Journal of Steroid Biochemistry, 1976, Vol. 7, pp. 241–247. Pergamon Press. Printed in Great Britain

COMPARISON OF THE BINDING OF [2,4,6,7-^{3}H] ESTRADIOL-17β [$E_2{}^3H$] TO THE IMMATURE RAT UTERUS UNDER *IN VIVO*, *IN VITRO* AND "CELL-FREE" CONDITIONS—I. SOME CHARACTERISTICS OF THE CYTOPLASMIC ESTRADIOL–RECEPTOR COMPLEX

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(*Received* 4 *August* 1975)

SUMMARY

A comparative study was done on the cytoplasmic estradiol–receptor complex obtained after *in vivo* infusion of $E_2{}^3H$ or after whole tissue incubation in the presence of $E_2{}^3H$ or after "cell-free" incubation with $E_2{}^3H$ of cytosol prepared in buffers of varying ionic strengths.

Sucrose density gradients containing 0·3 M KCl showed that cytosol after *in vivo* infusion sedimented as a distinct peak in the "4S" region whereas after either "whole-tissue" or "cell-free" incubations in the presence of $E_2{}^3H$ both "4S" and "5S" peaks were present simultaneously, provided the incubation was done at higher temperatures.

Under "cell-free" conditions, a comparison of the dissociation kinetics of cytosol estradiol–receptor complexes showed two first-order components irrespective of the buffer utilized (k_1D:1 to 2×10^{-4} s^{-1} and k_2D:1 to 2×10^{-5} s^{-1}). However, the quantitative importance of the two components was different depending on the presence of NaCl. The rapidly dissociating component was 3 or 4 times more important than the slowly dissociating component in the absence of salt, whereas in the presence of salt, the slowly dissociating component was twice the rapidly dissociating component. Similarly, the apparent dissociation constants were the same in Tris–EDTA and Tris–NaCl buffers ($1{\cdot}5 \times 10^{-10}$M). Sucrose density gradients revealed the presence of aggregates in the presence of NaCl.

These observations tend to show that experimental procedures may have a preponderant influence on some characteristics of the cytosol receptor, when analyzed in a "cell-free" system, and which appear to be more complex as compared to *in vivo* conditions.

INTRODUCTION

A large body of experimental evidence cited in literature suggests that an early and fundamental step in the sequence of events which mediate the biological activity exerted by steroid hormones is the intracellular binding of the hormone to a specific "receptor" molecule in the cytoplasmic fraction of the cells of target tissues [1–3]. The steroid–receptor complex thus formed in the cytoplasm is then translocated to the cell nucleus by a temperature-dependent activation process, inducing a conformational modification of the complex, which would potentiate its entry in the nucleus [1, 4–6].

Different molecular entities of the cytosol estradiol–receptor protein have been characterized by their sedimentation patterns after centrifugation on sucrose density gradients; but the exact relationship between these different forms and their physiological significance is still ill-understood [2, 7]. Since the status, native or experimentally induced, of the cytoplasmic estradiol–receptor complex can have a direct bearing on the translocation process and eventually on the resultant status of the nuclear complex formed, it was considered worthwhile to study and compare some of the physicochemical parameters of the cytoplasmic estradiol-receptor complex formed under *in vivo*, *in vitro* and in "cell-free" experimental conditions.

Under "cell-free" experimental conditions, the effect of various buffers and the influence of their ionic strength on the resulting cytoplasmic receptors were studied, in order to gain a better insight into the experimental factors and artefacts which could possibly modify the apparent physicochemical parameters which in turn could have a direct impact on the formation of the nuclear complex.

MATERIALS AND METHODS

Animals. Wistar R immature female rats 28–30 days old, weighing between 45–50 g were used.

Chemicals. All chemicals utilized were of analytical grade and were used without any further purification. [2,4,6,7-^{3}H] 17β estradiol [$E_2{}^3H$], S.A. 85–105 Ci/mmol and [^{14}C] 17β estradiol ($E_2{}^{14}C$), S.A. 50–60 mCi/mmol were obtained from New England Nuclear and were checked for radiochemical purity on Sephadex LH 20 (Pharmacia) microcolumns. Instagel (Packard Instruments) was utilized for all radioactivity countings.

