Nuclear oestrogen receptors in rat liver

Development of assay conditions, characterization and the translocation of oestrogens *in vivo*

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A method for the determination of specific oestrogen-receptor binding sites in rat liver nuclei is described. Nuclear receptors showed a high affinity for oestradiol ($K_d \approx 3 \times 10^{-9}$ M), a low capacity, and a distinct specificity for substances with known oestrogenic and anti-oestrogenic activity. No sex differences were seen in the concentrations of nuclear receptors from either vehicle- or ethynyloestradiol-pretreated rats. Only a limited number of binding sites could be extracted with 0.4 M-KCl. The remaining sites, which were solubilized by sonication and treatment with deoxyribonuclease I, sedimented at 3–4 S. Of four oestrogens tested (oestradiol, ethynyloestradiol, diethylstilboestrol, tri-*p*-anisylchloroethylene), ethynyloestradiol was the most effective translocation agent *in vivo*, nuclear uptake occurring at doses below 1 µg/rat; changes in salt extractability of nuclear receptors occurred at doses lower than those required to achieve absolute increases in nuclear receptor concentrations.

The fact that oestrogens can modify the synthesis and secretion of many hepatic proteins has been recognized for many years (Song *et al.*, 1969). Although Nasjletti & Masson (1972), using isolated oestrogen-perfused rat liver, were able to show that the synthesis of at least one such protein, renin substrate, was affected by direct oestrogen action on the liver, and although the presence of classical oestrogen receptors has subsequently been demonstrated in this organ (Chamness *et al.*, 1975; Viladiu *et al.*, 1975; Eisenfeld *et al.*, 1976), there is still considerable doubt as to whether other oestrogen effects are mediated in this manner. For instance, it is now generally accepted that the wide-ranging effects of oestrogens on hepatic drug and steroid metabolism are presumably not mediated by hepatic oestrogen receptors, but indirectly via changes in the secretion of pituitary hormones (Colby, 1980).

In order to gain further insight into which oestrogen actions on the liver are primary and which are secondary, we decided to conduct a thorough investigation into the amounts of nuclear oestrogen receptors under different endocrine conditions. In particular we were interested in determining whether certain dose-dependent effects on hepatic steroid metabolism correlate with demonstrable transloca-

tion of the receptors into the nucleus. In this paper we describe the development of the nuclear receptor assay, the receptor properties, and the dose-dependent translocation *in vivo* of various oestrogens.

Materials and methods

[2,4,6,7(n)-³H]Oestradiol-17β (sp. radioactivity 85–110 Ci/mmol) was supplied by Amersham-Buchler, Braunschweig, Germany. Dextran T70 was from Deutsche Pharmacia, Freiburg, Germany. Bovine serum albumin and γ-globulin were from Behringwerke, Marburg, Germany. Dexamethasone, diethylstilboestrol and ethynyloestradiol were purchased from Sigma, München, Germany. All other unlabelled commercially available steroids and analytical grade reagents were obtained from Merck, Darmstadt, Germany. All biochemicals were obtained either from Serva, Heidelberg, or Boehringer, Mannheim, Germany. Tamoxifen and monohydroxytamoxifen were generously supplied by Dr. A. H. Todd (ICI, Macclesfield, Cheshire, U.K.); tri-*p*-anisylchloroethylene, *E*- and *Z*-clomiphene citrate by Dr. W. L. Albrecht (Merrell-National Laboratories, Cincinnati, OH, U.S.A.); nafoxidine hydrochloride by Dr. P. W. O'Connell (Upjohn Co., Kalamazoo, MI, U.S.A.); nitromifene by Dr. R. Wolf (Parke-Davis, München, Germany); cyproterone

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acetate by Professor Dr. F. Neumann (Schering, Berlin, Germany); flutamide by Dr. R. Neri (Schering Corp., Bloomfield, NJ, U.S.A.); and 2-hydroxy-oestradiol and 2-methoxyoestrone by Professor Dr. R. Knuppen (Lübeck, Germany).

Animals

Specific-pathogen-free male and female Sprague-Dawley/NIH/HAN rats were obtained from the Zentralinstitut für Versuchstierkunde, Hannover, Germany. Groups of animals aged 60–120 days were maintained on a 12h-light/12h-dark schedule. Animals were fed on a standard pellet diet (Altromin 1320, Lage, Germany) and water *ad libitum*.

