J. steroid Biochem. Vol. 23, No. 3, pp. 279–289, 1985
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0022-4731/85 $3.00 + 0.00
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IN VIVO OESTROGENICITY AND BINDING CHARACTERISTICS OF α-ZEARALANOL (P-1496) TO DIFFERENT CLASSES OF OESTROGEN BINDING PROTEINS IN RAT LIVER

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(*Received* 14 *December* 1984)

Summary—It is now well established that the mycotoxin zearalenone and some of its derivatives possess oestrogenic activity. In the present study, the binding characteristics of [^{3}H]zearalanol (P-1496) to different classes of sites including [1] the oestrogen receptor, [2] the higher capacity lower affinity (HCLA) sites, [3] the antioestrogen sites and [4] a new class of binding sites apparently specific for P-1496 were examined in rat liver. Analysis of the binding by sucrose density gradient centrifugation confirmed that P-1496 binds to the oestrogen receptor but not to the higher capacity lower affinity sites for oestradiol-17β. Furthermore, saturation experiments using partially-purified fractions showed that P-1496 binds to the oestrogen receptor with an affinity very similar to that of oestradiol-17β (apparent dissociation constants ranged from 0.1–0.3 nM). Competition studies using partially purified cytosolic oestrogen receptor suggested that P-1496 binds to a second high affinity site distinct from the oestrogen receptor. This binding site was further characterized as selective for P-1496 by saturation analysis following the complete occupancy of oestrogen receptor by oestradiol-17β. The *in vitro* binding characteristics of P-1496 were then compared with *in vivo* effects on concentrations of serum triglycerides. Treatment of ovariectomized female rats daily with 1.5 or 2 mg P-1496/kg body weight resulted in marked increases in the concentrations of serum triglycerides associated with the very low density lipoprotein (VLDL) fraction. Dose–response studies indicated that there was no sex difference with respect to the dose necessary to produce significant increases in serum triglycerides. The present study shows striking similarities between the binding of P-1496 and oestradiol-17β to liver oestrogen receptor *in vitro*. However, differences are observed with respect to their binding to other cytoplasmic components of liver. In addition, although P-1496 is capable of eliciting *in vivo* oestrogenic effects in liver, it is much less potent than oestradiol-17β.

INTRODUCTION

Liver is now accepted as a target organ for oestrogens. Binding sites that comply with the term "oestrogen receptors" have been located in cytosolic [1–5] and nuclear [6, 7] fractions from liver. Furthermore, oestradiol-17β bound to these sites has been shown to translocate to the nucleus both *in vitro* [7] and *in vivo* [6]. A variety of hepatic responses to *in vivo* administration of oestrogen appear to be mediated through the oestrogen receptor [8–13]. The responsiveness of serum triglycerides is of particular relevance because of their possible importance in some of the adverse side effects produced by oestrogen-containing oral contraceptives. Several studies have shown elevation of serum triglycerides by oestrogens in both humans [14–16] and animals [13, 17–21]. In the rat, these changes have been associated mainly with the VLDL fraction of serum [13, 19, 21] and they are caused primarily by increased secretion from the liver rather than a decreased utilization [17–19].

The compound α-zearalanol (P-1496) has pharmaceutical and agricultural uses [22–24]. It is a synthetic derivative of the mycotoxin zearalenone (P-1492) which is a β-resorcylic acid lactone produced by various species of *Fusarium* [25]. P-1496 was found to be the most oestrogenic of the zearalenone derivatives but less oestrogenic than oestradiol-17β as demonstrated by *in vivo* uterotropic assays [26] as well as *in vitro* studies on the binding to oestrogen receptor in uterus [26] and liver [27]. In the present study we examined binding characteristics of this compound in cytosol from rat liver focusing on oestrogen receptor as well as other sites distinct from the receptor. The availability of [^{3}H]P-1496 of high specific activity has permitted a more accurate determination of binding properties compared to previous studies. Furthermore, an attempt was made to compare such *in vitro* characteristics to *in vivo* effects of P-1496 on concentrations of serum triglycerides.

EXPERIMENTAL

Animals

Adult (70–80-days old) Sprague–Dawley rats were used and maintained on a schedule of 14 h light

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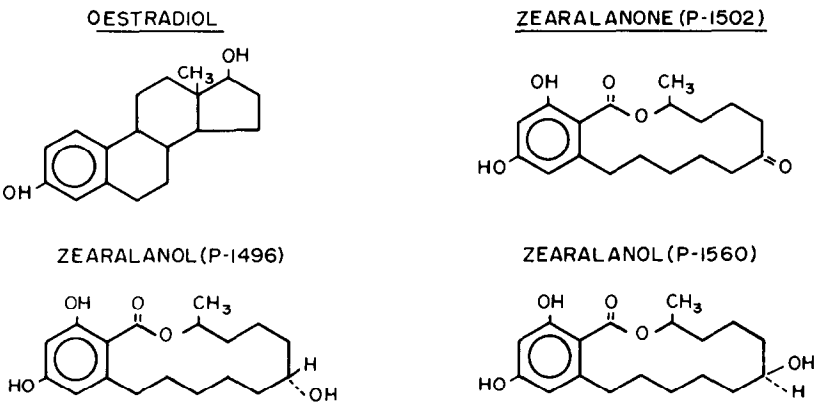

Fig. 1. Structural formulae of P-1496 and related zearalenone derivatives.

and 10 h darkness. Food and water were available *ad libitum*.

For *in vivo* experiments, female rats were ovariectomized at 54 days of age. All treatments were started at 61 days of age and lasted for 14 days unless stated otherwise. Rats were injected subcutaneously, on a daily basis, with the appropriate dose of P-1496 dissolved in 0.1 ml of propylene glycol. Control rats were treated with vehicle alone. Animals were fasted for 14–16 h and subsequently killed by decapitation, 22–24 h after the last injection. Blood was collected and livers and uteri excised and chilled on ice.

Buffers and chemicals

The following buffers were used: 10 mM Tris, 1 mM EDTA and 1 mM dithiothreitol adjusted to pH 7.4 at 4°C (buffer A); 0.5% (w/v) activated, untreated charcoal powder and 0.05% (w/v) dextran (clinical grade) in buffer A (DCC solution).

