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Effects of Progesterone on Nuclear and Cytosol Steroid Receptor Levels in the Oestrogen-stimulated Uterus: Comparison of the Sheep and Mouse*

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Abstract

Methods for the measurement of nuclear receptors for oestradiol (E_2) and progesterone (P) in mouse uterus and sheep endometrium have been established. Scatchard analysis of nuclear receptors gave the following dissociation constants (nM) and binding site concentrations (pmol steroid/mg DNA): E_2 receptor, mouse 3·76 and 2·68, sheep 2·03 and 5·37; P receptor, mouse 5·77 and 2·52, sheep 5·34 and 3·58. The effects of a single injection of P on nuclear and cytosol levels of E_2 and P receptors have been contrasted in these tissues from E_2-treated mice and sheep. In both species, P treatment resulted, in 30–120 min, in depletion of its own cytosol receptor and accumulation of its nuclear receptor. A significant reduction of cytosol E_2 receptor was seen only in the mouse at 24 h. P decreased the nuclear E_2-receptor level in the mouse at 2–8 h, but had no such effect in the sheep. The results indicate that the anti-uterotrophic action of P in mouse uterus is caused by an early direct effect of P on nuclear E_2-receptor retention, and appear also to explain why P is not anti-uterotrophic in sheep uterus.

Introduction

Progesterone (P) does not decrease the weight of the uterus in ovariectomized ewes that have received daily oestradiol (E_2) treatments, whereas in E_2-stimulated laboratory rodents P is markedly anti-uterotrophic (Stone *et al.* 1978; Miller *et al.* 1979). It is well established that P blocks the replenishment of uterine cytosol E_2 receptor in E_2-treated rodents (Hsueh *et al.* 1976; Coulson and Pavlik 1977), but in the rat this action of P appears to occur too slowly (8–24 h) to account for the rapid effect of a single injection of P on uterine weight (Okulicz *et al.* 1981). In the E_2-stimulated ewe, P has a variable but sometimes marked effect on uterine cytosol E_2-receptor level, depending upon the doses of steroids used and the time intervals between steroid treatments and receptor measurement (Koligian and Stormshak 1977; Stone *et al.* 1978; Miller *et al.* 1979; Stone *et al.* 1979). These results in the sheep support the idea that anti-uterotrophic actions of P in rodents are not directly related to or a consequence of P effects on cytosol E_2-receptor replenishment (Koseki *et al.* 1977; Miller *et al.* 1979; De Sombre and Lyttle 1980; Okulicz *et al.* 1981).

Recently it has been shown that a single P treatment causes an early decrease in uterine nuclear E_2-receptor level in both hamsters and rats, this response being sufficiently prompt (2–4 h) to provide a possible explanation of the anti-uterotrophic

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action of P in these species. This early P action in the nucleus is dependent upon *de novo* RNA and protein synthesis and may represent a fundamental mechanism for P modulation of E_2 action (Evans *et al.* 1980; Evans and Leavitt 1980*a*, 1980*b*; Okulicz *et al.* 1981). In a previous comparative study, we described several effects of P on the E_2-stimulated uterus of the sheep and mouse (Miller *et al.* 1979; Stone *et al.* 1979). Uterine cytosol E_2- and P-receptor levels were determined at 12 or 24 h after a single P treatment or after the last of more than one P treatment. We now report studies carried out to establish procedures for measuring nuclear E_2 and P receptors in the uteri of sheep and mice. In order to further understand the possible relationships between P effects on uterine steroid receptor levels and uterine metabolic activity in these two species, we have examined in some detail the early time course of the effects of a single P injection on both the nuclear and cytosol levels of E_2 and P receptors.

Materials and Methods

Animals and Hormone Injections

Mature Merino ewes were purchased from commercial flocks and QS strain mice were obtained from the University's animal house. Animals were bilaterally ovariectomized via mid-ventral (sheep) or dorsolateral (mice) laparotomy under Nembutal (sheep) or tribromoethanol (mice) anaesthesia 4–8 weeks before experiments and received priming injections of 40 μg (sheep) or 1 μg (mice) of E_2 every 2 weeks, the last priming injection being given 12–14 days before day 1 of any experiment.

E_2 and P were dissolved in peanut oil and administered by separate injections subcutaneously (mice, 0·1 ml) or intramuscularly (sheep, 1·0 ml). In general, animals received treatment injections of E_2 (mouse, 0·1 μg; sheep, 40 μg) at about 0900 h on days 1 and 2 and at this time on day 3 were either killed or received further injections of the vehicle, E_2 alone, P alone (mouse, 1·0 mg; sheep 12 mg), or E_2 + P. The killing times in relation to hormone treatments are indicated in particular experiments. Mice were killed by cervical dislocation and sheep by bleeding and section of the cervical spinal cord.

Uteri were dissected from animals promptly after killing, weighed and placed in crushed ice. Mice tissues were processed essentially immediately, and sheep tissues were transported from Camden to Sydney with a maximum of 2 h between killing and initial homogenization. Unless specified, all procedures were carried out at 0–2°C. Sufficient mouse uteri were pooled to yield samples of 0·4–0·5 g per replicate. For sheep, all measurements were done on 0·5-g aliquots of caruncular endometrium only.

