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SLOWLY EXCHANGEABLE POOL OF ESTRADIOL IN THE RAT UTERUS

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SUMMARY

Chase experiments, alternating infusions with ^{14}C and ^{3}H labelled estradiol, were performed in adult rats. After infusion, purified nuclei were extracted from uterine homogenates and the $^{3}H/^{14}C$ ratio was determined in cytosol and nuclear fractions. It was shown that {4^{14}C}estradiol exchanged progressively with {6,7^{3}H}estradiol in all subcellular fractions, but at a much slower rate than in the plasma.

The retardation was more evident in the Tris-EDTA-KCl extract (S_{III}) of the nuclear preparations than in other soluble "receptors" from the cyctosol (S_I) or the particulate fraction (S_{II}). The final residual precipitate of the nuclear fraction contained a limited pool of estradiol, more slowly exchangeable than in other fractions.

These data confirm the extensive recycling of estradiol within the uterine cell. They also show in the nucleus, the existence of a more slowly exchangeable pool, of limited capacity, which appears to be possibly a final but still reversible step in the hormone distribution within the cell.

The implication of this limited pool in the hormonal activity is briefly discussed.

CHASE experiments, alternating long-term intravenous infusion with radioinert estradiol and short-term infusion with {6,7^{3}H}estradiol, or vice versa, have shown the existence of a relatively rapid exchange-process between plasma and tissue hormone[1]. The limiting factors in this exchange mechanism were the blood-flow through the organ (hence the supply in circulating hormone to be exchanged per unit time) [2, 3] and the recycling of tissue hormone within the cell [1]. Indeed, estradiol taken up by the uterus is rapidly distributed throughout the subcellular fractions and diluted with the pre-existing pools, without obvious modification in their respective sizes[1, 2]. It then participates in the dynamic equilibrium which appears to be continuous between the intracellular pools and the plasma at equilibrium. All subcellular pools however do not attain the steady state with the same rapidity. The particulate fraction appeared to show a relative delay in the establishment of this equilibrium [1, 2]. In order to have a better insight into the cellular dynamics, chase experiments were performed by alternating {^{14}C} and {6,7^{3}H}estradiol-17β infusions with different time sequences.

MATERIALS AND METHODS

The materials and the methods were essentially the same as described previously [1, 2].

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{4[14]C}estradiol (50 mCi/mmol, NEN) was checked for radiochemical purity (at least 97 %) by Celite or Sephadex LH_{20} column chromatography.

Purified nuclei were prepared by two different methods:

Method A (*adapted from Widnell et al* [4–6])

Two uteri were homogenized in 2 ml of sucrose 0·32 M, containing 3 mM $MgCl_2$ and 14 mM 2-mercaptoethanol (homogenizing medium) in an all glass homogenizer (Potter) as described previously [2]. After filtering the homogenate and washing the filter with adequate amount of distilled water to give a final sucrose molarity of 0·25 M, the filtrate was layered on top of 1 ml homogenizing medium. After 10 min-centrifugation at 700 *g*, at 4°C, the supernatant was recovered (S_I, or cytosol fraction). The particulate fraction was then rehomogenized in 2 ml sucrose 2·4 M, containing 1 mM $MgCl_2$ and centrifuged for 10 min at 50·000 *g*. The supernatant was recovered (S_B). The precipitate was once again resuspended in 4 ml sucrose 2·4 M and centrifuged at 50·000 *g* for 45 min. Three layers were usually obtained: a plug on top of the sucrose (pellet C), the sucrose layer (S_D) and the "nuclear" precipitate. The latter was then successively extracted with Tris-EDTA buffer, pH 8·2 (S_{II}) and Tris-EDTA-KCl pH 8·5 (S_{III}) as previously described [2]. The last precipitate (Ppt) was counted for radioactivity as such.

Method B

Two uteri were homogenized in 2 ml homogenizing medium in a Teflon–Glass homogenizer (Potter). The homogenate was filtered and the filter washed as described previously. This first filtrate was called: homogenate 1. The non-filtrable residue was rehomogenized in the same solution in an all-glass homogenizer and filtered. The filter was washed as above. This second filtrate was called: homogenate 2. Homogenates 1 and 2 were layered separately on 1 ml of homogenizing medium and centrifuged at 700 *g* for 10 min. The supernatants (cytosol fractions) were recovered (S_I^1 and S_I^2). The precipitate of homogenate 1 was resuspended in 0·5 ml of 30 % sucrose; 0·25 ml of the suspension was layered on top of a 4·5 ml continuous 30–60 % sucrose gradient and centrifuged for 15 min at 800 *g*. 24 fractions were collected. On monitoring the fractions for radioactivity, a peak was usually found in the fractions 6–14, which were pooled, diluted with an equal volume of homogenizing medium and centrifuged for 15 min at 2·400 *g*.

