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DYNAMIC EXCHANGE OF {6, 7³H}ESTRADIOL-17β AT THE SUBCELLULAR LEVEL IN RAT UTERUS

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SUMMARY

In a previous work it was shown that {6, 7³H}estradiol, administered as a continuous intravenous infusion to adult rats, was taken up by the uterus and partitioned rapidly between cytosol and particulate fractions, where it was bound to specific "receptors". The rapid passage to the particulate fraction was followed by a slower equilibration leading to a constant ratio of radioactivity of about 25 and 75% in cytosol and particulate fractions respectively. The dynamic equilibrium which was suggested from these data was further analyzed by chase-experiments. Adult rats were first submitted to 3 h-intravenous infusions with radioinert estradiol, at a rate of 200 ng/h, in order to saturate the uterine binding sites; the infusion was then switched over to {6, 7³H} estradiol at the same rate, for 1–180 min.

Uterine radioactivity increased progressively; however, contrary to plasma free radioactivity which had reached its equilibrium level after 45 min, the steady state was not achieved after 180 min. Subcellular partition of the labelled hormone in the uterus however was effected in a similar way as during radioactive infusions alone, without a preliminary saturation of the uterus with radioinert hormone. Binding to the "receptor" was manifest after only 2 min of {6, 7³H}estradiol infusion.

In vivo–in vitro chase experiments were performed by alternating 3 h-intravenous infusion with {6, 7³H}estradiol and 2–60 min incubation of the uteri with 5×10^{-9} M radioinert estradiol, or vice versa. Estradiol bound to the uterus *in vivo* was slowly exchanged with the hormone from the medium. In course of the incubation the subcellular distribution of the former did not change. The subcellular distribution of the latter showed a slightly higher proportion in the cytosol up to 20 min of incubation.

These data show that estradiol taken up by the uterus remains exchangeable with the circulating hormone. Also, a continuous dynamic exchange of estradiol exists between cytosol and particulate fractions, resulting in an intracellular recycling of the hormone. The particulate fraction however shows some retardation in the complete equilibration with the cytosol suggesting the existence in the former of a more slowly exchangeable pool.

THE FATE of estradiol-17β in the uterine cell has been the subject of a number of studies. It was proposed that estradiol taken up by the cell was first associated to a cytosol "receptor" and was subsequently translocated to the nucleus [1–5]. This passage from the cytosol to the nucleus involved some degree of "consumption" of the cytosol "receptor" sites. Indeed, a decrease of cytosol binding ability has been described after estrogen injection in immature rats and after *in vitro* incubation of immature rat uteri in the presence of estradiol [6, 7].

When estradiol was administered to adult rats via a constant intravenous infusion, it was shown that the passage to the particulate fraction of the cell was very rapid, and that the relative concentration of estrogen in the cytosol and in the particulate fractions eventually came to an equilibrium, notwithstanding the continuation of estradiol infusion and of the increase in uterine total estradiol concentration [8].

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Hereby, a dynamic equilibrium between the subcellular components was suggested, and it was further studied by *in vivo* and *in vitro* chase experiments, as described in the present investigation.

MATERIALS AND METHODS

The materials and the techniques used have been described earlier [8, 9]. Radioinert estradiol-17β (Sigma) was crystallised thrice in ethanol–water before use. The chemical purity was checked by celite column partition chromatography, Sephadex LH_{20} column chromatography and the U.V. absorption spectrum. *In vitro* incubations were performed in Krebs–Ringer solutions pH 7·4, containing 1 g/l of glucose and 30 g/l of BSA.

RESULTS

1. *Subcellular exchange of estradiol at equilibrium concentration of the hormonal pools.* In vivo *experiments*

Groups of adult rats were infused intravenously for 3 h with radioinert estradiol-17β, at a rate of 200 ng/h. At this infusion rate, the uterine binding sites were saturated [9, 10] and the estrogen pools in the plasma and in the tissue were in equilibrium [8–10]. The infusion was then switched over to {6, 7^{3}H}estradiol at the same rate for 1–180 min. The animals were sacrificed at 1, 2, 5, 10, 15, 20, 30, 45, 60, 120 and 180 min after the start of the radioactive infusion. Total unconjugated radioactivity was determined in plasma. Total radioactivity was measured in whole uteri and also in the particulate and in the cytosol fractions after separation. A group of animals was infused with radioactive estradiol only for 360 min, to serve as controls for the group of animals infused sequentially with radioinert estradiol for 180 min and radioactive estradiol for 180 min.