[1,2-^{3}H]Zearalanol (P-1496) (41–45 Ci/mmol) was purchased from Amersham Corp., Arlington Heights, Ill., U.S.A. [2,4,6,7-^{3}H] Oestradiol-17β (91–115 Ci/mmol) and [*N*-methyl-^{3}H]tamoxifen (87 Ci/mmol) were obtained from New England Nuclear Corp., Boston, Massachusetts, U.S.A. Radioinert P-1496 (α-zearalanol), P-1560 (β-zearalanol) and P-1502 (zearalenone) (Fig. 1) were a gift from International Minerals and Chemical Corp., Terre Haute, Indiana, U.S.A. Radioinert steroids and diethylstilbestrol (DES) were obtained from Steraloids, Wilton, Pennsylvania, U.S.A. Nafoxidine was kindly donated by the Upjohn Co., Kalamazoo, Michigan, U.S.A. Purity of radioactive and radioinert steroids was greater than 99%. Biofluor was purchased from New England Nuclear Corp. Reagents for automated determination of cholesterol (enzymatic) and triglycerides were purchased from Dow Chemical Co. (Indianapolis, Indiana).

Quantitation of triglycerides in lipoprotein classes

Serum lipoproteins were separated into three fractions (HDL, LDL and VLDL) using the micromethod described by Bronzert and Brewer[28]. Serum was prepared by centrifugation of blood at 1500 *g* for 30 min at 4°C. Aliquots (175 μl) of serum were added to duplicate sets of tubes (Beckman), one set containing 13.8 mg of solid KBr. The latter was dissolved by gentle shaking of the samples. Both sets of tubes were subsequently centrifuged in a Beckman Type 42.2 Ti rotor at 170,000 *g* for 2.5 h at 4°C. After centrifugation, lipoprotein fractions were isolated [13] and analyzed [29] using a Gilford 3500 Analyzer.

Preparation of cytosolic fractions

Animals were killed by decapitation. Livers were excised quickly and placed on ice. All subsequent procedures were performed at 0–4°C. Two livers were pooled, minced finely using scissors, weighed and then homogenized in buffer A (20%, w/v unless stated otherwise) by 5 strokes with a Teflon pestle using a Potter–Elvehjem homogenizer. Cytosol was prepared by centrifugation of the crude homogenates at 100,000 *g* for 60 min. Avoiding the fat layer, the supernatant was removed and used as the cytosolic fraction. Protein concentrations were determined by the method of Lowry *et al.*[30] and are given in the figure legends. In some cases, cytosolic oestrogen receptor fractions were partially purified by ammonium sulfate precipitation as described previously [31]. Precipitates were resuspended in buffer A to a volume equal to that of the original cytosol.

Detection of cytoplasmic P-1496-binding components

Specific binding of P-1496 in whole cytosol was saturated after incubation for 4 h with 10 nM [^{3}H]P-1496 (final concentration) at 0–4°C and was stable up to 18 h (results not shown). Hence, an incubation time of 4–6 h was routinely used for sedimentation analysis of [^{3}H]P-1496 binding in whole cytosol. Similar results have been observed for cytosolic binding of [^{3}H]oestradiol-17β [32].

Sedimentation properties of cytosolic binding proteins were analyzed by incubating (6 h at 4°C) aliquots (200 μl) of whole cytosol with 200 μl of [^{3}H]P-1496 (30 nM, final concentration). In some cases the incubation mixtures included radioinert

oestradiol-17β or P-1496 (3 μM, final concentration). Following incubation, mixtures were added to DCC pellets prepared from 200 μl of DCC solution. The resulting mixtures were vortexed and left on ice for 15 min. They were then centrifuged (800 *g* for 10 min) and 200 μl aliquots of the supernatant layered on 5–20% sucrose gradients (4.4 ml) prepared in buffer A. Gradients were centrifuged (18 h at 4°C) in a SW-60 swinging bucket rotor at 100,000 *g*. Bovine serum albumin (4.6 S) was used as a marker. Following centrifugation, gradients were fractionated and each fraction (approx 100 μl) assessed for radioactivity.

Binding of [^{3}H]P-1496 was determined quantitatively in resuspended ammonium sulfate precipitates by incubation (15–17 h at 0–4°C) with an equal volume (100 μl) of labelled compound (0.2–60 nM) in buffer A. Assays were performed in duplicate in the presence (non-specific binding) and absence (total binding) of 100-fold excess of radioinert oestradiol-17β (results not shown) or P-1496. After incubation, unbound radiolabelled compound was removed by treatment (20 min at 0–4°C) with DCC solution (200 μl). Charcoal was then sedimented (800 *g* for 10 min) and aliquots (200 μl) of supernatant assessed for radioactivity. The efficiency of DCC in removing unbound radioactivity was 99.6%. Specific binding was determined as the difference between total and non-specific binding in the sample. Binding data were then analyzed according to Scatchard[33]. Curved plots were resolved to linear components by computer analysis as described previously [34].

The following procedure was used to quantify non-receptor sites for P-1496: Ammonium sulfate precipitate resuspended in buffer A (6 ml) was preincubated (2 h at 0–4°C) with oestradiol-17β (1 μM, final concentration). Aliquots of this mixture were subsequently incubated (15–17 h at 0–4°C) with an equal volume (100 μl) of [^{3}H]P-1496 (1.6–93 nM) in buffer A. Assays were performed in duplicate in the presence (non-specific binding) and absence (total binding) of 100-fold excess of radioinert P-1496. After incubation with [^{3}H]P-1496, unbound radiolabelled compound was removed by treatment with DCC and bound radioactivity determined as described under saturation analysis. Binding data were then analyzed according to Scatchard [33].

Specificity studies

Aliquots (100 μl) of resuspended ammonium sulfate precipitates were incubated (15–17 h at 0–4°C) with an equal volume of [^{3}H]P-1496 (15 nM, final concentration) in buffer A. Incubations were performed in duplicate in absence or presence of varying concentrations of radioinert competitor (1- to 2500-fold). After incubation, unbound [^{3}H]P-1496 was removed by treatment with DCC solution and bound radioactivity measured as described above.

