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Interaction of the Anti-Oestrogen, Nafoxidine Hydrochloride, with the Soluble Nuclear Oestradiol-Binding Protein in Chick Liver

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Nafoxidine hydrochloride (Upjohn, 11100A) injected with oestradiol into immature chicks inhibits the hormone-induced increase in [^{3}H]oestradiol-binding activity in salt extracts of liver nuclei as well as the subsequent production by liver of egg-yolk phosphoprotein. Substantial inhibition of both oestradiol-induced responses is seen when nafoxidine is given in a dose approximately equimolar with that of oestradiol. *In vitro* nafoxidine competitively inhibits binding of [^{3}H]oestradiol in nuclear extracts. The K_i for the inhibition is 43 nM, which indicates an affinity of nafoxidine for the binding protein about 4% of that of oestradiol. The inhibitory action of nafoxidine *in vivo* thus is more potent than the relative binding affinity determined *in vitro* might indicate. One possible explanation is that the primary site of nafoxidine action is at a point proximal to nuclear receptor interaction. Nafoxidine injected alone into the chick does not induce phosphoprotein synthesis, but it does increase [^{3}H]oestradiol-binding activity in extracts of liver nuclei to a limited extent. No differences in the properties of the oestradiol-binding activity in extracts from nafoxidine-treated chicks or from oestradiol-treated chicks were detected. Chick liver cytosol does not contain detectable high-affinity oestradiol-binding activity. A low-affinity oestradiol-binding component with a sedimentation coefficient of 3.5S was found, but it was unaffected by treatment of chicks with either nafoxidine or oestradiol. The results suggest a difference in the mechanism of oestradiol action in the chick liver and in the widely studied rat uterus, on which the usual model for oestradiol action is largely based.

Non-steroidal anti-oestrogens have been useful probes in the investigation of the mechanism of action of oestradiol. Some of the earliest indications of the physiological relevance of oestradiol–cytosol receptor interaction came from experiments with anti-oestrogens (Jensen, 1966). These and other studies resulted in the current model for oestradiol action in the mammalian uterus, in which the hormone initially binds a specific high-affinity cytosol receptor, the complex undergoes a transformation from a 4S to a 5S species and migrates to the nucleus, where it interacts with specific acceptor sites in the chromatin and stimulates specific transcriptional events (Jensen *et al.*, 1974; O'Malley & Means, 1974).

The anti-oestrogen nafoxidine hydrochloride (1-{2-[*p*-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]ethyl}pyrrolidine hydrochloride; Upjohn 11100A) is a diphenyldihydronaphthalene derivative which has been widely used in mammalian systems (Jensen & DeSombre, 1973). It binds both the cytoplasmic and nuclear forms of oestradiol receptor in rat uterus (Rochefort *et al.*, 1972*a*). It exhibits both agonistic and antagonistic effects in rat uterus, and recent work suggests that a site of the antagonistic action is in the blockage or delay in replenishment of the cytoplasmic oestrogen receptor (Clark *et al.*, 1974; Katzenellenbogen & Ferguson, 1975; Capony & Rochefort, 1975).

In the chick liver, oestradiol induces the formation of the egg-yolk phosphoprotein precursor vitellogenin (Clemens, 1974; Deeley *et al.*, 1975). Particular interest in the mechanism of oestradiol action in this tissue derives from the fact that, with one exception (Arias & Warren, 1971), no groups have been able to demonstrate the presence of a typical high-affinity cytosol receptor for oestradiol (Mester & Baulieu, 1972; Ozon & Bellé, 1973; Lazier, 1975). They were, however, able to show specific high-affinity binding of oestradiol in salt extracts of liver nuclei. Such activity is also present in an insoluble nuclear residue (Lebeau *et al.*, 1974) and in chromatin (Gschwendt & Kittstein, 1974). Treatment of immature chicks or of roosters with oestradiol results in a substantial increase in each of these binding activities preceding the production of vitellogenin. Rooster liver cytosol contains only a low-affinity binding protein for oestradiol (Gschwendt, 1975*a*).

Studies on anti-oestrogen action in chick liver were thus undertaken in an attempt to clarify the apparent difference in the interaction of oestradiol in this tissue and in the rat uterus. Indeed, two reports show that nafoxidine is anti-oestrogenic in a single injection in the chick and is not itself oestrogenic (Gschwendt, 1975*b*; Lazier, 1975). This contrasts with the situation in the rat (Clark *et al.*, 1974). Gschwendt (1975*b*) noted the inhibitory action of

nafoxidine on the rooster liver oestradiol-binding sites in chromatin. Here we examine the effect of nafoxidine in the presence or absence of oestradiol on the salt-soluble nuclear oestradiol-binding activity. Preliminary abstracts dealing with some of the findings have been presented elsewhere (Lazier & Alford, 1975, 1976*b*).

