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SUBCELLULAR DISTRIBUTION AND BINDING OF {6,7-^{3}H} ESTRADIOL IN RAT UTERUS, AT EQUILIBRIUM, AFTER LONG-TERM INTRAVENOUS INFUSION

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SUMMARY

In the course of a 3 h-infusion of adult rats with {6,7-^{3}H} estradiol, the radioactivity in the uterus was rapidly distributed between the cytosol and the particulate fraction. More than 50% was associated with the latter after 5 min of infusion; this proportion increased to a constant value of about 75% after 3 h.

On sucrose density gradient containing 0·4 M KCl, the radioactivity in the cytosol was found associated with a 4–5 S "receptor". On KCl free gradient, the bound radioactivity appeared in the form of aggregates and of a 4–5 S peak. From the particulate fraction, two distinct constituents were obtained: a 3·5–4 S binder, extracted with Tris-EDTA, pH 8·2, and a 4–5 S binder extracted thereafter with Tris-EDTA-KCl pH 8·5. The 3·5–4 S, Tris-EDTA extractable "receptor" was more labile than the 4–5 S cytosol or the 4–5 S nuclear "receptor". Its physiological significance is not yet clear. A significant amount of radioactivity remained associated with the residual precipitate. The data show that estradiol is rapidly distributed in the subcellular fractions and a constant partitioning between cytosol and particulate fraction is established, whatever the level of uterine concentration of the hormone. This suggests the existence of a dynamic exchange between the binding constituents at the subcellular level.

INTRODUCTION

IT HAS been known for several years that estradiol-17β is taken up by specific target organs and retained there for a longer period than in non target tissues[1]. Prior to this, it was shown that the uptake is dependent on the plasma concentration of the hormone and, when a steady state was achieved after a constant infusion of the tracer for at least 3 hrs, a hyperbolic relationship can be shown between plasma and uterine concentrations of the hormone[2, 3]. This relationship gave rise to the definition of a "biological exchange constant", which expresses the plasma estradiol level at half saturation of the tissue. The saturation level was called the "tissue capacity" and was estimated to be 2×10^{-8} moles/kg of wet tissue weight[4–6]. The presence of estradiol binding components in the uterus ("receptors") has been described by several authors[7–9]. Two types of "receptors" were identified: one in the cytoplasm of immature or ovariectomised rats and the other in the nucleus of estrogen treated animals. Recently, an estradiol binding protein fraction possessing a very low capacity and high affinity was detected in uterine nuclei from estrogen untreated rats[9, 10]. In a preliminary note, we reported the presence of another estradiol binding component present in the particulate fraction of rat uterus homogenate[11].

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In the present report we describe the bound state of {6,7-^{3}H} estradiol in subcellular fractions of the rat uterus, at equilibrium, after long-term infusion of the tracer. It was observed that estradiol-17β was distributed in a constant proportion between the cytosol and the particulate fraction and that this distribution was essentially dependent on the binding of the hormone to three specific "receptors": one in the cytosol, and two others extracted from the particulate fraction.

MATERIALS AND METHODS

Materials

Wistar R rats were used. Immature animals were 28 days old and weighed 45–50 g. Adult animals were 3 months old and weighed 150 g.

{6,7-^{3}H} estradiol-17β, 50 Ci/mmol was checked for radio-chemical purity by celite or sephadex LH_{20} column chromatography.

Methods

Infusion technique, tissue extraction and radioactivity counting were performed as described previously[4, 5]. The infusion rate was between 180–200 ng/h in adult rats, and about 100 ng/h in immature animals. These infusion rates were utilized in order to obtain almost complete saturation of the uterine binding sites[5].

Cell fractionation was performed by a method adapted from Widnell *et al.* [12–14], which made it possible to obtain purified nuclei by further processing the whole tissue-homogenate. All manipulations were carried out at 0–4°C unless otherwise stated.

