the prostate; depresses the 5α-reductase, arginase, and acid phosphatase activities in the prostate; and affects lipid and carbohydrate metabolism (10, 11, 20, 29, 31–33, 42). Although the observed effects in many respects are similar to estrogenic effects, several experimental and clinical results indicate that estramustine phosphate affects normal and neoplastic prostate tissue in a way that cannot be attributed solely to its antigonadotropic or weak estrogenic properties (15, 16, 30, 38).

Plym Forshell and Nilsson (35), using labeled compounds (1 to 10 mg/kg body weight), found considerably higher levels of radioactivity in the rat ventral prostate following i.v. administration of estramustine phosphate than after administration of estradiol phosphate or estradiol. The main metabolite of estramustine phosphate in the ventral prostate was identified as its dephosphorylated congener estramustine. Estramustine was also the main metabolite in peripheral human blood after i.v. or p.o. (single dose) administration of estramustine phosphate (34).

Using estramustine of high specific radioactivity, Forsgren and Högberg (8) and Heisaeter (17) have shown the presence of a macromolecule in the cytosol from the rat ventral prostate that binds estramustine. This macromolecule has a molecular weight of 40,000 to 50,000, a Stokes' radius of 2.9 nm, a frictional ratio of 1.2, a sedimentation coefficient of 3.5 to 4S, and an isoelectric point of 5 (9).

Since this macromolecule appeared to occur in substantial quantities in the rat ventral prostate, it was conceivable that it might play an important role in the tissue localization of estramustine and in the mechanism of action of estramustine phosphate in prostatic tissue. We have therefore investigated the binding kinetics and ligand specificity of this macromolecule as well as estramustine uptake in different organs.

MATERIALS AND METHODS

Chemicals

The radioactive compounds used were [³H]estramustine ([2,4,6,7⁻³H]estradiol, 3-N-bis(2-chloroethyl)carbamate; 80 to 107 Ci/mmol), [³H]estradiol ([2,4,6,7⁻³H]estradiol, 91.3 Ci/mmol), and [³H]dihydrotestosterone ([1,2,4,5,6,7⁻³H]dihydrotestosterone; 80 Ci/mmol). [³H]Estramustine was synthesized at AB Leo, and [³H]dihydrotestosterone was obtained from New England Nuclear Chemicals GmbH, Dreieich, Germany. The compounds were routinely purified before use to at least 99% purity by chromatography on Sephadex LH-20 using the solvent systems toluene/methanol.
Preparation of Cytosol

Preparation of Cytosol

at a setting of 34 to 36 for 6 periods of 5 sec with intervals of

and succinylated.


tohomogenizer (type I, sodium salt). Cellulose (Cellex B, 410) was purchased

New Brunswick Scientific, Edison, N.J., and washed according to the method of Alberts and Hemnick (1) before use. The

(0.001 M EDTA, 0.1 mM dithioerythritol, 10% (v/v) glycerol, 0.1% (w/v) gelatin, and 0.05% (w/v) Dextran T 70). All buffers were adjusted to pH 7.2 to 7.4 at 25°C.

For all experimental purposes, glass-distilled, deionized wa-

In Vivo Studies

Distribution Studies. Two experiments were performed, one with [3H]estradiol and the other with [3H]estradiol. In each experiment, 2 groups of animals (5 in each) were used, one killed 30 min and the other 2 hr after i.p. administration of the tracer dissolved in acetone, 100 μl/animal containing 35 μCi (0.44 nmol; 0.8 μg/kg body weight) of [3H]estradiol or 43 μCi (0.47 nmol; 0.5 μg/kg body weight) of [3H]estradiol. The animals were bled to death under ether anesthesia. Various tissues were dissected out and stored at −30°C until analyzed. Duplicate samples (100 to 500 μl) of blood plasma and dissected tissues, with the exception of the epiphysis, pituitary gland, and thyroid gland, were dried in Combusto-Cones for combustion by means of a Packard Tri-Carb Model 306 sample oxidizer. The recovery was checked by use of corresponding tissue samples from untreated animals containing known amounts of [3H]estradiol or [3H]estradiol. The entire epiphysis, pituitary gland, and thyroid gland were dissolved at 50°C in 0.5 to 1 ml of Soluene and counted for radioactivity after addition of 10 ml of InstaGel/0.5 M HCl (9/1, v/v).

