A NEW ASSESSMENT OF SUBCELLULAR DISTRIBUTION OF BOUND ESTROGEN IN THE UTERUS.*

David Williams and Jack Gorski

Departments of Physiology and Biophysics, and Biochemistry
University of Illinois, Urbana, Illinois 61801

Received August 4, 1971

SUMMARY: The experiments to be described demonstrate that the levels of estradiol-receptor complex† present in uterine cytosol after whole tissue incubations at 0° or 37° are much lower than previous estimates. This discrepancy appears to be due to free estrogen trapped extracellularly which binds to the receptor upon disruption of the cells during homogenization. The time course of estradiol binding to cytosol and nuclear fractions at 37° shows that the amount of bound estradiol in the cytosol is very small relative to the total bound estradiol, and suggests that the transfer of estradiol binding sites from cytosol to nucleus is a very rapid process. Incubations at 0° indicate that the entry of estradiol into the uterine cell is markedly temperature-dependent.

While working with free cell suspensions (1) prepared from the immature rat uterus, we made two observations that were not consistent with previously reported data concerning the interaction of estradiol with the uterus in vitro: 1) during incubations at 37°, the levels of estradiol-receptor complex in the cytosol fraction were much lower than have been reported in intact tissue (2-4), while the levels of estradiol-receptor complex in the nucleus were essentially the same; and 2) after incubations at 0°, very little estradiol-receptor complex could be detected, in contrast to older reports (2-4) which indicated that this complex was readily formed in the cytosol fraction of the intact uterus during incubations with estradiol at 0°. This communication demonstrates that, in studies of whole uterine tissue, previous estimates of specifically bound estradiol in the cytosol of the uterus after 0° or 37° incubations have been overestimated by as much as 10-fold because estradiol which is trapped in the tissue during the incubation becomes bound to the cytosol binding protein during homogenization.

METHODS

Incubations--Uteri were excised from 20-24 day old Holtzman rats and incubated in Eagle's HeLa medium (2-3 uteri/4 ml) at 0° or 37° under an atmosphere of 95% O_2-5% CO_2. The uteri were equilibrated at the appropriate temperature and

*Supported by National Institutes of Health Grants HD 04828 and GM 43656, and the Ford Foundation.

†The term estradiol-receptor complex is used here to describe the specific estradiol binding protein(s) of the uterus.

then ^{3}H-estradiol-17β (^{3}H-E_2) (40 Ci/mM, New England Nuclear) or non-radioactive estradiol-17β (E_2) (Mann Research) was added. Incubations were terminated by transferring the flasks to an ice bath or transferring the uteri to 10 ml of cold 0.01 M Tris-HCl - 0.0015 M Na_2EDTA, pH 7.3 (TE buffer). All subsequent operations were carried out at 0-4°.

Homogenization and tissue fractionation--Homogenization in TE buffer was as described previously (2). Centrifugation of the homogenate at 800 x g for 20 min yielded the low speed supernatant (LSS), which was subsequently centrifuged at 226,000 x g for 45 min to yield the high speed cytosol fraction (HSS). The 800 x g pellet was washed 4 times in 5 ml TE buffer, each wash followed by centrifugation at 800 x g for 10 min to yield the washed nuclear fraction.

Determination of bound ^{3}H-E_2--Bound ^{3}H-E_2 in the HSS was determined by gel filtration on 7 x 0.6 cm Sephadex G-25 columns at 0-4° or by a modification of the hydroxylapatite procedure described by Erdos (5). Both procedures gave identical results. Bound ^{3}H-E_2 in the nuclear fraction was determined by ethanol extraction of the washed nuclear fraction (2). Where indicated, bound ^{3}H-E_2 in the nuclear fraction was determined by gel filtration subsequent to extraction of the washed nuclear pellet with 0.05 M Tris-HCl - 0.0015 M EDTA - 0.4 M KCl, pH 7.3 (TKE buffer).

RESULTS AND DISCUSSION

The ability to distinguish between the binding of estradiol occurring during the tissue incubation and that occurring during the homogenization is

Table I. Apparent and actual levels of bound estradiol (E_2) in the cytosol and nucleus after incubation at 37°.

