THE EFFECT OF ACTINOMYCIN D ON THE BINDING OF TRITIATED OESTRADIOL BY THE VAGINA OF THE OVARIECTOMIZED MOUSE

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Abstract

The binding of [^{3}H]oestradiol-17β by whole mouse vaginal tissue *in vivo*, and by subcellular fractions of mouse vaginal homogenates *in vivo* and *in vitro*, was examined at varying times after an intravaginal injection of the antimetabolite, actinomycin D. Under conditions of almost complete inhibition of protein synthesis the average receptor half-life was $24 \cdot 2 \pm 3 \cdot 2$ hr, contrasting with the previously reported value of 5–6 days for the soluble oestrogen receptor of the rat uterus. The cytoplasm to nucleus transfer of the oestrogen receptor complex was inhibited by actinomycin D. No effect was observed on the sedimentation characteristics of the receptor and there was no indication of receptor subunit accumulation.

Prolonged actinomycin D administration failed to abolish completely the oestrogen binding and there were indications that under these conditions the tissue was able partly to overcome the effects of the inhibitor.

I. INTRODUCTION

The binding of oestrogens to specific target organ proteins, referred to as oestrogen receptors, is now well documented. This appears to involve an initial interaction with an extranuclear receptor followed by a temperature-dependent transfer of the oestrogen receptor complex to the nucleus (see Gorski *et al.* 1968; Baulieu *et al.* 1971 for reviews). Injection of the antimetabolite actinomycin D at 1 and 25 hr prior to an intravaginal injection of [^{3}H]oestradiol-17β decreases the total tissue retention of radioactivity by slightly more than 50% (Stone 1971), suggesting a receptor half-life of less than 25 hr. Sarff and Gorski (1971), however, have estimated that the half-life of the oestrogen receptor prepared from rat uterine cytosol is 5–6 days. This contrasts with a previously estimated half-life of 20–22 hr for the general soluble proteins of this tissue fraction (Gorski and Notides 1969).

The experiments of this paper have examined more closely the affinity of [^{3}H]oestradiol-17β for receptors of the mouse vagina at varying times after the intravaginal administration of actinomycin D. The binding *in vivo* by the whole tissue and by nuclear and 105,000 *g* supernatant fractions prepared therefrom, and *in vitro* by the supernatant fraction, was studied. With the doses and the route of administration of the inhibitor that were chosen no obvious harmful systemic effects were observed even with repeated 24-hr administration and it had previously been shown the ultrastructure of the epithelial cells appeared normal with no signs of toxic effects or disintegration (Pollard 1970).

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II. Materials and Methods

(a) *Solutions*

[2,4,6,7-^{3}H]oestradiol-17β (specific activity 370 μCi/μg) was obtained from the Radiochemical Centre, Amersham, England. The purification before use and preparation for intravaginal administration has been previously described (Stone and Baggett 1965*a*). Actinomycin D (Calbiochem) was dissolved in 10% propylene glycol in distilled water, or in distilled water alone, to give the required concentration for injection in 10 μl. For the *in vitro* experiment aliquots of the [^{3}H]oestradiol solution and of ethanolic stock solutions of non-radioactive oestradiol (Sigma Chemical Co.) were dried under nitrogen and the residue dissolved in 0·01M Tris–0·1M KCl–0·001M EDTA buffer (referred to as TKE buffer) (pH 7·4).

(b) *Animals*

Randomly bred mice of the QS strain bred in this laboratory and of weight 20–25 g were ovariectomized, primed 1–2 weeks later with 1·0 μg of subcutaneously injected oestradiol-17β, and used 7–14 days after priming. Within each experiment mice were randomized to experimental groups. Intravaginal applications were made with an Agla micrometer syringe. Mice were killed by cervical dislocation. For the *in vivo* oestradiol uptake studies the vaginal lumen was rinsed through *in situ* from the cervix with 1 ml of 0·9% NaCl and the rinses discarded. The tetrazolium assay (Martin 1960) was used to follow the effects of the inhibitor treatments on the oestrogenic response as previously described (Stone 1971), except that the mice were killed 22 hr after oestrogen administration.