Buffers. The buffers utilized were: Tris–HCl (10 mM Tris + HCl) pH 7·4; Tris–HCl–NaCl (10 mM Tris + HCl + 0·15 M NaCl) pH 7·4; Tris–EDTA (10 mM Tris + 1·5 mM EDTA) pH 7·4; Tris–EDTA–NaCl (10 mM Tris + 1·5 mM EDTA—0·15 M NaCl) pH 7·4; Tricine–NaCl (10 mM Tricine + 0·15 M NaCl) pH 7·4.

I. *Preparation of cytoplasmic receptors.* The three different experimental conditions were:

(1) Binding of $E_2{}^3H$ *in vivo*: 6–12 animals were subjected to constant infusions with $E_2{}^3H$ at 180 ng/h for 4 h, to reach a constant concentration in the tissues and to attain saturation of the receptors as described previously [8]. At the conclusion of the infusion, the animals were sacrificed and the uteri removed and cleaned of adhering fat and mesentery, and the cellular fractionation was done in Tris–HCl buffer [9] to obtain the cytoplasmic receptors in the form of estradiol–receptor complex.

(2) Binding of $E_2{}^3H$ by whole uteri *in vitro*: Whole uteri from 6–12 animals were isolated and after removal of adhering fat and mesentery, were slit open lengthwise and cut into smaller slices. The incubation under constant shaking was conducted at 0–4°C or 22°C for 1 h in 2–5 ml of Krebs–Ringer Phosphate (K–R) buffer pH 7·4 containing 1 g% of glucose, in the presence of 3×10^{-9}M $E_2{}^3H$. In earlier studies [9], it was shown that at this hormone concentration in the medium the tissue uptake of $E_2{}^3H$ attained the saturating level observed *in vivo*. At the conclusion of the incubation, uteri were removed and blotted with filter paper and rinsed in K–R buffer. This operation was repeated three times and after a final blotting, the tissues were fractionated as described above, in Tris–HCl buffer.

(3) Binding of $E_2{}^3H$ to cytosol for "cell-free" studies: Uteri from 6–12 animals were removed after sacrifice and placed on an ice-cooled glass plate. The tissues were cleaned of fat and mesentery and the cellular fractionation in adequate buffers was done as described above. The cytosol was then incubated in the presence of 2×10^{-9}M $E_2{}^3H$ at 0–4°C for 1 to 2 h.

II. *Assay of specific binding to cytosol preparations.* Samples (0·2 ml) of labeled cytosol were assayed for specific binding by the Dextran-coated charcoal adsorption technique [10]. 0·5 ml of Dextran-coated charcoal suspension (250 mg % charcoal + 25 mg % Dextran, suspended in corresponding buffer) was added to the incubation mixture and stirred on a vortex for 15 s. The mixture was allowed to repose for 15 min at 0–4°C, and centrifuged at 800 ***g*** for 10 min. The supernatant, containing the bound $E_2{}^3H$, was counted for radioactivity.

III. *Kinetics of dissociation.* Cytosol prepared in appropriate buffer was incubated with 2×10^{-9} M of $E_2{}^3H$ at 0–4°C for 1 h and at 22°C for 30 min. The labelled cytosol was stripped with a concentrated Dextran-coated charcoal suspension (1/8th vol. of 1·5 g % charcoal, 150 mg % Dextran); $E_2{}^{14}C$ was then added in a large molar excess (100–300-fold) and exchange was assayed on 0·2 ml aliquots by measuring the remaining $E_2{}^3H$ in the bound form by the charcoal method, after different incubation times at 22°C. Control samples were processed in parallel to estimate the degree of stability of the estradiol receptor complex under the experimental conditions. The residual non-specific binding of $E_2{}^3H$ in the charcoal supernate was corrected for in each sample by the following relationship:

$$\text{Factor of non-specific binding} = \frac{\text{d.p.m. } {}^{14}C \text{ in the charcoal supernate}}{\text{d.p.m. } {}^{14}C \text{ in the charcoal precipitate}}$$

d.p.m. ^3H non-specifically bound

= d.p.m. ^3H in the precipitate × Factor of non-specific binding.

d.p.m. ^3H specifically bound

= Total d.p.m. ^3H in the supernate − d.p.m. ^3H non-specifically bound

The corrected data were then plotted on a semi-logarithmic scale.

IV. *Binding constant at equilibrium.* 0·2 ml samples of the cytosol prepared in appropriate buffer were incubated with increasing quantities of $E_2{}^3H$ in triplicates at 22°C for 30 min followed by 1 h at 0–4°C. At the conclusion of the incubation, Dextran-coated charcoal suspension was added to the samples and the bound $E_2{}^3H$ was determined in the supernate. The data were then analyzed according to Scatchard [11]. When necessary corrections were made according to the method of Rosenthal [12].