Chemicals and Buffers

[6,7-^{3}H]Oestradiol (specific activity 54 Ci/mmol) and [1,2-^{3}H]progesterone (specific activity 43 Ci/mmol) were obtained from the Radiochemical Centre (Amersham, U.K.), and [17*a*-methyl-^{3}H]promegesterone (R5020) (specific activity 87 Ci/mmol) and unlabelled R5020 were obtained from New England Nuclear Corp. (Boston, U.S.A.). All other chemicals were A.R. grade.

Aqueous samples were counted in toluene–Teric X–10 (2 : 1 v/v) containing 2·8 g of diphenyloxazole (PPO) and 70 mg of 1,4-bis(5-phenyloxazol-2-yl)benzene (POPOP) per litre whereas non-aqueous samples were counted in toluene containing 3 g of PPO and 100 mg of POPOP per litre. Buffer 1 contained 30 mM Tris-HCl, 1 mM EDTA, 12 mM monothioglycerol and 30% glycerol (v/v), pH 7·5, and was used in the initial homogenization for nuclear receptors; buffer 2 contained 10 mM Tris-HCl, 1 mM EDTA, 12 mM monothioglycerol, pH 7·5, and was used to dissolve steroids for nuclear receptor assay and for nuclear charcoal solutions; buffer 3 was buffer 1 plus 0·5 M KCl and was used for the extraction of nuclear receptor; buffer 4 was buffer 2 containing 10% glycerol (v/v) and was used for washing the nuclear pellet before extraction. All these buffers were prepared fresh the day before use and where specified were supplemented with 10 mM Na_2MoO_4 (Nielsen *et al.* 1977; Noma *et al.* 1980). Buffer 5 contained 0·25 M sucrose, 10 mM Tris-HCl, 1·5 mM EDTA, 5 mM NaN_3, 10 mM Na_2MoO_4, 10% glycerol (v/v), pH 7·4, and was used for tissue homogenization

in cytosol receptor assays; buffer 6 was buffer 5 without sucrose and was used for cytosol charcoal solutions and to dissolve steroids for cytosol receptor assay. Dextran/charcoal solutions contained 5 g of charcoal (Norit A; Mathesen, Coleman and Bell, Norwood, Ohio) and 0·5 g of dextran (T/70 Pharmacia, Uppsala, Sweden) per litre for nuclear assays and 5·5 g of charcoal and 0·55 g of dextran per litre for cytosol assays.

Cytosol Receptor Assay

All tissue was minced finely with scissors and in addition, sheep endometrium was pushed through a tissue press (pore size 0·5 mm). Aliquots (0·5 g) were homogenized in 12 vol. of buffer 5 using an Ultra-Turrax (Janke and Kunkel, Staufen, West Germany) homogenizer at 180 V using three 10-s bursts with 30 s between bursts. The homogenate was centrifuged for 10 min at 20 000 *g* and the pellet saved for DNA estimation. The final cytosol was prepared by centrifugation of the supernatant for 60 min at 105 000 *g*. Endogenous free or weakly bound steroid was removed with charcoal/dextran (10 : 1) for 10 min, removing the charcoal by centrifugation at 2000 *g* for 10 min. The levels of E_2 and P cytosol receptors were measured as described previously (Miller *et al.* 1977, 1979). In brief, for E_2 receptor, aliquots of cytosol were incubated with [^{3}H]E_2 (final concentration 10 nM) in the presence or absence of a 100-fold excess of unlabelled stilboestrol. Exchange with any endogenous bound steroid was effected at 30°C for 20 min. Free steroid was removed with charcoal as above and the receptor was measured following agar gel electrophoresis at low temperature (Wagner 1972). For the P receptor, aliquots were incubated with cortisol (final concentration 1 μM) for 30 min at 0°C to reduce interference by any corticosteroid-binding globulin. Incubations were continued for 18 h at 0°C with [^{3}H]R5020 (final concentration 10 nM) with and without a 100-fold excess of unlabelled R5020 to allow exchange with any endogenous bound steroid. Incubates were then treated with charcoal and analysed by agar gel electrophoresis as for the E_2 receptor.

Nuclear Receptor Assay

For both receptors, the *in vitro* exchange procedures of Chen and Leavitt (1979) and Evans *et al.* (1980) were essentially followed. Tissue was prepared as for the cytosol receptors and homogenized in 10 vol. of buffer 1. The homogenate was centrifuged at 800 *g* for 15 min and the 'nuclear pellet' fraction washed three times with 10 vol. of buffer 4, resuspending with a Teflon pestle. An aliquot of the final suspension was taken for DNA estimation. Nuclear receptor was extracted at 0°C from the final pellet using 7 vol. of buffer 3 and resuspending every 5 min for 1 h. The extract was collected after centrifugation at 20 000 *g* for 40 min. Nuclear receptor was measured in triplicate 300-μl aliquots of the extract, which were incubated with ^{3}H-labelled steroid (final concentration 10 nM) with or without a 100-fold excess of the appropriate unlabelled compound (stilboestrol, P or R5020) to give a total volume of 500 μl. For E_2 receptor, optimal conditions for exchange were found to be 60 min at 30°C (see Results). For P receptor, the method of Chen and Leavitt (1979) was basically followed and thus [^{3}H]P was used as the ligand in most experiments. Exchange was carried out for 18 h at 0°C as the receptor was heat labile. Free steroid was removed by a 1-min treatment with 500 μl of charcoal solution. For Scatchard analysis (Scatchard 1949), increasing concentrations of labelled steroid (0·625–20 nM) were used with and without a 100-fold excess of unlabelled compound.