(c) *Tissue Preparation and Measurement of Radioactivity*

Radioactivity estimates for whole vaginae were made as previously described (Stone 1971). For tissue fractionation studies, the vaginae were dissected onto ice, minced with scissors, and homogenized at 0°C in TKE buffer with an all-glass Dual tissue grinder (Kontes Glass Co.) using a motor-driven pestle. For the experiments of Tables 1 and 2, the homogenate was centrifuged at 800 *g* and the pellet resuspended twice using a teflon pestle. The final pellet, referred to as the nuclear fraction, was extracted and the level of radioactivity estimated as described previously (Stone 1971). The combined supernatants were centrifuged at 4°C for 1 hr at 105,000 *g* using a type 40 rotor in a Spinco L265 Bultracentrifuge. The final pellet was discarded and aliquots of the supernatant were taken for radioactivity estimation. For the experiment of Table 3, the entire homogenate was centrifuged at 105,000 *g* as above and the pellet discarded.

Binding in the supernatant was studied by charcoal absorption and by density-gradient centrifugation. For charcoal absorption, aliquots of the supernatants were incubated for 10 min at 4°C with 1 ml of TKE buffer containing 0·5% charcoal (Norit A; Matheson, Coleman, and Bell) and 0·005% dextran C (Sigma Chemical Co.). The whole was centrifuged and the supernatant, representing the protein-bound oestradiol, was decanted and the radioactivity estimated. Density-gradient centrifugation was carried out as previously described (Stone 1971), except that the centrifugation was continued for 16 hr and bovine serum albumin, Cohn Fraction V (Commonwealth Serum Laboratories), was used as a reference standard for the calculation of approximate sedimentation coefficients (Martin and Ames 1961).

III. Results

In the first experiment 1·2 μg of actinomycin D was administered intravaginally 1, 3, 9, or 27 hr prior to 30 pg of [^{3}H]oestradiol-17β. Control animals received [^{3}H]oestradiol-17β but not actinomycin D. Within each treatment five groups, each of five animals, were killed 1 hr after the administration of the oestradiol. In one group total radioactivity in each organ was measured while tissues in the remaining four groups were separately pooled, nuclear and 105,000 *g* supernatant fractions prepared, and aliquots of each fraction taken for radioactivity estimation. Binding was studied in replicate aliquots of each supernatant by charcoal absorption. The

effect of the treatments and injection of the vehicle alone (no oestradiol) on the 22-hr tetrazolium response was also measured with five mice per group. The dose of actinomycin D used was considerably higher than the 0·3 μg used in previous studies (Stone and Baggett 1965*a*; Stone 1971). Pilot tests had indicated that with actinomycin D from the present source the increase was required for inhibition of metabolic activity and oestrogen binding.

TABLE 1

EFFECT OF ACTINOMYCIN D ON [^{3}H]OESTRADIOL RETENTION

Actinomycin D (1·2 μg) was administered intravaginally at varying times before 30 pg of [^{3}H]oestradiol-17β. At 1 hr the vaginal retention, the distribution of radioactivity between 105,000 *g* supernatant and nuclear fractions of vaginal homogenates, and oestradiol binding by the supernatant fraction were measured. At 22 hr the tetrazolium response was measured

Actinomycin D pretreatment time (hr)	Whole tissue		Tissue homogenate		
	Total radioactivity (% of injected)	Tetrazolium response ($A_{495\ nm}$)	Total in fractions (% of injected)	Supernatant fraction (% of recovered)	Binding (% of incubated)
(A)	(B)	(C)	(D)	(E)	(F)
1	41·9	0·023	37·8	28·6	33·9
3	39·3	0·016	35·2	28·3	36·2
9	37·4	0·016	—	—	28·2
27	17·1	0·014	14·7	43·0	15·4
Control	38·3	0·198	35·0	30·6	38·6
No oestradiol	—	0·011	—	—	—

Summary of analyses of variance†

Source of variation	Variance ratios				
	B	C	D	E	F
1 *v.* 3 hr	0·77 (1)	0·06 (1)	0·09 (1)	1·13 (1)	0·15 (1)
1+3 hr *v.* 27 hr	81·13** (1)	0·47 (1)	120·34***(1)	105·87*** (1)	14·60** (1)
Error mean square	*22·65* (12)	*353·31* (12)	*4·39* (8‡)	*11·97* (8‡)	*70·45* (9)

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

† Number of degrees of freedom given in parentheses. ‡ One missing observation.

