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Some *in vivo* Interactions of Oestradiol with the Mouse Vagina

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Abstract

Within 8 min after the intravaginal administration of 25 pg of [^{3}H]oestradiol ([^{3}H]E2) an equilibrium appeared to have been established between E2 in the 'nuclear' and 'soluble' compartments of vaginal homogenates. When an initial injection of [^{3}H]E2 was followed 4 or 64 min later by an intravaginal injection of unlabelled E2 there was little effect on the retention of the initial dose at 4 min after the second dose and a highly significant decrease in retention at 64 min. However, the distribution of the initial dose between the two compartments was little affected at any time studied. When the second injection contained E2 labelled with ^{3}H or ^{14}C an unexpectedly high proportion of this dose was retained at 4 min. With the [^{14}C]E2 an increase in the proportion of the second dose in the nuclear compartment was observed 64 min after injection. The results suggested considerable recycling of E2 or the receptor complex between the two tissue compartments.

It was not possible to differentiate significantly the sedimentation behaviour of the nuclear and soluble receptors when both were centrifuged in buffer containing 0·4M KCl. The nuclear receptor showed no change in sedimentation coefficient between high salt (0·4M KCl) and low salt (0·01M KCl) conditions.

Introduction

The interaction of oestradiol with oestrogen-responsive tissues involves an initial binding to an extranuclear receptor and transformation of the receptor complex followed by translocation of the complex to the nucleus (for reviews see Gorski *et al.* 1968; Baulieu *et al.* 1971; Jensen *et al.* 1973). Transformation of the receptor is apparently obligatory for the subsequent stimulation of nuclear RNA synthesis (Jensen *et al.* 1972, 1973; Mohla *et al.* 1972). Although most of the studies have involved uterine preparations, those that have examined in some detail oestrogen interactions with vaginal receptors have shown the expected vaginal retention and subcellular distribution of radioactivity following intravaginal administration of [^{3}H]oestradiol (Martin 1964; Martin and Baggett 1964; Stone 1971). However, a difference in the net half-life of the receptors of rat uterus and mouse vagina is apparent (Sarff and Gorski 1971; Stone and Pollard 1973).

In the experiments of this paper some *in vivo* interactions of [^{3}H]oestradiol with the mouse vagina following intravaginal administration have been further studied. It was hoped that this local approach might overcome some problems inherent in *in vitro* studies and also in *in vivo* studies with systemic administration, where the tissue is subject to changing blood levels of the steroid.

Materials and Methods

Solutions

[2,4,6,7-^{3}H]-17β-oestradiol (specific activity 370 μCi/μg) (hereafter designated [^{3}H]E2) and [4-^{14}C]-17β-oestradiol (specific activity 188 μCi/mg) ([^{14}C]E2) were obtained from the Radiochemical Centre, Amersham, England. The purification before use and preparation for intravaginal injection has been previously described (Stone and Baggett 1965*a*). Non-radioactive 17β-oestradiol (E2) (Sigma Chemical Co.) solutions were prepared from ethanolic stocks kept at 5°C. Intravaginal injections were made as previously described in 5 μl (Stone and Pollard 1973).

Table 1. Uptake of tritiated oestradiol by mouse vagina

Mice received intravaginal injections of 25 pg of [^{3}H]E2 and were killed 1, 2, 4, 8, 16, 32 or 64 min later. Radioactivity was measured in the nuclear and soluble fractions of vaginal homogenates and in 0·4M KCl extracts of the nuclear fractions (see Methods). Results are means of two replicates and for each time within each replicate pooled tissue from 20 animals was used

Time after injection (min)	Tissue radioactivity: A. Total (% of injected)	B. Nuclear fraction (% of recovered)	C. Nuclear extract (% of B)
1	64·8	42·5	55·8
2	65·6	52·7	55·6
4	53·3	55·2	58·6
8	50·8	62·1	56·9
16	58·0	68·5	57·7
32	56·1	67·8	64·5
64	44·9	63·7	58·9

Summary of analyses of variance

Source of variation	Degree of freedom	Variance ratios: A	B	C
Replicates	1	0·07	0·03	0·01
Time				
Linear	1	22·41**	31·60**	5·90
Quadratic	1	0·19	7·08*	0·02
Remainder	4	2·38	0·43	1·84
Error	6	*17·57*	*26·00*	*8·16*

* $0{\cdot}05 > P > 0{\cdot}01$. ** $0{\cdot}01 > P > 0{\cdot}001$.