V. *Sucrose-density gradient centrifugation.* As described previously [9] 5–20% linear sucrose density gradients were prepared in adequate buffers containing 0·3 M KCl. When sucrose (0·25 M) was present in the homogenizing medium, 8·5–28·5% linear sucrose gradients were used.

VI. *Protein measurements* were done on aliquots of the cytosol sample according to the technique of Lowry [13].

RESULTS

I. *The dissociation kinetics and the apparent dissociation constant of the receptor.* (a) In Fig. 1 are shown the results of dissociation kinetics measured at 22°C, carried out on estradiol–receptor complexes obtained after "cell-free" labeling of the cytosols, prepared in various buffers. Protein concentrations were adjusted to 0·8 mg/ml by diluting the cytosol preparation with the corresponding buffer. In all the four buffers utilized: Tris–HCl, Tris–EDTA, Tris–HCl–NaCl, and Tricine–NaCl, the dissociation patterns of the complexes were similar, showing typical two first-order kinetics components. The dissociation kinetics were roughly comparable in all four cases (Table 1).

However, from Fig. 1 it is evident that the influence of the presence of NaCl in the media for processing

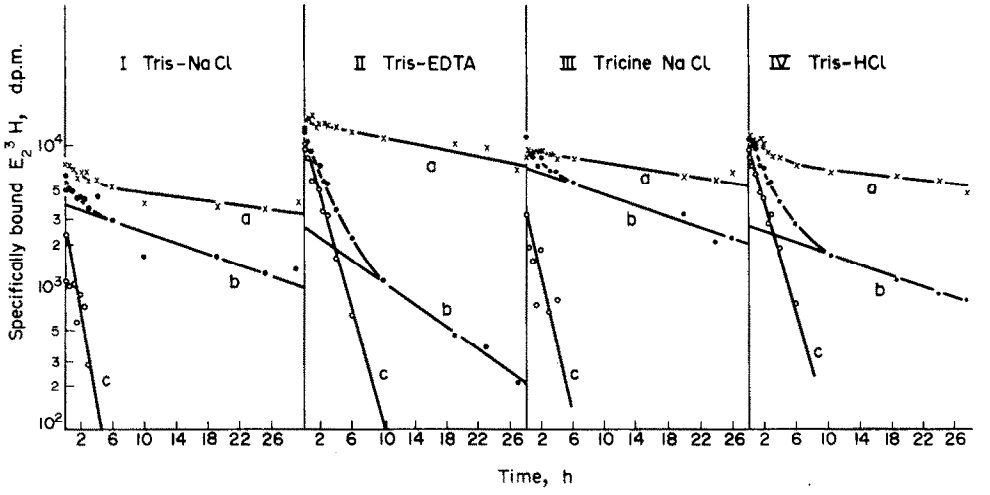

Fig. 1. Dissociation kinetics of the estradiol–receptor complex. Estradiol–receptor complexes were formed by incubating cytosols prepared respectively in: I. Tris–NaCl pH 7·4; II. Tris–EDTA pH 7·4; III. Tricine–NaCl pH 7·4; IV. Tris–HCl pH 7·4; for 1 h at 0–4°C with 2×10^{-9}M $E_2{}^3H$; the incubation was continued further at 22°C for 30 min. After removal of free steroid by charcoal treatment and dilution of the treated cytosol to a protein concentration of 0·8 mg/ml with corresponding buffer, a large molar excess (100–300-fold) of [^{14}C] labeled estradiol was added and the dissociation allowed to proceed at 22°C with constant stirring.

At the indicated time intervals 0·2 ml aliquots were taken and the bound $E_2{}^3H$ was assayed by the Dextran-coated charcoal technique. Corrections were made for non-specific binding (see Materials and Methods), and the data plotted on a semi-logarithmic scale. The dissociation curves were decomposed and the *kD* for the first (a) and the second (b) components were computed. Upper curve (c): control samples processed identically without addition of [^{14}C] labelled estradiol.

the cytosol is reflected by an inversion of the relative importance of the two phases: (i) the first phase, after correction for interference by the second phase, when extrapolated to zero hour shows that quantitatively more $E_2{}^3H$ receptor complex contributed to this phase, in buffers without NaCl than in buffers with NaCl (segment c Fig. 1). (ii) the second phase when extrapolated to zero hours shows that inversely a higher proportion of $E_2{}^3H$–receptor complex contributed to this phase, in the presence of NaCl (segment b, Fig. 1).