Analysis of Radioactivity Found in Prostate Cytosol following Labeling in Vivo. Four rats were given i.p. injections of 20 μCi (0.25 nmol) of [3H]estradiol in 100 μl of acetone. Two hr later, the ventral prostate glands were removed, pooled, and homogenized. The cytosol was prepared, and protein-bound radioactivity was recovered by gel chromatography on a 1.4- x 27-cm column of Ultrogel AcA-54 (separation range, 6,000 to 70,000). Fractions of 0.5 ml were collected and measured for absorption at 280 nm and for radioactivity. Radioactive
peaks were collected, and the radioactive ligands were characterized by TLC after extraction of the aqueous phase with diethyl ether. After mixing with unlabeled estradiol, estrone, estramustine, and the nitrogen mustard derivative of estrone (LS 271), the samples were analyzed on aluminum oxide TLC sheets developed in chloroform/methanol (99/1, v/v). Unlabeled standards were run simultaneously on both sides of the samples and visualized by spraying with concentrated sulfuric acid/absolute ethanol (70/30, v/v) and drying at 110° for 5 min. The sample lanes (2 cm wide) were divided into 1-cm pieces, which were extracted for 20 min with 1 ml of ethyl acetate in counting vials and counted for radioactivity after addition of 10 ml of InstaGel.

In Vitro Studies

In all in vitro incubation studies except in case of the DNA-binding experiments, the cytosol protein concentration used was 1 to 20 µg/ml. The incubations were performed in the presence of 2 mg BSA per ml or 0.1% (w/v) gelatin in order to avoid adsorption of ligand or binding of protein to the glass wall of the incubation tube. For each test sample, a background sample without cytosol was run and corrected for.

Separation of Free and Bound Ligand. In the in vitro experiments, the DCC adsorption technique was usually applied to remove unbound and weakly bound ligand from the incubated cytosol sample. In this procedure, the incubated sample (200 or 300 µl) was cooled (0°) and treated with 0.5 ml of ice-cold DCC suspension consisting of 1.5% charcoal (washed with water to remove fines, dried at 120° for 24 hr, and stored in a desiccator) in DCC buffer. Following addition of the DCC suspension to the sample, the mixture was vortexed for 1 sec and stored for 20 min at 0°. After centrifugation at 3000 × g for 20 min at 0-4°, a 200- or 300-µl portion of the supernatant, containing the DCC-resistant estramustine/protein complex, was counted for radioactivity.

Rate of Association. The rate of association between the estramustine-binding protein and [3H]estramustine was studied at 5 temperatures (0°, 15°, 22°, 30°, and 37°) by measuring the amount of DCC-resistant ligand/protein complex formed at different times following incubation of duplicate samples of cytosol (200 µl, 10 µg protein, and 2 mg BSA per ml TEK(01)DG buffer) with 10 nM [3H]estramustine. No corrections were made for rates of degradation or dissociation.

Rates of Dissociation and Degradation. Cytosol samples (10 µg protein and 2 mg BSA per ml TEK(01)DG buffer) were incubated with 5 nM [3H]estramustine for 18 hr at 15° in the absence or presence of a 250-fold excess of unlabelled estramustine. Each incubation mixture was divided into 2 equal portions. One portion of each kind was used to study the dissociation rate of the estramustine/protein complex. In order to remove unbound ligand, these portions were treated with DCC suspension (1% charcoal) for 5 min at 0° and centrifuged at 12,000 × g for 5 min at 2°. The supernatants were checked for radioactivity (zero time sample), and unlabeled estramustine (in absolute ethanol) was added to a final concentration of approximately 10 µM in order to inhibit reassociation of dissociated [3H]estramustine. The decrease of bound radioactivity was followed at the same temperatures as used in the association study. By subtracting the nonspecific binding of [3H]estramustine (i.e., binding in the presence of a 250-fold excess of ligand) from the total binding of [3H]estramustine, the specific binding at different times was calculated.