Animals and Tissue Preparation

Randomly bred mice of the QS strain were ovariectomized, primed and randomized to experimental groups as described previously (Stone 1971). For tissue fractionation studies pooled vaginae were homogenized in 0·01M tris–0·01M KCl–0·001M EDTA buffer (pH 7·4) (referred to as buffer A) and 800 *g* pellets ('nuclear' fractions) and 105 000 *g* supernatants ('soluble' fractions) were prepared (Stone and Pollard 1973). The nuclear fractions were extracted by homogenization or suspension in 0·01M tris–0·4M KCl–0·001M EDTA buffer (pH 8·5) (referred to as buffer B) and the extract separated from the residue by centrifugation at 31 000 *g* for 30 min. [^{3}H]E2 binding by the nuclear extracts, the soluble fraction and bovine serum albumin, Cohn fraction V (Commonwealth Serum Laboratories), was studied by charcoal absorption and density-gradient centrifugation (Stone 1971; Stone and Pollard 1973). *p*-Hydroxymercuribenzoate (PHMB) (Sigma Chemical Co.) was used to check the specificity of the binding (Stone 1971). Radioactivity estimates on whole vaginae, on aliquots of aqueous tissue fractions and on particulate fractions were made as previously described

(Stone 1971). In the double label experiments counts were corrected to disintegrations per minute using [^{3}H]palmitic acid and [^{14}C]benzoic acid as internal standards.

Results

The vaginal uptake of injected [^{3}H]E2 was measured in the experiment described in Table 1. Total vaginal radioactivity showed a significant linear decrease over the time studied and at 64 min 44·9% of the injected dose remained in the tissue. During the first 8 min the proportion of the recovered tissue radioactivity that was associated with the nuclear compartment increased and thereafter showed no significant change with time. The proportion of the nuclear fraction that could be extracted with a single treatment with buffer B showed no significant change over the experimental period. Within each replicate the binding in the nuclear extract, as measured by charcoal absorption, did not change with time but was significantly less in the second replicate ($75{\cdot}8 \pm 3{\cdot}6$ *v.* $63{\cdot}1 \pm 1{\cdot}4\%$; $P < 0{\cdot}05$). Binding in the soluble fraction, not examined in the first replicate, was significantly less than binding by the respective nuclear extract ($63{\cdot}1 \pm 1{\cdot}4$ *v.* $47{\cdot}9 \pm 1{\cdot}6\%$; $P < 0{\cdot}01$). Sucrose density-gradient studies on the nuclear extract showed a single radioactivity peak with a sedimentation coefficient of $4{\cdot}2 \pm 0{\cdot}15$ S and this did not change significantly ($4{\cdot}6 \pm 0{\cdot}1$ S; $P > 0{\cdot}05$) after dialysis for 20 h against 2000 times the volume of buffer A and centrifugation in gradients prepared in buffer A. The sedimentation of the soluble receptor ($7{\cdot}3 \pm 0{\cdot}12$ S) decreased after dialysis against buffer B ($4{\cdot}3 \pm 0{\cdot}12$ S). In each case the radioactivity peak after dialysis could not be differentiated significantly from the protein peak as judged by the absorbance at 220 nm, and there was no real indication of aggregation.

Table 2. Binding of [^{3}H]E2 by, and sedimentation coefficients of, vaginal nuclear and soluble oestrogen receptors in the presence and absence of *p*-hydroxymercuribenzoate

The tissues used were pooled vaginae from 80 mice killed 8 min after receiving 25 pg of [^{3}H]E2 intravaginally. Binding of radioactivity was measured by charcoal absorption and sedimentation coefficients were measured after centrifugation for 16 h at 4°C at 140 000 *g* (av.) through a 5–20% linear sucrose gradient in the appropriate buffers

Fraction	Bound radioactivity (% of incubated)	Sedimentation coeff. (S)	Fraction	Bound radioactivity (% of incubated)	Sedimentation coeff. (S)
Nuclear extract	70·1	4·2	Soluble	67·1	7·0
+ PHMB	6·6	—	+ PHMB	2·1	—
Dialysed nuclear extract	60·0	4·1	Albumin	5·2	4·6
+ PHMB	8·5	—	+ PHMB	6·0	4·6

The specificity of the binding after incubation with PHMB was examined in the experiment of Table 2. Pooled vaginal tissue obtained 8 min after intravaginal injection of [^{3}H]E2 contained 44·1% of the injected dose, and of this 57·9% was associated with the nuclear fraction. Buffer B extracted 68·9% of the radioactivity in the nuclear fraction. In both charcoal absorption and density-gradient centrifugation studies (Table 2) addition of PHMB severely inhibited the binding of [^{3}H]E2 in the soluble fraction and in the nuclear fraction before and after dialysis. Again the sedimentation pattern of the nuclear extract was not changed after dialysis. Binding of [^{3}H]E2 to albumin either in the presence or absence of PHMB could not be demon-

strated with charcoal absorption due to the high affinity of the charcoal, but on gradient centrifugation no effect of PHMB was observed. In each case the radioactivity sedimented with a peak corresponding to 4·6 S.