In Fig. 2 are represented the exchange data in terms of d.p.m.^{3}H exchanged/total d.p.m.^{3}H, in function of time. The two upper curves a and b show that exchange was faster and quantitatively more important in buffers without NaCl than in buffers containing NaCl (curves c and d). This shows that the state of the estradiol–receptor complex is dependent on the buffers utilized to process them and that exchangeability is modified by the presence of NaCl in the medium.

In order to test if the kinetics of exchange of the estradiol–receptor complex were not modified at 22°C in function of time, exchange was initiated at different times after cytosol was prepared and maintained at 22°C. For this experiment, ovariectomized adult rats (3 months old weighing 145–150 g) were utilized one day post ovariectomy, in order to have sufficient cytosol required for the numerous measurements. Figure 3 illustrates the data obtained. The exchange curves showed similar patterns no matter after what lapse of time the exchange was initiated. The parallelism of the exchange curves therefore shows that in course of the time interval (180 min) at 22°C, the estradiol–receptor complex had not undergone any spontaneous modification. The insert to Fig. 3 shows that when exchange initiation times were normalized to

Table 1. Dissociation rate constants (*kD*) of $E_2{}^3H$-Cytosol receptor complex from immature rat uteri (in s^{-1})

Buffer	*	k_1D	k_2D
Tris–NaCl	0·8	$1{\cdot}9 \times 10^{-4}$	$1{\cdot}2 \times 10^{-5}$
Tris–EDTA	0·8	$1{\cdot}2 \times 10^{-4}$	$2{\cdot}4 \times 10^{-5}$
Tricine–NaCl	0·8	$1{\cdot}5 \times 10^{-4}$	$1{\cdot}2 \times 10^{-5}$
Tris–HCl	0·8	$1{\cdot}3 \times 10^{-4}$	$1{\cdot}2 \times 10^{-5}$

* Protein concentration, mg/ml.

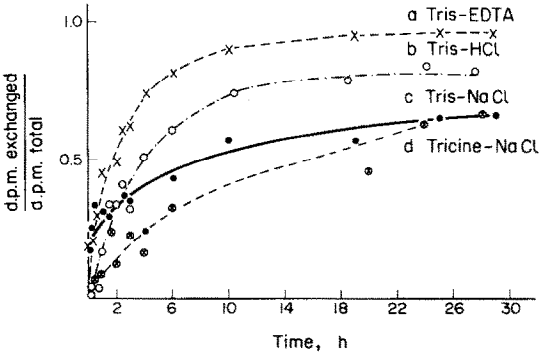

Fig. 2. Comparative exchange of $E_2{}^3H$ with [^{14}C] labeled estradiol. The data were obtained from the experiments shown in Fig. 1. *Abscissa*: time in h. *Ordinate*: d.p.m. Exchanged/d.p.m. Total.

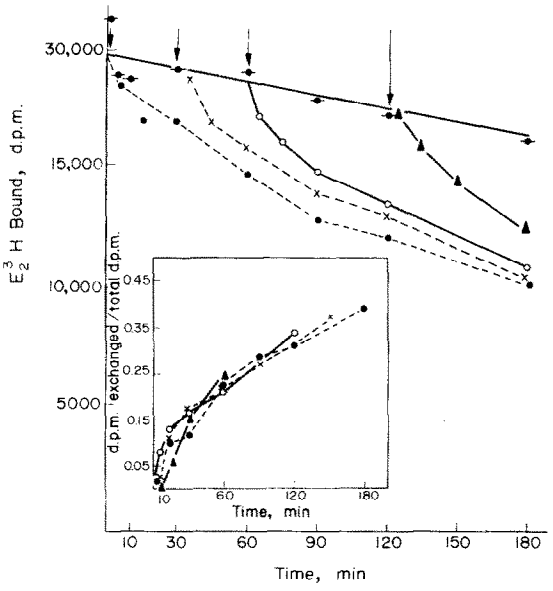

Fig. 3. Exchange of $E_2{}^3H$ with unlabeled estradiol at 22°C from the complex formed in Tris–EDTA–NaCl buffer. Cytosol from ovariectomized adult rats (3 months old, weighing 145–150 g) one day post-ovariectomy, prepared in Tris–EDTA–NaCl buffer pH 7·4 was incubated in the presence of 3×10^{-9}M $E_2{}^3H$ at 0–4°C. Free steroid was removed by charcoal adsorption. Protein concentration was 5·6 mg/ml. Incubation was pursued at 22°C. A large molar excess (221-fold) of unlabeled estradiol was added at the indicated time intervals (↓) and the bound $E_2{}^3H$ was assayed by the charcoal technique. *Ordinate* of insert d.p.m. Exchanged/Total d.p.m.; *Abscissa* of insert time in minutes after addition of unlabeled E_2.