Protein, DNA and Statistical Analysis

Protein in nuclear extracts and cytosols was measured by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard and making allowance for the differing Tris content of the buffers. DNA was measured using the diphenylamine method of Burton (1956), using calf thymus DNA as the standard. Any potential interference by sodium molybdate in these colorimetric assays was accounted for by its inclusion in the appropriate standards. Results are expressed as picomoles of steroid bound per milligram of DNA or per milligram of protein, and were analysed by Duncan's multiple range test (Steel and Torrie 1960) or by analysis of variance, where appropriate.

Results

Preliminary experiments were designed to optimize assay conditions, in particular the assay of receptors in mouse uteri. Initially, the effect of molybdate addition to

buffer 5, for preparation of mouse cytosol receptors, was examined. Animals were killed after 2 days of E_2 treatment and triplicate cytosol fractions, each from 0·5 g of pooled uterine tissue, were prepared. Supplementing the buffer with molybdate yielded a significant increase ($P<0·01$) in the amount of each receptor measured (E_2 receptor, $7·54\pm0·28$ *v.* $5·48\pm0·28$; P receptor, $5·49\pm0·26$ *v.* $3·69\pm0·17$). Accordingly, in subsequent experiments, buffer 5 supplemented with molybdate was used for the assay of cytosol receptors unless otherwise specified.

Table 1. Effect of different buffers used in the assay of cytosol and nuclear oestradiol and progesterone receptors in mouse uterus

Results are expressed as picomoles of steroid bound per milligram of DNA and are means ±s.e. of three tissue pools. ** Highest estimate for each parameter ($P<0·01$). For details of injection schedule see text

Buffer	Concn of cytosol receptor for:		Concn of nuclear receptor for:	
	Oestradiol	Progesterone	Oestradiol	Progesterone
5 $+Na_2MoO_4$	$7·58\pm0·56$**	$2·64\pm0·44$**	$1·076\pm0·028$	$1·120\pm0·042$
1 $+Na_2MoO_4$	$5·70\pm0·19$	$1·68\pm0·17$	$1·884\pm0·092$**	$0·722\pm0·024$
1 $-Na_2MoO_4$	$4·57\pm0·12$	$1·30\pm0·03$	$1·380\pm0·126$	$1·530\pm0·038$**

A further examination of buffer effects was studied in the experiment of Table 1. All animals received two days of E_2 treatment. On day 3 one group of animals received no steroid and was used to compare buffers 1 and 5 supplemented with molybdate and buffer 1 not supplemented in the assay of E_2 and P cytosol receptors. For each buffer, triplicate 0·5-g portions of pooled uteri were used. Two other groups of mice received either 0·1 μg of E_2 or 1 mg of P on day 3 and were killed 90 min later. Uteri from these allowed a comparison of the above buffers in the assay of the E_2 and P nuclear receptors. For each comparison, duplicate portions of 0·5 g of pooled uterine tissue were used. E_2 exchange was carried out at 30°C for 60 min.

Table 2. Effect of time and temperature of incubation on exchange of the salt-extracted nuclear oestradiol receptor

Results are expressed as mean ±s.e. of picomoles of steroid bound per milligram of DNA. Triplicate incubations are from three separate tissue pools. Means with the same superscript are not significantly different ($P>0·05$)

Time (min)	Concn of nuclear oestradiol receptor at:		
	25°C	30°C	37°C
15	$0·872\pm0·015^{a}$	$1·511\pm0·024^{b}$	$1·727\pm0·023^{c}$
30	$1·081\pm0·044^{a}$	$1·790\pm0·023^{c,d}$	$1·883\pm0·032^{d,e}$
60	$1·475\pm0·013^{b}$	$1·967\pm0·020^{e}$	$1·824\pm0·036^{c,d}$

For the cytosol receptors, buffer 5 and molybdate proved most satisfactory, whereas buffer 1 with molybdate and buffer 1 without molybdate were optimal for the E_2 and P nuclear receptors, respectively ($P<0·01$). In subsequent experiments, E_2 and P cytosol and E_2 nuclear receptors were measured in buffers containing molybdate and P nuclear receptors in buffers containing no molybdate.