Purity of [^{3}H]P-1496

The purity of [^{3}H]P-1496 was determined periodically using an HPLC system (Beckman models 112, 165, 340, 421). An aliquot of stock [^{3}H]P-1496 (7.2 μg/ml) was diluted 100 times with a solution of methanol containing radioinert P-1496, P-1560 and P-1502 (50 μg/ml of each) and 15 μl (0.15 μCi) of the resulting mixture were applied onto an ultrasphere-ODS column (15 cm × 4.6 mm i.d.) previously equilibrated in 53% aqueous methanol. A linear gradient was then applied (53–70% methanol in water) over a period of 30 min. Fractions were collected using an LKB Superac Fraction collector. Purity was expressed as the percentage of radioactivity recovered coeluting with radioinert P-1496 (monitored by measuring the absorbance at 260 nM). Using this method, P-1496 was clearly separated from its related compounds P-1560 (the β-isomer of P-1496) and P-1502. It was found that stock solutions of [^{3}H]P-1496 were greater than 95% radiochemically pure.

Competition for antioestrogen binding sites

Liver from female rats was homogenized in buffer A (10%, w/v) and centrifuged at 14,500 *g* for 30 min. Avoiding the fat layer, the supernatant was removed and diluted 2.5-fold before use. Aliquots (250 μl) of diluted supernatant were incubated (17 h at 0–4°C) with 125 μl of [^{3}H]tamoxifen (15 nM, final concentration) and either 125 μl of competitor (1.5 μM, final concentration) or buffer alone. [^{3}H]Tamoxifen and competitors were dissolved in buffer A containing 0.2% BSA. After incubation, unbound [^{3}H]tamoxifen was removed by treatment (20 min at 0–4°C) with DCC solution (0.5 ml). Charcoal was then sedimented (800 *g* for 10 min) and aliquots (0.5 ml) of supernatant assessed for radioactivity.

RESULTS

Effect of P-1496 on uterine weights

An increase in uterine wet weight is a commonly used indicator of *in vivo* oestrogenicity of a compound. Hence uteri of treated female animals were weighed routinely and provided a comparison with the oestrogenic effects of P-1496 on liver. Administration of P-1496 (2 mg/kg body weight) to ovariectomized female rats for 2 weeks resulted in approx 7-fold increase in uterine wet weight. Significant increases were evident at all time points including 1 day of treatment (Fig. 2A). Moreover, dose–response data indicated doses as low as 0.05 mg P-1496/kg body weight for 2 weeks were sufficient to produce a small but significant trophic response. However, the effect was more prominent with doses higher than 0.5 mg/kg body weight (Fig. 2B).

Changes in serum triglycerides

The oestrogenic response of liver to the *in vivo*

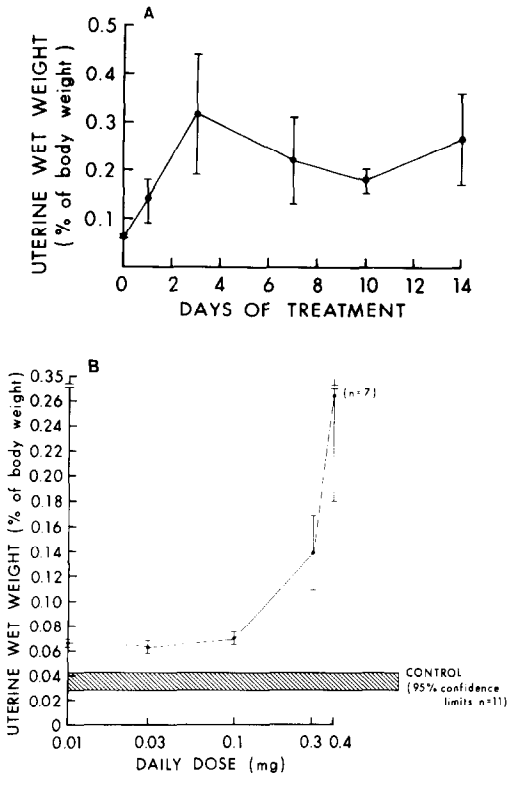

Fig. 2. Effects of P-1496 on uterine weights. Experimental details in (A) and (B) are the same as in Fig. 4 and Fig. 3 respectively.

administration of P-1496 was tested by measurement of the triglyceride concentrations in whole serum and the VLDL fraction. Consistent with previous observations in this laboratory using oestradiol-17β [13], no significant changes were associated with the triglyceride content of HDL and LDL fractions. Thus, average levels (mg/dl serum) and standard deviations in LDL and HDL, respectively, were 13.3 ± 5.5 and 27.4 ± 3.2 ($N = 7$) for controls (ovariectomized females treated with vehicle), and 17.5 ± 22.2 and 34.5 ± 11.2 ($N = 7$) for treated animals (2 mg P-1496/kg/day, for 14 days). Dose–response experiments on ovariectomized female rats showed that a dose greater than 0.5 mg/kg body weight was required to produce a significant effect on total serum triglycerides (Fig. 3A) as well as triglycerides associated with the VLDL fraction (Fig. 3B). A time course experiment was conducted on ovariectomized female rats (Fig. 4). This experiment showed that significant changes in triglyceride concentrations occurred after 7 days of daily treatment (2 mg P-1496/kg body weight).

Experiments conducted in this laboratory [21] showed a pronounced sex difference in the responsiveness of triglyceride levels to oestradiol-17β. Therefore, dose-dependent effects of P-1496 were also determined in adult male rats (Table 1) and the data indicated that responsiveness in males was similar to that observed in females (Fig. 3).

Binding of P-1496 in whole cytosol from male and female rat liver

The cytoplasmic binding of [^{3}H]P-1496 was analyzed by sucrose-density gradient centrifugation (Fig. 5). Such analysis showed two peaks of radioactivity corresponding to sedimentation coefficients of 4–5 S and 8–9 S, respectively. The latter peak was completely displaced by 100-fold excess of P-1496 or oestradiol-17β and is believed to represent [^{3}H]P-1496 bound to oestrogen receptor. The first peak was displaced to some extent by 100-fold excess P-1496 (but not by 100-fold excess oestradiol-17β).