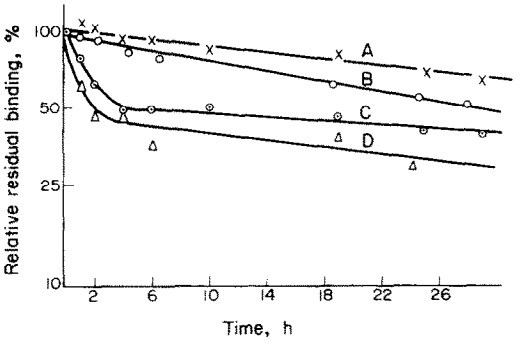

Fig. 5. Comparative stability of the estradiol–receptor complex formed in different buffers on treatment with Dextran-coated charcoal at 0–4°C. Estradiol–receptor complexes were formed in Tris–EDTA (A), Tris–HCl (B), Tris–NaCl (C) and Tricine–NaCl (D) buffers, as described in the legend to Fig. 1. Protein concentration was 0·8 mg/ml. 0·2 ml samples were subjected to charcoal treatment under constant shaking during periods extending from 15 s to 28 h, at 0–4°C. At the indicated time intervals, the samples were centrifuged at 800 *g* for 10 min and the supernate counted for bound radioactivity. The data were plotted on a semi-logarithmic scale.

time 0, all the four exchange curves superimposed themselves and showed the quantitative similitude of exchange in function of time.

(b) The results of saturation analysis of the cytosol receptors in Tris–EDTA and Tris–HCl–NaCl buffers are presented in Fig. 4a and 4b, according to the method of Scatchard, as described under Materials and Methods. The linearity of the saturation data in both buffers, after resolution of the experimental curve for non-specific binding demonstrates a single class of high affinity, low capacity binding sites. The apparent dissociation constant calculated from the corrected linear components gave reasonably similar values. $1{\cdot}6 \times 10^{-10}$ M and $1{\cdot}5 \times 10^{-10}$ M respectively, which compare favorably with values cited in the literature [3, 14–16].

At low $E_2{}^3H$ levels, an apparent positive cooperative effect was observed. However, Fig. 4a (circles with a cross) shows that a longer preincubation time at 22°C reduced to a certain extent this apparent cooperative effect.

II. *Stability of the estradiol–receptor complex in the presence of Dextran-coated charcoal at* 0–4 *C.* Estra-

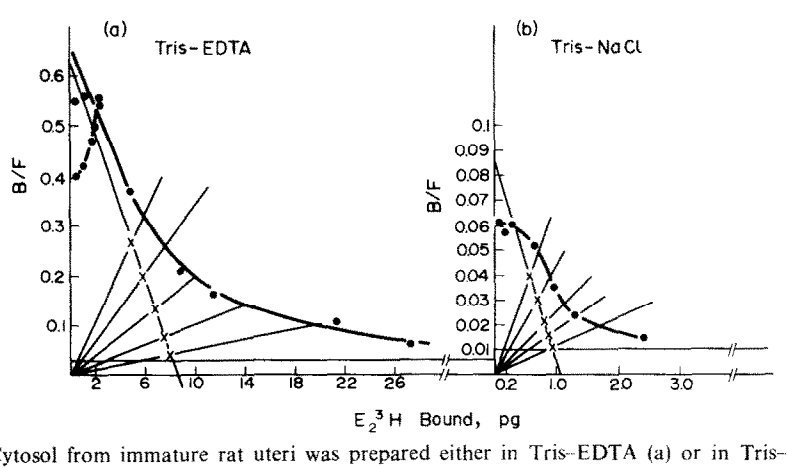

Fig. 4. Cytosol from immature rat uteri was prepared either in Tris–EDTA (a) or in Tris–NaCl (b) buffers. Protein concentration was 6·0 mg/ml. 0·2 ml aliquots of the cytosols were incubated first at 22°C for 30 min and thereafter at 4°C, for 1 h with increasing amounts of $E_2{}^3H$, ranging from 4×10^{-11}M to 2×10^{-8}M; the amount of bound $E_2{}^3H$ was assayed by the charcoal technique. The data were plotted according to Scatchard, and the corrections for non-specific binding was done by the method of Rosenthal. In Fig. 4a, the circles with a cross show the data obtained from samples with low $E_2{}^3H$ levels, that were incubated for 1 h at 22°C before incubation at 0–4°C for 1 h.