Two uteri were minced with scissors and successively homogenized in 2 ml of 0·32 M sucrose solution, containing 3 mM $MgCl_2$ and 14 mM 2-mercaptoethanol, in an all glass homogenizer. Care was taken to avoid heating in course of homogenisation by keeping the homogenizer in an ice bath, and rotating the pestle at a slow speed between 150 and 200 rpm. Six strokes of about 10 sec each, intermitted by a 5 sec interval were usually sufficient to achieve complete homogenisation of both uteri. The pH of the homogenizing medium varying between pH 6 and 7·4 was found to have no influence on the subcellular distribution of the infused radioactive hormone. For facility, the pH of this medium was then set at 6·6.

The homogenate was filtered through one layer of nylon bolting cloth (John Staniar Ltd, Manchester, England, 110 Mesh) and the filter-cloth was washed with 0·5 ml of distilled water. The filtrate, usually containing 6–12 mg/ml of protein, was centrifuged at 700 *g* for 10 min. The resulting low-speed supernatant S_I was considered as the "cytosol" fraction. When purified nuclei were not processed the remaining "particulate" fraction, which contained nuclei, cell membranes and myofibrillar structures was further homogenized in 1 ml of Tris (10 mM)-EDTA (1·5 mM) buffer pH 8·2, in order to remove residual soluble radioactive material which could contaminate the high salt extract (see below). The homogenate was centrifuged at 106·000 *g* for 15 min and the supernatant (S_{II}) was recovered. The pellet was then rehomogenized in 1 ml of 0·4 M KCl in Tris-EDTA, pH 8·5 and a third centrifugation was performed at 106·000 *g* for 15 min. The supernatant (S_{III}) (high salt extract) was separated and the precipitate (Ppt) recovered. 0·2 ml of S_I was diluted with an equal volume of distilled water

to reduce the sucrose concentration of the suspension and layered on 4·5 ml of 5–20% sucrose gradient either in water or in Tris (10 mM), EDTA (1·5 mM), KCl (0·4 M) buffer. 0·4 ml of S_{II} and S_{III} were layered on 5–20% sucrose gradient containing either Tris-EDTA or Tris-EDTA and KCl at the above mentioned molarities. All gradients were then centrifuged at 135·000 *g* for 16 h. 28 fractions were collected by piercing the bottom of the tubes.

For preparation of purified nuclei the "particulate" fraction was processed as described later[14].

RESULTS

1. *Subcellular distribution of* {6,7-^{3}H} *estradiol, during the process of hormonal equilibrium between plasma and uterus*

Groups of adult rats were infused for 5–180 min with {6,7-^{3}H} estradiol-17β at a rate of 200 ng/h. At various times, the animals were sacrificed and plasma and uteri were collected, extracted and partitioned into free and conjugated fractions as described previously[4, 5, 15]. Portions of the uteri were fractionated into "cytosol" and "particulate" fractions as described under methods and radioactivity was counted in both subcellular fractions.

Figure 1 shows that total uterine radioactivity (consisting of over 85% {6,7-^{3}H}estradiol-17β)[4, 5, 15], increased progressively to attain a constant level at the end of 3 h of infusion. Plasma level of unconjugated radioactivity (consisting of over 80% estradiol-17β)[4, 16] increased more rapidly to reach equilibrium within 1 h. Hence, plasma to uterus ratio was high after a few minutes of infusion and fell rapidly thereafter. After 5 min of infusion, while the total uterine concentration was still very low, the particulate fraction of the uterus contained already more than 50% of the total radioactivity. This proportion increased in a regular manner to attain 73% after 3 h of infusion.

2. *Subcellular distribution of* {6,7-^{3}H} *estradiol in uterus at equilibrium*

Table 1 gives the per cent distribution of {6,7-^{3}H} estradiol in subcellular fractions of adult and immature rat uteri, after 3 h-infusion of the tracer at several rates. S_I, S_{II}, S_{III} and precipitate (Ppt) were obtained as described under methods.