The binding of [^{3}H]P-1496 in the 4–5 S region was quantitatively similar in male and female rats (Fig. 5B). This finding is in contrast to the properties of [^{3}H]oestradiol-17β in this region [27] and confirms the results of competition studies [27] which suggested that P-1496 does not bind to the oestradiol-17β HCLA sites.

Studies using partially-purified cytosolic receptor

Liver oestrogen receptor was partially purified from cytosol by ammonium sulfate precipitation and this fraction was used to examine binding of [^{3}H]P-1496 by Scatchard analysis and competitive binding experiments. Using this partially purified fraction, the nonspecific binding was reduced significantly making quantitation of specific binding more accurate. Saturation analysis of binding was carried out routinely using overnight incubations at 0–4°C. Analysis of the binding data from female preparations revealed a curved plot (Fig. 6). This was resolved by a non-linear squares method [34] into two linear components implying the presence of two types of binding sites with apparent dissociation constants of 0.26 and 7.62 nM and capacities of 25 and 51 fmol/mg protein, respectively. A parallel experiment with [^{3}H]oestradiol was carried out using either excess oestradiol or P-1496 to determine non-specific binding. Scatchard analysis of the data revealed a single type of binding sites with an apparent dissociation constant of 0.24 nM and a capacity of 17 fmol/mg protein (Fig. 7). These values on [^{3}H]-oestradiol binding are in agreement with previous findings [31, 35].

Competition of [^{3}H]P-1496 binding in resuspended ammonium sulfate precipitate

Experiments were conducted using 15 nM final concentration of [^{3}H]P-1496 and a variety of steroidal and non-steroidal compounds were tested. Figure 8 shows the competition by P-1496, P-1560 (the β-isomer of P-1496), P-1502, oestradiol-17β and DES for [^{3}H]P-1496 binding. Progesterone, corticosterone and 5α-dihydrotestosterone (DHT) were also tested at a range of concentrations from 0.05–75 μM (results not shown) but those compounds had no pronounced effect on [^{3}H]P-1496 binding even at high concentrations. As seen in Fig. 8, the best competitor

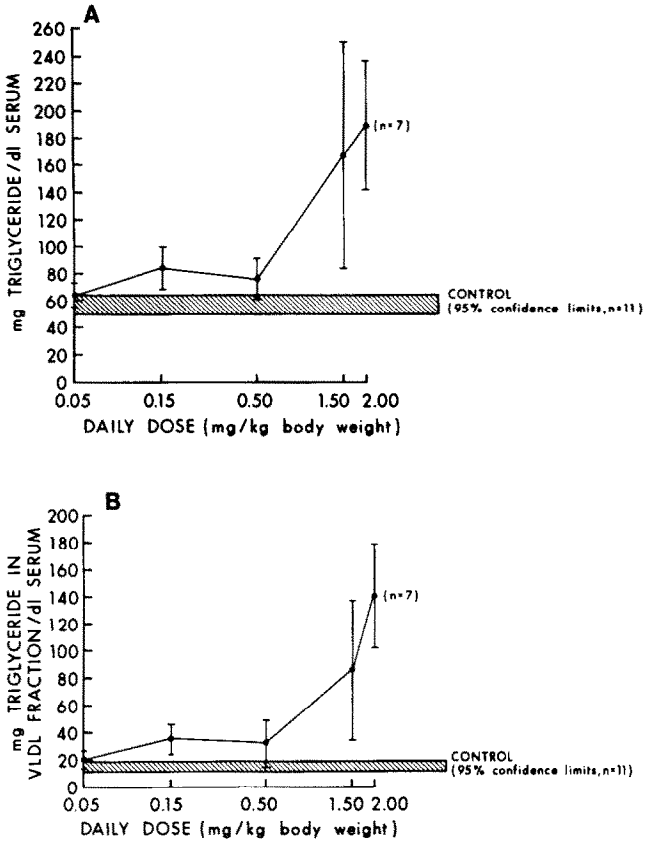

Fig. 3. Effects of P-1496 on serum triglycerides. Adult female rats were ovariectomized at 54-days of age. One week later they were injected s.c. with varying doses of P-1496 in 0.1 ml of propylene glycol for 14 consecutive days. Control animals were treated with vehicle alone. All animals were fasted 14–16 h prior to being killed. Serum was prepared and fractionated as described under Experimental. Results (mean ± SD, $N = 4$ except for the highest dose where $N = 7$) represent the triglyceride concentrations in total serum (A) and in the VLDL fraction (B).

of [^{3}H]P-1496 binding was P-1496 itself. P-1560 and P-1502 also competed but at higher concentrations. The finding that P-1496 is a much better competitor than DES or oestradiol-17β provides indirect but nevertheless additional evidence for the presence of a second P-1496 site that was suggested from the saturation experiments.

High affinity binding of [^{3}H]P-1496 to a site distinct from the oestrogen receptor

The presence of a second high affinity site for P-1496 distinct from the oestrogen receptor was examined further in resuspended ammonium sulfate precipitate fractions which were preincubated (2 h at 0–4°C) with 1 μM oestradiol-17β (final concentration) to ensure saturation of the oestrogen receptor according to experiments characterising the antioestrogen site [36]. This excess oestradiol would also saturate any HCLA sites contaminating the purified fraction. Binding of [^{3}H]P-1496 was investigated by saturation analysis as described in Experimental. Specific binding data from such an experiment indicated saturable binding and resulted in a single straight line when analyzed according to Scatchard[33] (Fig. 9).

These data suggest the presence of a single type of binding site distinct from the oestrogen receptor with an apparent dissociation constant of 8.5 nM and with a capacity of 27.4 fmol/mg protein. Moreover, specific binding of [^{3}H]oestradiol-17β when examined under similar conditions, was approx 2% of the specific binding by [^{3}H]P-1496.