The effect of time and temperature of incubation on the E_2 nuclear exchange reaction was examined in the experiment of Table 2. Animals received 0·1 μg of E_2 90 min before being killed and nuclear extracts were incubated at 25, 30 and 37°C for 15, 30 or 60 min. Exchange appeared to be incomplete at 25°C at all times and there was little difference between 30 and 37°C with either 30 or 60 min of incubation. In a further study (not shown), no further exchange was observed when incubations were continued for 2 h at 30°C. In subsequent experiments, all E_2 nuclear exchange was carried out at 30°C for 60 min.

Table 3. Relative specific binding of [^{3}H]progesterone ([^{3}H]P) and [^{3}H]R5020 ([^{3}H]R) by the mouse uterine nuclear progesterone receptor and the effect of cortisol (C)

Results are expressed as mean ±s.e. of femtomoles of steroid remaining after dextran/charcoal treatment of 300 μl of nuclear extract, and are derived from three separate tissue pools from both P-injected and uninjected animals

Incubation conditions	Progesterone injected		No progestetone injected	
	Total specific binding	Difference from control	Total specific binding	Difference ftom control
Control 1[A]	329·9±17·2	—	110·9±3·0	—
[^{3}H]P+C	323·6±15·9	6·3	116·4±4·6	−5·5
[^{3}H]P+P	104·6±9·3	225·3	56·3±1·2	54·6
[^{3}H]P+P+C	113·7±8·2	216·2	63·0±0·5	47·9
Control 2[B]	406·1±38·5	—	167·0±3·5	—
[^{3}H]R+R	225·6±15·8	180·5	115·0±2·4	52·0
[^{3}H]R+R+C	242·6±16·2	163·5	122·0±2·6	45·0

[A] [^{3}H]P only. [B] [^{3}H]R only.

To examine the relative merits of using [^{3}H]P or [^{3}H]R5020 for the exchange assay of the nuclear P receptor, and to examine possible interference from any contaminating corticosteroid-binding globulin in the nuclear extracts, the experiment of Table 3 was carried out. Mice received either the vehicle or 1 mg of P on day 3 and were killed 90 min later. For both labelled steroids, incubations of the salt-extracted nuclear fraction were continued for 18 h at 0°C in the presence or absence of a 100-fold excess of the corresponding non-labelled compound. Cortisol was included in some incubations. Results are expressed as femtomoles of steroid remaining per incubate after charcoal treatment.

In no case did cortisol significantly depress binding, and it was thus not included in subsequent assays of nuclear P receptor. Using [^{3}H]R5020, there was a slight decrease in specific binding (as measured by the difference between total binding and binding obtained in presence of an excess of the unlabelled compound), an increase in non-specific binding and an increased variability. Accordingly, [^{3}H]P was used in later experiments.

Scatchard analyses of the mouse E_2 and P nuclear receptors were carried out as described in Materials and Methods and are shown in Fig. 1*a*. Animals were killed 2 h after an injection of 0·1 μg of E_2 or 1 mg of P on day 3. Dissociation constants (K_d) and binding site concentrations (pmol/mg DNA) for the E_2 and P receptors were 3·76 nM and 2·68, and 5·77 nM and 2·52, respectively.

To show that depletion of cytosol receptor is accompanied by an increase in nuclear receptor level, the experiments of Tables 4 and 5 were carried out. Animals

were killed 0, 0·5, 1, 2, 4 or 8 h after receiving 0·1 μg of E_2 (Table 4) or 1 mg of P (Table 5) on day 3. E_2 and P cytosol receptor levels and E_2 or P nuclear receptor levels were measured, using three tissue pools per replicate. By 0·5 h following E_2 injection, there was a significant rise in nuclear E_2-receptor level, which reached a

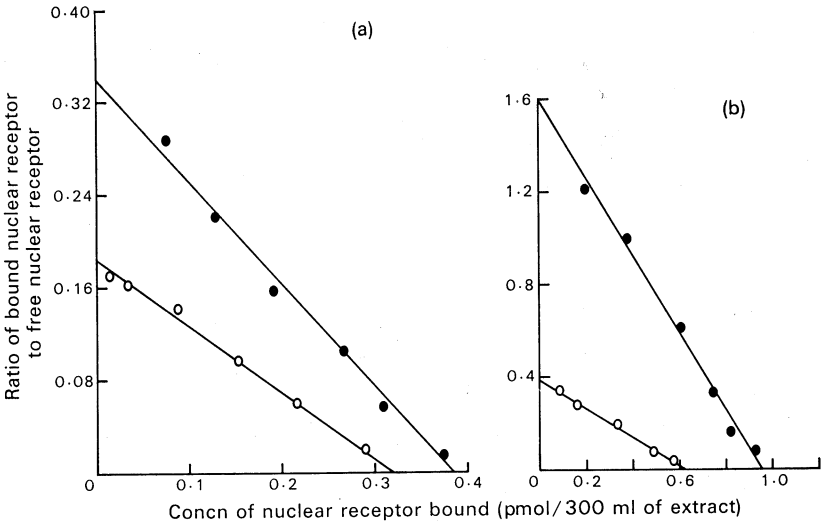


Fig. 1. Scatchard analysis of the nuclear oestradiol (E_2, ●) and progesterone (P, ○) receptors of mouse uterus (*a*) and sheep caruncular endometrium (*b*). Calculated dissociation constants (nM) and binding site concentrations (pmol steroid/mg DNA) are:

Tissue	Dissociation constant		Binding site concn	
	E_2	P	E_2	P
Mouse	3·76	5·77	2·68	2·52
Sheep	2·03	5·34	5·37	3·58

Table 4. Effect of a single injection of oestradiol on the cytosol oestradiol and progesterone and nuclear oestradiol receptor concentrations in mouse uterus

Results are expressed as mean ± s.e. of picomoles of steroid bound per milligram of DNA. Three replicates per group. Within each parameter, means with the same superscript are not significantly different ($P > 0·05$)

Time (h)	Concn of cytosol receptor for: Oestradiol	Progesterone	Concn of nuclear progesterone receptor
0	$6·28 \pm 0·40^{c}$	$4·43 \pm 0·22^{a,b}$	$0·54 \pm 0·10^{a}$
0·5	$6·71 \pm 0·39^{c}$	$4·55 \pm 0·40^{a,b}$	$1·36 \pm 0·18^{b}$
1	$4·42 \pm 0·18^{b}$	$4·18 \pm 0·17^{a}$	$2·88 \pm 0·09^{d}$
2	$2·81 \pm 0·24^{a}$	$4·25 \pm 0·26^{a}$	$2·38 \pm 0·10^{c,d}$
4	$1·60 \pm 0·15^{a}$	$5·67 \pm 0·19^{b,c}$	$2·11 \pm 0·15^{c}$
8	$2·84 \pm 0·27^{a}$	$6·70 \pm 0·42^{c}$	$1·19 \pm 0·17^{b}$

maximum 1–2 h after injection and was still significantly greater than the control value at 8 h. Cytosol E_2-receptor level was lowest between 2 and 8 h, and cytosol P-receptor level showed a significant increase by 8 h.

Following the injection of P, there was a significant rise in nuclear P-receptor level by 0·5 h and this also reached peak levels around 1–2 h after injection. By

8 h, the level was still significantly greater than the control value. There was no change in E_2 cytosol receptor level over 8 h, and P cytosol receptor level fell by 0·5 h and then continued to decline slowly.

Table 5. Effect of a single injection of progesterone on the cytosol oestradiol and progesterone and nuclear progesterone receptor concentrations in mouse uterus

Results are expressed as mean ± s.e. of picomoles of steroid bound per milligram of DNA. Three replicates per group. Within each parameter, means with the same superscript are not significantly different ($P > 0{\cdot}05$)

Time (h)	Concn of cytosol receptor for: Oestradiol	Progesterone	Concn of nuclear progesterone receptor
0	$9{\cdot}60 \pm 0{\cdot}18^a$	$4{\cdot}80 \pm 0{\cdot}08^d$	$0{\cdot}027 \pm 0{\cdot}010^a$
0·5	$9{\cdot}09 \pm 0{\cdot}31^a$	$1{\cdot}76 \pm 0{\cdot}08^c$	$0{\cdot}227 \pm 0{\cdot}010^b$
1	$8{\cdot}06 \pm 0{\cdot}13^a$	$1{\cdot}45 \pm 0{\cdot}15^b$	$0{\cdot}386 \pm 0{\cdot}044^c$
2	$9{\cdot}46 \pm 0{\cdot}27^a$	$1{\cdot}41 \pm 0{\cdot}06^b$	$0{\cdot}477 \pm 0{\cdot}014^c$
4	$7{\cdot}63 \pm 1{\cdot}10^a$	$1{\cdot}25 \pm 0{\cdot}09^b$	$0{\cdot}217 \pm 0{\cdot}045^b$
8	$8{\cdot}33 \pm 1{\cdot}89^a$	$0{\cdot}87 \pm 0{\cdot}02^a$	$0{\cdot}188 \pm 0{\cdot}001^b$

To examine conditions for the assay of E_2 and P cytosol and nuclear receptors in sheep caruncular endometrium, three ewes were treated with E_2 on days 1 and 2. On day 3, one animal was killed as a control with no further steroid treatment; the others received E_2 or P and were killed 2 h later. Cytosol receptor level for E_2 and P was measured in each animal to examine the effect of the inclusion of molybdate or to check that receptor depletion had occurred following steroid treatment. For

Table 6. Effect of molybdate addition and the injection of oestradiol or progesterone on the measured levels of cytosol oestradiol and progesterone receptors in caruncular endometrium of sheep

Results are expressed as picomoles of steroid bound per milligram of DNA. Two replicates per group. Significance of difference from control ($+Na_2MoO_4$): * $0{\cdot}05 > P > 0{\cdot}01$; ** $0{\cdot}01 > P > 0{\cdot}001$

Treatment	Addition of Na_2MoO_4	Concn of oestradiol receptor	Concn of progesterone receptor
Control	−	9·35**	3·24*
	+	13·58	4·73
Oestradiol	+	8·04**	4·77
Progesterone	+	12·12	2·03**

the nuclear E_2 receptor, the effect of molybdate addition to the buffer and varying the time and temperature of exchange were measured. For the nuclear P receptor, the effect of molybdate together with the effect of cortisol addition were examined. For both nuclear receptors, Scatchard plots were carried out as described in Materials and Methods. In the case of P, only a five-point assay was possible because of insufficient tissue.