Table 1. Subcellular distribution of {6,7-^{3}H}-estradiol at equilibrium

Infusion rates	Per cent total activity (mean ± s) S_I	S_{II}	S_{III}	Ppt
Adult rats				
5 ng/h*	27·9	9·2	16·0	46·9
50 ng/h*	25·3	6·2	19·6	48·6
180 ng/h†	23·7 ± 3·4 ($n = 19$)	14·1 ± 4·2 ($n = 7$)	26·7 ± 5·0 ($n = 7$)	33·5 ± 6·9 ($n = 7$)
500 ng/h*	28·5	13·8	16·2	43·9
Immature rats				
6 ng/h‡	41	13	28	18
50 ng/h‡	39	8	25	27

*Subcellular fractionation of one pool made up of portions of 6 uteri.

†Subcellular fractionations of *n* individual uteri (mean ± 1 S.D.).

‡Subcellular fractionation of one pool of 6 uteri.

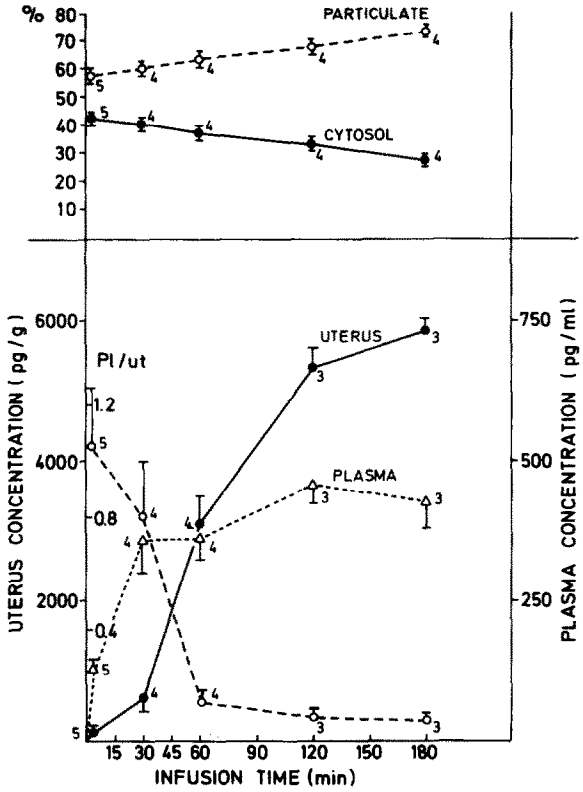

Fig. 1. Concentration of total radioactivity in the uterus and of total free radioactivity in the plasma of adult rats, expressed in terms of weight equivalent of {6,7-^{3}H} estradiol during continuous intravenous infusion of the labelled hormone at a rate of 180 ng/h. The ratio of plasma/uterus concentrations (PI/ut) is also indicated (lower part of the graph).

The relative distribution of the radioactivity in the cytosol and the particulate fractions of the uterus is indicated as % of the total radio-concentration in the tissue homogenate (upper part). Each point is the mean ± S.D.M. of indicated number of animals.

At an infusion rate of 180 ng/h, an average of 23·7% was found in the cytosol (S_I) of adult rats. The phase of the estrus cycle did not appear to influence the distribution of the infused hormone between cytosol and particulate fraction: 21·8, 23·1, 21·5 and 24·1% in the cytosol fraction in proestrus, estrus, metestrus and diestrus respectively (mean of 3 experiments). At lower or higher rates of infusion, the subcellular distribution was also similar. The concentration in the cytosol was higher (about 40%) in immature animals (Table 1). A significant amount of radioactivity in the particulate fraction was not extractable either by Tris-EDTA, or by Tris-EDTA-KCl buffers, and remained in the precipitate (Ppt), especially in adult animals.