Figure 1 shows that plasma level of unconjugated radioactive estrogens increased rapidly and reached its plateau level in about 45 min. The shape of this curve was similar to the one obtained when the labelled hormone was infused, without prior saturation with radioinert component [8]. Uterine radioactivity level increased much more slowly and did not reach equilibrium by 180 min. This can be accounted for by the fact that the uterine sites were already saturated with radioinert estradiol [11, 12]. However, the subcellular partition of the labelled hormone between the cytosol and the particulate fractions was very rapidly achieved, despite a minor proportion of radioactive compared to the total estradiol in the tissue. The relative concentration of labelled hormone in the cytosol was already less than 50 % from the first minute of infusion and diminished to an equilibrium level of 25–30 %, whereas the radioactivity still increased steeply in the tissue as a whole.

The binding of the tracer to various subcellular fractions is configurated in Fig. 2. The three types of uterine "receptors", S_I (cytosol) and S_{II} and S_{III} (particulate) [8], were analyzed by ultracentrifugation on 5–20 % sucrose gradient containing KCl (0·4 M). On isolation of the three "receptors" 60 min after the start of the tracer infusion, one obtained their characteristic sedimentation patterns [8]. After 2 min, the peaks corresponding to S_I and S_{III} were already evident. The peak corresponding to S_{II} was also perceptible, although the general pattern was somewhat distorted due to an excessive amount of unbound radioactivity.

Chase experiments with {6, 7^{3}H}estradiol were also performed in immature rats previously infused with radioinert estradiol for 3 h at a rate of 100 ng/h.

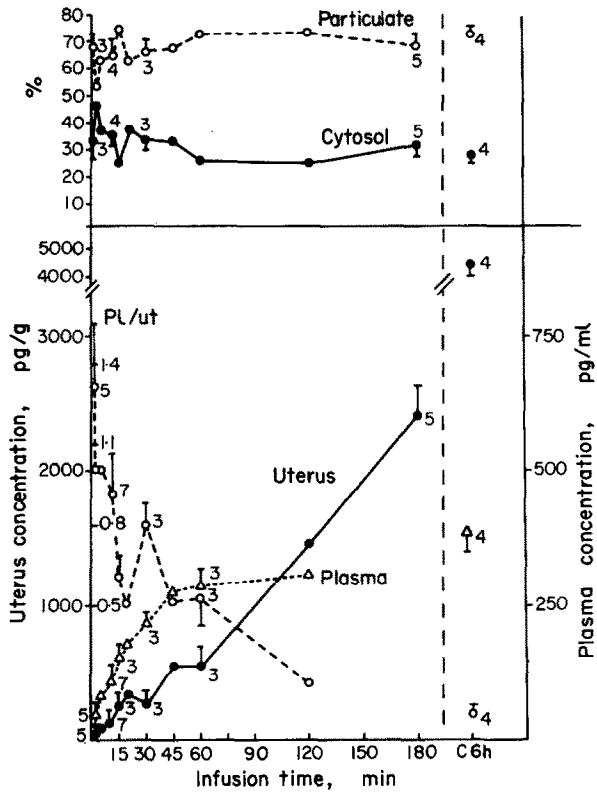

Fig. 1. Concentration of total radioactivity in the uterus and of total free radioactivity in the plasma of adult rats, expressed in terms of weight equivalent of {6, 7³H}estradiol during continuous intravenous infusion of the labelled hormone at a rate of 200 ng/h. The ratio plasma/uterus concentrations(Pl/Ut) is also indicated. The infusions with {6, 7³H} estradiol were preceded by a 3 h-infusion with radioinert estradiol at the same rate (lower part of the graph). The radioactive distribution in the cytosol and the particulate fractions of the uterus is indicated as % of the total radioconcentration in the tissue homogenate (upper part).

On the right part of the graph, results from control animals, submitted to 6 h- (C 6 h) intravenous infusion with {6, 7³H}estradiol, not preceded by infusion with radioinert hormone, are indicated.

Each point is the mean ± S.D.M. of indicated numbers of animals. Points without S.D.M. are means of 2 animals.