The remaining portion of each incubation mixture was used to examine the degradation rate of the estramustine-binding protein at the same temperatures as used for the association and dissociation studies. At various times, duplicate samples (200 µl) were analyzed for protein-bound radioactivity. Specifically bound [3H]estramustine was calculated as described above.

Enzymatic Digestion. The effect of various enzymes on the [3H]estramustine/protein complex was examined. Samples of cytosol (0.5 ml, 10 µg protein, and 4 mg BSA per ml TEK(01)DG buffer) were labeled at 0° for 18 hr with 0.5 nM [3H]estramustine. Following addition of 1 mg enzyme in 0.5 ml TEK(01)DG buffer to each sample, incubation was performed at 37° for 30 min. After cooling on ice, a portion of each incubation mixture was counted for total radioactivity, and the remaining part was examined for protein-bound radioactivity by filtration through a Sephadex G-25 Medium column (0.8 × 12.5 cm) in TEK(01)DG buffer using Blue Dextran 2000 as a marker.

Effect of Various Factors on the Binding of [3H]Estramustine. The binding of [3H]estramustine by prostate cytosol was examined between pH 4 and pH 9. Cytosol samples prepared in TEK(01)DG buffer were diluted with 2 volumes of 0.1 M acetate buffer (pH 3.6 to 5.6), 0.1 M phosphate buffer (pH 6.0 to 7.6), or 0.1 M Tris-HCl buffer (pH 7.4 to 8.8), giving samples with varying pH containing 10 µg cytosol protein and 2 mg BSA per ml. Incubation was performed at 15° for 18 hr with 200-µl samples in the presence of 10 nM [3H]estramustine. Protein-bound ligand was determined by the DCC technique.

Samples (200 µl) containing 10 to 20 µg cytosol protein and 2 mg BSA per ml were also incubated with 1 to 5 nM [3H]estramustine in the presence of varying amounts of N-bromosuccinimide, p-hydroxymercuribenzoate, dithiothreitol, absolute ethanol, and KCl.

Ligand Specificity and Binding Characteristics of the Estramustine-binding Protein. The specificity of the estramustine-binding protein was studied by incubation of duplicate samples (200 µl, 1.6 µg cytosol protein per ml TEK(01)DG buffer containing 0.1% gelatin) with 10 nM [3H]estramustine in the presence of various unlabeled steroids and steroid derivatives in concentrations ranging from 1 nm to 45 µM. After 18 hr at 15°, the bound radioactivity was determined by DCC treatment. The result was expressed as percentage of the amount of radioactivity bound in the absence of unlabeled competitor and plotted following logistic transformation against the logarithm of the ratio between unlabeled competitor and labeled marker according to the method of Rodbard et al. (36). The resulting displacement curve was considered parallel to the standard curve of estramustine when p > 0.05 when tested on the assumption of nonparallelism. "Parallelism" was considered to represent competitive inhibition of [3H]estramustine binding. Comparison between the different concentrations required for 50% inhibition of [3H]estramustine binding was made only in case of curves parallel to the standard curve. Nonparallelism (p < 0.05) was interpreted as the presence of different binding sites on the protein for the competing compound and estramustine and/or that the displacing effect was caused by other factors than pure competition for the estramustine-binding site. Maximal specific binding and apparent dissociation constant were calculated according to the method of Scatchard (37) correcting for nonspecific binding according to the method of...
Chamness and McGuire (4).