Preparation of liver nuclei

Purified cell nuclei were isolated from liver homogenates as described by Blobel & Potter (1966). Centrifugation was done at 100 000 *g* for 60 min at 3°C. The nuclei were resuspended and washed twice in STKM buffer [250 mM-sucrose/50 mM-Tris/HCl (pH 7.5)/25 mM-KCl/5 mM-MgCl₂] and centrifuged at 1600 *g* for 10 min. Nuclei were finally resuspended in 5 ml of STKM buffer containing 0.2% (final concn.) Triton X-100. Nuclei were counted with a haemocytometer.

Electron microscopy

Nuclei were prepared for electron microscopy as described by Tamulevicius *et al.* (1979).

Assay of specific oestrogen-binding sites in nuclei

Oestrogen-binding sites in nuclei were determined by an exchange assay adapted from Anderson *et al.* (1972). To optimize the various parameters of the assay system, preliminary studies were performed (see the Results section). Basically, samples of nuclear suspension were incubated at various temperatures and for various lengths of time with labelled oestradiol either alone or with a 100-fold excess of unlabelled oestradiol. At 4°C only occupation of previously unoccupied binding sites occurs, whereas at 37°C endogenous oestradiol bound to the receptor is exchanged with labelled oestradiol and occupation of available binding sites occurs with [³H]oestradiol. The use of excess unlabelled oestradiol measures non-specific binding, whereas labelled steroid alone measures total binding. The concentration specifically bound is calculated as the difference between total and non-specific binding. After incubation, as described under 'Standard assay conditions' (see the Results section), the nuclear samples were placed in an ice bath, after which unbound oestradiol was removed by washing the nuclei four times as follows. Nuclei were centrifuged at 1500 *g* for 10 min at 2°C and resuspended in 1 ml of STKM buffer containing 0.2% Triton X-100 and 0.2% gelatin. (In some experiments 1% bovine

serum albumin was originally used, but gelatin was found to be equally effective and much more economical.) After centrifugation the nuclei were twice resuspended and washed in 1 ml of STKM buffer/0.2% gelatin and finally resuspended in 1 ml of STKM buffer. Portions of the suspensions (0.9 ml) were transferred to conical counting vials (Milli 3PE; Baker Chemicals, Gross-Gerau, Germany) and sedimented down. Pellets were dissolved in 0.2 ml of Lumasolve overnight and counted for radioactivity in 2.5 ml of Lipoluma scintillation 'cocktail' (Baker Chemicals).

Salt extraction

For salt-extraction purposes, 0.5 ml of the washed nuclear suspension (1 ml) was treated as described under 'Standard assay conditions' (see the Results section) and measured directly. Further 0.4 ml samples were centrifuged at 1500 *g* for 10 min at 2°C and the nuclei resuspended in 0.4 ml of 10 mM-Tris/HCl (pH 7.5)/1 mM-dithioerythritol/0.5 mM-bacitracin (TDB buffer) at 4°C (Roy & McEwen, 1978). To each sample 0.4 ml of TDB buffer containing 0.8 M-KCl was added, mixed, incubated for 30 min at 4°C with shaking and then centrifuged at 25 000 *g* for 30 min at 4°C. The supernatant was discarded and the chromatin-bound residue extracted with 1 ml of ethanol overnight. The ethanol extract was decanted and the residue re-washed with 1 ml of ethanol. Both extracts were combined and counted for radioactivity.