Table 3. Displacement of oestradiol from vaginal tissue

Animals received a first injection of 25 pg of [^{3}H]E2 followed at the specified times later by a second injection of 25 pg (experiment 1) or 2·5 ng (experiment 2) of E2 (see text for full experimental details). R = radioactivity

T_1[A]	T_2[A]	Dose of E2 (pg or ng)	Experiment 1: I. Total tissue R (% of injected)	II. Total tissue R (% of injected)	Experiment 2: III. Total recovered R (% of injected)	IV. Nuclear fraction R (% of III)
4	4	0	73·4	65·8	66·1	69·7
		25 or 2·5	66·6	64·9	55·0	69·2
4	64	0	60·9	73·5	65·9	75·7
		25 or 2·5	26·3	48·6	38·1	74·2
64	4	0	64·1	57·9	54·4	79·7
		25 or 2·5	65·2	55·2	57·5	78·7
64	64	0	72·6	63·0	61·2	74·7
		25 or 2·5	23·8	35·9	34·7	73·1

Summary of analyses of variance

Source of variation	D.F.	Variance ratios: I	II	D.F.	Variance ratios: III	IV
Dose (D)	1	51·33***	12·48**	1	79·57***	1·30
Time of killing (K)	1	47·53***	9·26**	1	22·46***	0·02
Time of second dose (S)	1	0·02	8·28**	1	6·11*	18·94***
D × K	1	39·15***	11·64**	1	44·08***	0·17
D × S	1	0·26	0·14	1	4·96*	0·03
K × S	1	2·55	0·76	1	0·02	29·01
D × K × S	1	3·17	0·02	1	3·41	0·00
Error	32	*96·57*	*134·2*	24	*24·29*	*8·06*

[A] T_1 = time (min) of second injection after initial one; T_2 = time (min) of killing after second injection.

* $0·05 > P > 0·01$. ** $0·01 > P > 0·001$. *** $P < 0·001$.

Table 3 shows the results of two experiments that attempted to examine the turnover of E2 in the vagina as indicated by the ability of unlabelled E2 to displace [^{3}H]E2 already in the tissue. Mice received an intravaginal injection of 25 pg of [^{3}H]E2 4 or 64 min prior to an injection of either the vehicle alone or 25 pg (experiment 1) or 2·5 ng (experiment 2) of unlabelled E2. The animals were killed 4 or 64 min following the second injection. In experiment 1 only the total tissue radioactivity was measured while in experiment 2 the distribution of radioactivity between nuclear and soluble fractions of vaginal homogenates prepared from five pooled tissues was also measured. In both experiments there was an overall effect of the second injection to decrease the total retention of [^{3}H]E2 by the vagina and this effect was far greater when the animals were killed 64 min after the second dose. With the lower dose (25 pg) the time of the second injection had no effect but with the higher dose, as expected, less retention was seen in those animals receiving the second injection 64 min after the first. In the tissue

fractions only one significant treatment effect was observed. With the longer time after the second injection there was a small but significant increase in the proportion of tissue radioactivity associated with the nuclear fraction.

These results suggested firstly that, although the receptors would have been near saturation in the early period following the second injection, a high proportion of this dose was initially retained by the tissue as the displacement was not great until the later times, and secondly that if the displacement by the free steroid was not similar in the two tissue compartments there was considerable turnover of [^{3}H]E2 between the two compartments.

Table 4. Displacement of oestradiol from vaginal tissue: ^{3}H labelling

Mice received a first injection of 25 pg of [^{3}H]E2 followed at the specified times by a second injection of vehicle alone or 2·5 ng of E2 containing 25 pg of [^{3}H]E2 (see text for full experimental details)

T_1[A]	T_2[A]	Second dose (ng)	Total tissue radioactivity: I. As % of initial dose	II. As % of total dose
4	4	0	71·0	71·0
		2·5	83·5	45·0
4	64	0	64·1	64·1
		2·5	36·2	19·5
64	4	0	62·0	62·0
		2·5	80·0	43·2
64	64	0	54·4	54·4
		2·5	36·5	19·7

Summary of analyses of variance

Source of variation	D.F.	Variance ratios: I	II
Dose (D)	1	1·58	233·63***
Time of killing (K)	1	74·11***	60·90***
Time of second dose (S)	1	3·19	6·29*
D × K	1	38·95***	17·98***
D × S	1	1·62	4·37*
K × S	1	0·06	0·03
D × K × S	1	0·14	0·12
Error	32	*93·52*	*41·28*

[A] T_1 = time (min) of second injection after initial one; T_2 = time (min) of killing after second injection.
* $0·05 > P > 0·01$. *** $P < 0·001$.