Table 6 shows the effect of molybdate addition in the assay of both cytosol receptors. As observed for the mouse, higher levels of both receptors were measured in the presence of molybdate and this was included in subsequent cytosol assays in sheep tissue. An unexplained high level of cytosol E_2, but not P, receptor was

measured in this experiment. The decline in E_2 cytosol receptor level after E_2 injection was accompanied by an increase in nuclear E_2 receptor concentration (Table 7). Again, addition of molybdate had a beneficial effect. Incubation for 60 min at 30°C was chosen for subsequent assays. With the nuclear P receptor (Table 8), inclusion of cortisol was without significant effect and molybdate proved deleterious and was omitted in subsequent assays of this parameter.

Table 7. Effect of molybdate addition and variation of the time and temperature of exchange in the assay of sheep nuclear oestradiol receptor

Results are expressed as picomoles of steroid bound per milligram of DNA. Incubations in triplicate. Means with the same superscript are not significantly different ($P>0\cdot05$)

Treatment	Addition of Na_2MoO_4	Temp. (°C)	Time (min)	Concn of oestradiol receptor
Control	+	30	60	$1\cdot393\pm0\cdot022^a$
Oestradiol	−	30	60	$3\cdot623\pm0\cdot046^b$
Oestradiol	+	30	30	$4\cdot100\pm0\cdot089^c$
Oestradiol	+	30	60	$4\cdot940\pm0\cdot140^d$
Oestradiol	+	30	90	$5\cdot237\pm0\cdot123^d$
Oestradiol	+	37	60	$5\cdot024\pm0\cdot126^d$

Scatchard analyses (Fig. 1*b*) yielded dissociation constants and binding site concentrations (pmol/mg DNA) of 2·03 nM and 5·37, and 5·34 nM and 3·58 for the E_2 and P nuclear receptors, respectively.

Table 8. Effect of molybdate and cortisol addition in the assay of sheep nuclear progesterone receptor

Results are expressed as picomoles of steroid bound per milligram of DNA. Incubations in triplicate. Means with the same superscript are not significantly different ($P>0\cdot05$)

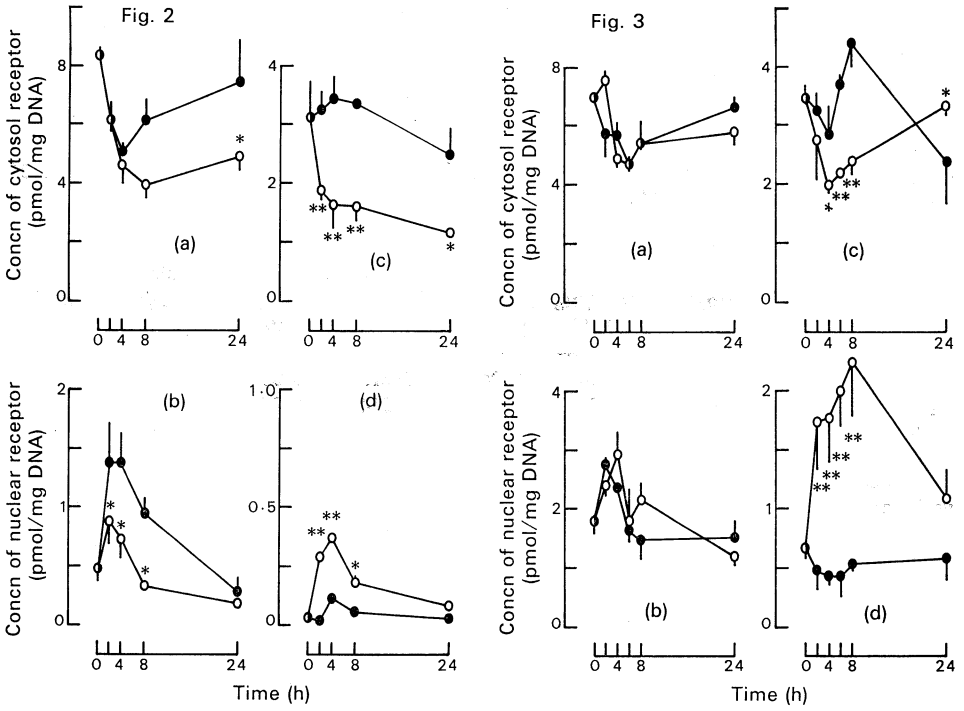
Treatment	Addition of Na_2MoO_4	Addition of cortisol	Concn of progesterone receptor
Control	−	−	$0\cdot334\pm0\cdot038^a$
Progesterone	−	−	$2\cdot711\pm0\cdot129^c$
Progesterone	−	+	$2\cdot683\pm0\cdot098^c$
Progesterone	+	−	$1\cdot486\pm0\cdot017^b$

In an attempt to explain whether the anti-oestrogenic activity of P in the mouse uterus and the lack of such activity in the ewe might be explained by species differences in the effect of P on nuclear E_2-receptor retention, the experiments of Figs 2 and 3 were carried out. Animals received E_2 on days 1 and 2 and were killed 0, 2, 4, 6 (sheep only), 8 or 24 h after an injection of E_2 or E_2+P on day 3. The level of cytosol and nuclear receptors for E_2 and P were measured.