Figure 2 shows the sedimentation patterns of S_I, S_{II} and S_{III}, on sucrose density gradients, obtained sequentially from the uterus of one adult rat. In this experiment the low-speed supernatant (S_I) was further centrifuged at 106·000 *g* for 60 min and the small precipitated fraction, containing mitochondrias and microsomes, was extracted with Tris-EDTA buffer. The sedimentation profile of this extract on sucrose gradient appears on the same figure (MICR.). It can be seen that S_I

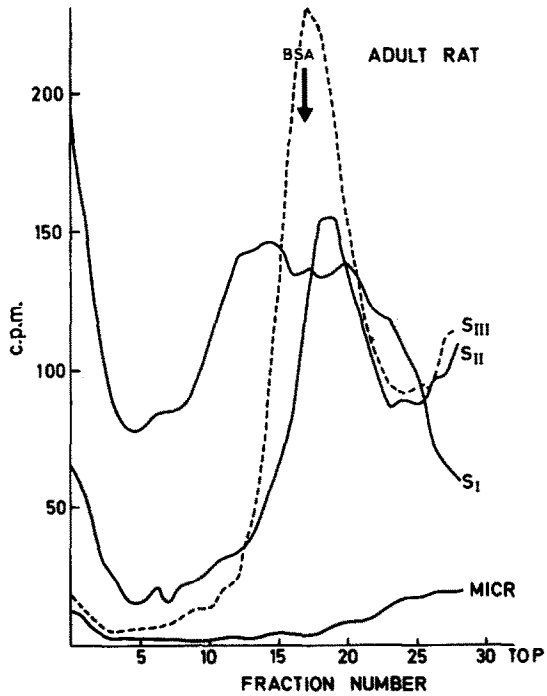

Fig. 2. Sedimentation profile of uterine radioactive fractions isolated from one adult rat uterus after a 3 h-intravenous infusion with {6,7-^{3}H} estradiol (180 ng/h). S_I (cytosol), S_{II} (Tris-EDTA extract from the particulate fraction) and MICR (Tris-EDTA extract from the high speed precipitate of the cytosol fraction) were centrifugated for 16 h at 135·000 *g* on 4·5 ml 5–20% sucrose gradient. S_{III} (Tris-EDTA-KCl extract from the particulate fraction) was centrifuged in the presence of 0·4 M KCl in the gradient. BSA: Bovine Serum Albumin.

was distributed in two areas: a heavy fraction of aggregates at the bottom of the tube and a rather large peak in the area of the BSA. S_{II} appeared as a sharp band slightly lighter than BSA. S_{III} gave one single peak, slightly ahead of BSA. No significant radioactive peak was found in the MICR. fraction. The sedimentation patterns of S_I, S_{II} and S_{III} obtained from uteri of animals in various phases of the estrus cycle were not different.

3. *Effect of high ionic concentration* (KCl: 0·4 M) *on sedimentation characteristics of the uterine "receptors"*

Figure 3 shows the migration profiles on a 5–20% sucrose density gradient containing 10 mM Tris, 1·5 mM EDTA 0·4 M KCl of S_I, S_{II} and S_{III} obtained from the uterus of one adult rat, infused for 3 h with {6,7-^{3}H} estradiol at a rate of 180 ng/h. The three "receptors" gave each a peak in the BSA region. The relative sedimentation coefficients observed were the following: $S_{III} > S_I > S_{II}$. This sequence was systematically obtained in other similar ultracentrifugation experiments. Sedimentation coefficients calculated from 7 experiments were $4{\cdot}47 \pm 0{\cdot}23$, $3{\cdot}80 \pm 0{\cdot}47$ and $4{\cdot}69 \pm 0{\cdot}33$ for S_I, S_{II} and S_{III} respectively, with reference to BSA having a sedimentation coefficient of 4·6 S. The differences between S_{II} and S_I or S_{III} were highly significant ($p < 0{\cdot}005$).