The relative percentage of total radioactivity associated with the cytosol fraction, 2 and 30 min after the start of the infusion, were 44 and 43 % respectively. In control animals, infused for 3 h with {6, 7³H} estradiol alone, the percentage of radioactivity in the cytosol was 39 %. The subcellular binding of exchanged hormone in immature animals was determined in one group of 6 rats infused first for 3 h with {6, 7³H} estradiol at a rate of 26 ng/h followed by a further 3 h infusion with radioinert estradiol at a rate of 200 ng/h. The residual radioactivity in the cytosol fraction of the pooled uteri, at the end of the experiment, represented 38 % of the total. The radioactivity in the cytosol (S_I) and in the nuclear KCl extract (S_{III}) presented the characteristic sedimentation patterns on a sucrose KCl gradient (Fig. 3a). The reverse experiment was done by infusing unlabelled estradiol (200 ng/h) in the first place followed by {6, 7³H}estradiol (26 ng/h). In this

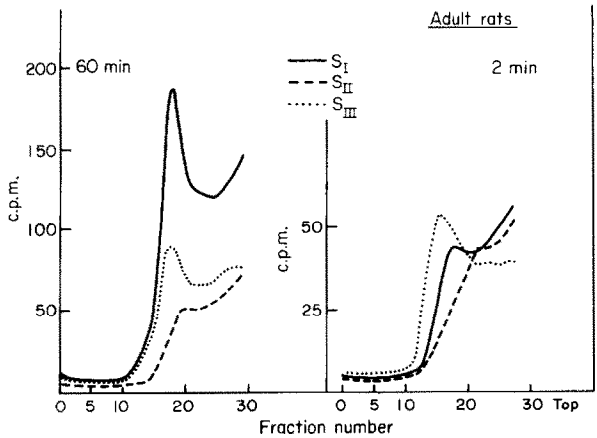

Fig. 2. Sedimentation profiles of uterine radioactive fractions isolated from adult rat uteri after 2 or 60 min-intravenous infusions with {6, 7³H}estradiol at a rate of 200 ng/h, preceded by a 3 h-infusion with radioinert estradiol at the same rate. S_I (cytosol), S_{II} (Tris-EDTA extract from the particulate fraction) and S_{III} (Tris-EDTA-KCl extract from the particulate fraction) were centrifuged for 16 h at 135·000 *g* on 4·5 ml 5–20 % sucrose 0·4 KCl gradients.

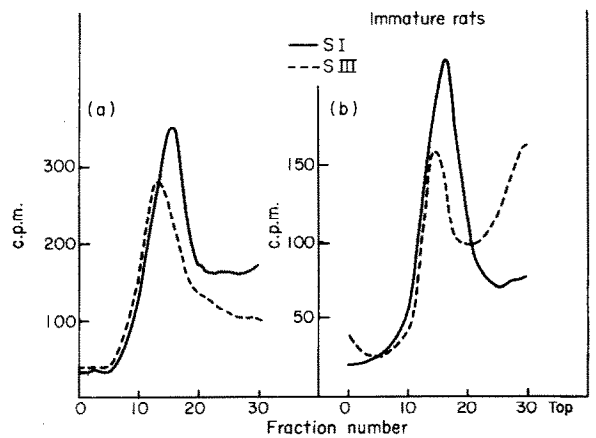

Fig. 3. Sedimentation profiles of radioactive S_I and S_{III} fractions (see Fig. 2) of immature rat uteri on sucrose-KCl gradients. (a) 3 h-intravenous infusion with {6, 7³H}estradiol (26 ng/h), followed by a 3 h-intravenous infusion with radioinert estradiol (200 ng/h). (b) 3 h-intravenous infusion with radioinert estradiol (200 ng/h) followed by a 3 h-intravenous infusion with {6, 7³H}estradiol (26 ng/h).

case the percentage of radioactivity in the cytosol fraction was 33 %. The sedimentation patterns of S_I and S_{III} were similar to those in the first experiment (Fig. 3b).

2. *Subcellular exchange of estradiol at equilibrium.* In vitro *experiments*

(a) In vitro *uptake and subcellular distribution of* {6, 7³H} *estradiol.* In order to analyse the subcellular exchange of estradiol *in vitro*, it was necessary to look for an adequate concentration of the hormone in the incubation medium. Therefore, adult and immature uteri were incubated for 2 h at 37°C in Krebs–Ringer-

glucose medium, with or without BSA (3 g%) in the presence of increasing concentrations of {6, 7³H}estradiol (from 5×10^{-11} to 3×10^{-6} M).