In the mouse (Fig. 2) those animals injected with E_2 alone showed, by 2 h, a significant rise ($P<0\cdot01$) in the level of nuclear E_2 receptor and a corresponding decrease in cytosol E_2-receptor level. The inclusion of progesterone resulted in a significant depression in nuclear E_2-receptor level for at least 8 h ($P<0\cdot05$), with no effect on

the level of cytosol E_2 receptor until 24 h. As expected, in these animals progesterone injection caused an increase in nuclear P-receptor level and a decrease in cytosol P-receptor level.

In the ewe (Fig. 3), E_2 treatment again caused a significant increase ($P<0{\cdot}05$) in nuclear E_2-receptor level by 2–4 h but progesterone was without significant effect on this parameter at all times. The level of nuclear and cytosol P receptor rose and fell, respectively, as expected following P injection.



Figs 2 and 3. Effect of a single injection of oestradiol (E_2, ●) or oestradiol plus progesterone (E_2+P, ○) on the level of cytosol (*a*) and nuclear (*b*) receptors for E_2, and cytosol (*c*) and nuclear (*d*) receptors for P in mouse uterus (*2*) and in sheep caruncular endometrium (*3*). Three replicates per group. Results are expressed as picomoles of steroid bound per milligram of DNA. Vertical bars indicate 1 s.e. Standard errors not shown fall within the points as drawn. E_2+P *v.* E only, ** $P<0{\cdot}01$; * $0{\cdot}01 < P < 0{\cdot}05$.

It was of interest to see if the ability of P to reduce nuclear E_2-receptor level in the mouse uterus required an elevated level of cytosol P receptor and its translocation to the nucleus. It was felt that a possible model to answer this question might be the uterus of the untreated animal where cytosol E_2-receptor level is adequate for a response to E_2 but cytosol P-receptor level is very low.

In the experiment of Table 9, E_2 and P cytosol and E_2 nuclear receptor levels were measured in untreated control animals, and 4 h after a single injection of E_2 ($0{\cdot}1$ μg) or E_2+P (1 mg). Because of the rather large mass of tissue required for this study, cytosol and nuclear receptors were measured from the same tissue pools using the nuclear receptor buffers. Three tissue pools, each of $0{\cdot}4$ g, were used for each tissue group.

E_2 alone significantly increased uterine weight at 4 h, an effect unaltered by progesterone. As expected, E_2 treatment reduced cytosol E_2-receptor level and again the small additional effect of P was not significant. Notwithstanding a variation in the significance of the actions of P, the apparent effects were of a similar order for each parameter, except for uterine weight. The percentage effect of P on nuclear E_2-receptor level was much smaller than in Fig. 3.

Table 9. Effect of a single injection of oestradiol (E_2, 0·1 μg) or oestradiol plus progesterone (E_2+P, 1 mg) on uterine weight and on the levels of cytosol E_2 and P receptors and nuclear E_2 receptor in the untreated mouse uterus

Animals were killed 4 h after treatment with the vehicle or steroid. Receptor results are expressed as picomoles of steroid bound per milligram of DNA, or per milligram of cytosol protein (cytosol receptors), or per milligram of DNA, or per milligram of nuclear extract protein (nuclear receptor). Three tissue pools per replicate. Summary of the analyses of variance of the uterine weight and receptor concentrations is given in the second half of the table. * $0·05 > P > 0·01$; ** $0·01 > P > 0·001$; *** $0·001 > P$

Treatment		Uterine weight (mg)	Concentration of cytosol receptor for: Oestradiol (pmol/mg DNA)	Oestradiol (pmol/mg protein)	Progesterone (pmol/mg DNA)	Progesterone (pmol/mg protein)	Concn of nuclear oestradiol receptor (pmol/mg DNA)	Concn of nuclear oestradiol receptor (pmol/mg protein)
Control		29·0	2·504	0·940	0·218	0·081	0·171	0·169
E_2		40·8	0·586	0·174	0·214	0·064	0·781	0·681
E_2+P		39·0	0·460	0·121	0·184	0·048	0·634	0·487
Source of variation	d.f.	Mean square values for above parameters						
E_2 *v.* E_2+P	1	0·83	0·023	0·004	0·001	0·0004	0·032**	0·057***
Control *v.* E_2+(E_2+P)	1	39·20***	7·838***	1·255***	0·001	0·0012*	0·575***	0·344***
Error	6	6·08	0·040	0·006	0·003	0·0002	0·002	0·001

Discussion

Molybdate, which has been reported to stabilize and prevent activation of cytosol receptors for the sex and other steroids (Noma *et al.* 1980), was also effective in this study in increasing the binding of E_2 and P by cytosol receptors of sheep and mouse uteri. In addition, molybdate appeared to have a stabilizing effect on the salt-extracted nuclear E_2, but not P, receptor in the uteri of both species. This effect may explain the apparent better tolerance of the nuclear E_2 receptors to *in vitro* exchange at 37°C than that observed by Korach (1979) and Evans *et al.* (1980). Otherwise, the *in vitro* characteristics of the two nuclear receptors were generally in agreement with data published for these and other species (Zava *et al.* 1976; Koligian and Stormshak 1977; Chen and Leavitt 1979; Korach 1979; Evans *et al.* 1980).

