BINDING OF PROGESTERONE BY RAT UTERUS *IN VITRO*

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SUMMARY: Rat uteri and abdominal muscle were incubated with ^{3}H-progesterone. Non-specific tritium uptake was seen in both tissues, but evidence for specific binding was found only in the uterus. Pretreatment with β-estradiol increased uterine binding. In the presence of progesterone, deoxycorticosterone, 19-nor-progesterone, testosterone and several synthetic progestational agents tritium uptake by the uterus was reduced significantly. β-Estradiol, corticosterone or the 5α-pregnane uterine metabolites of progesterone did not reduce tritium uptake.

Physiological activity is measurable on three levels; *in vivo*, intact tissue *in vitro*, and in cell-free preparations. In most cases information derived from studies on all three levels has proved to be fruitful. In this communication we wish to report *in vitro* experiments with rat uteri which suggested that specific progesterone receptors do, in fact, exist, and encouraged us to proceed to more extensive experiments with cell-free uterine cytosols (1,2,3).

MATERIALS AND METHODS

7α-^{3}H-Progesterone (6.7 Ci/mM) and 1,2-^{3}H-corticosterone (31.7 Ci/mM) were purchased from Amersham-Searle and were checked for purity by thin layer chromatography on silica gel in chloroform-acetone 9:1. Steroids were purchased from Sigma Chemical Co., St. Louis, Mo. Progesterone in aqueous suspension (Lipo-Lutin) was a gift from Parke, Davis & Co.

Immature rats (22-23 days old) of the CFE strain (Carworth) were housed under conditions of controlled temperature (25.5°C) and lighting (14L; 10D). They were anesthetized with Nembutal and decapitated; the uteri were removed, dissected free of fat, and weighed. Uteri weighing more than 50 mg were bisected at the junction of the two horns and slit open. Smaller uteri were incubated intact. The uteri and samples of abdominal muscle of similar weight

were placed into 25 ml Erlenmeyer flasks containing 5 ml KRP (calcium-free Kreb-Ringer phosphate [pH 7.2] containing 200 mg% glucose), or in TRIS (0.05M TRIS buffer [pH 7.4] containing 0.01M KCl and 0.0015M $MgSO_4$).

The flasks were placed in a Dubnoff metabolic shaking incubator at 37°C. After 30 minutes the medium, containing blood and tissue debris, was sucked off and replaced with fresh medium containing 0.1μCi 7α-^{3}H-progesterone dissolved in medium (final concentration of ^{3}H-progesterone was 3×10^{-9}M).

After incubation the tissues were rinsed twice in 5 ml ice-cold medium and blotted dry with filter paper. In obtaining the data for Fig. 1, the tissues were rinsed in 5 ml medium and transferred to 25 ml Erlenmeyer flasks containing 5 ml fresh medium for a further incubation of 60 minutes, to remove ^{3}H-progesterone not firmly bound to the tissue.

Measurement of radioactivity. Total tissue tritium was measured by dissolving the tissues in Soluene (Packard Instrument Co.) in counting vials. After 24 hours at room temperature, 10 ml counting fluid were added (5 g PPO and 0.5 g dimethyl POPOP per liter toluene). After 24 hours standing in the refrigerator to allow chemiluminescence to subside samples were counted in a Nuclear Chicago Model 6848 scintillation counter. Quench correction was made by the addition of and internal standard (^{3}H-toluene) and results were expressed as DPM per mg fresh weight of tissue, or as DPM per μg DNA. When ^{3}H-progesterone uptake was related to the DNA content of the uterus, the uteri were halved and one-half was incubated with ^{3}H-progesterone while the other half was used for DNA determination. When untreated immature rats were used the right and left halves of uteri from 2 rats were pooled for incubation and chemical analysis. DNA was determined by the method of Webb and Levy (4). All DPM values reported in the tables are means ± standard error of 6 incubations.

Hormonal pretreatment. In some experiments (Tables 1 and 4), immature rats (22-23 days) were injected with β-estradiol benzoate (Progynon, Schering) in sesame oil, 1 μg in 0.1 ml sc once daily for 2 days, or with progesterone

(aqueous suspension) 2.5 mg in 0.1 ml sc or in a single injection 25 mg in 1 ml ip.