Competition for antioestrogen binding sites in rat liver

Because of the presence of high concentrations of antioestrogen binding sites in rat liver [36, 37], we also examined the possibility that the P-1496 second site was the antioestrogen site. Hence, an experiment was performed using the 14,500 *g* supernatant from rat liver which contains high amounts of antioestrogen binding sites [36]. Incubations included [^{3}H]tamoxifen alone or in the presence of 100-fold excess competitor. The competitors oestradiol-17β and nafoxidine were used as negative and positive controls, respectively. This study (Table 2) demonstrated that P-1496 or related compounds (namely

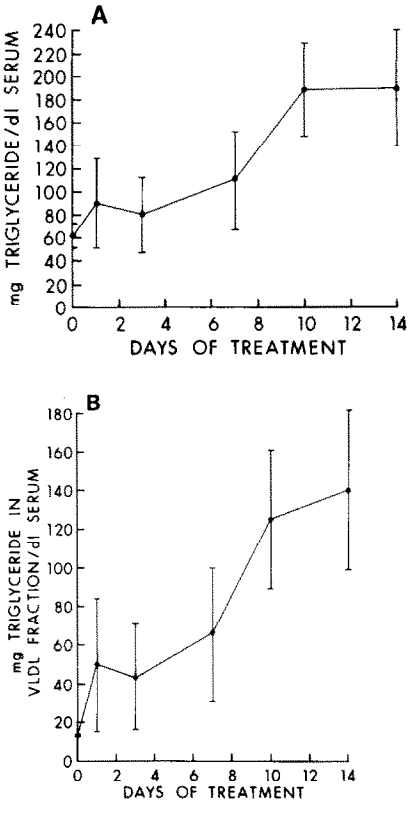

Fig. 4. Time-course of P-1496 effects on serum triglycerides. Adult female rats were ovariectomized at 54 days of age. One week later they were injected s.c. with P-1496 (2 mg/kg body weight/day) in 0.1 ml of propylene glycol for the number of days indicated. All animals were fasted 14–16 h prior to being killed. Serum was prepared and fractionated as described under Experimental. Results (mean ± SD, $N = 5$ except for the 14 days of treatment where $N = 7$) represent triglyceride concentrations in total serum (A) and in the VLDL fraction (B).

P-1560 and P-1502) do not compete for the [^{3}H]tamoxifen binding in rat liver.

DISCUSSION

The studies reported in this paper characterize the binding properties of [^{3}H]P-1496 to rat liver oestrogen binding proteins including evaluation of interactions with (1) the oestrogen receptor, (2) the HCLA sites (3) the antioestrogen sites and (4) a new class of high affinity sites apparently specific for P-1496 but distinct from receptor.

Table 1. Effect of P-1496 on serum triglyceride levels of male rats

Dose of P-1496 (mg/kg/day)	Triglyceride concentration (mg/dl of serum)	
	Serum	VLDL Fraction
0	71.4 ± 15.6	36.4 ± 14.9
1	106.4 ± 12.4*	72.5 ± 12.5*
2	147.4 ± 29.8*	115.8 ± 24.9*
5	106.9 ± 25.2†	79.9 ± 20.1‡

*$P < 0.001$ compared to control (Student's paired t-test).
†$P < 0.1$ compared to control (Student's paired t-test).
‡$P < 0.02$ compared to control (Student's paired t-test).
Values represent the mean ± SD ($N = 5$). Animals were treated for 2 weeks. Control animals received vehicle alone.

Previous studies have shown specific binding of P-1496 to oestrogen receptor from various tissues. In the uterus, the apparent dissociation constant (K_d) for P-1496 was calculated indirectly from competition experiments and also directly by saturation analysis of [^{3}H]P-1496 binding to uterine cytosol. These experiments suggested that P-1496 had an approx 10-fold lower affinity for the oestrogen receptor than oestradiol-17β [26, 38]. Studies on rat mammary gland also came to similar conclusions [39]. In the liver, from competition experiments with radioinert P-1496, it was suggested that the affinity of this compound for the oestrogen receptor was 3-fold lower in comparison to oestradiol-17β [27]. Our study using sedimentation and Scatchard analysis verifies that P-1496 binds to oestrogen receptor in the liver with high affinity. Scatchard analysis of [^{3}H]P-1496 binding to partially purified cytosol revealed a high affinity binding component with an apparent dissociation constant (K_d) and a capacity very similar to that of the oestrogen receptor for oestradiol-17β. The same results were obtained whether specific binding of [^{3}H]P-1496 was computed by using 100-fold excess oestradiol-17β or P-1496. The present findings that P-1496 can bind to oestrogen receptor *in vitro* with the same affinity as oestradiol-17β are in contradiction with earlier studies [26, 38]. The discrepancy with previously published data on P-1496 binding in the liver [27] can probably be explained by the fact that in this earlier study, affinity constants were calculated indirectly, from competition experiments. Differences with affinity constants quoted for other tissues may also be due to the use of crude cytosolic fractions which might metabolize P-1496 to less oestrogenic compounds [27, 40–42].

In addition to the oestrogen receptor, cytosol from rat liver contains other sites that bind oestradiol-17β with higher capacity and lower affinity (HCLA sites). These are present in much higher concentrations in males compared to females, have a sedimentation coefficient of 4–5 S, and bind steroidal oestrogens as well as androgens, but do not bind non-steroidal oestrogens like DES [31, 32, 43–46]. The following evidence suggests that P-1496 does not bind to these sites: (a) there was no sex difference in the amount of [^{3}H]P-1496 associated with the 4–5 S region of sucrose gradients; (b) binding of [^{3}H]P-1496 in this region was not displaced by radioinert oestradiol-17β in either males or females. These results are in agreement with those of Powell-Jones *et al.*[27] who showed that the binding of [^{3}H]oestradiol-17β in the 4–5 S region was not reduced by P-1496.