The level of both nuclear receptors in sheep endometrium was higher than that in whole mouse uterus. This was true for both baseline levels, i.e. at 0 h on day 3 before any further steroid treatment, and following E_2 or P injection on day 3. Although this may be an artefact of the assay procedure, it may reflect differences in tissue receptor concentration or species differences in adrenal steroid secretion,

which has been suggested to influence receptor replenishment (Cidlowski and Muldoon 1978) and basal nuclear receptor level (Jungblut *et al.* 1978). A real species difference in receptor concentration may explain, in part, the higher sensitivity, on a body weight basis, of the sheep to both steroids.

Following treatment with a single injection of either steroid on day 3, there was the expected depletion of the respective cytosol receptor and accumulation of nuclear receptor. The pattern of change was only examined in some detail in the mouse where the nuclear P receptor was significantly elevated by 0·5 h, reached a peak level at 1–2 h and was still above control values at 8 h. This pattern is generally similar to that described for the hamster (Chen and Leavitt 1979).

For the mouse, nuclear E_2-receptor retention also continued for at least 8 h and the overall pattern agreed well with early observations (Stone 1963) on the uptake of radioactivity by the uterus following *in vivo* administration of [^{3}H]E_2. There is a dramatic contrast, however, with the results of Korach (1979) who found a peak level at 1 h, a return to control values by 4 h and a secondary rise at 8 h. It is not clear if the anomalies are wholly explained by differences in the state of the animals, i.e. E_2-treated adult ovariectomized females *v.* immature females, and in the methods of steroid administration. Juliano and Stancel (1976), using the immature rat uterus, also reported a short-term retention of nuclear E_2 receptor, and Anderson *et al.* (1972) found little change between 1 and 6 h after E_2 injection.

In both the ewe and the mouse, P administration to E_2-treated animals reduced the level of its own cytosol receptor, an effect still significant at 24 h (Figs 2 and 3). In the ewe, P caused a small but non-significant decrease in cytosol E_2-receptor level at 24 h (Fig. 3). This relatively modest effect has been previously noted (Miller *et al.* 1979; Stone *et al.* 1979). In the mouse, P had no effect on cytosol E_2-receptor level over the first 8 h but did cause a significant decrease at 24 h. The magnitude of the effect was somewhat less than previously observed (Miller *et al.* 1979). It appears that the effect of P on cytosol E_2-receptor replenishment represents in the mouse, but not in the ewe, a relatively minor component of a generalized inhibition of uterine protein synthesis (Miller *et al.* 1979, 1982). It also appears that this effect on E_2-receptor replenishment is not the explanation for the anti-oestrogenic effects of P in the rat or mouse uterus (Miller *et al.* 1979; Walters and Clark 1980).

Concomitant with its lack of effect on cytosol E_2-receptor level in the ewe, P, at all times, was without effect on nuclear E_2-receptor level (Fig. 3). Under these conditions, P caused a mild stimulation of *in vitro* protein synthesis at 24 h (Miller *et al.* 1979, 1982). By contrast, P in the mouse brought about a prompt and highly significant decrease in nuclear E_2-receptor level (Fig. 2), an effect now also described for the hamster (Evans *et al.* 1980; Evans and Leavitt 1980*b*) and rat (Okulicz *et al.* 1981). This decrease in nuclear E_2 receptor in the mouse showed a close temporal relationship with rapid effects on uterine weight and *in vitro* RNA synthesis (Miller *et al.* 1982).

Although P was able to reduce nuclear E_2-receptor level in the unstimulated uterus of the mouse (Table 9), the effect was minor relative to that seen in E_2-stimulated animals that already had an elevated level of cytosol P receptor. This indicates that to obtain anti-oestrogenic responses to P there is, as expected, a necessary involvement of P with its cytosol receptor and translocation of the steroid–receptor complex to the nucleus.

The present results, together with those of Evans *et al.* (1980), Evans and Leavitt (1980*b*) and Okulicz *et al.* (1981), appear to provide a basic explanation for the anti-uterotrophic activity of P in such species as the hamster, rat and mouse and the lack of such activity in the ewe. At least for the hamster, the inhibitory effects of P require changes in RNA and protein synthesis (Evans and Leavitt 1980*a*). Perhaps, in the ewe, the 'linkage' mechanism by which the translocated P receptor initiates such changes in the nucleus is lacking. Elucidation of this mechanism should provide a further understanding of steroid hormone action.

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