Materials and Methods

Chemicals

[2,4,6,7-^{3}H]Oestradiol-17β(114Ci/mmol) was obtained from New England Nuclear Corp., Montreal, Quebec. Radiochemical purity, monitored by t.l.c., was greater than 98%. [^{14}C]Formaldehyde was from New England Nuclear Corp. Unlabelled steroids, bovine serum albumin and puromycin dihydrochloride were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Nafoxidine hydrochloride was a generous gift of the Upjohn Co., Kalamazoo, MI, U.S.A. The charcoal–dextran suspension was prepared from Norit A (Fisher Chemical Co., Fairlawn, NJ, U.S.A.) and Dextran T70 (Pharmacia, Montreal, Quebec, Canada).

Animals and injections

Male Cobb chicks (1–3 weeks old) were obtained from a local hatchery. Oestradiol, puromycin and nafoxidine were all dissolved in propylene glycol for injection (intraperitoneally) at concentrations of 25, 25 and 50mg/ml respectively. Doses were 2.5mg/100g body wt. for oestradiol, 2.5mg/100g for puromycin and 5.0mg/100g for nafoxidine, unless otherwise indicated.

Preparation of cell extracts

The crude nuclear pellet was prepared by centrifugation in sucrose-containing buffers (A and B) as described previously (Lazier, 1975). Nuclear extracts for sucrose-gradient centrifugation were prepared in the same buffers supplemented with thioglycerol (10mM) as recommended by Harrison & Toft (1975). This did not appear to affect the activity or stability of [^{3}H]oestradiol binding.

The washed crude nuclear pellets were homogenized in buffer C (0.5M-KCl/1.5mM-EDTA/10mM-Tris/HCl, pH7.4) (1g of tissue/ml). DNA in the nuclear homogenate was determined by the method of Burton (1970) and is expressed as μmol of nucleotide. The homogenate was frozen for at least 1h at −20°C, thawed and centrifuged at 37000*g* for 30min. The supernatant thus obtained is the nuclear extract. Washing the nuclear pellet with Triton X-100 solutions did not affect the yield of oestradiol-binding activity (per unit of DNA), indicating that the salt-soluble binder is actually located in the nuclei (Best-Belpomme *et al.*, 1975).

Cytosol was prepared by centrifugation of the initial 700*g* supernatant at 100000*g* for 1h.

Determination of [^{3}H]oestradiol binding

All cell extracts were preincubated with an equal volume of charcoal–dextran suspension (0.25% Norit A, 0.0025% dextran in 10mM-Tris/HCl/1.5mM-EDTA, pH7.4) for 30min at 37°C. Lazier (1975) showed that this treatment was necessary in extracts from oestradiol-treated chicks to expose specific sites for assay subsequently by incubation with [^{3}H]oestradiol at 2°C. Rochefort *et al.* (1972*a*) used the procedure to remove endogenous bound and unbound nafoxidine from uterine cell fractions. The nuclear oestradiol-binding protein of chick liver is stable throughout incubation at 37°C.

Incubation of the charcoal-treated extracts (0.6ml) with [^{3}H]oestradiol (0.25–2.5nM) was for 16h at 2°C. Unlabelled diethylstilboestrol (1000-fold excess) was included in parallel assay mixtures for determination of non-specific binding. After the incubation at 2°C, an equal volume of charcoal–dextran suspension (concentration as above) was added and after 30min the mixtures were centrifuged at 3000*g* for 10min. The supernatants were added to Aquasol (New England Nuclear Corp.) and the radioactivity corresponding to the bound [^{3}H]oestradiol was determined in a Nuclear–Chicago Isocap 300 liquid-scintillation spectrometer (efficiency approx. 35%). All data are given as specific [^{3}H]oestradiol binding, ascertained by subtracting the value for non-specific binding from that for total binding obtained on incubation with [^{3}H]oestradiol alone.

Preparation of serum and phosphoprotein P analysis

The procedures were exactly as described previously (Lazier, 1975).

Sucrose-gradient centrifugation

Sucrose (5–20%, w/v) gradients were prepared in 4.0ml polypropylene tubes (Martin & Ames, 1961). Stock solutions (0.5 and 28%, w/v) of sucrose in 10mM - Tris/HCl/1mM - EDTA/10mM - thioglycerol, pH7.5, were used to prepared low-salt gradients by using a Buchler gradient former. High-salt gradients were prepared by including 0.5M-KCl in both of the stock sucrose solutions. Cytosol (0.3ml), incubated for 30min at 2°C with 1nM-[^{3}H]oestradiol, was layered on the gradient, which was then centrifuged in a Spinco SW.56 rotor at 40000rev./min for 16h. Fractions (two drops each) were obtained by piercing the bottom of the tube. The fractions were added to Aquasol and radioactivity was determined as described above.