These possibilities were examined using E2 labelled with either ^{3}H (Table 4) or ^{14}C (Table 5) for the second injection. In the experiment of Table 4 the vehicle alone or 2·5 ng of E2 containing 25 pg of [^{3}H]E2 were administered intravaginally 4 or 64 min after the initial injection of 25 pg of [^{3}H]E2. The animals were killed 4 or 64 min after the second injection. Total tissue radioactivity was measured and is expressed as a percentage of the initial dose or of the total dose. By 4 min after the second

injection there was no indication of displacement by this injection and a considerable proportion of the second dose was still in the tissue. As expected, at 64 min displacement by the second dose, which had a lower specific activity, had decreased the tissue radioactivity. These effects explain the lack of a significant dose effect and the significant dose × killing time interactions in the appropriate analyses. The timing of the second dose relative to the initial one had little effect on the subsequent retention.

Table 5. Displacement of oestradiol from vaginal tissue: ^{14}C labelling

Mice received an initial injection of 25 pg of [^{3}H]E2 followed at the specified times later by a second injection of vehicle only or 2·5 ng of [^{14}C]E2 (see text for full experimental details). Some non-significant interactions are pooled in the analyses of variance. R = radioactivity

			Experiment 1: Tissue R (% of injected)		Experiment 2: I. Recovered R (% of injected)		Experiment 2: II. Nuclear fraction R (% of recovered)	
T_1[A]	T_2[A]	Second dose (ng)	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C
4	4	0	73·0	—				
		2·5	57·9	27·2				
4	16	0	68·3	—				
		2·5	52·5	3·0				
4	64	0	63·9	—				
		2·5	28·8	1·3				
64	4	0	61·8	—	51·2	—	76·1	—
		2·5	53·4	43·0	51·6	19·0	75·9	30·9
64	16	0	61·4	—				
		2·5	48·0	3·6				
64	64	0	59·3	—	52·4	—	74·2	—
		2·5	24·2	1·4	22·1	0·8	72·7	62·5

Summary of analyses of variance of ^{3}H radioactivity

Experiment 1: Source of variation	D.F.	Variance ratio	Experiment 2: Source of variation	D.F.	Variance ratios: I	II
Second dose	1	99·37***	Second dose	1	54·42***	0·60
Time of second dose	1	8·67**	Time of second dose	1	49·06***	5·09*
Time of killing			Interaction	1	57·77***	0·39
Linear	1	48·14***				
Quadratic	1	4·09*	Error	12	*16·21*	*5·11*
Interactions	7	1·02				
Error	48	*63·40*				

[A] T_1 = time (min) of second injection after initial one; T_2 = time (min) of killing after second injection.

* $0·05 > P > 0·01$. ** $0·01 > P > 0·001$. *** $P < 0·001$.

In the experiment of Table 5 the vehicle alone or 2·5 ng of [^{14}C]E2 was administered intravaginally 4 or 64 min (experiment 1) or 64 min (experiment 2) after an initial intravaginal injection of 25 pg of [^{3}H]E2. The animals were killed 4, 16 or 64 min (experiment 1) or 4 or 64 min (experiment 2) after the second injection when total tissue ^{3}H and ^{14}C (experiment 1) or the distribution of ^{3}H and ^{14}C between the nuclear and soluble fractions of five pooled vaginal homogenates (experiment 2) were measured. By 4 min after the second injection there was generally a small decrease in the tissue

[^{3}H]E2 and, although a considerable proportion of the second dose remained in the tissue, the greater part of the tissue radioactivity, but not of course the tissue E2, was due to the initial dose. By 64 min the second injection of E2 had markedly decreased the retention of [^{3}H]E2, but the ^{3}H again represented the greater part of the tissue radioactivity. Again, in the nuclear fraction 64 min following the second dose, there was a slight but significant decrease in the proportion of tissue [^{3}H]E2 associated with the nuclear fraction while the proportion, but not the total, of [^{14}C]E2 in the nuclear fraction had increased.