Competition by non-radioactive steroids. Non-radioactive steroids used in competition experiments were dissolved in absolute ethanol (1 mg per ml) and were diluted with incubation medium to the desired concentration. 7α-^{3}H-Progesterone (3 x 10^{-9}M) and non-radioactive steroids at 10, 10^2, or 10^3 times this concentration were incubated together and thus had equal access to binding sites.

RESULTS AND DISCUSSION

On incubation of rat uterus with ^{3}H-progesterone the tritium content of tissue increased with time but the rate of uptake slowed appreciably after 60 minutes (Fig.1A). When incubated with increasing concentrations of progesterone

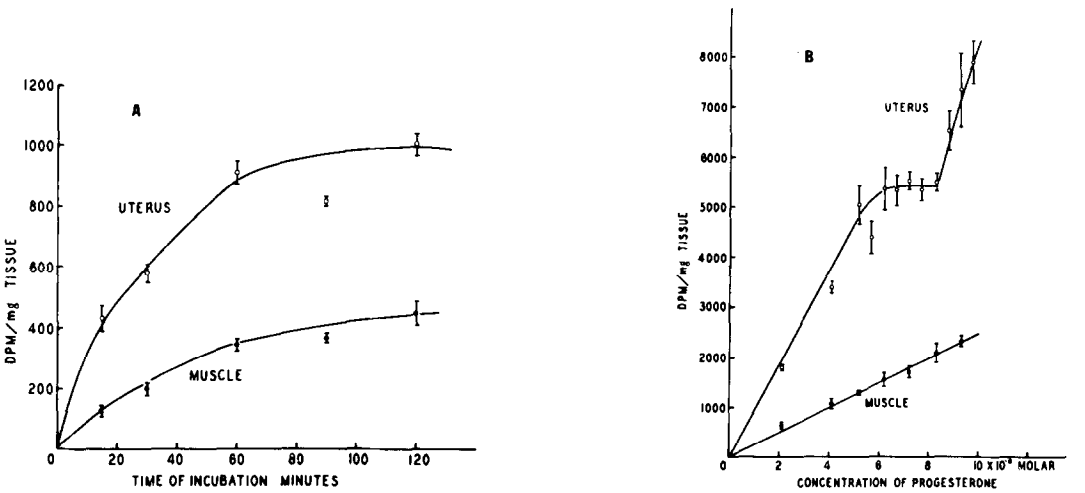

FIGURE 1

Uptake of tritium by rat tissues on incubation with 7α-^{3}H-progesterone. Experimental details given in Materials and Methods. *A*, As a function of Time. Each flask contained 0.1 μCi ^{3}H-progesterone in KRP. *B*, As a function of progesterone concentration (2 to 9.8 x 10^{-8}M cold progesterone in KRP with 0.1 μCi ^{3}H-progesterone for each 10^{-8} moles of progesterone).

uptake of tritium was proportional to progesterone concentration up to 5 x 10^{-8}M. A plateau was seen from 5 to 8 x 10^{-8}M, after which the uterine tritium content increased linearly (Fig.1B). On the other hand, uptake by muscle was continuously proportional to concentration throughout the range tested. This difference in the behavior of the two tissues suggests the presence in the uterus of a saturable specific receptor, not present in muscle. At concentrations above 8 x 10^{-8}M in the uterine incubations, and at all concentrations in the muscle incubations the linear uptake is explicable in terms of non-specific low affinity binding.

Table 1 compares the uptake of ^{3}H-progesterone by muscle and uteri from

TABLE 1. 7α-^{3}H-Progesterone uptake *in vitro* in control and estradiol benzoate treated rats.

Experiment	Pretreatment	DPM/mg Uterus	DPM/mg Muscle	DPM/ g DNA in uterus
1	none	181 ± 5	117 ± 4	16.5 ± 1
	β-estradiol benzoate	242 ± 11	94 ± 5	49.0 ± 3*
2	none	341 ± 18	110 ± 4	27.2 ± 2
	β-estradiol benzoate	310 ± 17	127 ± 6	44.6 ± 3*

*P < 0.01 (analysis of variance)

Results of two typical experiments are shown. Tissues were removed 24 hours after the last injection. Incubations with ^{3}H-progesterone in TRIS buffer were followed by a 60 minute wash in fresh medium.