The precipitates obtained from the pools 6–14 (particulate 1) which mostly contained the purified nuclei, and from the homogenate 2 (particulate 2) were then extracted with Tris-EDTA pH 8·2 (giving the supernatants S_{II}^1 and S_{II}^2), and Tris-EDTA-KCl pH 8·5 (giving the supernatants S_{III}^1 and S_{III}^2 and the precipitates Ppt^1 and Ppt^2) as previously described [2].

Comparison of methods A *and* B

RNA, DNA [7, 8] and proteins [9] were measured in the "nuclear" particulate fractions obtained by both methods from pools of adult rat uteri. Table 1 gives the results obtained.

It can be seen that in our hands, although method A was suitable for preparation of liver nuclei, it did not yield a substantial purification of nuclei from uteri homogenates, in so far as RNA/DNA or protein/DNA ratios were taken as

Table 1. Comparison of 2 methods of nuclear preparation (see text) from pools of adult rat uteri

	Method A				Method B			
					Homogenates		Particulate fractions	
	Pool n°	Homogenate	Pellet C	"Nuclear" Precipitate	Pool n°	1	2	1	2
RNA/DNA	1	0·70	0·64	0·46	a	0·68	0·47	0·21	0·43
	2	0·60	0·60	0·50	b	0·67	0·47	0·22	0·41
	3	0·80	—	0·69	c	0·72	0·50	0·23	0·44
	4	0·47	0·85	0·41					
	5	0·68	—	0·47					
	6	4·3*	—	0·25*					
Prot./DNA	1	12·1	8·6	4·2	a	20·6	11·0	3·9	—
	2	13·4	17·7	10·3	b	17·0	9·7	4·9	8·0
	3	19·1	—	7·8	c	18·6	7·9	4·5	14·8
	4	14·8	8·6	6·1					
	5	9·2	—	4·8					
	6	144·0*	—	3·3*					

*Liver.

criteria of degree of purification. Method B gave better and more reproducible nuclear preparations (particulate fraction 1).

RESULTS

1. *Exchange of estradiol between plasma and uterine subcellular pools*

Groups of 10–12 adult rats were first infused for 3 h with {4^{14}C}estradiol at a rate of 200 ng/h. The infusion was then continued at the same rate with {6,7^{3}H}-estradiol, for varying times from 1 to 180 min. At the end of the infusions plasma and uteri were collected; the ^{3}H and ^{14}C content was determined in the pools of the corresponding subcellular fractions of all uteri (S_I, S_{II}, S_{III}, Ppt) and in the metabolites (E_1, E_2 conjugates) of the pooled plasma samples. It was found necessary to pool the corresponding uterine fractions and the plasma extracts of all the animals in order to accumulate sufficient counts in the ^{14}C channel. Counting error was kept below 3 % for each reported result. Results are summarized in Table 2.

The table shows that the relative distribution of ^{14}C(% ^{14}C) in the subcellular fractions of the uterus remained unchanged, whatever the duration of chasing with {6,7^{3}H}estradiol (1–180 min). The latter was rapidly distributed throughout the subcellular fractions. After 1 min only, more than half of the tritium was associated with the particulate fractions (S_{II}, S_{III} + Ppt) inspite of the very low concentration of {6,7^{3}H}estradiol in the tissue at this moment (0·27 % of the total, ^{3}H + ^{14}C, estradiol). However, the ^{3}H/^{14}C ratio shows that the relative amount of {6, 7^{3}H}estradiol was higher in the cytosol (S_I) and lower in the particulate fractions (S_{II}, S_{III} and Ppt). The lowest ratio was found in the S_{III} fraction (about three times less than in the cytosol).