Figure 4 gives the log–log correlation between concentration of tritiated estradiol in the uterus (in ng/g) at the end of the incubation time and the molar concentration of the tracer in the medium. Unlike *in vivo* experiments, where the plasma concentration with respect to the uterine concentration gave a hyperbolic correlation [9] (see Fig. 4), the *in vitro* data presented an almost linear correlation throughout the range of concentrations, in the presence or in the absence of BSA in the medium for both immature and adult rats.

At low concentrations in the medium (below 10^{-9} M) {6, 7³H}estradiol concentration in the uterus was lower than the corresponding values for the *in vivo* experiments. When the concentration in the medium was between 10^{-8}–10^{-9} M, {6, 7³H}estradiol concentration in the uterus superceded the saturation value of the uterine binding sites (tissue capacity) shown by the plateau levels in *in vivo* experiments. The presence of BSA decreased the uptake of estradiol by 3–4 times at low and by 5–8 times at high medium concentrations. In the presence of BSA, the ratio of tissue (moles/kg) to medium (moles/l) concentrations decreased from 3 in adult and 6 in immature rats at low medium concentration, to about 1 in both groups at high medium concentration.

The differences observed between *in vivo* and *in vitro* uptake of {6, 7³H} estradiol suggest that unspecific binding occurred *in vitro*. Figure 5 shows the sedimentation patterns of S_I and S_{III} fractions isolated after incubation of adult rat uteri in the presence of 5×10^{-7}, 5×10^{-8} or 5×10^{-9} M {6, 7³H}estradiol, and 3 g% BSA. At the lowest molarity, the characteristic pattern was observed for both "recep-

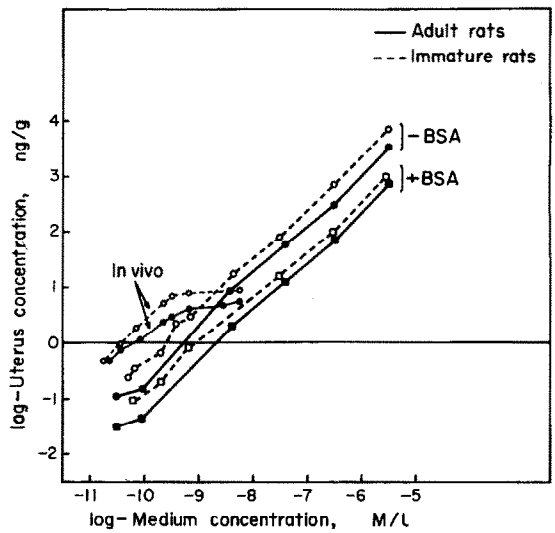

Fig. 4. Log–log relationship between radio concentration of adult (-) or immature (...) uteri (in ng of {6, 7³H}estradiol per g of wet tissue) and molar concentration of {6, 7³H} estradiol in the medium at the end of 2 h- *in vitro* incubation at 37°C in Krebs–Ringer solution, pH 7·4, containing 1 g/l of glucose, with (+ BSA) or without (− BSA) bovine serum albumin (3 g%) in the medium. Radio-concentration of adult or immature rat uteri, at the end of 4 h-intravenous infusions with {6, 7³H}estradiol at increasing rates are indicated as comparison; plasma concentration of {6, 7³H}estradiol is considered as the "medium" concentration (*in vivo*) (calculated from De Hertogh *et al.* [9].

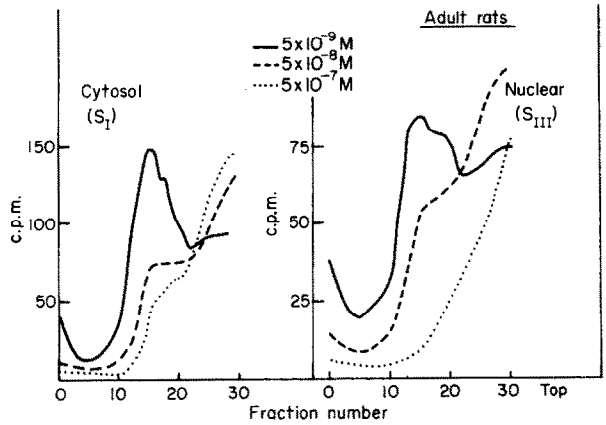

Fig. 5. Sedimentation profiles of radioactive S_I and S_{III} fractions (see Fig. 2) of adult rat uteri, incubated for 2 h at 4°C in Krebs–Ringer glucose-BSA medium pH 7·4 in the presence of increasing molarities of estradiol and a constant concentration of {6, 7³H} estradiol in the medium. BSA was present at a concentration of 3 g %. S_I and S_{III} fractions were centrifuged on sucrose-KCl gradient at 135·000 *g* for 16 h.