	DPM/Uterus ± S.E.M.*		
Sample**	$-E_2$	$+E_2$***	$+E_2$†
Cytosol	47,116 ± 2,515	16,407 ± 1,106 (P<.01)‡	1,166 ± 262
Nucleus	75,633 ± 1,380	70,923 ± 503 (NS)‡	1,603 ± 112

* Standard error of the mean.
** Incubation conditions: 3 uteri/4 ml Eagle's HeLa medium, 30 min, 37°, 10^{-8} M ^{3}H-E_2.
*** Uteri exposed to a second 30-min incubation at 0° with 10^{-6} M E_2 or homogenized directly in 10^{-6} M E_2.
† 10^{-6} M E_2 added simultaneously with 10^{-8} M ^{3}H-E_2 at the beginning of the 37° incubation.
‡ Statistical significance (P) or non-significance (NS) determined by Student's t Test.

based on the slow dissociation of the estradiol-receptor complex at 0° (6, and see below). The rationale is that the addition of excess E_2 before homogenization will result in competition for unfilled binding sites but will have little effect on binding sites previously occupied with ^3H-E_2. The experiment described in Table I shows that after a 30-min incubation at 37° with 10^{-8} M ^3H-E_2, a subsequent incubation for 30 min at 0° with 10^{-6} M E_2 causes a significant decrease in assayable cytosol-bound ^3H-E_2 but has no effect on nuclear-bound ^3H-E_2. If the second incubation with excess E_2 is eliminated but the tissue is homogenized in TE buffer containing 10^{-6} M E_2, the same decrease in assayable cytosol-bound ^3H-E_2 is seen, indicating that the observed competition occurs during or after the homogenization itself. Extraction of nuclear-bound ^3H-E_2 with TKE buffer and subsequent determination of bound ^3H-E_2 by gel filtration again shows no effect of the homogenization in excess E_2 on the amount of bound ^3H-E_2 in the nucleus.

The experiments described in Fig. 1 and Table II give assurance that the observed competition between ^3H-E_2 and E_2 during the homogenization is not due

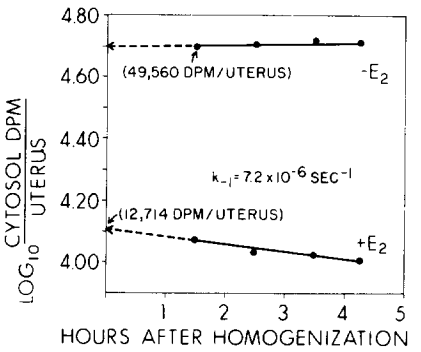

Fig. 1. Determination of dissociation rate constant. Uteri were incubated 30 min at 37° with 10^{-8} M ^3H-E_2. After thorough washing, one group of uteri was homogenized in TE buffer ($-E_2$) and the other in TE buffer + 10^{-6} M E_2 ($+E_2$). The HSS was prepared and the amount of bound ^3H-E_2 in both samples followed as a function of time.

to either; 1) exchange occurring from the time of homogenization to the time of assay (90-120 min), or 2) an enhanced exchange occurring during the homogenization itself.

When the amount of bound estradiol in the cytosol was followed as a function of time with and without a 100-fold excess of cold estradiol, dissociation occurred at a slow rate (Fig. 1). The dissociation rate constant determined from the $+E_2$ curve is in agreement with published values (6), and the difference between the $+E_2$ and $-E_2$ samples cannot be explained by extrapolation of the $+E_2$ curve back to the time of homogenization. The experiment

Table II. Effect of homogenization on bound estrogen.

I. 15 uteri incubated 30 min at 0° with 8 x 10^{-9} M ^{3}H-E_2, uteri homogenized and the LSS divided into 3 equal portions.

II. 10 uteri incubated 30 min at 0° with 8 x 10^{-9} M ^{3}H-E , followed by 30 min at 0° with 8 x 10^{-7} M E_2. Uteri divided into 2 equal groups.

Sample	Description	CPM/Aliquot
A	HSS from I prepared and assayed	5,150
B	LSS from I rehomogenized, HSS prepared and assayed	4,859
C	LSS from I rehomogenized with uteri from II, HSS prepared and assayed	6,044
D	Uteri from II homogenized, HSS prepared and assayed	1,879
B + D		6,738
B + D - (Calculated Exchange)		6,396
B + D - (Calculated Exchange) - C		352
A - D		3,271

described in Table II demonstrates that an enhanced exchange does not occur during the homogenization itself. If an enhanced exchange had occurred when uteri from group II were homogenized in LSS from group I (sample C), significantly less bound ^{3}H-E_2 whould have resulted than the sum of the individual samples (B + D). As can be seen in Table II, the sum of B + D is only slightly greater than C, and if one accounts for the actual exchange that should have occurred from the time of rehomogenization to the time of assay (120 min), the difference between sample B + D and C (352 CPM) does not explain the difference between samples A and D (3,271 CPM). Similarly, if pre-labeled LSS is merely rehomogenized in excess E_2, no more exchange occurs than can be explained by the dissociation rate constant and the time elapsed from the addition of excess E_2 to the time of assay. We conclude from these studies that the decrease in assayable cytosol-bound ^{3}H-E_2 observed when uteri are homogenized in excess E_2 reflects simultaneous competition for unfilled binding sites between E_2 and ^{3}H-E_2 trapped in the tissue during the incubation. Extensive washing of the tissue before homogenization does not eliminate the difference, suggesting that the trapped ^{3}H-E_2 is weakly bound to the mass of tissue protein or partitioned into lipid components of the uterus. Homogenization in the absence of excess E_2 results in the formation of apparent estradiol-receptor levels in the cytosol greatly in excess of the amount actually formed during the incubation.