Results are shown in Table 1. As the fractions for all of the 9-hr pretreatment replicates were lost during extraction the analyses only include the results from the other times. All pretreatments decreased the tetrazolium response essentially to no-oestradiol levels and there were no significant differences between times. Although there was no effect after 3 hr pretreatment, by 27 hr the total tissue radioactivity, the radioactivity recovered in the fractions, and the binding in the supernatant fraction were all significantly inhibited to less than 50% of control levels. The difference between the total radioactivity and that recovered in the fractions probably represents preparation losses and radioactivity associated with the mitochondrial and microsomal fractions. The proportion of the recovered radioactivity that was associated with the supernatant fraction was significantly greater at 27 hr and agreed with previous findings (Stone 1971).

Table 2 shows the results of a similar experiment which measured the effect of 9 and 27 hr pretreatment with actinomycin D on the above parameters. Part of the experiment was done in replicate. Both treatments again decreased the tetrazolium response to low levels. By 9 hr there was a significant decrease in tissue radioactivity, both in whole tissue and the total fractions, and this effect was greater at 27 hr. There was no significant effect on the distribution of radioactivity or the binding in the supernatant until the later time.

TABLE 2

EFFECT OF ACTINOMYCIN D ON [^{3}H]OESTRADIOL RETENTION

Actinomycin D (1·2 μg) was administered intravaginally either 9 or 27 hr before 30 pg of [^{3}H]oestradiol-17β. At 1 and 22 hr the parameters of Table 1 were measured

Actinomycin D pretreatment time (hr) (A)	Whole tissue		Tissue homogenate		
	Total radioactivity (% of injected) (B)	Tetrazolium response ($A_{495\ nm}$) (C)	Total in fractions (% of injected) (D)	Supernatant fraction (% of recovered) (E)	Binding (% of incubated) (F)
Replicate 1					
Control	47·8	0·141	42·9	29·3	35·1
9 hr	30·7	0·038	34·1	24·9	31·6
27 hr	17·8	0·026	24·1	38·1	19·0
Replicate 2					
Control	—	—	35·7	27·5	46·0
9 hr	—	—	28·0	26·3	44·7
27 hr	—	—	13·6	42·0	34·2

Summary of analyses of variance†

Source of variation	Variance ratios				
	B	C	D	E	F
Control *v.* 9 (*A*)	8·16* (1)	15·27** (1)	7·91* (1)	3·82 (1)	0·59 (1)
Control+9 *v.* 27 (*B*)	17·12** (1)	13·56** (1)	41·81*** (1)	108·05*** (1)	23·27*** (1)
Replicate (*R*)	—	—	11·11** (1)	0·95 (1)	27·38*** (1)
R×*A*	—	—	0·03 (1)	1·20 (1)	0·13 (1)
R×*B*	—	—	0·60 (1)	2·67 (1)	0·36 (1)
Error mean square	*89·58* (12)	*540·9* (12)	*34·0* (18)	*8·43* (18)	*37·41* (1)

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

† Number of degrees of freedom given in parentheses.

The results of the experiments described above might be explained, in part, by an action of actinomycin D on the transport of oestradiol into the vaginal tissue rather than on receptor synthesis. In the experiment of Table 3, [^{3}H]oestradiol binding was therefore studied in isolated vaginal supernatants prepared from mice 1, 3, 9, or 27 hr after an intravaginal injection of 1·2 μg of actinomycin D. Tissues from 20 mice were pooled for each time and the 105,000 *g* supernatant prepared as described above. Duplicate aliquots, each representing this fraction from one mouse, were incubated with 25 pg of [^{3}H]oestradiol and buffer or 0·5 or 50 ng of unlabelled

oestradiol-17β, at 4°C for 2 hr prior to the addition of the charcoal–dextran. Binding in the supernatant showed a significant linear decrease with increasing time after actinomycin D treatment. The significant quadratic dose effect and the dose–time interaction are, in part, due to the non-specific binding which, although small, becomes a relatively greater proportion of the total at low binding levels.