Figure 4 gives the sedimentation patterns on sucrose gradients of S_I, S_{II}

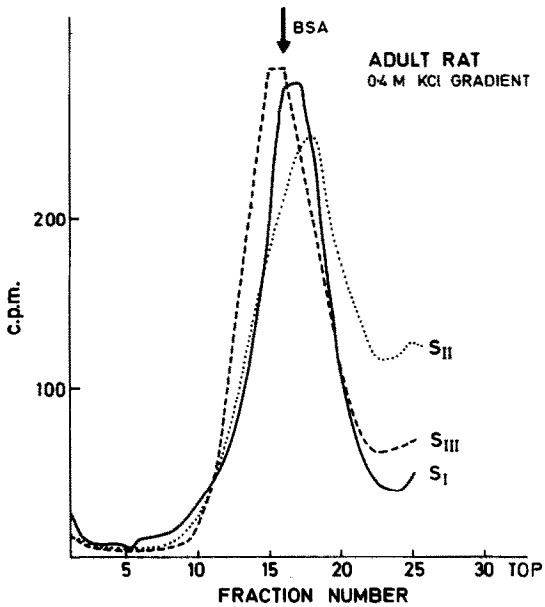

Fig. 3. Sedimentation patterns of uterine radioactive S_I, S_{II} and S_{III} fractions and BSA (see Fig. 2) in 0·4 M KCl 5–20% sucrose gradient. One adult rat was infused for 3 h with {6,7-^{3}H} estradiol at a rate of 180 ng/h.

and S_{III} obtained from immature rat uteri, at the end of a 3 h infusion with {6,7-^{3}H}-estradiol at a rate of 100 ng/h. They were comparable with those of adult rats both in the absence or in the presence of 0·4 M KCl.

4. *Effect of temperature on the binding to the uterine "receptors"*

Five immature rat uteri were homogenized in sucrose in the usual manner, and portions of the filtered homogenate were incubated for 1 h at 4 and 37°C in the presence of 7×10^{-10} M {6,7-^{3}H} estradiol.

Figure 5 shows the sedimentation pattern of S_I isolated from these homogenates at the end of the incubation period. Plasma of these animals was also incubated in parallel at both temperatures. It was observed that the binding of {6,7-^{3}H} estradiol in the uterine cytosol, which appeared on the gradient as aggregates and as a peak in the 4–5 S region in the 4°C incubation, was lost at 37°C. While the 4–5 S peak, representing the binding of {6,7-^{3}H}-estradiol to the plasma, was not influenced by the temperature of incubation.

Figure 6 shows the influence of 5 min incubation at different temperatures on the relative binding of {6,7-^{3}H} estradiol to S_I, S_{II} and S_{III}, isolated from adult rat uteri, after 3 h infusion with 180 ng/h of {6,7-^{3}H} estradiol. Aliquots of S_I, S_{II}, S_{III} were diluted in 1·5 volumes of 10 mM Tris-HCl buffer pH 8, containing 1 mM EDTA and 250 mM sucrose. 0·2 ml of the diluted fractions were incubated for 5 min at increasing temperatures. At the end of the incubation time, 0·5 ml of charcoal suspension (250 mg charcoal and 2·5 mg dextran in 100 ml of the same buffer) was added, shaken and the mixture centrifuged at 2·500 rpm for 10 min. Portions (0·4 ml) of the supernatants were counted. Countings obtained from the 4°C incubated aliquots were considered as 100% binding. Between 30 and 40°C, an important loss of binding was observed, which was comparatively more rapid

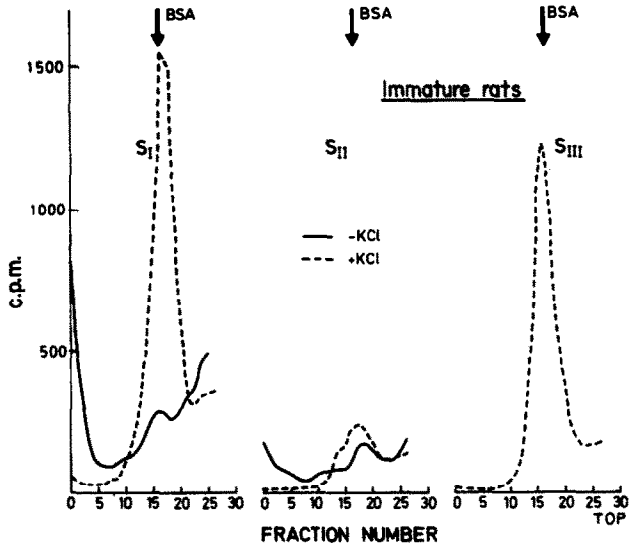

Fig. 4. Sedimentation patterns of radioactive S_I, S_{II} and S_{III} fractions (see Fig. 2) obtained from a pool of immature rat uteri at the end of 3 h-intravenous infusion with {6,7-^{3}H} estradiol at a rate of 100 ng/h. Solid line: KCl free 5–20% sucrose gradient; dotted line: 0·4 M KCl gradient.