Using the procedure of adding excess E_2 after incubation and prior to homogenization, we have examined the levels of ^{3}H-E_2 actually bound during tissue incubations under different conditions. Figure 2A shows the time course of ^{3}H-E_2 binding to cytosol and nuclear fractions at 37° with 10^{-8} M ^{3}H-E_2.

Bound ^3H-E_2 in the cytosol rapidly attains an apparent steady state level which comprises only 5-10% of the total bound ^3H-E_2 in the tissue after the first few minutes of incubation. This result is in contrast to previous studies (2) at 10^{-8} M ^3H-E_2 which showed a large initial increase in cytosol-bound ^3H-E_2 followed by a slow decrease as the complex apparently moved to the nucleus. A comparison of these data and those published previously (2) is shown in Fig. 2B,

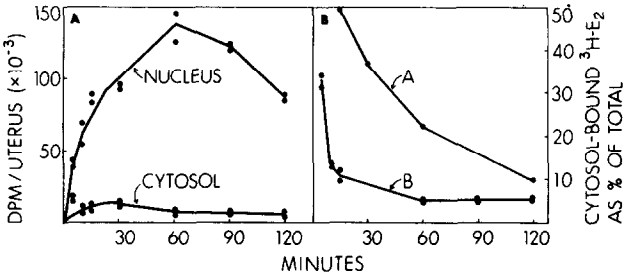

Fig. 2. (A) Time course of ^3H-E_2 binding to cytosol and nuclear fractions at 37°. (B) Values for nuclear binding used to construct curve A have been multiplied by a factor of 1.67 to account for the efficiency of extraction with TKE buffer as indicated in reference (2).

where the levels of bound ^3H-E_2 in the cytosol are expressed as a per cent of the total bound ^3H-E_2 (cytosol + nucleus). Examination of earlier data (curve A) suggests that ^3H-E_2 is rapidly bound to cytosol binding sites but that the redistribution to the nucleus is a slow process. The new data

Table III. Effect of temperature on apparent and actual levels of bound estrogen (E_2) formed during 5-min incubations.

10^{-9} M ^3H-E_2				10^{-8} M ^3H-E_2			
		DPM/Uterus*				DPM/Uterus*	
°C	Sample	-E_2**	+E_2**	°C	Sample	-E_2**	+E_2**
0	Cytosol	3,974	268	0	Cytosol	34,904	5,554
0	Nucleus	1,250	20	0	Nucleus	6,381	1,618
0	Total	5,224	288	0	Total	41,285	7,172
37	Cytosol	8,932	2,514	37	Cytosol	104,198	18,145
37	Nucleus	6,915	6,140	37	Nucleus	39,882	39,344
37	Total	15,847	8,654	37	Total	144,080	57,489

* Values are means of duplicate incubations. -E_2, uteri homogenized in TE buffer; +E_2, uteri homogenized in TE buffer + 10^{-6} M E_2.

** Non-specific binding has been subtracted from all values. Non-specific binding was determined from a parallel set of incubations to which the appropriate concentration of ^3H-E_2 and a 100-fold excess of cold E_2 was added simultaneously at the initiation of the incubations at both temperatures.

presented here (curve B) show that the actual level of bound ^3H-E_2 in the cytosol rapidly becomes a relatively small fraction of the total; suggesting that once an estradiol-receptor complex is formed in the cytosol, it rapidly moves to the nuclear compartment.