TABLE 3

EFFECT OF ACTINOMYCIN D ON [^{3}H]OESTRADIOL BINDING *IN VITRO*

Actinomycin D (1·2 μg) was administered intravaginally at varying times prior to killing. Oestrogen binding was studied in incubations of 105,000 *g* supernatant fractions of the vaginal homogenates with [^{3}H]oestradiol-17β and varying levels of unlabelled oestradiol-17β

Actinomycin D pretreatment time (hr)	Binding (% of incubated) at oestrogen level of:		
	0	0·5 ng	50 ng
Control	32·1	16·5	6·1
1	32·5	16·2	5·8
3	27·7	14·1	5·3
9	21·8	9·3	4·0
27	14·4	7·6	2·6

Summary of analysis of variance

Source of variation	D.F.	Mean square	Variance ratio
Dose			
Linear (D_L)	1	2362·57	1952·54***
Quadratic	1	47·53	39·28***
Time			
Linear (T_L)	1	516·81	427·12***
Dose × time			
$D_L \times T_L$	1	182·78	151·06***
Remainder	7†	1·84	1·52
Error mean square	24	*1·21*	—

*** 0·001 > *P*. † Non-significant single D.F. effects pooled as remainder.

Further aliquots of the supernatant fraction from the control animals and the 9- and 27-hr treatments, each representing the fraction from four mice, were incubated with 100 pg of [^{3}H]oestradiol at 4°C for 2 hr and portions subjected to density-gradient centrifugation as described in Section II(*c*). The gradient profile is given in Figure 1 and shows that the amount of the [^{3}H]oestradiol which was associated with the 7·5S component decreased with increasing time after actinomycin D treatment. As noted by Sarff and Gorski (1971) there was neither an inhibitor effect on the sedimentation constant of the receptor nor any tendency for subunit accumulation.

The effect of extended actinomycin D treatment on the vaginal response to [^{3}H]oestradiol was also studied. Intravaginal applications of 2·4 μg of actinomycin D were given every 24 hr and 30 pg of [^{3}H]oestradiol were given 24, 48, 72, or 96 hr after initiation of the inhibitor treatment. Total tissue retention of radioactivity at 1 hr and the tetrazolium response at 22 hr after the oestradiol were measured. Results are shown in Table 4. Although there was again an approximate 50% inhibition by

24 hr, the binding was subsequently not completely abolished. The significant quadratic effect of time in the analysis suggests that the inhibitory effects of actinomycin D

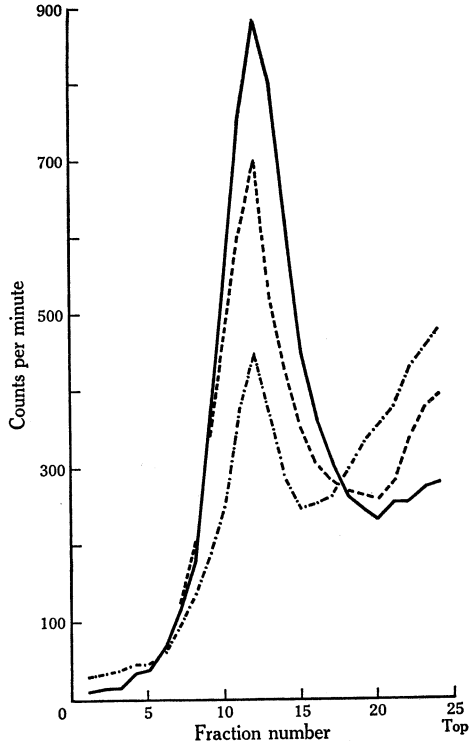

Fig. 1.—Density gradient centrifugation profile of 105,000 *g* supernatant of mouse vaginal homogenate labelled *in vitro* with [^{3}H]oestradiol and prepared from animals which received 1·2 μg of actinomycin D at 9 hr (– – –) and 27 hr (—·—·—) before killing. —— Control.