Sedimentation analysis of salt-non-extractable binding protein

Nuclei from rats pretreated with ethynyloestradiol were prepared as described under 'Standard assay conditions' (see the Results section). Portions (1 ml) of the nuclear suspension were incubated with 0.8 ml of 52.5 nM-[³H]oestradiol alone or containing 5.25 μM-unlabelled oestradiol for 30 min at 37°C. The nuclei were centrifuged as described above, except that 2 ml of the respective buffers were used. After the final centrifugation, nuclei from four to six respective incubations were pooled, taken up in a small volume of STKM buffer and centrifuged at 1500 *g* at 2°C. The pellets were resuspended in 6 ml of TDB buffer, followed by the addition of 6 ml of TDB buffer/0.8 M-KCl. After standing for 30 min at 0°C, the chromatin fraction was obtained after centrifugation at 105 000 *g* for 30 min at 4°C. After the addition of 0.2 ml of TKM buffer, the pellet was sonicated (100 W; 5–10 s at 0°C) and mixed with 0.3 ml of TKM buffer containing 1.5 mg of deoxyribonuclease I (1500 units) and 0.2 ml of STKM buffer containing 52.5 nM-[³H]oestradiol alone or containing 5.25 μM-unlabelled oestradiol and incubated at 37°C for 30 min. The suspensions were then centrifuged at 25 000 *g* for 30 min at 4°C.

The supernatant was decanted into tubes containing pelleted dextran-coated charcoal (from 1 ml of 0.5% Norit A/0.05% Dextran T70 in STKM buffer) and vortex-mixed. The charcoal was removed after 2 min by centrifugation at 1500 *g* for 10 min. The charcoal treatment was repeated, after which 0.2 ml of the resulting supernatant was transferred to a linear 5–20% (w/v) sucrose gradient in TKM buffer. This was centrifuged at 149 000 *g* for 18 h at 4°C in a SW 50.1 rotor. Fractions (approx. 0.18 ml) were collected from the bottom of the tubes. Bovine serum albumin (1%) and γ -globulin (1%) were used as markers.

Translocation studies with oestradiol, ethynyl-oestradiol, diethylstilboestrol or tri-p-anisylchloroethylene: total specific receptor sites and chromatin-associated sites

Oestradiol, ethynyl-oestradiol, diethylstilboestrol or tri-*p*-anisylchloroethylene, at doses in the range 1–1000 μ g, were administered intraperitoneally 45 min before isolation of nuclei from male rats. Nuclei were treated as described for specific oestrogen binding-site determination and salt extraction (see under 'Standard assay conditions' in the Results section).

Competition studies

The liver nuclei from male rats treated with 100 μ g of ethynyl-oestradiol 45 min before investigation were used. All details were as described for nuclear-receptor concentration determination without KCl extraction, except that the labelled oestradiol was added in 50 μ l of STKM buffer and the competing ligands (molar ratios competitor: oestradiol = 0, 0.1, 1.0, 10, 100 and 1000) were given in a further 50 μ l of STKM buffer.

A log-logit linear transformation was performed (Rodbard *et al.*, 1969) and the relative 50% competition ratio (RCR 50; the ratio of the concentration of antagonist and oestradiol needed to decrease the binding of labelled oestradiol by 50%; see Blondeau *et al.*, 1978) calculated.

Statistical analysis

Statistical analyses were performed by using SAS Institute (Cary, NC, U.S.A.) programs for Duncan's multiple range test. The significance level was set at $P < 0.01$ unless otherwise stated.

Results

In order to establish 'Standard assay conditions', the following parameters were determined. (All experiments described in the first three sections were performed with nuclei from rats injected with 100 μ g of ethynyl-oestradiol 1 h before being killed.)

Effect of [³H]oestradiol concentration

Fig. 1 shows the effect of different [³H]oestradiol concentrations on the amount of bound oestradiol (fmol/incubation) in terms of total, specific and non-specific binding. For these experiments incubations were carried out at 37°C for 30 min. For routine measurements of specific nuclear receptor content in the 'Standard assay', a near-saturating concentration of 15 nM-[³H]oestradiol was used.

Effect of incubation time and temperature

At 4°C, no significant increase in the binding of oestradiol occurred with time of incubation (Fig. 2) for either male or female rat liver nuclei. The amount of oestradiol bound is presumably due to occupation of unoccupied receptor-binding sites. Increasing the temperature resulted in an enhanced uptake of oestradiol, leading to a maximal exchange after 3.5 h at 25°C or 30 min at 37°C in both sexes. In both cases no difference in the absolute amounts of bound oestradiol could be observed. A rapid exchange was also seen when the nuclei incubated at 4°C and 15°C were subsequently transferred to a 37°C water bath. Longer periods of incubation at 37°C led to a fall in specific binding capacity. For the 'Standard assay', an incubation time of 30 min and temperature of 37°C were chosen.