Discussion

Characteristics of vaginal oestrogen receptors examined in these experiments agree in a number of respects with those described for uterine receptors. However, in contrast to the results of Jensen *et al.* (1969) and Giannopoulos and Gorski (1971*b*), it was not possible to differentiate significantly the sedimentation of the nuclear and soluble receptors under high salt conditions. It is not clear whether this represents a real difference or a difference in methodology. It was also not possible to change the sedimentation of the nuclear receptor under low salt (0·01M KCl) conditions (Erdos 1968).

Soon after the entry of E2 into the vagina a stable distribution appeared to have been established between E2 in the nuclear and soluble compartments. Although the values obtained may have included an artefact due to non-specific association induced by the homogenization procedure (Williams and Gorski 1971), the significant change with time indicates that this is a real effect. Non-specific binding would also be expected to be low under these conditions (Stone and Baggett 1965*b*). This stable distribution has also been noted with *in vivo* studies following systemic administration (Toft and Gorski 1966), although under these conditions the situation is complicated by a changing level of steroid presented to the tissue. The *in vivo* situation seems to differ from the *in vitro* one (Musliner *et al.* 1970; Giannopoulos and Gorski 1971*a*) where the transfer of the steroid–receptor complex is more gradual and almost complete. In *in vitro* studies any loss of nuclear binding with time is not associated with a concomitant increase in binding in the soluble fraction (Giannopoulos and Gorski 1971*a*). This may reflect degeneration *in vitro* of any mechanism associated with replenishment of soluble receptor binding, or argue against recycling of the steroid or receptor complex or both. Such recycling is, however, suggested by the present displacement experiments where, under conditions of marked decrease in retention of the initial dose, little change was seen in its subcellular distribution. Specific direct labelling of the receptor in the nucleus appears not to occur (Clark and Gorski 1969) and, if the difficulty in displacement of E2 from the receptor which is seen *in vitro* is also encountered *in vivo*, the results would suggest dissociation of E2 from the receptor prior to, or associated with, movement back to the soluble compartment. The observed increase in the proportion of the second dose in the nuclear fraction might be expected under these conditions. A turnover time of approximately 24 h for the vaginal cytosol receptor (Stone and Pollard 1973) would suggest that new receptor synthesis would contribute little to the binding at these times.

Further studies to examine possible recycling of E2 and the receptor complex within the tissue compartments and also the mechanisms by which E2 is lost from the tissue *in vivo* are being carried out.

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References

Baulieu, E. E., *et al.* (1971). *Recent Prog. Horm. Res.* **27**, 351.
Clark, J. H., and Gorski, J. (1969). *Biochim. Biophys. Acta* **192**, 508.
Erdos, T. (1968). *Biochem. Biophys. Res. Commun.* **32**, 338.
Giannopoulos, G., and Gorski, J. (1971*a*). *J. Biol. Chem.* **248**, 2524.
Giannopoulos, G., and Gorski, J. (1971*b*). *J. Biol. Chem.* **248**, 2530.
Gorski, J., Toft, D., Shymala, G., Smith, D., and Notides, A. (1968). *Recent Prog. Horm. Res.* **24**, 45.
Jensen, E. V., Suzuki, T., Numata, M., Smith, S., and DeSombre, E. R. (1969). *Steroids* **13**, 417.
Jensen, E. V., Mohla, S., Gorell, T., Tanaka, S., and DeSombre, E. R. (1972). *J. Steroid Biochem.* **3**, 445.
Jensen, E. V., Mohla, S., Brecher, P. I., and DeSombre, E. R. (1973). *Adv. Exp. Med. Biol.* **36**, 60.
Martin, L. (1964). *J. Endocrinol.* **30**, 337.
Martin, L., and Baggett, B. (1964). *J. Endocrinol.* **30**, 41.
Mohla, S., DeSombre, E. R., and Jensen, E. V. (1972). *Biochem. Biophys. Res. Commun.* **46**, 661.
Musliner, T. A., Chader, G. J., and Villee, C. A. (1970). *Biochemistry* **9**, 4448.
Sarff, M., and Gorski, J. (1971). *Biochemistry* **10**, 2557.
Stone, G. M. (1971). *Aust. J. Biol. Sci.* **24**, 1023.
Stone, G. M., and Baggett, B. (1965*a*). *Steroids* **5**, 495.
Stone, G. M., and Baggett, B. (1965*b*). *Steroids* **6**, 277.
Stone, G. M., and Pollard, I. (1973). *Aust. J. Biol. Sci.* **26**, 201.
Toft, D., and Gorski, J. (1966). *Proc. Natl. Acad. Sci. U.S.A.* **55**, 1574.
Williams, D., and Gorski, J. (1971). *Biochem. Biophys. Res. Commun.* **45**, 258.

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