**Binding to DNA-cellulose.** The interaction of DNA with the estramustine/cytosol protein complex was studied using DNA-cellulose synthesized according to the method of Alberts and Herrick (1) by means of calf thymus DNA. Samples [1 ml, 8 to 10 mg cytosol protein per ml TEK(01)DG buffer] were incubated for 18 hr at 0° with 1 to 10 nm [3H]estramustine or [3H]dihydrotestosterone in the absence or presence of a 100-fold excess of unlabeled estramustine or dihydrotestosterone. Portions (5 ml) of DNA-cellulose suspension containing approximately 1200 μg of DNA per g of cellulose (dry weight) were used to prepare columns with a bed volume of about 1.5 ml. After the columns were washed overnight with TEK(15)DG buffer, the incubation mixtures were applied on top of the columns. When the samples had been adsorbed on to the DNA-cellulose, the flow was stopped. The columns were incubated for 2 hr at 0° and washed with 10 ml TEK(15)DG buffer at a rate of 4 to 5 ml/hr. The columns were eluted with 7.5 ml TEK(40)DG buffer containing 0.2 mg BSA per ml. Samples were also run on columns of pure cellulose to evaluate the background binding of radioactivity.

**RESULTS**

**In Vivo Studies**

**Distribution Studies.** The results from the studies on the tissue distribution of [3H]estramustine and [3H]estradiol are shown in Chart 1. A clear difference is seen in the distribution patterns of the 2 compounds and/or their metabolites. Thirty min following administration of the tracer, the pancreas, epiphysis, lung, adrenal gland, seminal vesicle, and brain cortex showed a higher uptake of estramustine than of estradiol. Some of these organs, such as the lung, adrenal gland, and seminal vesicle, also retained estramustine more efficiently than they retained estradiol. Four tissues showed increased concentrations of estramustine at 2 hr compared to 30 min: ventral prostate; preputial gland; submaxillary gland; and thyroid gland. Although the uptake of estradiol by the ventral prostate at 30 min was as high as the uptake of estramustine, the concentration of estradiol in the same tissue at 2 hr was insignificant. These results indicate that the rat ventral prostate is capable of accumulating estramustine but not estradiol.

**Protein-bound Metabolites of Estramustine in Prostate Cytosol.** Chromatography on Ultrogel AcA-54 of prostate cytosol recovered from rats 2 hr following administration of [3H]estramustine showed a major radioactive peak eluted at a position corresponding to a molecular weight of 40,000 to 50,000 (Chart 2). The fractions from 19.5 ml to 32.5 ml were pooled and analyzed by TLC. The main radioactive peak had a mobility corresponding to that of estramustine. A smaller amount of radioactivity travelled as the estramustine-congener LS 271 (Chart 3).

**In Vitro Studies**

**Rate of Association.** The association rate of [3H]estramustine with the prostate cytosol protein was very high at 37° (Chart 4) and almost as high at 30°. At 37°, however, the estramustine/protein complex was rapidly degraded, which created a sharp maximum in bound radioactivity following incubation for 10 min. At 30°, decreased binding was observed after 2 hr. The association rate decreased with decreasing temperature. Equilibrium was reached at 22° after 2 hr, at 15° after 8 to 12 hr, and at 0° after 20 hr. At 0°, however, only 25% of the maximal binding was achieved. For practical reasons, cytosol labeling was carried out at 15° for 18 hr in most experiments.

**Rates of Dissociation and Degradation.** The rate of dissociation of the estramustine/protein complex increased rapidly with temperature, with half-lives of about 210, 80, 9, and 4 min at 15°, 22°, 30°, and 37°, respectively (Chart 5). The half-lives and dissociation rate constants are summarized in Table 1. At 0°, no dissociation was found even after 24 hr. Since the dissociation rates were high compared to the degradation rates (see below), no corrections were made for the latter. The estramustine-binding protein in rat ventral prostate cytosol...
Estramustine in Rat Ventral Prostate

Chart 2. Chromatography of [3H]estramustine-labeled prostate cytosol on Ultrogel AcA-54. Cytosol was prepared from ventral prostate glands 2 hr after administration of 0.25 nmol [3H]estramustine to each of 4 rats. The first UV-absorbing peak at an elution volume of 16.5 ml corresponds to the exclusion volume of the column.