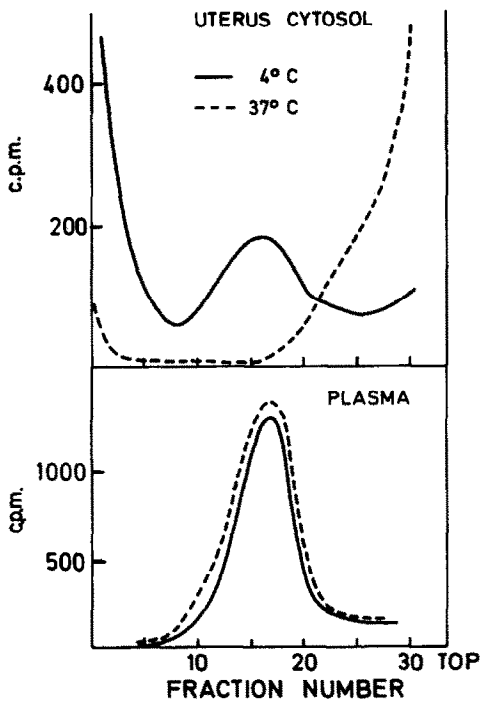

Fig. 5. Sedimentation patterns on 5–20% sucrose gradient of cytosol radioactive fraction after 1 h-incubation at 4°C (solid line) or 37°C (dotted line) of immature rat uteri homogenates in the presence of $7 \times 10^{-10}\mu$ {6,7-^{3}H} estradiol (upper part).
Rat plasma was incubated and centrifuged under similar conditions (lower part).

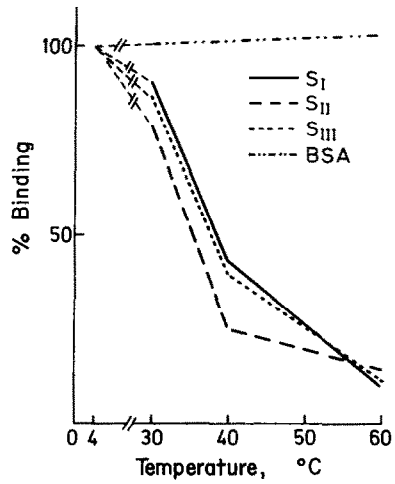

Fig. 6. Effect of 5 min incubation at increasing temperatures on radioactivity binding to S_I, S_{II} and S_{III} uterine fractions (see Fig. 2) isolated from adult rat at the end of 3 h-intravenous infusions with {6,7-^{3}H} estradiol at a rate of 180 ng/h. Dextran coated charcoal was used for adsorbing unbound material. The bound radioactivity at 4°C was considered as 100% binding. BSA was incubated under the same conditions in the presence of {6,7-^{3}H}-estradiol.

for S_{II}, and parallel for S_I and S_{III}. Under similar circumstances BSA showed no such loss of binding with increasing temperature.

The relatively rapid loss of binding observed for S_{II} was also repeatedly found in cellular fractions preserved for longer periods of time. After 24 h, unlike S_I and S_{III}, the binding ability of the S_{II} fraction was usually lost.

5. Effect of Tris-EDTA buffer pH on the extraction of S_{II} from the particulate cell fraction

The particulate fraction obtained from adult rat uteri, infused during 3 h with {6.7-^{3}H}-estradiol (180 ng/h), was homogenized in Tris (10 mM), EDTA (1·5 mM) buffer, pH 7·4. The homogenate was centrifuged at 106·000 *g* for 15 min, the supernatant was recovered and layered on a 5–20% sucrose gradient containing 0·4 M KCl (Fig. 7, curve 1); the precipitate was further homogenized in Tris-EDTA buffer, pH 8·2. After 15 min centrifugation at 106·000 *g* a second supernatant was recovered (Fig. 7, curve 2) and the precipitate was rehomogenized in Tris-EDTA buffer pH 8·5. After collecting a third supernatant (Fig. 7, curve 3), the residual precipitate was finally homogenized in Tris-EDTA buffer, pH 8·5, containing 0·4 M KCl. This last supernatant was also layered on sucrose gradient (Fig. 7, curve 4). A 4–5 S peak was found only in the second Tris-EDTA extract (pH 8·2, S_{II}) and in the Tris-EDTA-KCl extract (pH 8·5, S_{III}).