Effect of receptor concentration on specific binding of oestradiol

In both male and female rats, the specific binding of oestradiol increased linearly with the concen-

Fig. 1. Dependence of [³H]oestradiol binding on oestradiol concentration

Nuclei were incubated with various concentrations of [³H]oestradiol with or without a 100-fold excess of oestradiol at 37°C for 30 min. The amount of bound oestradiol (fmol/incubation) was measured as described in the Materials and methods section. $\blacktriangle$, Total binding; $\bullet$, specific binding; $\blacksquare$, non-specific binding.

Fig. 2. Time curves of specific oestrogen binding at various temperatures

Nuclei were obtained from ethynyloestradiol-treated male and female rats as described in the Materials and methods section and incubated with 15 nM-[³H]oestradiol alone and with a 100-fold excess of oestradiol for various lengths of time. The data are presented as percentage of the maximal uptake (9170 ± 1790 binding sites/nucleus).

tration of nuclei (i.e. specific oestradiol-binding protein) over a wide range, up to 10^7 nuclei/incubation. For the 'Standard assay' 0.25 ml portions of nuclear suspension containing approx. 5×10^6 nuclei were used.

Specifically bound oestradiol as a function of the time interval after injection of ethynyloestradiol

Both males and females were injected intraperitoneally with 100 µg of ethynyloestradiol and

nuclei were isolated at various times thereafter. The maximal amount of specifically bound oestradiol was always observed between 30 and 60 min after administration of the steroid for both males and females. After 1 h, the nuclear receptor content fell rapidly, to reach about 20% of the maximum uptake after 2–5 h. For the 'Standard assay', a 45 min interval after ethynyloestradiol administration was chosen.

Specifically bound oestradiol as a function of oestrogen dose

To determine the dose and type of oestrogen capable of translocating the maximal number of oestrogen-binding sites into the nucleus, four different oestrogens (oestradiol, ethynyloestradiol, diethylstilboestrol and tri-*p*-anisylchloroethylene) were administered 45 min before isolation of the nuclei. Ethynyloestradiol proved to be the most effective. Even doses as low as 1 µg significantly raised the concentration of specific binding sites (Table 1). In contrast, the other oestrogens tested were far less effective, the first significant increase occurring at doses greater than 10 µg.

Effect of Triton X-100 on liver nuclei

By including the non-ionic detergent Triton X-100 in the nuclear suspension, the number of measurable binding sites in the nuclei could be increased by 50%. This enhancement was due to an increase in the detection of specific binding sites only, non-

specific binding being unaffected by this treatment. Electron microscopy showed that Triton X-100 treatment almost completely detached the nuclear envelope. Despite the lack of a membranous system, the nuclei remained intact throughout the incubation and subsequent washes. Although some extrusion of the nuclear matrix was observed during the last washes, this was not associated with a loss of specific binding. Cytoplasmic contamination of the nuclei was determined by assaying lactate dehydrogenase as a marker enzyme, and was shown to be less than 0.003%.

Standard assay conditions

From the results obtained, the following procedure was developed. On the day of experiment, rats received intraperitoneal injections of either 100 µg of ethynyloestradiol in 0.1 ml of 0.9% NaCl/ethanol (1:1, v/v), or vehicle alone for the control groups. Animals were decapitated exactly 45 min later, their livers rapidly removed, minced with scissors, and placed in ice-cold 0.9% NaCl and processed as soon as possible. Samples (0.25 ml) of nuclear suspension containing approx. 5×10^6 nuclei were incubated at 4°C or 37°C for 30 min with 0.1 ml of 52.5 nM-[³H]oestradiol (final concn. 15 nM) with or without a 100-fold excess of unlabelled oestradiol. The subsequent procedures are as described under 'Assay of specific oestrogen-binding sites in nuclei'. With these standard conditions the following basic results were obtained (Table 2). No

Table 1. *Specifically bound oestradiol determined by exchange assay as a function of oestrogen dose*
Oestrogens were administered intraperitoneally 45 min before nuclear isolation. Results are means $\pm$ s.d. ($n = 6$).
Statistical significance: * $P < 0.05$; ** $P < 0.01$.