tors". At higher concentrations, large amounts of radioactive estradiol appeared on top of the gradients, and the 4 or 5 S peaks were masked. The percentages of uterine radioactivity in the cytosol fractions were 38, 41 and 52 % at 5×10^{-9}, 5×10^{-8} and 5×10^{-7} μ respectively. On the basis of these experiments {6, 7³H} estradiol concentration in the medium was usually set at 3 to 5×10^{-9} M for ulterior *in vitro* studies.

(b) *In vitro exchange of estradiol with subcellular pools.* Two simultaneous experiments were carried out in order to study the *in vitro* exchange of subcellular estrogen pools. In a first set of experiments adult rats were infused with {6, 7³H} estradiol for 3 h at a rate of 200 ng/h, in order to saturate the binding sites. The uteri were then excised, sliced and immediately incubated at 4°C from 2 to 60 minutes with radioinert estradiol at a concentration of 5×10^{-9} M in Krebs–Ringer solution containing glucose and albumin. In a second set of experiments, radioinert estradiol was infused and the incubation medium contained ³H estradiol, other conditions remaining unchanged. At the end of the incubation times, the uterine slices were carefully washed three times in the incubation medium containing no estradiol, blotted and processed for cytosol and particulate fractions.

Table 1 gives the percentages of radioactivity in the subcellular fractions on the one hand, and the total radioactive estradiol in the tissue and in the incubation medium (in ng/g of incubated uteri) on the other hand. It can be seen that the proportion of radioactivity in the cytosol (S_I) remained unchanged despite the progressive washing out of labelled hormone into the incubation medium (Table 1a). Conversely, the radio-active estradiol taken up by the tissue from the incubation medium (Table 1b), was rapidly partitioned between cytosol and particulate fractions, although the cytosol proportion remained higher. After 20 min of incubation, about 10 % of the uterine estradiol had been exchanged with the medium.

3. {6, 7³*H*}*estradiol distribution in uterus homogenates*

Homogenates of uteri of adult or immature rats in 0·32 M sucrose were

Table 1. *In vitro* exchange of estradiol in the uterus*

Incubation time (min)	Concentration of {6, 7³H}estradiol: Tissue (pg/g)	Concentration of {6, 7³H}estradiol: Medium (pg/g of incubated tissue)	Subcellular distribution (%): Cytosol (S_I)	Particulate: S_{II}	Particulate: S_{III}	Particulate: Ppt
A. 3 h-Infusion with {6, 7³H}E_2 (200 hg/h). Incubation with radioinert E_2 (5×10^{-9} M)						
2	–	–	29	10	20	41
2	–	–	30	12	24	35
10	–	–	29	13	24	34
20	–	–	30	9	26	35
60†	–	–	25	10	24	41
2	4·800	150	29 (27)‡	8 (8)	25 (19)	38 (46)
10	3·960	200	29 (26)	9 (9)	29 (28)	33 (37)
20	4·200	460	31 (37)	10 (10)	26 (23)	32 (30)
B. 3 h-Infusion with radioinert E_2 (200 hg/h). Incubation with {6, 7³H}E_2 (5×10^{-9} M)						
2	120	–	50	11	15	23
2	–	–	47	17	17	19
10	230	–	49	14	15	23
20	400	–	38	12	14	36

*Results were obtained from single rat experiments.
†Pool of two animals.
‡In parenthesis: results for non-incubated horn of same animal.

incubated for 1 h at 4°C in the presence of trace amounts of {6, 7³H}estradiol. At the end of the incubation time, the subcellular fractions (S_I, S_{II}, S_{III}) were obtained.

Figure 6 shows the sedimentation characteristics of S_I, S_{II} and S_{III} fractions on sucrose-KCl density gradients. S_I yielded the usual pattern of radioactivity distribution, but no binding peak was found, either for S_{II} or for S_{III}. The residual precipitate contained a minor amount of radioactivity. A similar distribution of the radioactivity was observed when adult rat uteri were homogenized in the presence of trace amounts of {6, 7³H}estradiol.