TABLE 4

EFFECT OF PROLONGED ACTINOMYCIN D ON [^{3}H]OESTRADIOL RETENTION

Actinomycin D (2·4 μg) was administered intravaginally at 24-hr intervals for 24, 48, 72, and 96 hr before 30 pg of [^{3}H]oestradiol-17β. At 1 hr the vaginal retention of radioactivity and at 22 hr the tetrazolium response were measured

Actinomycin D pretreatment time (hr)	Tissue radioactivity (% of injected)	Tetrazolium response ($A_{495\ nm}$)	Summary of analyses of variance			
			Source of variation	D.F.	Variance ratio: tissue radioactivity	Variance ratio: tetrazolium response
Control	42·8	0·177	Time			
24	19·5	0·011	Linear	1	10·83**	2·60
48	11·9	0·024	Quadratic	1	9·22**	0·02
72	5·7	0·041	Cubic	1	1·19	0·09
96	11·0	0·077	Error mean			
No oestradiol	—	0·033	square	15†	*22·48*	*543·6*

** $0{\cdot}01 > P > 0{\cdot}001$. † One missing observation.

were being overcome by 96 hr. A similar lessening of the inhibition at the later time was seen in the tetrazolium response.

IV. Discussion

In the present experiments the average time of actinomycin D pretreatment required to decrease the binding of [^{3}H]oestradiol to 50% of control levels was $24{\cdot}2 \pm 3{\cdot}2$ hr. This is in reasonable agreement with the half-life for the total soluble proteins of the rat uterus (Gorski and Notides 1969) but contrasts with the value of 5–6 days reported for the oestrogen receptor from this organ (Sarff and Gorski 1971). Although the turnover times of mammalian proteins can differ widely (Russell and Snyder 1969), such a difference in this instance is difficult to reconcile with the number of similarities between the oestrogen receptors of the rat and mouse uterus and vagina (Stone and Baggett 1967), and possibly also with the similarities of oestrous cycle length in these species. One major difference between the approach used in the present experiments and that used by Sarff and Gorski (1971) was the timing of the inhibitor treatment in relation to the [^{3}H]oestradiol. These authors treated animals with unlabelled oestradiol-17β followed at varying times by the inhibitor. The ability of the cytosol fraction to bind [^{3}H]oestradiol was then studied. The results of Sarff and Gorski (1971) suggest that prior binding of oestradiol by the receptor might afford some protection to the rate of receptor degradation. Thus the difference in turnover times might be explained in part by the observations of Berlin and Schimke (1965) that the apparent half-life of a protein is an exponential function of its rate of degradation and only a linear function of its rate of synthesis.

By 1 hr after intravaginal injection, the time chosen to examine retention and binding, a stable distribution of oestradiol between the cytoplasmic and nuclear compartments has been established (Stone and Pollard 1972). Due to the as yet unexplained effect of the inhibitor on the distribution of [^{3}H]oestradiol within the vagina, an examination of only the cytosol binding may not give a true indication of the inhibition. Within the limits of the fractionation procedure the distribution observed probably represents an indication of the relative specific binding by these two tissue fractions rather than an artefact of non-specific binding induced by the homogenization (Williams and Gorski 1971). Non-specific binding of steroids by the vagina and uterus is very low by 1 hr after local administration (Stone and Baggett 1965*b*).

The failure of extended actinomycin D treatment to abolish and maintain a complete inhibition of the [^{3}H]oestradiol retention and the tetrazolium response is of interest. The lowest retention of [^{3}H]oestradiol noted at 72 hr is still above that seen with the non-oestrogenic steroids (Stone and Baggett 1965*b*). The tendency of the oestradiol retention and the tetrazolium response to return to normal levels may represent an immunological response to the maintained response levels of the inhibitors. If not, a further complication in the turnover of the oestrogen receptor is indicated.

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