Chart 4. Rate of association between [3H]estramustine and the binding protein in rat ventral prostate cytosol at various temperatures. The concentrations of ligand and protein were 10 nM and 10 µg/ml, respectively. At each time, protein-bound [3H]ligand was determined by use of the DCC technique. Corrections for dissociation and degradation were not made.

Chart 3. TLC analysis of radioactivity extracted from the main radioactive peak eluted from the Ultrogel AcA-54 column (cf. Chart 2). The mobilities of standards are indicated E2, estradiol; E1, estrone; LS 275, estramustine; LS 271, 17-dehydroestramustine.

Chart 5. Rate of dissociation of [3H]estramustine/protein complex in rat ventral prostate cytosol. The preformed complex was incubated in the presence of 10 µM unlabeled estramustine at various temperatures. At each time, protein-bound [3H]ligand was determined by use of the DCC technique. Correction for degradation of protein was not made.

Effects of pH, Enzymes, and Chemicals. Binding of estramustine to rat ventral prostate cytosol occurred with a broad pH optimum between pH 7 and 8.5 with maximum binding at pH 7.8 (Chart 7).

DNase I and RNase A had no significant effects on the binding of estramustine, whereas subtilopeptidase A completely destroyed the estramustine/protein complex. Streptomyces griseus and pancreatic crude protease decreased the binding by 85 and 45%, respectively. These results indicate that the estramustine-binding agent is a protein. Binding of [3H]estramustine decreased by 50% when labeling of the cytosol was performed in the presence of 1 to 2 mM N-bromosuccinimide or p-hydroxymercuribenzoate. Dithiothreitol also influenced the binding of [3H]estramustine giving a 35% decrease at 5 mM and a 55 to 60% decrease at 10 mM. The binding was uninfluenced by 10% absolute ethanol or 0.6 M KCl.

Ligand Specificity of the Estramustine-binding Protein. The efficiency of various steroids and steroid derivatives in displacement of 10 nM [3H]estramustine from the estramustine-binding protein in ventral prostate cytosol (1.6 µg of protein per ml) is shown in Chart 8 and in Tables 2 and 3.

Most of the tested estramustine congeners carrying the
Rates of dissociation and degradation of the estramustine/protein complex in rat ventral prostate cytosol

<table>
<thead>
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<th>Temperature</th>
<th>$t_{1/2}$ (min)</th>
<th>$k_1$ (min⁻¹)</th>
<th>$t_{1/2}$ (min)</th>
<th>$k_1$ (min⁻¹)</th>
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<tbody>
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<td>15°</td>
<td>210</td>
<td>0.003</td>
<td>5010</td>
<td>0.0001</td>
</tr>
<tr>
<td>22°</td>
<td>80</td>
<td>0.009</td>
<td>1750</td>
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<tr>
<td>30°</td>
<td>9</td>
<td>0.08</td>
<td>360</td>
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</tr>
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<td>37°</td>
<td>4</td>
<td>0.16</td>
<td>85</td>
<td>0.0085</td>
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</table>

Chart 6. Rate of degradation of $[^{3}H]$estramustine/protein complex in rat ventral prostate cytosol. After 18 hr at 15° C, the samples containing 10 nM $[^{3}H]$estramustine (with or without a 250-fold excess of unlabeled estramustine) and 10 μg of cytosol protein per ml were further incubated at various temperatures. At each time, protein-bound $[^{3}H]$-ligand was determined by use of the DCC technique.

Chart 7. Effect of pH on the binding of $[^{3}H]$estramustine in rat ventral prostate cytosol. Cytosol samples (10 μg cytosol protein per ml) were incubated with 10 nM $[^{3}H]$estramustine at 15° C for 18 hr at various pH values. Protein-bound $[^{3}H]$-ligand was determined by the DCC technique.