The experiments described in Table III are concerned with the effect of temperature on the apparent and actual levels of bound ^3H-E_2 formed during brief tissue incubations. The data indicate that during brief incubations at 0°, the uterus accumulates considerable ^3H-E_2, but that little of the accumulated ^3H-E_2 is actually bound to intracellular binding sites until the tissue is homogenized. Comparison of the actual levels of bound ^3H-E_2 formed at 0° and 37° shows the tremendous temperature-dependency of this process. The data presented here and the observation that ^3H-E_2 is rapidly bound in cell free systems at 0° (7) suggest that the entry of estradiol into the uterine cell is the process which exhibits the marked temperature-dependency in tissue incubations. Numerous investigators (2-4) have employed brief tissue incubations at 0° with ^3H-E_2 followed by incubations at 37° to evaluate the effect of temperature on the movement of the estradiol-receptor complex into the nucleus. It is apparent that these experiments do not permit an accurate evaluation of the temperature-dependency of this transfer process because of the low levels of bound ^3H-E_2 actually present in the cell at the conclusion of the brief 0° incubations. Longer incubations at 0° permit increasing amounts of ^3H-E_2 to penetrate the cell, but the process is remarkably slow. After 60 min at 0° with 8×10^{-9} M ^3H-E_2, only 15-20% of the binding sites are filled relative to the same time period at 37°.

Figure 3 shows the result of sucrose gradient centrifugation of cytosol

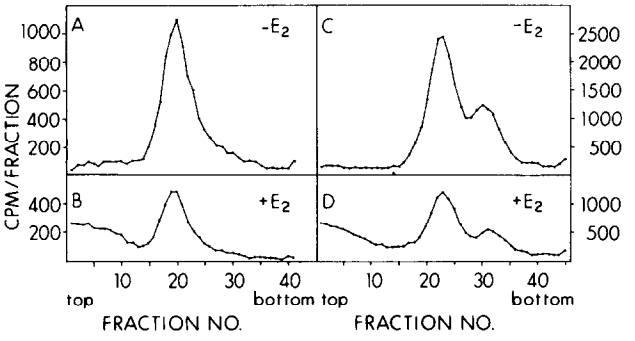

Fig. 3. Sucrose gradient analysis of actual and apparent bound estradiol in the cytosol fraction. 100-μl samples of HSS were layered on 5-20% sucrose gradients containing TE buffer (A and B) or TKE buffer (C and D). TE gradients of 4.8 ml were centrifuged at 149,000 x g (av.) for 16.5 hr using a Beckman SW-50.1 rotor. TKE gradients of 3.8 ml were centrifuged at 246,000 x g (av.) for 22 hr using a Beckman SW-56 rotor. $+E_2$, 100-fold excess E_2 added to tissue before homogenization in TE buffer; $-E_2$, homogenization in TE buffer.

under low salt and high salt conditions after incubation of the tissue for 60 min at 0^{o} with 8 x 10^{-9} M ^{3}H-E_2. The low salt gradients (A and B) show the characteristic 8S peak which is reduced by about 60% when excess E_2 is added to the tissue before homogenization. The high salt gradients (C and D) show the salt-dissociated 4S and 6S peaks previously described (8). The addition of excess E_2 before homogenization appears to reduce both binding moieties to the same extent, suggesting that neither form is preferentially labeled at 0^{o}. Similarly, after incubation of the tissue at 37^{o}, both cytosol forms appear to be reduced to the same extent by the addition of excess E_2 before homogenization (gradients not shown).

The above results show that previous data from this and other laboratories concerning the concentration of bound estradiol in the cytosol of the uterus after whole tissue incubations are in error. These new data indicate that, once formed, the estradiol-receptor complex is rapidly transferred to the nuclear compartment. Whether or not this transfer to the nuclear compartment is temperature-dependent in intact cells is not clear from these experiments. Experiments in cell free systems which have used temperature-dependence as a criterion for physiological significance may need further scrutiny.

REFERENCES

1. D. Williams and J. Gorski, Proc. of the 53rd Mtg. of the Endocr. Soc., A-147 (1971).

2. G. Giannopoulos and J. Gorski, J. Biol. Chem., 246, 2524 (1971).

3. E. V. Jensen, T. Suzuki, T. Kawashima, W. E. Stumpf, P. W. Jungblut and E. R. DeSombre, Proc. Nat. Acad. Sci. U.S.A., 59, 632 (1968).

4. G. Shyamala and J. Gorski, J. Biol. Chem., 244, 1097 (1969).

5. T. Erdos, M. Best-Belpomme and R. Bessada, Anal. Biochem., 37, 244 (1970).

6. T. Erdos, R. Bessada, M. Best-Belpomme, J. Fries, D. Gospodarowicz, M. Menahem, E. Reti and A. Veron, in Advances in the Biosciences, Proc. of the Schering Workshop on Steroid Hormone Receptors, Berlin, 1970, Pergamon Press (in press).

7. D. Toft, G. Shyamala and J. Gorski, Proc. Nat. Acad. Sci. U.S.A., 57, 1740 (1967).

8. G. Giannopoulos and J. Gorski, J. Biol. Chem., 246, 2530 (1971).