DISCUSSION

It was previously shown that equilibrium between plasma and uterus {6,7-^{3}H} estradiol was obtained after 3 h-intravenous infusion of the hormone at a constant rate[4, 5, 16]. This equilibrium could be obtained at both low and high rates of infusion and was shown to be a function of the plasma estradiol concentration. Hereby, a "biological exchange constant" was defined as the equilibrium plasma

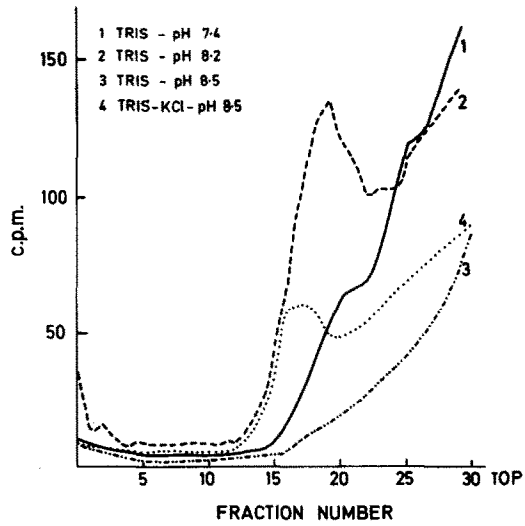

Fig. 7. Sedimentation patterns on 5–20% sucrose gradient containing 0·4 M KCl of 4 radioactive extracts obtained successively from the particulate fraction of adult rat uteri with Tris-EDTA buffers of increasing pH values. The last buffer[4] contained also 0·4 M KCl. These uteri were obtained after a 3 h-intravenous infusion with {6,7-^{3}H}-estradiol at a rate of 180 ng/h.

concentration at half saturation of the uterine binding sites. It was estimated to be 3×10^{-10} M in adult and $1{\cdot}8 \times 10^{-10}$ M in immature rats[2, 4, 5].

The establishment of this equilibrium concentration was investigated in the present study. The plasma concentration reached equilibrium in about 30 min. Whereas, uterine concentration continued to increase until 180 min when saturation was obtained at a concentration of about 2×10^{-8} moles/kg wet weight (tissue capacity[4–6]). This lag in achieving equilibrium is most probably due to the limitation in the uterine blood flow. Indeed, from the 30th to the 60th min of infusion, the uterine concentration of {6,7-^{3}H} estradiol increased from 600 to 3·100 pg/g. This represented the total amount present in 15 ml of blood, hence a minimal blood flow rate of 0·5 ml/g/min. Blood flow in castrated rats is of the order of 0·3 ml/g/min, and increases to 0·8 ml/g/min after estrogen stimulation [17]. Hence, the increase in uterine estradiol concentration represented probably the largest part of estradiol in the blood flowing through the tissue. Therefore, plasma binding of estradiol in the rat did not appear to prevent uptake, when uterine binding sites were not saturated.

The distribution of estradiol within the cell appears to be very rapid, if we consider the cytosol and the particulate fractions. More than 50% of the radioactivity appeared in the latter after 5 min of infusion, when less than 2% of the binding sites were saturated with {6,7-^{3}H} estradiol. After 3 h, when all binding sites were saturated, the percentage in the cytosol had dropped to about 25%. This value then remained constant, up to the 6th hour of infusion*, while total uterine concentration also remained constant[4, 5]. Hence, rapid distribution of estradiol throughout the cell fractions occurs in adult rats, as in ovariectomised

*See accompanying papers.

or immature animals[18, 19]. Relative distribution between cytosol and particulate fraction then slowly comes to equilibrium at 25 and 75% respectively.