Sucrose gradients were also prepared from high- or low-salt stock sucrose solutions which contained [^{3}H]oestradiol (1 nM). Samples (0.2 ml) of nuclear extract were layered on the gradient, and centrifugation and collection of fractions were as described for conventional gradients. Bound [^{3}H]oestradiol in the fractions was determined by the charcoal-adsorption technique used by Harrison & Toft (1975). Charcoal–dextran suspension (0.4 ml) was added to each fraction, and after 1 min at 2°C the mixture was centrifuged at 3000*g* for 10 min. The charcoal–dextran suspension was more concentrated (0.5% charcoal, 0.05% dextran) than that used in other experiments reported here, and the exposure time to charcoal was briefer (1 min). However, preliminary experiments showed that the same amount of specific [^{3}H]oestradiol binding was measured by the two techniques.

Sedimentation values were determined by comparison with an internal ^{14}C-labelled bovine serum albumin marker (Martin & Ames, 1961) prepared by the method of Rice & Means (1971).

Results

Nafoxidine inhibition of oestradiol-induced [^{3}H]oestradiol binding and of phosphoprotein production

Table 1 shows the inhibitory effect of nafoxidine given intraperitoneally with oestradiol in several dose combinations on the hormone-induced increase in [^{3}H]oestradiol-binding activity in salt extracts of nuclei and on phosphoprotein production. The dose of oestradiol is similar to that found necessary for induction of phosphoprotein production by other investigators (Beuving & Gruber, 1971; Gschwendt & Kittstein, 1974; Deeley *et al.*, 1975). Table 1 (*a*) shows that nafoxidine given in a dose twice that of oestradiol (w/w, or 1.2:1 on a molar basis) almost completely inhibited both responses at the times examined. With the maximum effective dose of oestradiol (10 mg/100 g; Lazier, 1975), 50% inhibition of phosphoprotein production at 64 h was seen with an equal dose of nafoxidine (w/w, or a molar ratio of nafoxidine to oestradiol of 0.6:1) (Table 1*b*). This contrasts with the usual doses required in the uterus, where anti-oestrogen/hormone molar ratios of 25–50 are generally used (Clark *et al.*, 1974; Katzenellenbogen & Ferguson, 1975). Table 1 also shows that nafoxidine itself did not induce phosphoprotein synthesis.

Effect of nafoxidine in vitro on the nuclear [^{3}H]oestradiol-binding protein

Nafoxidine added *in vitro* to nuclear extract prepared from livers of oestradiol-treated chicks inhibited binding of [^{3}H]oestradiol in a competitive manner. Fig. 1 shows a double-reciprocal plot for specific [^{3}H]oestradiol binding in the absence and presence of three concentrations of nafoxidine. A plot of the slopes versus inhibitor concentration (Fig. 1 inset) yields a straight line, suggesting simple linear competitive inhibition. The K_I for nafoxidine was

Table 1. *Anti-oestrogenic action of nafoxidine: effect of time and dose*

Specific binding of [^{3}H]oestradiol (1 nM) in nuclear extracts of liver, and serum phosphoprotein P contents were assayed as described in the Materials and Methods section. Each group represents the mean ±S.E.M. for duplicate determinations on three or four individual chicks. The numbers in parentheses refer to the dose of oestradiol or nafoxidine (mg/100 g body wt).

Treatment and dose	Time (h)	[^{3}H]Oestradiol bound (fmol/μmol of DNA)	Serum phosphoprotein P (μg/ml)
(*a*)			
Oestradiol (2.5)	15	48.1 ± 14.0	—
Oestradiol (2.5)+nafoxidine (5.0)	15	8.2 ± 4.2	—
Oestradiol (2.5)	48	47.6 ± 9.9	32.6 ± 8.6
Oestradiol (2.5)+nafoxidine (5.0)	48	16.9 ± 3.9	~1.0
Oestradiol (10)	48	92.0 ± 11.1	51.8 ± 14.0
Oestradiol (10)+nafoxidine (20)	48	16.9 ± 4.0	2.0 ± 0.1
(*b*)			
Oestradiol (10)	24	61.1 ± 14.0	—
Oestradiol (10)+nafoxidine (10)	24	48.8 ± 18.1	—
Oestradiol (10)	48	92.0 ± 11.1	51.8 ± 14.0
Oestradiol (10)+nafoxidine (10)	48	15.3 ± 4.4	13.8 ± 7.0
Oestradiol (10)	64	59.5 ± 4.6	118.3 ± 4.3
Oestradiol (10)+nafoxidine (10)	64	17.7 ± 4.0	60.5 ± 7.0
(*c*)			
Vehicle control	48	9.2 ± 1.2	~1.0
Nafoxidine (5.0)	48	17.4 ± 2.8	~1.0
Nafoxidine (10.0)	64	—	~1.0