Chart 8. Competition of various steroid mustard derivatives for binding of $[^{3}H]$estramustine in rat ventral prostate cytosol. The resulting inhibitory curves are linearized by plotting logit Y versus log X, where Y is the ratio between the amount of $[^{3}H]$estramustine bound in the presence and the absence of unlabeled competitor, and X is the ratio between concentration (conc.) of unlabeled competitor and concentration of $[^{3}H]$estramustine incubated, respectively. Each point represents the mean of duplicate samples. For compound code number, see “Materials and Methods.” A, LS 275 (•), LS 271 (○), LS 289 (△), LS 451 (x), and LS 299 (φ). The curve of LS 2179 coincided with that of LS 275 and is not drawn. B, LS 275 (•), LS 452 (○), LS 524 (△), LS 2035 (x), LS 7275 (φ), and LS 451 (x), and LS 299 (φ).
Table 2
Relative binding affinity of various estrogen nitrogen mustard derivatives for the estramustine-binding protein in rat ventral prostate

Cytosol samples (200 μl, 1.6 μg protein per ml) were incubated at 15° for 18 hr with 10 nM [3H]-estramustine in the presence of varying amounts (1 nM to 45 nM) of unlabeled estrogen nitrogen mustard derivatives. Bound radioactivity was analyzed by use of the DCC technique described in “Materials and Methods.” For compounds giving curves not parallel (p < 0.05) with that of estramustine (LS 275), relative binding affinity (RBA) was not calculated.

\[
RBA = \frac{\text{Excess of estramustine required for 50% inhibition}}{\text{Excess of competitor required for 50% inhibition}}
\]

Under the experimental conditions used, the concentration of estramustine required for 50% inhibition was 20 nM; i.e., 2-fold excess and saturation of the estramustine binding sites were accomplished at approximately 100 nM estramustine (cf Chart 9).

<table>
<thead>
<tr>
<th>R₁</th>
<th>R₂</th>
<th>R₃</th>
<th>Relative binding affinity</th>
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<tr>
<td>LS 271</td>
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<td>1.16</td>
</tr>
<tr>
<td>LS 275</td>
<td>O</td>
<td>H</td>
<td>1.00</td>
</tr>
<tr>
<td>LS 289</td>
<td>OCCH₃</td>
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<tr>
<td>LS 299</td>
<td>OP(OH)₂</td>
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<td>LS 358</td>
<td>OCCH₂CH₃</td>
<td>H</td>
<td>NC*</td>
</tr>
<tr>
<td>LS 470</td>
<td>OCC(CH₃)₂</td>
<td>H</td>
<td>NC</td>
</tr>
<tr>
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<td>OCN(CH₂CH₂Cl)₂</td>
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<td>LS 2035</td>
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<tr>
<td>LS 2179</td>
<td>H</td>
<td>OCCH₃</td>
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* NC, not calculated.

yielded a mean apparent Kᵦ of about 17 nM [16.6 ± 2.6 (S.E.); n = 7]. The mean value of the maximal binding capacity (Bₑₘₐₓ) was about 5 nmol/mg cytosol protein (4.6 ± 0.2; n = 7). A representative analysis is shown in Chart 9.

Binding of the Estramustine/Protein Complex to DNA-Cellulose. Practically no estramustine/protein complex was retained by DNA-cellulose (Chart 10). In agreement with reported results (27), however, the dihydrotosterone/androgen receptor complex was significantly retained on the DNA-cellulose column.

DISCUSSION

The present investigation shows that the ventral prostate of the rat accumulates estramustine more efficiently than estradiol. Furthermore, the retention of estramustine was more significant in the prostate than in most other tissues studied, although the preputial gland, submaxillary gland, and thyroid gland also showed retention of the drug. The most probable explanation for this capacity of the ventral prostate to accumulate estramustine is the occurrence in the gland of an agent that has a high capacity to bind the drug. As mentioned above, this estramustine-binding agent was partially characterized in a previous paper as a macromolecule with a molecular weight of 40,000 to 50,000 (9).