Experiments performed on partially purified receptor fraction suggested that the lack of binding of P-1496 to the HCLA sites was not the only difference in cytosolic binding between P-1496 and oestradiol-

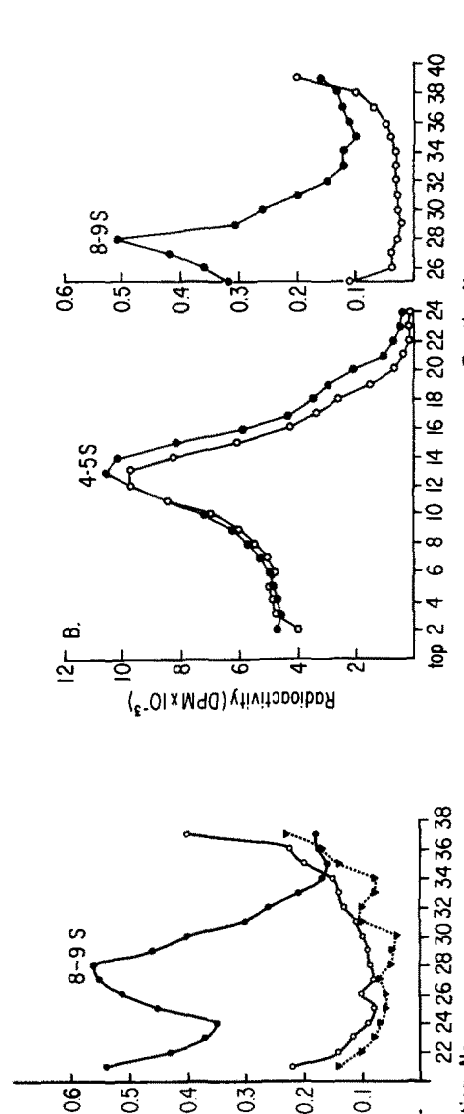

Fig. 5. Sucrose density gradient analysis of [^{3}H]P-1496 binding in whole cytosol prepared from female (A) and male (B) liver. Samples of cytosol were incubated (6 h at 0–4°C) with 200 μl of [^{3}H]P-1496 (30 nM, final concentration) in the absence (●) or presence of 100-fold excess P-1496 (▲) or oestradiol-17β (○). Following treatment with DCC, aliquots (200 μl) of supernatant were analyzed on 5–20% sucrose gradients. Protein concentrations of cytosol were 16 and 18 mg/ml for A and B, respectively. Similar results were obtained from three separate experiments. Gradients have been separated into top and bottom regions with different scales.

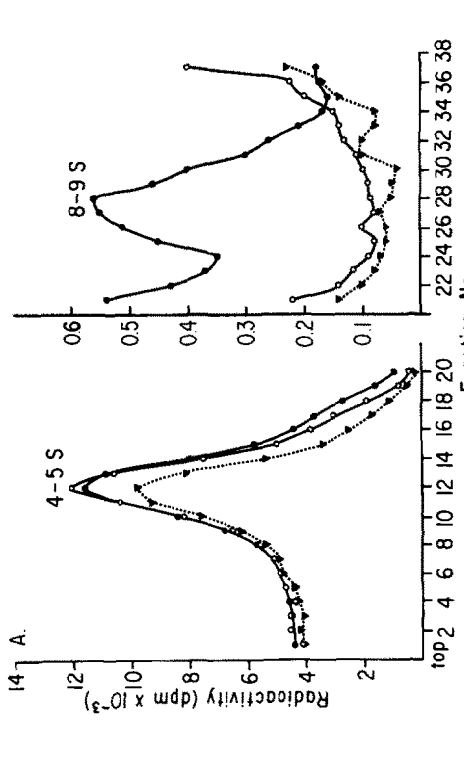

Fig. 6. Saturation analysis of [^{3}H]P-1496 binding in rat liver cytosol. Samples of resuspended ammonium sulfate precipitate from female rat liver were incubated (15–17 h at 0–4°C) with an equal volume (100 μl) of [^{3}H]P-1496 (0.1–12 nM, final concentration) in the absence (○) or presence (▲) of 100-fold excess P-1496. Following treatment with DCC, bound radioactivity was measured. Values obtained for specific binding (●) were plotted according to Scatchard[33]. The protein concentration of cytosol was 16 mg/ml. Similar results were obtained from three experiments.