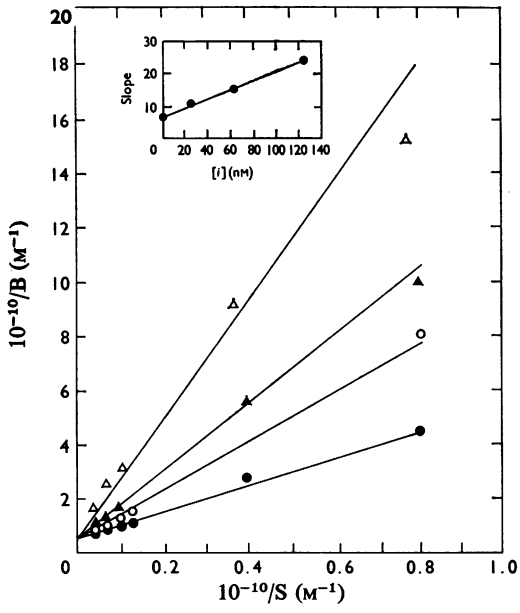

Fig. 1. *Double-reciprocal plot for nafoxidine inhibition of [^{3}H]oestradiol-binding activity in vitro*

Salt extract of liver nuclei was prepared 20h after injection of oestradiol. Specific binding of various concentrations of [^{3}H]oestradiol in the absence and presence of three concentrations of nafoxidine was determined as described in the Materials and Methods section. ●, [^{3}H]Oestradiol alone; ○, +25nM-nafoxidine; ▲, +62.5nM-nafoxidine; △, +125nM-nafoxidine. 1/B, Reciprocal of bound oestradiol; 1/S, reciprocal of [^{3}H]oestradiol added. Inset: a plot of the slopes from the double-reciprocal plot $\left[\frac{K_d}{n}\left(\frac{1+i}{K_i}\right)\right]$ versus inhibitor concentration (i).

43nM, reflecting an affinity of nafoxidine for the receptor which is about 4% that of oestradiol. This contrasts sharply with the inhibitory potency of nafoxidine *in vivo* (see Table 1).

Effects of nafoxidine alone: time-course for the increase in [^{3}H]oestradiol-binding activity and the effect of puromycin

As was apparent in Table 1 and has been shown earlier (Lazier, 1975; Gschwendt, 1975*b*), nafoxidine itself does not induce phosphoprotein production. However, given *in vivo* it does result in a small but significant increase in [^{3}H]oestradiol binding in liver nuclear extracts. Fig. 2 shows the extent of the increase in extracts prepared at various times after nafoxidine injection. A comparable dose of oestradiol gave a much earlier and more extensive increase in nuclear-binding activity (Lazier, 1975). The early

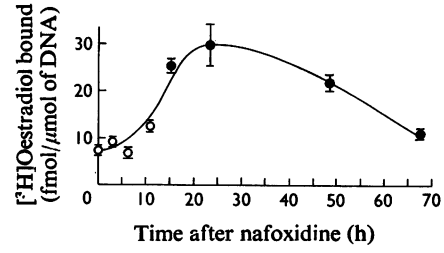

Fig. 2. *Time course of the increase in [^{3}H]oestradiol-binding activity in salt extracts of liver nuclei after nafoxidine injection*

Specific binding of 1nM-[^{3}H]oestradiol was measured as described in the Materials and Methods section. Each point represents the mean ±S.E.M. for extracts from at least three individual chicks, assayed in duplicate.

(1½h) increase in [^{3}H]oestradiol binding in nuclear extracts found after oestradiol injection was inhibited by cycloheximide and actinomycin D (Lazier, 1975). It was much more difficult to demonstrate an effect of translation or transcription inhibitors on the nafoxidine effect, because of the marked toxicity of these drugs in chicks (LeCount & Grey, 1972), and the relatively long exposure time required before a measurable nafoxidine response is seen. Cycloheximide given in the maximum non-lethal dose 1h before nafoxidine had a marginal effect on the increase in [^{3}H]oestradiol binding measured 16h after nafoxidine (results not shown). Puromycin was less toxic over the required 16h time-period. Table 2 shows that a single dose of puromycin 1h before nafoxidine partly inhibited the effect of nafoxidine. Two doses of puromycin, 1h before and 10h after nafoxidine, were inhibitory, but a single dose of puromycin 10h after nafoxidine was ineffective. Thus protein synthesis may be important early in the response of chick liver to nafoxidine.

Properties of the nafoxidine-increased [^{3}H]oestradiol-binding activity

It was important to establish whether or not the [^{3}H]oestradiol-binding activity increased by nafoxidine in nuclear extracts was the same activity as that increased by oestradiol itself. Scatchard (1949) plots of the specific [^{3}H]oestradiol-binding activity in nuclear extracts from nafoxidine-treated and oestradiol-treated chicks gave K_d values of 1.8nM and 1.7nM respectively. Somewhat lower values have been reported for the oestradiol-induced activity by several groups (Mester & Baulieu, 1972; Ozon & Bellé, 1973; Lazier, 1975). It is our experience, however, that liver preparations from different flocks of chicks may

Table 2. *Effect of puromycin on the nafoxidine-induced increase in nuclear [^{3}H]oestradiol-binding activity*
Puromycin was injected at the times shown relative to nafoxidine injection. All chicks were killed 15h after nafoxidine. Specific binding of [^{3}H]oestradiol (1nM) was determined in liver nuclear extracts as described in the Materials and Methods section. Data are expressed as mean values ±S.E.M. for duplicate determinations on three separate liver preparations.