When {6,7^{3}H}estradiol was infused for a longer period of time, the proportion of tritium slowly equilibrated with the ^{14}C in all the subcellular fractions; ^{3}H/^{14}C ratio however, confirmed the much slower equilibration in the S_{III} fraction. After 3 hours of {6,7^{3}H}estradiol infusion, a complete equilibration was obtained between

Table 2. Exchange of {6, 7^{3}H}estradiol with {4^{14}C}estradiol in plasma and uterine subcellular fractions. {4^{14}C}estradiol was infused during 3 h. {6, 7^{3}H}estradiol was infused during 1–180 min. Rate of infusions: 200 ng/h

Expt n°*	Infusion time of E_2-6, 7^{3}H	Uterus					Plasma				Muscle
		Total	S_I	S_{II}	S_{III}	Ppt	Total	E_1	E_2	Conj.	Total
1	1 min										
	% ^{14}C	100	22	9	31	38	—	—	—	—	—
	% ^{3}H	100	38	14	17	32	—	—	—	—	—
	^{3}H/^{14}C†	1·9	3·5	3·2	1·1	1·8	25	6·4	44	4·7	—
	% E_2 as ^{3}H‡	0·27	0·51	0·47	0·16	0·26	3·5	0·9	6·1	0·6	—
2	1 min										
	^{3}H/$_{14}$C	5·1	9·9	5·6	2·2	3·6	64	17	92	11	31
3	5 min										
	% ^{14}C	100	21	5	33	41	—	—	—	—	—
	% ^{3}H	100	48	7	19	26	—	—	—	—	—
	^{3}H/^{14}C	9	20	13	5	6	—	—	—	—	—
	% E_2 as ^{3}H	2·0	4·2	2·8	1·0	1·2	—	—	—	—	—
4	15 min										
	% ^{14}C	100	24	7	21	49	—	—	—	—	—
	% ^{3}H	100	35	5	17	42	—	—	—	—	—
	^{3}H/^{14}C	8	12	6	7	7	62	53	160	13	
	% E_2 as ^{3}H	8	11	6	7	7	40	37	62	12	—
											—
5	45 min										
	% ^{14}C	100	21	9	26	45	—	—	—	—	—
	% ^{3}H	100	31	10	19	40	—	—	—	—	—
	^{3}H/^{14}C	14	20	16	10	12	68	38	194	36	—
	% E_2 as ^{3}H	17	22	19	13	15	50	37	76	35	—
6	180 min										
	% ^{14}C	100	19	7	28	46	—	—	—	—	—
	% ^{3}H	100	19	7	27	47	—	—	—	—	—
	^{3}H/^{14}C	34	31	34	32	33	—	—	—	—	—
	% E_2 as ^{3}H	49	46	49	46	48	—	—	—	—	—

*Each experiment was carried out on the pool of uteri from 12 rats infused similarly.
†Isotopic ratio (radioactivity ^{3}H/radioactivity ^{14}C).
‡% of total weight of radioactive estradiol (^{3}H + ^{14}C) present as {6, 7^{3}H} estradiol.

^{3}H and ^{14}C in all the fractions, despite the persistence of about 50 % of ^{14}C labelled estradiol. In the plasma, the relative concentration of {6,7^{3}H}estradiol increased much more rapidly than in the uterus. After 45 min less than 25 % of the plasma estradiol was still ^{14}C labelled estradiol. This observation confirms the extensive recycling of estradiol within the uterus and the existence of a dynamic equilibrium between all the subcellular fractions.

2. *Slowly exchangeable pools in the nuclear fractions*

(a) *KCl-extractable pool.* Groups of 10–12 adult rats were sequentially infused first with {4^{14}C}estradiol during 3 h and then with {6,7^{3}H}estradiol for time periods of 1–180 min as described above. Nuclei were purified according to

Table 3. $^{3}H/^{14}C$ ratio of subcellular fractions (method A) of adult rat uteri, isolated after 3 h-infusion with {$4^{14}C$}estradiol (200 ng/h) followed by infusions during 1–180 min (200 ng/h), or *in vitro* incubation of total uterus or uterus homogenates with {$6,7^{3}H$}estradiol