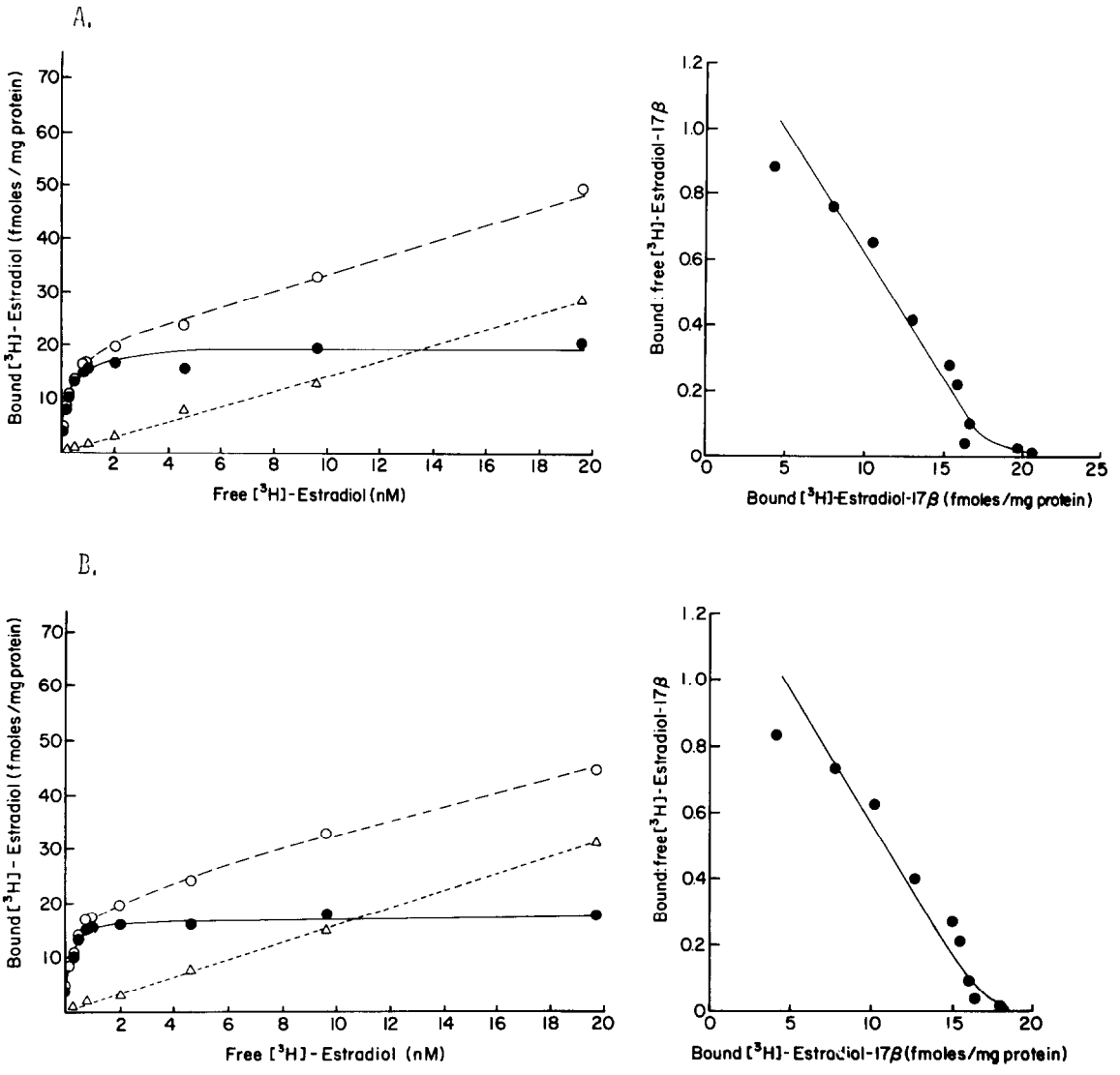

Fig. 7. Saturation analysis of [^{3}H]estradiol-17β binding in rat liver cytosol. Experimental details are as described in Fig. 6 except that [^{3}H]estradiol-17β was used instead of [^{3}H]P-1496. Incubations were carried out in the absence (○) or presence (▲) of 100-fold excess estradiol-17β (A) and P-1496 (B). The final concentration of [^{3}H]estradiol-17β was varied from 0.1–20 nM. The protein concentration of cytosol was 21 mg/ml.

17β. First, competition experiments showed that the binding of [^{3}H]P-1496 was not displaced as well by radioinert oestradiol-17β or DES compared to radioinert P-1496, particularly in the range of 1–250-fold excess competitior concentrations. Second, use of 100-fold excess oestradiol-17β or DES in [^{3}H]P-1496 saturation analysis experiments always resulted in non-specific plots that were curved instead of linear (data not shown). Third, Scatchard analysis of specifically bound [^{3}H]P-1496 (computed using radioinert P-1496 to determine non-specific binding) revealed curved graphs. These could be resolved into two components one of which corresponded to the oestrogen receptor in terms of affinity and capacity of binding and a second site which has a 10-fold higher K_d than oestrogen receptor for P-1496. Moreover, the second site was not detected when Scatchard analysis was performed using [^{3}H]oestradiol-17β. These observations taken together suggest a number of possibilities: (a) the binding of P-1496 to oestrogen receptor sites exhibits negative co-operativity; this would result in curved Scatchard plots and the appearance of 2 types of binding sites [47]; (b) P-1496 binds to a second site distinct from the oestrogen receptor. The second possibility was tested further by saturation analysis of [^{3}H]P-1496 binding following saturation of the oestrogen receptor with radioinert oestradiol-17β. Measurement of specific binding of [^{3}H]oestradiol-17β under these conditions resulted in very low values which were negligible compared to those obtained for [^{3}H]P-1496. Analysis of the binding data for [^{3}H]P-1496 suggested the presence of a

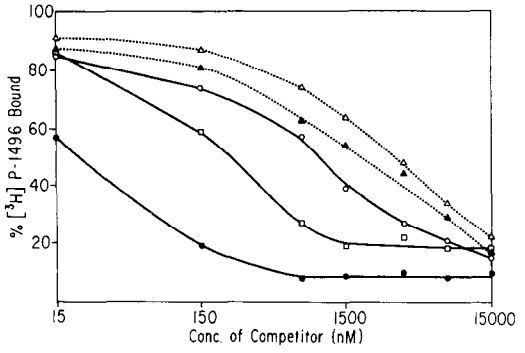

Fig. 8. Competition by oestradiol-17β, DES and the zearalenone derivatives for [^{3}H]P-1496 binding sites in rat liver cytosol. Samples of redissolved ammonium sulfate precipitate from female liver cytosol were incubated (15–17 h at 0–4°C) with a constant concentration of [^{3}H]P-1496 (15 nM final concentration) and various concentrations of oestradiol-17β (▲), DES (△), P-1496 (●), P-1560 (□) or P-1502 (○). Following treatment with DCC, bound radioactivity was measured. Protein concentration of cytosol was 18 mg/ml.

Table 2. Competition of zearalenone derivatives for antioestrogen binding sites in rat liver

Competitor	% Reduction of control
Nafoxidine	53
Oestradiol-17β	0
P-1496	3
P-1560	0
P-1502	0

Samples of diluted 14,500 *g* supernatant were incubated (17 h at 0–4°C) with [^{3}H]tamoxifen (15 nM, final concentration) alone or in the presence of 100-fold excess competitor. Following DCC treatment, samples were assessed for bound radioactivity. Results are presented as the percentage reduction of control values (control = binding in the absence of competitor). The protein concentration of diluted supernatant was 3.5 mg/ml.

single class of sites distinct from the oestrogen receptor. These sites bind P-1496 with an apparent dissociation constant of 8.5 nM and a finite capacity of 27 fmol/mg protein in female rats. These values are quite similar to those obtained by analyzing Scatchard plots from experiments where samples were not presaturated with 1 μM oestradiol [$K_d = 10.5 \pm 4.5$ (nM), $n = 50.5 \pm 44.5$ (mean ± SD, $n = 6$)]. The P-1496 binding sites have now been characterized further by full specificity studies that show that they are specific for P-1496 and its analogs and they do not bind oestrogens such as oestradiol, DES, oestriol or oestrone. They are also not a blood contaminant since they are not found in other tissues such as prostate, uterus, fast or slow muscle. It is certainly possible that these sites are not cytosolic *in vivo* as has been suggested for other binding sites namely the HCLA sites (or HASP) [48] and the oestrogen receptor [49, 50]. An attempt to characterize the subcellular localization of the P-1496 sites however has proven unsuccessful because of the high amount of non-specific binding (Mastri *et al.*, manuscript in preparation).