Treatment	[^{3}H]Oestradiol-binding activity (fmol/μmol of DNA)
Nafoxidine	21.7±0.5
Nafoxidine+puromycin (−1h)	11.1±2.4
Nafoxidine+puromycin (−1h, +10h)	6.0±2.1
Nafoxidine+puromycin (+10h)	25.1±2.4
Control	4.2±2.0

Table 3. *Specificity of [^{3}H]oestradiol-binding activity in salt extracts of liver nuclei from oestradiol-treated and nafoxidine-treated chicks*
Binding of 1nM-[^{3}H]oestradiol was determined as outlined in the Materials and Methods section. The potential competitor was added to the assay mixture in the concentration indicated. The results are given as the mean±range for duplicate determinations.

Addition	Concentration	[^{3}H]Oestradiol-binding activity in liver nuclear extracts (c.p.m.)	
		Oestradiol-treated chicks	Nafoxidine-treated chicks
None	—	6185±129	2941±48
Diethylstilboestrol	1μM	1726±46	1628±24
Oestradiol-17β	1μM	1563±70	1672±18
Oestradiol-17β	10nM	2625±103	2047±10
Oestradiol-17α	1μM	1579±12	1585±88
Oestradiol-17α	10nM	3903±38	2377±57
Progesterone	1μM	6231±197	2855±50
Testosterone	1μM	6655±40	3112±37
Cortisol	1μM	6665±41	3114±48
Nafoxidine	100nM	2620±24	1890±11
Nafoxidine	10nM	3801±19	2325±32

yield values for the K_d for [^{3}H]oestradiol which vary from 0.5 to 2.0nM.

The specificity of the [^{3}H]oestradiol-binding activity in liver nuclear extracts from nafoxidine- and oestradiol-treated chicks is compared in Table 3. Progesterone, cortisol and testosterone had no effect in either case, but oestradiol-17β, oestradiol-17α, diethylstilboestrol and nafoxidine all suppress the binding of [^{3}H]oestradiol by both types of extract. Oestradiol-17α was less effective than oestradiol-17β at a concentration of 10nM. The partial competition by oestradiol-17α has been observed earlier (Mester & Baulieu, 1972). In addition, Gschwendt & Kittstein (1974) showed that injection of oestradiol-17α into roosters resulted in an increase in [^{3}H]oestradiol-17β binding by liver chromatin and in phosphoprotein production. Chan & Common (1974) have suggested that oestradiol-17α is a major phenolic steroid in hen plasma.

Neither the [^{3}H]oestradiol-binding activity in liver nuclear extracts from oestradiol-treated chicks nor that in extracts from nafoxidine-treated chicks was reproducibly stable on centrifugation in conventional sucrose gradients. However, centrifugation in gradients containing an even distribution of [^{3}H]oestradiol did give consistent results. Such gradients have been reported by Harrison & Toft (1975) to give superior resolution of the oestradiol receptor from chick oviduct cytosol when compared with conventional sucrose gradients. Fig. 3 shows that both types of extract in high-salt gradients exhibit specific [^{3}H]oestradiol-binding activity with a sedimentation coefficient of 4.5S. In low-salt gradients a loss of about 30% of the activity in the 4.5S peak was seen for extracts from oestradiol-treated and nafoxidine-treated chicks (Lazier & Alford, 1976*a*). No other binding species appear, in contrast with the typical aggregation of the 5S nuclear uterine oestradiol receptor to an 8–10S species in low-salt conditions (Alberga *et al.*, 1970).

The temperature-sensitivity of the [^{3}H]oestradiol-binding activity was compared in both types of extract (Fig. 4). Similar inactivation curves were seen for both cases, with a half-life of 5 min at about 55°C. The nuclear oestradiol-binding protein of chick liver is relatively temperature-stable compared with

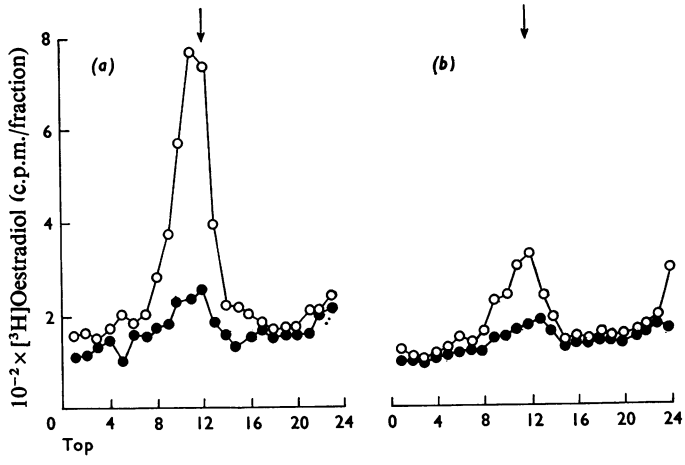

Fig. 3. *Sedimentation of the soluble nuclear [^{3}H]oestradiol-binding activity in sucrose gradients containing an even distribution of [^{3}H]oestradiol*