At equilibrium, {6,7-^{3}H} estradiol was found in four subcellular fractions: one in the soluble fraction (S_I) and three in the particulate fraction (S_{II}, S_{III} and residual precipitate). S_I (cytosol) and S_{III} (KCl extract of particulate fraction) were similar to the cytosol and nuclear receptors, described by several authors [7–9, 20].

On sucrose gradients, containing 0·4 M KCl, they gave a single peak of bound {6,7-^{3}H}-estradiol in the BSA region. S_I was slightly lighter (4·5 S) and S_{III} slightly heavier (4·7 S). However, in gradients without KCl, S_I gave mostly aggregates and a minor 4–5 S, peak.

We have not succeeded in obtaining a 8–9 S peak, except when small amounts of KCl were added (0·05–0·1 M). Under these circumstances, the binding peak may occupy any place on the gradient, from aggregates to a 4 S peak depending on the ionic concentration*.

Cytosol fractions obtained from adult rats infused with {6,7-^{3}H} estradiol at different stages of the estrus cycle gave comparable binding patterns on KCl-free gradients*. Subcellular distribution of the infused hormone between cytosol and particulate fraction was also similar.

Intermediate between the S_I (cytosol) and S_{III} (nuclear) binding fractions, we obtained a third "receptor", extracted from the particulate fraction with Tris-EDTA buffer, at pH 8·2. The relative binding of this fraction represented approximately 15% of the total uterine radioactivity. This "receptor" migrated as a 3·5–4 S peak (slightly lighter than S_I), both on KCl free, and on 0·4 M KCl gradients, differentiating it not only from S_I but also from S_{III}.

Furthermore, it was not obtained from the particulate fraction with Tris-EDTA pH 7·4, showing that it did not represent a residual cytosol contaminant. It is not a contaminant of the S_{III} either since KCl was needed for extracting the latter. Finally, the effect of temperature on its ability to bind estradiol also differentiated it from albumin or from a 4 S unspecific plasma binding substance. The extraction characteristics of this S_{II} "receptor" suggests that it is probably associated with the particulate fraction.

Whether it has a nuclear origin still remains to be confirmed because of the composite nature of the "particulate fraction" which contains other heavy constituants besides nuclei (cell membranes, myofibrillar structures). A Tris-EDTA (pH 8·2) soluble "receptor" (S_{II}) was extracted from purified nuclei[14], although in a lesser proportion as compared to the KCl extract (S_{III}) of these preparations.* Subcellular equilibrium of estradiol was much more rapid with S_{II} as compared to S_{III}, as shown in chase experiments.† Association of the S_{II} "receptor" with the myofibrillar structure is not likely, as it was present also in endometrium.* A 3·5 S, labile "receptor" of aldosterone of nuclear origin was found by Edelman in the kidney[21]. A nuclear 4 S binder of estradiol was found by Baulieu in the uterus, and considered as an artefact, because it was mostly present in *in vitro* experiments, where tissue damage could have occurred[22]. On the contrary, the S_{II} here described, was barely found in *in vitro* experiments,* and showed a lesser *in vitro* stability than S_I and S_{III}. The significance of this S_{II} binding is as yet unknown.

*Personal data.

†See accompanying papers.

In conclusion, {6,7-^{3}H}-estradiol distributed itself rapidly in the subcellular fractions of the uterus, whatever the level of uterine concentration of the hormone. A constant partitioning between cytosol and particulate fraction was then slowly established, and a state of equilibrium occured between plasma, uterine cytosol and uterine particulate fraction. In the cytosol, estradiol was bound mainly to a specific 4·5 S "receptor" (on KCl gradients), whereas in the particulate fractions it was found, in three different forms: one bound to a 3·8 S and labile Tris-EDTA extractable "receptor", a second bound to a 4·5 S Tris-EDTA-KCl extractable "receptor" and a third bound to the residual precipitate. This equilibrium status suggests that a dynamic exchange exists at the subcellular level. Further work was devoted to that particular study.

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