DISCUSSION

Estradiol taken up by the uterus is retained in the tissue for prolonged periods by virtue of the specific binding to the cytosol and nuclear "receptors". Several investigators have reported that the cytosol binding sites play an active role in the transport mechanism of estradiol from the cytosol to the nucleus [1–5], and that this mechanism involves a loss of cytosol binding capacity [6, 7]. This translocation of estradiol to the particulate fraction is rapid and in less than 5 min, more than 50% of uterine radioactivity is associated with this fraction [5, 8]. However, on prolongation of infusion of the labeled hormone it was shown that

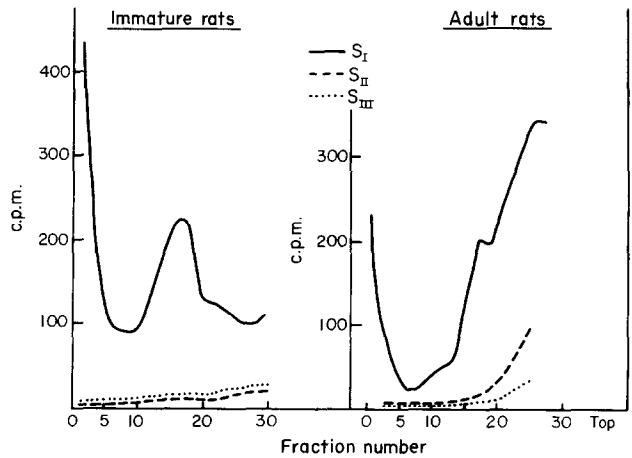

Fig. 6. Sedimentation profiles of S_I, S_{II} and S_{III} fractions (see Fig. 2) of immature or adult rat uteri after 1 h-incubation at 4°C of the homogenates (in 0·32 M sucrose, 3 mM Mg Cl_2, pH 6·6), in the presence of trace amounts of {6, 7³H}estradiol (about 10^{-10} M). Centrifugation of S_I and S_{II} was done on sucrose KCl free gradients; S_{III} was centrifuged on sucrose KCl gradient.

the relative concentration in the particulate fraction increased still further to attain a steady level of about 75 % of the total uterine concentration [8]. It was also shown earlier that the total estradiol concentration in the uterus also increased during the infusion, until saturation level was achieved at the end of 3 h [8, 9]. In the present study, equilibrium levels of estradiol in the tissue were obtained by infusing radioinert estradiol for 3 h prior to the radioactive estradiol infusion at the same rate. The labelled hormone also partitioned itself very rapidly between cytosol and particulate fractions, and more than 50 % of the radioactivity was associated with the latter from the very first few min of infusion. Thereafter, the equilibrium was slowly established between cytosol and particulate fractions in the ratio of 30–70 %. The total amount of estradiol in the uterus remained constant while the {6, 7³H}estradiol was infused [8, 9], although the concentration of the labelled hormone increased regularly but did not reach equilibrium level after 3 h. In the plasma, however, the equilibrium was achieved after 45 min only. A slower increase in total uterine radioactivity, while a normal distribution of the labelled hormone in the subcellular fractions persisted, may be explained by intratissular dilution of the labeled estradiol with the radioinert hormone. Hence, both radioactive and radioinert estradiol would be exchanged similarly with the plasma hormone, despite the different time sequence of their entrance in the tissue. This implies a rapid exchange of the intracellular pools with one another, and a recycling of the hormone within the cell, without decrease in binding capacity. The exchange between radioinert and radioactive hormone is further demonstrated by the sedimentation patterns of cytosol and particulate "receptors" 2 min after infusion of the labelled hormone. The radioactive hormone was manifestly bound to the S_I, S_{II} and S_{III}, as evidenced by distinct 4–5 S peaks on sucrose gradients containing KCl.

Chase experiments performed in immature animals also showed that {6, 7³H} estradiol was associated with the "receptors", whatever the time sequence of the infusion, radioinert first and radioactive estradiol afterwards or vice-versa. *In*

vitro incubations of immature or adult rat uteri with tritiated estradiol also showed a very rapid passage of the hormone into the particulate fraction, where it associated with the "receptors". The hormone concentration in the incubation medium must not however exceed 5×10^{-9} M, because above that concentration, the typical 4–5 S peaks on sucrose KCl-gradients are progressively masked by the accumulation of unbound radioactivity, due to overloading of the specific binding capacity of the tissue.