Nuclear extract (0.2ml) from oestradiol-treated (*a*) or nafoxidine-treated (*b*) chicks was applied to high-salt sucrose gradients containing an even distribution of 1nM-[^{3}H]oestradiol, as described in the Materials and Methods section. Chicks were injected 20h before death. ○, [^{3}H]Oestradiol; ●, [^{3}H]oestradiol in gradients run in the presence of 0.1 μM-diethylstilboestrol; arrow represents the internal ^{14}C-labelled bovine serum albumin marker.

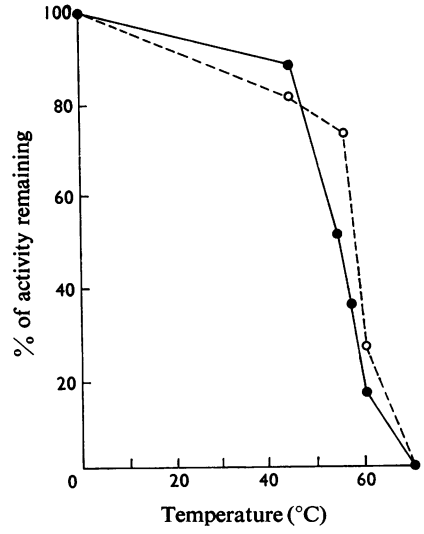

Fig. 4. *Temperature-sensitivity of [^{3}H]oestradiol-binding activity in nuclear extracts from oestradiol-treated or nafoxidine-treated chicks*

Extracts were preincubated for 5min at the indicated temperature, 0.6ml samples removed, cooled on ice and assayed in triplicate for specific binding of 2nM-[^{3}H]oestradiol as described in the Materials and Methods section. ●, Extract from oestradiol-treated chicks; ○, extract from nafoxidine-treated chicks. Chicks were injected 20h before death.

the nuclear receptor in uterus, where 50% of binding activity is lost after 5min at 45°C for receptor heated in the absence of oestradiol (Puca *et al.*, 1971).

Thus the high-affinity [^{3}H]oestradiol-binding activity in liver nuclear extracts from nafoxidine-treated chicks exhibits properties of [^{3}H]oestradiol affinity, sucrose-gradient sedimentation, hormone specificity and temperature sensitivity very similar to those for extracts from oestradiol-treated chicks.

Effect of nafoxidine on the low-affinity cytosol oestradiol-binding protein

Gschwendt (1975*a*) has characterized a low-affinity [^{3}H]oestradiol-binding protein in rooster liver cytosol which sedimented at about 4S in conventional sucrose gradients. We have confirmed his data for cytosol from immature chicks. On conventional sucrose gradients or gradients containing an even distribution of [^{3}H]oestradiol and an internal ^{14}C-labelled albumin marker, we find a single [^{3}H]oestradiol-binding species, sedimenting at 3.3–3.5S. Binding was not suppressed by inclusion of a 100-fold excess of diethylstilboestrol in the gradients. Fig. 5 shows conventional sucrose-gradient profiles for liver cytosol from control chicks and from chicks that had been given nafoxidine or oestradiol 20h previously. In rat uterus, exposure to nafoxidine for 23h results in marked depletion of the cytosol oestradiol receptor (Clark *et al.*, 1974). In the chick liver, however, injection of neither nafoxidine nor oestradiol had any

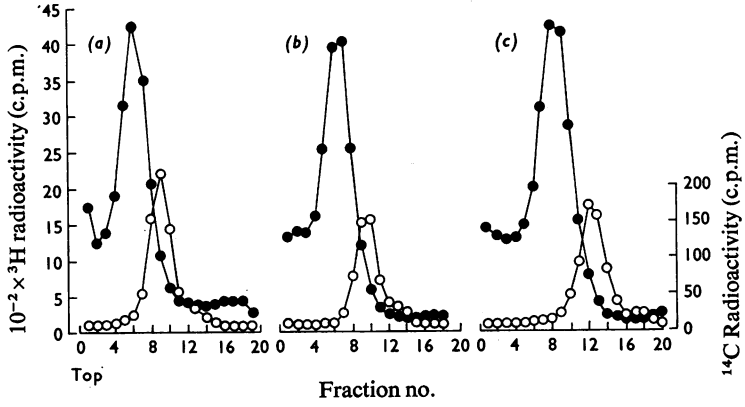

Fig. 5. *Sedimentation of cytosol on conventional sucrose gradients*
Cytosol was incubated for 30min at 2°C with 1nM-[^{3}H]oestradiol, and 0.3ml was applied to 5–20% sucrose gradients along with an internal ^{14}C-labelled albumin marker as described in the Materials and Methods section. (*a*) Liver cytosol from control chicks; (*b*) liver cytosol from oestradiol-treated chicks (20h); (*c*) liver cytosol from nafoxidine-treated chicks (20h). ●, [^{3}H]Oestradiol; ○, ^{14}C-labelled bovine serum albumin.