The mean weight of immature rat uteri was 31.2 ± 6.0 (36 observations). The mean weight of estrogen stimulated uteri was 119 ± 15.2 (36 observations).

untreated and β-estradiol benzoate treated rats. Expressed as DPM/mg of tissue there is no significant difference. Since estradiol treatment triples the uterine weight, the total uptake per uterus is therefore three-fold greater. When expressed in terms of the uterine DNA content it is seen that β-estradiol caused a two- or three-fold increase in tritium uptake. On the other hand, β-estradiol

had no effect on progesterone uptake by muscle. Our data are in accord with previous conclusions that β-estradiol induces the synthesis of a specific progesterone receptor (1,5,6).

The ability of various non-labelled natural steroids to compete with ^{3}H-progesterone for binding sites is shown in Table 2. Cold progesterone decreased

TABLE 2. Effect of non-radioactive steroids on the binding of 7α-^{3}H-progesterone by immature rat uterus *in vitro*

Steroid added	% of control	Incubation medium
Progesterone ($3.2 \times 10^{-8}M$)	86*	KRP
Progesterone ($3.2 \times 10^{-7}M$)	73**	KRP
Progesterone ($3.2 \times 10^{-6}M$)	53**	KRP
19-Norprogesterone ($3.3 \times 10^{-6}M$)	49**	TRIS
Deoxycorticosterone ($3.0 \times 10^{-9}M$)	62**	TRIS
Δ4-Pregnene-20α-ol-3-one ($3.2 \times 10^{-6}M$)	37**	TRIS
Δ4-Pregnene-20β-ol-3-one ($3.2 \times 10^{-6}M$)	43**	TRIS
5α-Pregnane-3,20-dione ($3.2 \times 10^{-6}M$)	95	TRIS
5α-Pregnane-3α-ol-20-one ($3.1 \times 10^{-6}M$)	98	KRP
Testosterone ($3.5 \times 10^{-6}M$)	67**	TRIS
Estradiol-17β ($3.7 \times 10^{-9}M$)	95	TRIS
Corticosterone ($2.9 \times 10^{-6}M$)	90	TRIS
Norgestrel ($3.2 \times 10^{-6}M$)	60**	TRIS
Dydrogesterone ($3.2 \times 10^{-6}M$)	79**	TRIS
Norethindrone ($3.3 \times 10^{-6}M$)	88	TRIS
Norethynodrel ($3.3 \times 10^{-6}M$)	91	TRIS
Chlormadinone Acetate ($2.4 \times 10^{-6}M$)	100	TRIS
Megestrol Acetate ($2.6 \times 10^{-6}M$)	72**	TRIS

*$p < 0.05$)
**$p < 0.01$) (analysis of variance)

All compounds were tested by combined incubation for 60 minutes in the concentrations indicated. Control incubations containing only 7α-^{3}H-progesterone ($3 \times 10^{-9}M$) were run with each group. A 60 minute wash in fresh medium followed the incubations.

Norethindrone = 19-nor-17α-ethinyl-testosterone
Norethynodrel = 17-hydroxy-19-nor-17α-pregn-5(10)-ene-20-yn-3-one
Norgestrel = 13β-ethyl-17α-ethinyl-17β-hydroxygon-4-ene-3-one
Chlormadinone acetate = 6-chloro-17-hydroxy-pregna-4,6,diene-3,20 dione acetate
Dydrogesterone = 9β,10α-pregna-4,6-diene-3,20-dione
Megestrol acetate = 17α-acetyl-6-dehydro-6-methylprogesterone

the tritium concentration in uterus significantly by either preincubation or combined incubation. The uptake in muscle was not changed by any compound tested. 19-Norprogesterone, deoxycorticosterone and both 20-isomers of Δ4-pregnene-20-ol-

3-one blocked tritium uptake as well as, or better than progesterone. Testosterone had a moderate and consistent effect while 17β-estradiol did not compete in the concentrations tested. The metabolites of progesterone in rat uterus (5α-pregnane-3,20-dione and 5α-pregnane-3α-ol-20-one) (7), 5β-pregnane-3,20-diol, cortisol and corticosterone were completely without effect.