Oestrogen	No. of specific oestradiol-binding sites/nucleus							
	Dose (μg)	...	0	1	5	10	100	1000
Oestradiol			701 $\pm$ 388	705 $\pm$ 287	398 $\pm$ 96	427 $\pm$ 320	2471 $\pm$ 903**	4904 $\pm$ 587**
Ethynyloestradiol			317 $\pm$ 174	2457 $\pm$ 714*	4589 $\pm$ 1210**	5001 $\pm$ 1207**	8929 $\pm$ 2380**	7161 $\pm$ 1701**
Diethylstilboestrol			542 $\pm$ 259	563 $\pm$ 146		1952 $\pm$ 560	3715 $\pm$ 1612**	4903 $\pm$ 1815**
Tri- <i>p</i> -anisylchloroethylene			305 $\pm$ 99	866 $\pm$ 223	591 $\pm$ 231	1071 $\pm$ 303	1727 $\pm$ 639**	2322 $\pm$ 1177**

Table 2. *Sex differences in specific oestradiol binding in adult rats*

Liver nuclei from both male and female adult rats, either vehicle- or ethynyloestradiol-treated, were assayed for binding to total, unoccupied and total chromatin-associated sites as described in the Materials and methods section. Animals were 106–110 days old. Results are means $\pm$ s.d. ($n = 8$).

	No. of specific binding sites/nucleus	
	Male	Female
Vehicle-treated		
Total sites	721 $\pm$ 301	588 $\pm$ 136
Unoccupied sites	235 $\pm$ 99	169 $\pm$ 39
Total chromatin-associated sites	236 $\pm$ 26	234 $\pm$ 59
Ethynyloestradiol-treated		
Total sites	6680 $\pm$ 1686	5970 $\pm$ 1847
Unoccupied sites	325 $\pm$ 96	398 $\pm$ 191
Total chromatin-associated sites	5324 $\pm$ 218	4680 $\pm$ 220

sex differences in 106–110-day-old adult rats, either vehicle- or ethynyloestradiol-treated, could be seen in the binding to unoccupied sites or in the total binding. Although the vehicle-treated rats showed a higher salt extractability (approx. 70–80%) of the chromatin-bound activity compared with ethynyl-oestradiol-treated rats (approx. 30%), again no sex differences could be observed.

By using the conditions described for routine determinations, the intra-test variation (8%) was calculated as mean s.d. from 36 determinations. The inter-test variation (10%) was calculated by determining the specific binding sites in six independently prepared nuclear suspensions from the same animal. These data were derived from rats pretreated with ethynyloestradiol. The inter-test variation was not significantly affected by the choice of reference parameter (g of liver, nucleus, mg of nuclear protein or mg of DNA).

Dissociation constants

Liver nuclei from ethynyloestradiol-treated male and female rats incubated with seven different concentrations of labelled oestradiol ranging from 0.8 to 45 nM exhibited the same affinity for oestradiol. Scatchard plots of the specific binding data were linear and thus did not show more than one class of binding sites, and gave dissociation constants (K_d) of $3.43(\pm 1.39) \times 10^{-9}$ M and $3.38(\pm 1.12) \times 10^{-9}$ M for male and female rat liver binding sites respectively.

Ligand specificity

The specific oestrogen-binding sites in adult male rat liver nuclei show a very high specificity for molecules with known oestrogenicity (Table 3). With the exception of diethylstilboestrol and monohydroxytamoxifen, non-steroidal oestrogens and

antioestrogens showed significantly less affinity for the binding sites than did steroidal oestrogens. No binding was observed with testosterone, 5 α -dihydro-testosterone, methyltrienolone, flutamide, progesterone, promegestone, cyproterone acetate, corticosterone or dexamethasone.

Sedimentation analysis

Sedimentation analysis of the salt-non-extractable binding protein showed a single radioactive peak sedimenting between 3 and 4 S [mean value for both sexes ($n = 6$) = 3.3; range = 3.2–3.8]. The radioactivity was completely displaced by a 100-fold excess of unlabelled oestradiol.