The binding capacity of the estramustine-binding molecule is resistant to treatment with RNase or DNase but is reduced or abolished following incubation with various proteases. These results indicate that the estramustine-binding macromolecule is a protein. Scatchard analysis of the binding data obtained from incubations of [3H]estramustine with prostatic cytosol suggests that the estramustine/protein complex has an apparent Kᵦ of 10 to 30 nM and that about 5 nmol of estramustine are bound per mg of total cytosol protein. Assuming that the estramustine-binding protein has one estramustine-binding site per molecule and that the molecular weight of the protein is 45,000, it can be calculated that the estramustine-binding protein accounts for about 20% of the total protein content of the rat ventral prostatic cytosol.

The estramustine-binding protein in rat ventral prostate seems to be quite specific for estramustine and closely similar compounds. None of the tested steroids lacking the nitrogen mustard moiety inhibited the binding of estramustine by more than 35% (progesterone in 4500-fold excess).
The results presented in this study and those reported earlier (8, 9, 17) clearly distinguish the estramustine-binding protein from the androgen receptor that occurs in rat ventral prostate cytosol. This receptor has a molecular weight of 270,000 to 280,000 (25) and a sedimentation coefficient of 7 to 8S (25). Furthermore, the androgen receptor has ligand specificity quite different from that of the estramustine-binding protein (22). Also, the estramustine/protein complex does not bind to DNA-cellulose, whereas such binding occurs in the case of all steroid receptors (26). Another characteristic of steroid receptors is their low tissue concentration (in the range of fmol/mg cytosol protein) which contrasts to the high tissue concentration of the estramustine-binding protein.

The apparent Kd of the estramustine-binding protein (10 to 30 nM), is of the same order of magnitude as the Kd's of the steroid-binding plasma proteins (41). However, no binding of estramustine corresponding to that of rat ventral prostate cytosol could be shown in rat plasma (9), indicating that the estramustine-binding protein does not originate from blood. Following i.v. injection of [3H]estramustine, Appelgren et al. (2), using an autoradiographic technique, found a time-dependent transport of radioactivity from the epithelial cells into the luminal secretory content of rat ventral prostate. This result indicates that the estramustine-binding protein is a secretory protein formed in the prostate cell and transported into the lumen of the prostate lobulus. The function of this major protein

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**Table 3**

Relative binding affinity of some non-estrogen nitrogen mustard derivatives and LS 298 for the estramustine-binding protein in rat ventral prostate

For further details, see legend to Table 2.

<table>
<thead>
<tr>
<th>R = C—N—(CH2CH2Cl)2</th>
<th>Lea no.</th>
<th>Relative binding affinity</th>
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</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>L5 524</td>
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<td></td>
</tr>
<tr>
<td>LS 298</td>
<td>NC*</td>
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</tbody>
</table>

* NC, not calculated.

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**Table 4**

Effect of various steroids and steroid conjugates on the binding of [3H]estramustine by rat ventral prostate cytosol

Cytosol samples (200 μl, 1.6 μg protein per ml) were incubated at 15° for 18 hr with 10 nM [3H]estramustine in the presence of varying amounts (1 μM to 45 μM) of unlabeled steroids and steroid conjugates. Following a treatment with DCC, the radioactivity bound in the presence of competitor was expressed as percentage of radioactivity bound in the absence of competitor. The table shows the cytosol binding of [3H]estramustine in the presence of 45 μM competitor, i.e., a 4500-fold excess of competitor in proportion to the concentration of [3H]estramustine. Estramustine gave 50% inhibition in 2-fold excess.