Because of evidence that the progesterone receptor in rat uterus might be similar or identical to corticosteroid-binding globulin (CBG) (8,9), the uptake of 1,2-^{3}H-corticosterone by rat uterus was tested in the same way as ^{3}H-progesterone (Table 3). There was very little uptake of tritium by the uterus and no

TABLE 3. Uptake of 1,2,-^{3}H-corticosterone *in vitro:* Comparison with 7α-^{3}H-progesterone

Tritiated Steroid	Preincubation*	60 Min Wash After Incubation	DPM/mg Uterus
Progesterone	Yes	Yes	261 ± 15
Corticosterone	Yes	Yes	11 ± 0.7
Progesterone	No	Yes	605 ± 22.0
Corticosterone	No	No	113 ± 2.3
Corticosterone	No	Yes	18 ± 2.4

Uteri from 22-23 day old rats were incubated with 0.1 μCi 1,2-^{3}H-corticosterone or 0.1 μCi 7α-^{3}H-progesterone in KRP.

*For 30 minutes in KRP.

effect of cold progesterone or corticosterone on this uptake. When the preincubation step was omitted, there was a consistent uptake of ^{3}H-corticosterone by the uterus, amounting to 15% of that of ^{3}H-progesterone under the same conditions. However, after a 60 minute wash in fresh incubation medium at 37°C, 90% of the corticosterone tritium bound to the uterus disappeared from from the tissue.

The uptake of 7α-^{3}H-progesterone *in vitro* was measured 5 and 10 minutes after the intraperitoneal injection of 25 mg of progesterone. The tritium content of the uterus was significantly lower than the control value at the time intervals tested indicating occupation of binding sites by cold progesterone (Table 4). A

TABLE 4. Effect of *in vivo* progesterone on the *in vitro* uptake of 7α-^{3}H-progesterone by rat uterus

Progesterone Dose, mg	Time after cold progesterone	DPM/mg	S.E.
0	-	656	± 26
25	5 min	447	± 46*
25	10 min	509	± 34*
0	-	299	± 5
2.5	24 hr	317	± 23
2.5	36 hr	337	± 12*
2.5	48 hr	328	± 20*

*P < 0.01 (analyses of variance).

Rats were injected with Lipo-Lutin (microcrystalline progesterone in aqueous suspension). After the time intervals indicated uteri were removed and incubated with 0.1 μCi 7α-^{3}H-progesterone in KRP.

smaller dose of progesterone (2.5 mg) injected subcutaneously had no effect after 60 minutes. After 24 hours the *in vitro* uptake of ^{3}H-progesterone was sometimes greater than in untreated control uteri and after 36 and 48 hours the uptake was always significantly greater than controls. By 72 hours the effect had disappeared. These observations remain to be explained.

Of the naturally occurring steroids tested deoxycorticosterone and testosterone competed significantly for specific binding sites (Table 2). It is interesting to speculate whether this can be related to the progestational activity of these compounds. As expected, some of the synthetic progestational agents tested depressed the uptake of ^{3}H-progesterone by the intact rat uterus, but none was as potent as progesterone. Correlation with *in vivo* activity was poor. Chlormadinone acetate did not reduce ^{3}H-progesterone uptake at all. On the other hand, the most potent progestational agent known, norgestrel, proved to be the best competitor. In these experiments with intact tissue, the ability of a compound to enter the uterine cell is a decisive factor, as well as its ability to bind to the receptor site. Currently we are attempting to obtain a stable preparation of the specific progesterone receptor, free of other binding proteins present in

uterine cytosols. When this is available we intend to re-assess the competitive activity of progestational agents for receptor binding sites.

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REFERENCES

1. Faber, L. E., Sandmann, M. L. and Stavely, H. E., *J. Biol. Chem.* 247,5648 (1972).
2. Faber, L. E., Sandmann, M. L. and Stavely, H. E., *J. Biol. Chem.* 247,8000 (1972).
3. Faber, L. E., Sandmann, M. L. and Stavely, H. E., *Endocrinology*, (in press).
4. Webb, J. M. and Levy, H. B., *J. Biol. Chem.* 213,107 (1955).
5. Toft, D. O. and O'Malley, B. W., *Endocrinology*,90,1041 (1972).
6. Corvol, P., Falk, R., Freifeld, M. and Bardin, C. W.,*Endocrinology*,90,1464 (1972).
7. Armstrong, D. T. and King, E. R., *Endocrinology*,89,191 (1971).
8. Milgrom, E. and Baulieu, E.-E., *Biochem. Biophys. Res. Comm.*,40,723 (1970).
9. Milgrom, E. and Baulieu, E.-E., *Endocrinology*,87,276 (1970).