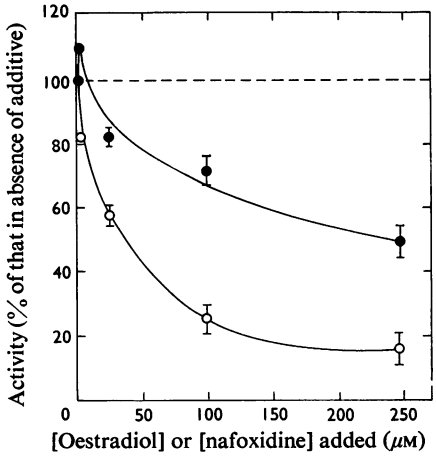

Fig. 6. *Competition of oestradiol and nafoxidine for [^{3}H]-oestradiol binding by cytosol*
Binding of 2nM-[^{3}H]oestradiol by cytosol (0.6ml) in the absence and presence of several concentrations of unlabelled oestradiol (○) or nafoxidine (●) was measured by the charcoal adsorption assay. Cytosol was prepared from 7-day-old chicks unexposed to exogenous oestradiol and was not preincubated with charcoal–dextran. Prior removal of endogenous steroids by charcoal–dextran incubation as described in the Materials and Methods section made no difference to the results. Each point represents the mean ±S.E.M. for triplicate determinations on three preparations.

apparent effect on the low-affinity oestradiol-binding protein of cytosol.

The effect of nafoxidine and of unlabelled oestradiol-17β on cytosol binding of [^{3}H]oestradiol was tested in a charcoal adsorption assay. Fig. 6 shows that very high concentrations of oestradiol-17β suppressed binding of 2nM-[^{3}H]oestradiol by cytosol. The concentration required to give 50% inhibition was 35μM. Nafoxidine was even less effective than oestradiol-17β; 250μM was required to give 50% inhibition.

Discussion

The inhibitory action of nafoxidine on oestradiol-induced vitellogenin synthesis by chick liver has been observed when nafoxidine is given with oestradiol (Lazier, 1975) or 30min before the hormone (Gschwendt, 1975*b*). We have taken serum phosphoprotein P content as a measure of vitellogenin synthesis, whereas Gschwendt (1975*b*) examined the vitellogenin protein by electrophoresis. Gschwendt (1975*b*) found that nafoxidine decreased the oestradiol stimulation of oestradiol-binding activity of isolated chromatin, and we show here that the anti-oestrogen also inhibits the oestradiol stimulation of the salt-soluble nuclear oestradiol-binding activity. Although the drug itself is not oestrogenic, its anti-oestrogenic potency is very marked, 50% inhibition of phosphoprotein synthesis being obtained by an anti-oestrogen/oestrogen molar ratio of about 0.6, by using the maximum effective dose of oestradiol. It is noteworthy that with this dose ratio, little inhibition of nuclear oestradiol-binding activity was seen at 24h, but at 48 and 64h the activity fell markedly. This suggests that nuclear receptor must be maintained in an adequate amount over a sustained period of time in order for a full phosphoprotein response to be obtained.

Gschwendt (1975*b*) reported that nafoxidine itself

stimulates the oestradiol-binding activity of rooster liver chromatin, paralleling our finding with the salt-soluble nuclear binding protein (Lazier, 1975). In an attempt to show whether or not nafoxidine influences the same binding protein as oestradiol, we examined several properties of the [^{3}H]oestradiol-binding activity in nuclear extracts from nafoxidine-treated and oestradiol-treated chicks. Both types of extract exhibit high-affinity binding of [^{3}H]oestradiol, similar hormone specificity, similar behaviour in high- and low-salt sucrose-gradient centrifugation and similar temperature-sensitivity. Although these techniques do not rule out subtle differences in the two oestradiol-binding activities, they show that the nafoxidine-induced activity is not grossly different from that induced by oestradiol. The explanation of the lack of oestrogenic activity of nafoxidine thus does not seem to lie in the production of markedly defective nuclear oestradiol receptor, but more likely in inadequate concentrations of the receptor being achieved over the required time period.