<table>
<thead>
<tr>
<th>Competitor (45 μM)</th>
<th>Bound [3H]estramustine (10 nM) (% of control set to 100)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No competitor</td>
<td>100</td>
</tr>
<tr>
<td>Progesterone</td>
<td>65</td>
</tr>
<tr>
<td>Estrone</td>
<td>70</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>75</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>80</td>
</tr>
<tr>
<td>Dehydroepiandrosterone</td>
<td>90</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>&gt;90</td>
</tr>
<tr>
<td>Dihydrotestosterone</td>
<td>&gt;90</td>
</tr>
<tr>
<td>Testosterone</td>
<td>&gt;90</td>
</tr>
<tr>
<td>19-Nortestosterone</td>
<td>&gt;90</td>
</tr>
<tr>
<td>5α-Androstane-3β,17β-diol</td>
<td>&gt;90</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>&gt;90</td>
</tr>
<tr>
<td>Cortisol</td>
<td>&gt;90</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>&gt;90</td>
</tr>
<tr>
<td>Dihydrotestosterone</td>
<td>&gt;90</td>
</tr>
<tr>
<td>Testosterone</td>
<td>&gt;90</td>
</tr>
<tr>
<td>17β-Estradiol, 3-sulfate</td>
<td>&gt;90</td>
</tr>
<tr>
<td>Estrone sulfate</td>
<td>&gt;90</td>
</tr>
<tr>
<td>Estrone glucuronide</td>
<td>&gt;90</td>
</tr>
<tr>
<td>17β-Estradiol, 17-glucuronide</td>
<td>&gt;90</td>
</tr>
<tr>
<td>17β-Estradiol, 3-glucuronide</td>
<td>&gt;90</td>
</tr>
</tbody>
</table>

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The apparent Kd of the estramustine-binding protein (10 to 30 nM), is of the same order of magnitude as the Kd's of the steroid-binding plasma proteins (41). However, no binding of estramustine corresponding to that of rat ventral prostate cytosol could be shown in rat plasma (9), indicating that the estramustine-binding protein does not originate from blood.

Following i.v. injection of [3H]estramustine, Appelgren et al. (2), using an autoradiographic technique, found a time-dependent transport of radioactivity from the epithelial cells into the luminal secretory content of rat ventral prostate. This result indicates that the estramustine-binding protein is a secretory protein formed in the prostate cell and transported into the lumen of the prostate lobulus. The function of this major protein
can only be speculated upon, but its quantitative importance makes it reasonable to assume that the protein may be an essential component of the seminal fluid. The capacity of the protein to bind estramustine and its close congeners with high affinity and high capacity may be only a fortuitous phenomenon but may also indicate that the protein has a ligand-binding site that binds a naturally occurring compound.

Several reports have been published on the binding of steroids to rat ventral prostate cytosol proteins sedimenting at 3 to 5S and with molecular weights of 45,000 to 70,000 (3, 5—7, 25, 26, 28, 39, 40). Karsznia et al. (19) in 1969 reported the presence in rat ventral prostate of a pregnenolone- and progesterone-binding protein sedimenting at 3.5S and with a molecular weight of 45,000 to 50,000. Simultaneously, Liao et al. (23) reported on a dihydrotestosterone-binding protein designated as "α-protein" in rat ventral prostate cytosol sedimenting at 3 to 3.5S that was distinct from the androgen receptor ("β-protein"). Recently, Heyns et al. (12—14) have described a protein in rat ventral prostate cytosol with "peculiar steroid-binding properties" and with a molecular weight of 51,000 and a sedimentation coefficient of 3.7S. The steroids that were bound with highest affinity were pregnenolone and androstenedione. It was calculated that this protein accounted for about 15% of the total protein in prostate cytosol. A high concentration of the protein was found in prostatic fluid, indicating that it is secreted by the prostate. The similarities between the estramustine-binding protein and the steroid-binding proteins described above are conspicuous.

The presence of a major estramustine-binding protein in the rat ventral prostate raises the question whether a similar protein occurs in the human prostate. If so, this would provide an explanation for the efficiency of Estracyt in the treatment of prostatic carcinoma; the estramustine-binding protein would concentrate estramustine from the blood and provide the tissue with large amounts of the drug. Studies are now in progress to investigate the possible existence of estramustine-binding protein in normal, hyperplastic, and carcinomatous human prostate.

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Binding Characteristics of a Major Protein in Rat Ventral Prostate Cytosol That Interacts with Estramustine, a Nitrogen Mustard Derivative of 17β-Estradiol

Björn Forsgren, Jan-Åke Gustafsson, Åke Pousette, et al.


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