It should be noted here that at low hormone concentration in the medium, the tissue concentration of radioactivity was lower than what was expected with reference to *in vivo* experiments where the plasma level of estradiol was considered as the "incubation" medium (see Fig. 4). The presence of binding proteins in the plasma would however suggest the possibility of lower relative uptake *in vivo*. These paradoxical results suggest that no efficient competition exists *in vivo* between plasma binding proteins (other than albumin) and uterine receptors, below the saturation level of the latter. It should also be noted that deep tissue layers and damaged areas of the uterus may have lost their binding ability *in vitro* as shown by Stumpf[13], and so decrease the total radio concentration in the tissue.

In vivo–in vitro chase experiments also showed a rapid exchange with both cytosol and particulate "receptors". Indeed, the relative distribution of radioactivity between cytosol and particulate fractions obtained after 3 h infusion with tritiated estradiol, remained unchanged, even after a 60 min *in vitro* incubation of the uteri in the presence of radioinert estradiol. During that time however, a significant amount of the radioactive hormone was replaced by unlabeled hormone from the medium. When the uteri were first loaded *in vivo* with cold estradiol and then incubated with radioactive hormone, the latter, although taken up by the tissue in a minor quantity, was rapidly distributed within all the cell fractions. After 2 min, 50% only remained in the cytosol; later this proportion decreased further although the total amount of exchanged {6, 7³H}estradiol increased in the tissue. The distribution of tritiated estradiol within the cell fractions was not however, due to an experimental artefact. Indeed, the incubation of homogenates of adult or immature uteri with tritiated estradiol did not permit, under our experimental conditions, to obtain a significant binding with the particulate fraction. Only the 4–5 S peak of the cytosol fraction was observed. This was also the case when uteri were homogenized in the presence of {6, 7³H}estradiol. Hence some active transport from cytosol to nucleus is necessary although overloading of the binding sites in both fractions can be obtained (see Fig. 5).

In conclusion, estradiol bound to the subcellular fractions of the uterus remains exchangeable with circulating hormone. Furthermore, it is rapidly exchanged between the subcellular fractions and a recycling appears to take place without loss of binding capacity neither in the cytosol, nor in the particulate fraction.

This dynamic equilibrium between cytosol and particulate fraction may explain why total depletion of cytosol binding does not occur, despite a long lasting or high rate inflow of hormone into the cell [8, 14, 15].

Whether the subcellular recycling of estradiol as described in the present work is accompanied by or is secondary to a recycling of the "receptor" or part of the "receptor" is debattable. Such sequence of events has been proposed by Ishii *et al.*[16] to account for their *in vitro* observation on cultured fibroblasts, of

triamcinolone uptake, nuclear translocation and release. This mechanism could satisfactorily explain the steady ratio of cytosol to nucleus bound, despite large variations in the total bound hormone [8, 15]. Free sites are apparently lacking in the nucleus [14]; nuclear binding will increase with increasing translocation of hormone-receptor complex from cytosol, secondary to increased level of circulating hormone. Total depletion of the hormone-receptor complex in the cytosol will however never occur [8, 14, 15]. This situation applies also to a physiological situation as the estrus cycle [15]. In the latter case also, nuclear binding increases with increasing estrogen secretion (proestrus) [15], although total binding capacity is not largely different from one phase to another [17]. Subcellular distribution of the hormone at saturation levels is also similar at all phases [8]. Hence, some equilibrium not only of the hormone, but also of the receptor sites, between cytosol and nucleus might occur as suggested by Ishii *et al.* (16) for triamcinolone. More direct evidence however is needed to ascertain the existence of such mechanism in the present model.

The higher proportion of hormone in the cytosol in the first few minutes of hormonal penetration in the tissue is seen whatever the state of hormonal saturation of the tissue. The subsequent decrease in this proportion is due to equilibration with subcellular pools whose absolute capacity remains unchanged.

However, it was always observed that the rapid passage of hormone from the cytosol to the particulate fraction was followed by a much longer equilibration time between all the fractions. Hence, the relative percentage of radioactivity in the S_{III} and the final precipitate fractions remained somewhat lower for a longer time when the tissue had been saturated with radioinert estradiol. This suggests that some part of the particulate fraction may display a slower exchangability with the others. The present experiments using tritium labeled and unlabeled estradiol did not permit us to confirm this point, and further experiments, using double isotope techniques were performed, and will be described later [12].

ACKNOWLEDGEMENTS

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