In view of the presence of high concentrations of antioestrogen sites in rat liver [36, 37] we evaluated binding of P-1496 to these sites. Our results clearly demonstrate that P-1496 does not bind to the antioestrogen site. This finding is consistent with the

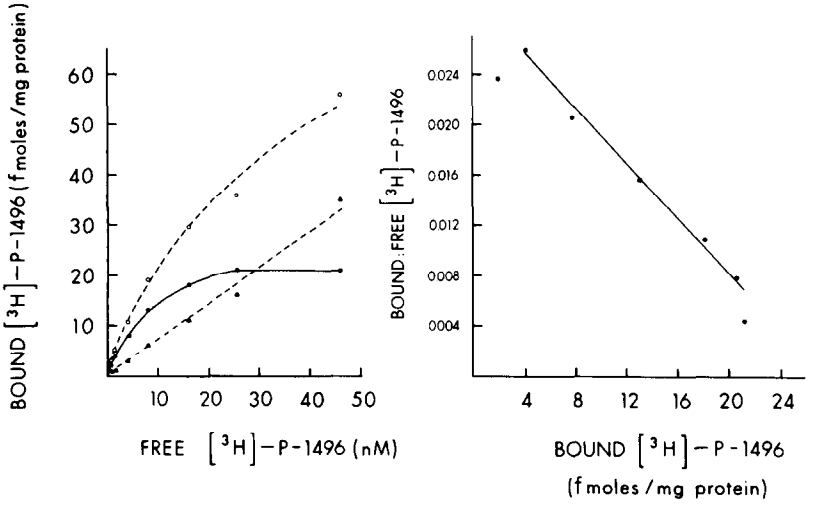

Fig. 9. Saturation analysis of [^{3}H]P-1496 binding in rat liver cytosol to sites distinct from the oestrogen receptor. Resuspended ammonium sulfate precipitate (6 ml) from female rat liver was preincubated (2 h at 0–4°C) with oestradiol-17β (1 μM, final concentration). Aliquots of this mixture were subsequently incubated (15–17 h at 0–4°C) with an equal volume (100 μl) of [^{3}H]P-1496 (1.6–93 nM) in the absence (○) or presence (▲) of 100-fold excess P-1496. Following treatment with DCC, bound radioactivity was measured. Values obtained for specific binding (●) were plotted according to Scatchard[33]. The protein concentration of cytosol was 19 mg/ml. These results are representative of two experiments.

structural requirements of the antioestrogen sites for the amine side chain [36].

The striking similarity between P-1496 and oestradiol-17β in their binding affinities to the oestrogen receptor coupled with qualitative differences in non-receptor sites prompted us to examine *in vivo* oestrogenic effects of P-1496 on the liver. The enhancement of serum triglycerides is a well recognized oestrogenic response [9, 13, 14, 17–21] and there is evidence to indicate that this effect is receptor mediated [9, 13]. The present investigation demonstrates elevations in serum triglycerides but doses of P-1496 required to produce this effect are approx 100-times greater than those of oestradiol-17β. Increases in serum triglycerides were associated with the VLDL fraction which is in agreement with previous studies on oestradiol-17β carried out in this laboratory [13, 21]. The relatively weak *in vivo* oestrogenicity of P-1496 has also been reported in the uterus [26]. Although a much greater amount of P-1496 compared to oestradiol-17β was required to elicit a specified increase in triglyceride levels, the time course of the changes was very similar to that obtained with oestradiol-17β [13]. Thus, significant changes in triglycerides were observed after 3–7 days of treatment. Assuming that the effect of P-1496 or oestradiol-17β on triglyceride levels is mediated through the oestrogen receptor, the weak responsiveness to P-1496 is quite intriguing. Although metabolic factors have not been examined, it is possible that they play an important part in this discrepancy. In addition, high affinity binding of P-1496 to protein components of rat liver other than receptor, such as those demonstrated in the present study may affect its *in vivo* availability to the oestrogen receptor.

Previous studies have compared triglyceride responsiveness to oestradiol-17β treatment in male and ovariectomized female rats [21]. Such comparison showed a remarkable sex difference in responsiveness with males requiring much higher doses of oestradiol-17β compared to the females. In the present investigation, a similar comparison using P-1496 showed no significant differences between males and females. Thompson and Lucier[21] have speculated that the sex difference in responsiveness to oestradiol-17β is due to either the abundant presence of HCLA sites in the male which could bind oestradiol-17β and diminish availability of ligand to the oestrogen receptor, and/or to a difference between males and females in the capacity to metabolize oestradiol-17β. Since P-1496 does not bind to the HCLA sites, our results would suggest that these sites play a major role in sexual dimorphism of hepatic responsiveness to oestradiol-17β although metabolic factors cannot be ruled out.

In summary, we have demonstrated that P-1496 can have both *in vivo* and *in vitro* oestrogenic effects on rat liver. However, the *in vivo* potency of P-1496 did not correlate with its *in vitro* high-affinity binding to the oestrogen receptor. It is possible that *in vivo* oestrogenicity is influenced by binding to other cytoplasmic components which are described in this paper. In particular, the presence of a second binding site to P-1496 and its role in hepatic responsiveness is currently under investigation.

Acknowledgements—We would like to thank Mr Zadock McCoy for technical assistance and Dr Beth Gladen for the analysis of Scatchard plots.

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