Dose-dependent chromatin-associated specific oestrogen binding after administration of different oestrogens

By extracting the nuclei with 0.4 M-KCl, it is possible to distinguish between two different classes of binding sites (extractable and non-extractable). Those binding sites that are not solubilized are generally assumed to represent the physiologically functional receptors. The dose-dependent increase in the number of salt-non-extractable binding (chromatin-associated) sites that occurs after exogenous steroid administration (Table 4) does not correlate well with the dose-dependent change in total specific binding-site concentration (Table 1). Whereas a well-defined dose-dependent relationship between ethynyloestradiol dose and increase in specific binding sites can be demonstrated (Table 1), the lowest dose already (1 μ g) leads to maximum non-extractability. A similar lack of correlation may be observed for the other oestrogens as well, with changes in KCl-extractability either preceding (in terms of dose) or leading (in terms of quantitative effects) absolute changes in binding-site concentrations.

Table 3. Relative 50% competition ratios (RCR 50 values) for steroids and non-steroidal oestrogens and antioestrogens competing with oestradiol for nuclear specific oestrogen-binding sites

Nuclei were obtained from ethynyloestradiol-treated male rats and incubated with 15 nM-[^{3} H]oestradiol and unlabelled competitor at the following molar ratios: 0, 0.1, 1, 10, 100 and 1000. Each experiment was performed three times and the specific binding data were pooled before RCR 50 calculation. Data are given as means, with 95% confidence limits in parentheses.

Ethynyloestradiol	0.11 (0.05–0.20)
Oestradiol	1.00 (0.71–1.40)
Diethylstilboestrol	2.57 (0.91–7.27)
Monohydroxytamoxifen	7.39 (4.42–12.3)
2-Hydroxyoestradiol	18.3 (16.1–21.1)
Oestriol	24.4 (15.3–38.9)
Oestrone	92.6 (79.6–117)
Nitromifene	208 (177–272)
Nafoxidine	328 (267–404)
E-Clomiphene	332 (290–398)
Tamoxifen	513 (481–574)
5 α -Androstane-3 β ,17 β -diol	~4000
Tri- <i>p</i> -anisylchloroethylene	~5000
2-Methoxyoestrone, Z-clomiphene,	~10000
5 α -androstane-3 α ,17 β -diol	

Table 4. *Dose-dependent chromatin-associated specific oestrogen binding after administration of different oestrogens* Oestrogens were administered intraperitoneally 45 min before nuclear isolation. Data are expressed as absolute values, and in parentheses as percentage of total nuclear specific oestradiol binding (see Table 1). Results are means $\pm$ S.D. ($n = 6$). Statistical significance: * $P < 0.05$; ** $P < 0.01$.

Oestrogen	Dose (μg)	Specific oestradiol-binding sites/nucleus						
		...	0	1	5	10	100	1000
Oestradiol			323 $\pm$ 163 (46%)	294 $\pm$ 126 (42%)	229 $\pm$ 150 (58%)	268 $\pm$ 183 (63%)	1853 $\pm$ 603** (75%)	4444 $\pm$ 942** (91%)
Ethnyloestradiol			118 $\pm$ 60 (37%)	1938 $\pm$ 571** (79%)	4154 $\pm$ 1218** (91%)	3683 $\pm$ 866** (74%)	6602 $\pm$ 1679** (74%)	5590 $\pm$ 1205** (78%)
Diethylstilboestrol			172 $\pm$ 51 (32%)	197 $\pm$ 66 (35%)		1595 $\pm$ 510 (82%)	3492 $\pm$ 1728** (94%)	4450 $\pm$ 1960** (91%)
Tri- <i>p</i> -anisylchloroethylene			86 $\pm$ 32 (28%)	495 $\pm$ 257 (57%)	439 $\pm$ 176 (74%)	792 $\pm$ 327* (74%)	1510 $\pm$ 677** (87%)	1586 $\pm$ 731** (68%)