	Infusion time (min)			*In vitro* incubation	
Cell fractions	1*	45*	180*	Whole tissue* (15 min; 5×10^{-9} M)	Uterus homogenate* (60 min; 5×10^{-10} M)
Cytosol (S_I)	—	18	2·2	132	24
Cytosol (106·000 *g*)	9·1	—	—	—	—
Precipitate	7·8	—	—	—	—
S_B (sucrose 2·4 M)	8·6	14	1·8	121	17
Pellet C	5·5	9·8	1·9	32	5·5
S_D (sucrose 2·4 M)	4·1	10·2		44	8·6
Nuclear fraction					
S_{II}	6·1	13·0	1·7	76	16
S_{III}	3·0	5·6	1·7	8	2·6
Ppt	5·1	8·4	1·8	lost	16

*Each experiment was carried out on the pool of uteri from 12 rats treated similarly.

method A (see methods). The results of the $^{3}H/^{14}C$ ratio in the subcellular fractions appear in Table 3.

In another experiment a group of animals was infused with {$4^{14}C$}estradiol for 3 h, and the uteri were then incubated *in vitro* for 15 min at 4°C in the presence of 5×10^{-9} M {$6,7^{3}H$}estradiol. In a third set of experiments, the animals were infused with {$4^{14}C$}estradiol, the uteri were homogenized and the homogenate was incubated for 60 min at 4°C in the presence of 5×10^{-10} M {$6,7^{3}H$}estradiol (Table 3).

From both *in vivo* and *in vitro* experiments, it appears that the S_{III} fraction from the purified nuclei contained a much lower $^{3}H/^{14}C$ ratio than the other fractions, resulting from a much slower exchange rate. However, this fraction also eventually equilibrated with the others (Table 3). $^{3}H/^{14}C$ ratios in the S_{II} fraction from the nuclear preparations were closer to the ratios in the cytosol.

When purified nuclei were obtained according to method B (see methods) after infusion with {$4^{14}C$}estradiol for 3 h followed by a 2 min infusion with {$6, 7^{3}H$}estradiol, essentially the same results were obtained (Table 4). The lowest $^{3}H/^{14}C$ ratio was found in the KCl extract of the most purified nuclear fraction (S^{1}_{III}).

(b) *Non extractable residual pool.* The estradiol exchange at the level of the nuclear residual precipitate (Ppt) was further investigated.

A group of 12 adult rats were injected intramuscularly with 30 ng (12 μc) of {$2, 4, 6, 7^{3}H$}estradiol at 5 p.m. The following day, after 16 h, the animals were submitted to 3 h-infusions with {$4^{14}C$}estradiol at a rate of 200 ng/h. Uteri were then processed in the usual manner and purified nuclei were obtained by method B. $^{14}C/^{3}H$ ratios (Table 4) show that the isotope equilibration was somewhat slower at the level of the final precipitates (Ppt^{1} and Ppt^{2}).

Table 4. $^3H/^{14}C$ Ratio of purified nuclei extracts of adult rat uteri (method B)* A: 3 h-intravenous infusion with {$4^{14}C$}estradiol (200 ng/h), followed by a 2 min-infusion with {$6,7^3H$}estradiol
B: Intramuscular injection of {$2, 4, 6, 7^3H$}estradiol (30 ng), followed, after 16 h, by a 3 h-infusion with {$4^{14}C$}estradiol (200 ng/h)
C: 4 h-intravenous infusion with {$4^{14}C$}estradiol (200 ng/h), followed by a 1 min infusion with {$6,7^3H$}estradiol, and a subsequent 30 min-infusion with radioinert estradiol (500 ng/h)

		A	B‡	C
Homogenate 1† (Teflon)	-Cytosol fraction (S_I^1)	14·9	13·5	6·3
	-Particulate fractions			
	"Nuclear fraction" (particulate 1)			
	$-S_{II}^1$	6·5	11·7	5·3
	$-S_{III}^1$	3·9	10·4	4·4
	$-Ppt^1$	lost	9·4	2·5
Homogenate 2† (glass)	-Cytosol fraction (S_I^2)	8·6	19·2	7·0
	-Particulate fraction 2			
	$-S_{II}^2$	5·8	17·1	5·2
	$-S_{III}^2$	4·5	18·1	5·2
	$-Ppt^2$	6·1	15·8	4·1

*Each experiment was carried out on the pool of uteri of 12 rats treated similarly.
†Uteri were homogenized in Teflon-glass homogenizer (homogenate 1), and the non filtrable residue was rehomogenized in glass-glass homogenizer (homogenate 2). See text.
‡$^{14}C/^3H$ ratio.