Lazier (1975) postulated that the increase in nuclear oestrogen receptor was an obligatory early action in the vitellogenic response in chick liver. The increase (at 1½h) was sensitive to actinomycin D and cycloheximide. Delaying the increase (with cycloheximide) resulted in a corresponding delay in phosphoprotein production. Both the extent of the phosphoprotein response and the increase in nuclear oestradiol-binding activity were proportional to the dose of oestradiol. Schneider & Gschwendt (1976) suggest that oestradiol treatment of roosters gives a rapid rise in soluble nuclear receptor (at 10min), followed by a decline and a second rise at 20min. The second rise was inhibited by cycloheximide. Joss *et al.* (1976) have demonstrated a very rapid rise in nuclear receptor, after injection of oestradiol via the portal vein, which was largely (but not completely) sensitive to cycloheximide. These experiments, and ours, agree in the important point that a stage dependent on protein synthesis is required to give the increase in nuclear oestrogen receptor, well before any vitellogenin production can be demonstrated by a very sensitive radio-isotopic technique (Bergink *et al.*, 1973). The mechanism for the increase in nuclear receptor could reflect synthesis of new receptor protein, or it could reflect an activation mechanism in which receptor was cleaved from a pre-receptor form by a rapidly turning-over peptidase. A small part of the increase may be accounted for by translocation of receptor from an unknown cell compartment.

Our data show that nafoxidine interferes with the oestradiol-induced increase in nuclear receptor content. The mechanism of nafoxidine inhibition appears to be more complex than solely direct blockade of the nuclear oestradiol-binding sites. This follows from our observation that the measured affinity of nafoxidine for the nuclear receptor *in vitro* is substantially less than that of oestradiol, whereas potent inhibition *in vivo* is obtained with equimolar doses of the drug and the hormone. The same discrepancy in the potency of nafoxidine *in vivo* and *in vitro* is apparent in the results of Gschwendt (1975*b*) for the effect of the drug on the oestradiol-binding sites in rooster liver chromatin. These observations can be explained in several ways. If the anti-oestrogenic action of the drug is in fact by competitive blockade of nuclear binding sites, it may be that much higher concentrations of nafoxidine relative to oestradiol are achieved in the liver cell through a difference in absorption or metabolism of the two compounds. It is also possible that nafoxidine is metabolized to a more potent inhibitor *in vivo*. An alternative explanation is that the primary site of nafoxidine action is not on the nuclear receptor itself, but at some point proximal to the nuclear receptor. Thus nafoxidine may interact with a pre-nuclear receptor present in some cell fraction other than the salt extract of nuclei, which has an affinity for nafoxidine equal to or greater than that of oestradiol. The nafoxidine–pre-nuclear receptor complex could not give the increase in nuclear receptor content as readily as can the oestradiol–pre-nuclear receptor complex. Nafoxidine itself therefore would give rise only to a limited amount of nuclear receptor but when given *in vivo* with oestradiol, the strong inhibitory effect of the drug would be obtained. A search for such a pre-nuclear receptor would be greatly facilitated by the use of radioactive nafoxidine, as would studies on the absorption, transport and metabolism of the drug. The competition studies reported here indicate that the putative pre-nuclear receptor is not present as a stable entity in cytosol. Another consideration is that nafoxidine could exert additional inhibitory mechanisms not related to the receptor system.

In rat uterus, the mediation of the oestradiol response by high-affinity cytosol receptor is widely recognized (Jensen *et al.*, 1974). The action of anti-oestrogens such as nafoxidine in depleting cytosol receptor has been shown by several groups (Rochefort *et al.*, 1972*a*, *b*; Clark *et al.*, 1974; Ruh & Ruh, 1974; Katzenellenbogen & Ferguson, 1975; Capony & Rochefort, 1975). Inhibition of replenishment may be the main focus of the anti-oestrogenic action. In studies in the rat on the inhibitory action of nafoxidine *in vivo* much higher doses of nafoxidine than of oestradiol are generally used (references cited above). Indeed Jensen *et al.* (1972) suggest that 100–1000-fold greater doses of nafoxidine than of oestradiol are required to see substantial inhibition *in vivo*. *In vitro* the affinity of nafoxidine for the uterine–cytosol oestrogen receptor is one-thirtieth that of oestradiol (Rochefort *et al.*, 1972*a*, *b*). Thus in the rat the anti-oestrogenic potency of nafoxidine *in vivo* more or less reflects the relative affinity of the drug for the cytosol receptor. In the chick, however, the drug is much more potent

than the affinity for the nuclear receptor *in vitro* might indicate. This difference in nafoxidine action, along with its lack of oestrogenic activity, and the absence of a typical cytosol receptor in chick liver underline a possible difference in the mechanism of oestradiol action in chick liver and the rat uterus. Sheridan (1975) has reviewed several other cases of steroid-hormone action in which saturable nuclear binding of hormone is seen in the absence of classical cytosol receptor. It is possible, however, that cytosol receptor has escaped detection owing to instability. If such an activity is eventually found in the chick the nafoxidine results could imply that replenishment of cytosol receptor is required in the course of a primary response to oestradiol. This does not appear to be the case in the rat (Clark *et al.*, 1974).

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