Discussion

The specific oestrogen-binding sites accumulating in the nucleus after administration of ethnyloestradiol show high affinity for oestradiol, a low capacity, and a distinct specificity for substances with known oestrogenic activity, and as such there can be little doubt that these binding sites represent putative oestrogen receptors translocated from the cytosol. Many of the properties of the receptor described in this study, including the dissociation constant and the specificity towards natural and synthetic oestrogens and other hormonal substances, are similar to those reported by other authors (Beers & Rosner, 1977; Chamness *et al.*, 1975; Eisenfeld *et al.*, 1976; Marr *et al.*, 1980; Powell-Jones *et al.*, 1980; Viladiu *et al.*, 1975). The binding specificity for triphenylethylene-type oestrogens and antioestrogens to the rat hepatic oestrogen receptor, which has previously received relatively little attention, was examined in detail in this study. We were able to confirm the relatively low binding affinity of nitromifene and nafoxidine (Chamness *et al.*, 1975), and show that two other antioestrogens, tamoxifen and *E*-clomiphene, have similar binding properties. *Z*-Clomiphene and tri-*p*-anisylchloroethylene are much less tightly bound, whereas the tamoxifen metabolite monohydroxytamoxifen is bound almost as well as oestradiol. The very-high-affinity binding of monohydroxytamoxifen has previously been demonstrated for oestrogen receptors obtained from calf and sheep uterus (Borgna & Rochefort, 1980).

As stated in the introduction, the reason for undertaking this investigation was to determine whether hepatic oestrogen receptors are involved either in the physiological sex differences seen in the activities of some steroid-hormone-metabolizing enzyme activities of the liver or in the changes in these activities after exogenous oestrogen administration.

The fact that no sex differences in the absolute concentrations of nuclear oestrogen receptors were found in vehicle-treated adult animals indicates that such receptors are unlikely to be involved in the maintenance of sex differences in hepatic enzyme activities and protein secretion under physiological conditions. Nor is it likely that these receptors are involved in oestradiol-induced 'feminization' of androgen-dependent activities in male animals, for such changes can be induced at oestradiol doses between 0.1 and 5 μg (Lax *et al.*, 1979), a dose too low to cause changes in the absolute concentrations of nuclear receptors. On the other hand, it has been demonstrated that administration of less easily metabolized oestrogens or higher doses of oestradiol do cause translocation of hepatic oestrogen receptors. Of the oestrogens tested for their translocating potential *in vivo*, ethnyloestradiol is by far the most efficient, causing significant increases at doses as low as 1 μg (approx. 3 $\mu\text{g}/\text{kg}$ body wt.). The differences in dose effectivity between ethnyloestradiol on the one hand and oestradiol and diethylstilboestrol on the other are much larger than can be explained in terms of affinity for the receptor (see Table 1), and presumably reflect differences in the extent of their hepatic metabolism. However, because the nuclear receptor concentrations were determined only at one fixed time interval (45 min after administration of the oestrogen), there must be some doubt about the low translocation potential of diethylstilboestrol and tri-*p*-anisylchloroethylene, since Kneifel & Katzenellenbogen (1981) have shown that non-steroidal oestrogens are translocated more slowly. This dose-dependent nuclear uptake of oestrogen receptors correlates well with that of the repression of hepatic 5 α -reductase activity (Lax *et al.*, 1979, 1981), an enzyme activity which is induced in males after administration of oestradiol doses insufficient to cause translocation. It may well be that the reaction of enzyme activities such as that of 5 α -reductase, which show biphasic dose

responses to oestrogen administration, represents antagonistic influences of oestrogens at the hepatic and central level.

As demonstrated by Powell-Jones *et al.* (1978), extraction of the nuclei with 0.4M-KCl resulted in only modest solubilization of the hepatic receptor sites. According to Clark *et al.* (1980), the KCl-non-extractable sites represent functional chromatin-bound (acceptor bound) receptors. The fact that ethynyloestradiol-treated rats show a different salt-extraction profile from that of vehicle-treated animals implies that ethynyloestradiol is binding to sites other than those binding steroid-receptor complexes entering the nuclei under physiological conditions. However, this phenomenon is not a unique property of ethynyloestradiol. When given in large enough doses, tri-*p*-anisylchloroethylene, diethylstilboestrol and even oestradiol decrease the effectiveness of salt extraction. Comparison of the data in Tables 1 and 4 reveals another interesting fact, namely that the decrease in salt extractability is not necessarily accompanied by a detectable increase in translocated cytoplasmic receptors. However, since it seems unlikely that the administration of an oestrogen could induce changes in the salt extractability of the nuclear receptors without entering the nucleus itself, it must be presumed that, provided that the oestrogen dose is low, some low-level translocation can occur without measurable accumulation of receptors in the nucleus.

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