A group of 12 adult rats were submitted to 4 h-intravenous infusions with {$4^{14}C$}estradiol at a rate of 200 ng/h. The infusions were then switched to {$6,7^3H$} estradiol for 1 min at the same rate, and afterwards to radioinert estradiol at a rate of 500 ng/h for 30 min. Table 4 shows that the slowest isotope equilibration occurred at the level of the final nuclear precipitate (Ppt^1).

DISCUSSION

Earlier experiments had shown that estradiol entering the uterine cell was rapidly exchanged with intracellular hormone, not only in the cytosol but also in the particulate fractions, without evident changes in the binding capacities of the "receptors" [1, 2]. This observation is confirmed here by the present study, using a double isotopic technique.

Indeed, the relative distribution of {$4^{14}C$}estradiol observed in the subcellular fractions of the adult rat uterus, at the end of a 3 h continuous infusion, was not significantly modified when the infusion was prolonged with {$6,7^3H$}estradiol at the same rate. The latter however progressively replaced the former in all the fractions. The kinetics of the replacement was somewhat more rapid in the cytosol fraction (S_I) where the percentage of tritium labelled estradiol was higher than in the binding constitutents of the particulate fractions (S_{II}, S_{III} and Ppt) in the first few minutes of {$6,7^3H$}estradiol infusion. Later, the increasing percentages of tritium labelled estradiol became similar in all the fractions, despite the persistance, after 3 h, of more than 50% of ^{14}C labelled estradiol (see Table 2). {$4^{14}C$}estradiol in the same time intervals, disappeared much more rapidly from the plasma where

it was replaced by {6,7^{3}H}estradiol. After 15 min, indeed, 62% of the plasma estradiol and only 8% of the uterine estradiol were tritium labeled.

This observation confirms the intensive recycling of estradiol within the uterine cell. The replacement of plasma {4^{14}C}estrone by tritium labelled hormone was slower as compared to plasma estradiol. This can be accounted for by the lower metabolic clearance rate of estrone in the rat [10] and the necessary time interval of conversion of estradiol to estrone in the blood. Similar delay was observed in the case of other metabolites of estradiol.

Among the extractable "receptors", the lowest ^{3}H/^{14}C ratio was found in the S_{III} fraction (KCl extract) and this became more evident when nuclei were purified (see Tables 2–4).

The non extractable final precipitate gave a ^{3}H/^{14}C ratio higher than the KCl extract (S_{III}), when the uteri were processed immediately at the end of the chasing infusion with {6,7^{3}H}estradiol (see Tables 2, 3 and 4A). This higher ratio was even more evident in '*in vivo*'–'*in vitro*' chase experiments (see Table 3). However when a further chasing was performed with radioinert estradiol (see Table 4C), the ^{3}H/^{14}C ratio was lower in the final precipitate than in the KCl. The data suggest that the final precipitate possesses a composite estradiol pool: one rapidly exchangeable with the circulating hormone and another limited pool of tightly bound hormone. The latter however was not solely responsible for the long lasting retention of estradiol in the uterus, since the major part of the {6,7^{3}H} estradiol found in the tissue, 16 h after injection, was almost proportionally exchanged with the chasing ^{14}C-estradiol in all the subcellular fractions (see Table 4B).

It can therefore be inferred that slowly exchangeable pools exist in the nuclei of adult uterine cells. One rather large pool is KCl-extractable and the other more limited and less exchangeable pool, is associated with the non-extractable nuclear precipitate. The latter appears as a possible last but still reversible step of the hormone distribution within the cell.

The existence in the nucleus of a very limited set of binding sites of high affinity has been reported by Alberga *et al.* [11] from "in vitro" experiments. Whether our observations of the existence of a limited pool of slowly exchangeable hormone in the nucleus is the *in vivo* expression of their *in vitro* finding [11] can not be ascertained because of the lack of quantitative estimation of the *in vivo* binding, as far as capacity and affinity are concerned.

The physiological implication of these observations may be twofold. Either the slowly exchangeable nuclear pool represents the final point of the estradiol distribution within the cell from which the hormonal activity will be functional or it acts merely as a reservoir of hormone available for binding to a limited number of active sites more directly related to the expression of the hormonal activity. The maintenance of nucleus bound hormone for several hours is indeed necessary to obtain the long term uterotrophic response [12].

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