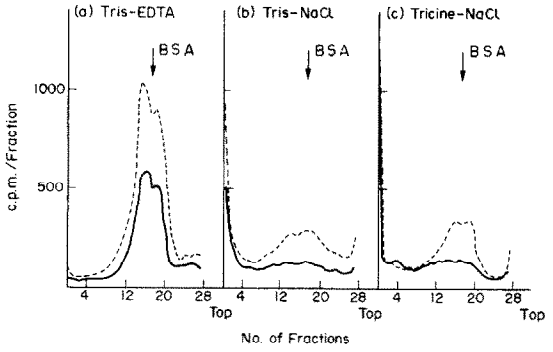

Fig. 6. Sedimentation profiles of estradiol–receptor complexes formed under "cell-free" conditions. Cytosols were prepared in the indicated buffer solutions and incubated at 0–4°C for 1 h in the presence of 3×10^{-9}M $E_2{}^3H$. Free steroid was removed by charcoal adsorption. After activation at 22°C for 30 min, 0·2 ml aliquots were layered on linear sucrose 5–20% density gradients, prepared in corresponding buffers containing 0·3 M KCl. *Upper curves*: activated cytosol. *Lower curves*: cytosol after exchange with unlabeled estradiol at 22°C for 1 h.

diol–receptor complexes were formed under "cell-free" experimental conditions in Tris–HCl, Tris–HCl–NaCl, Tris–EDTA and Tricine–NaCl respectively, and were subjected to the charcoal treatment during periods extending from 15 s to 28 h: 0·2 ml samples were incubated at 0–4°C, after addition of the Dextran-coated suspension prepared in respective buffer solutions and were maintained under constant agitation. Figure 5 shows that complexes formed in Tris–HCl and Tris–EDTA without NaCl showed a higher resistance to charcoal treatment than the complexes prepared in Tris–HCl–NaCl and Tricine–NaCl. The curves show a major difference in the early parts where complexes prepared in NaCl containing buffers show a higher lability of the binding in the presence of charcoal. After the first four hours a relative stabilization occurred in the two latter cases.

III. *Sucrose-density gradients of the estradiol–receptor complexes.* 0·2 ml aliquots of estradiol–receptor complexes formed in various buffers, were analyzed on sucrose-density gradients containing 0·3 M KCl. The $E_2{}^3H$–receptor complex formed under "cell-free" experimental conditions after activation at 22°C and stripping with charcoal, show that irrespective of the buffer utilized for preparing the receptors, both "4S" and "5S" peaks were present (Fig. 6, upper curves). The formation of aggregates was a characteristic evidenced by the gradient profiles. In the absence of salt (Fig. 6a), no aggregates were discernible whereas in the presence of NaCl (Fig. 6b and c) aggregates were invariably observed at the bottom of the centrifuge tubes. The lower curves in Fig. 6a, 6b and 6c show the sedimentation profiles of the temperature activated $E_2{}^3H$–receptor complexes after exchange with unlabeled estradiol at 22°C for 1 h. An effective chase was evidenced in all cases by a reduction of both "4S" and "5S" peaks. Whether this exchange occurred in a similar manner in the aggregates, was not quite apparent from the sedimentation profiles.

Under *in vitro* experimental conditions, (Fig. 7a) a distinct "4S" peak was obtained when the incubation was conducted at 4°C. On raising the temperature of incubation to 22°C, both "4S" and "5S" were obtained. Under *in vivo* experimental conditions, only the "4S" peak was observed (Fig. 7b).

DISCUSSION

Experimental factors contributing to the occurrence of multiple forms of estrogen–receptor complexes in the rat uterus have been reported by several investigators, such as concentration dependent aggregation [17], artefacts produced by use of polyanions [18], state of aggregation of the estradiol–receptor complex, depending on ionic strength [19, 20]. The present observations, made on some of the physicochemical characteristics of the cytoplasmic receptor of immature rat uteri prepared in various buffers also show the influence of the experimental conditions on the apparent complexity of the estradiol–receptor.

Basically, however, the estradiol-receptor complex appeared to be composed of one set of homogeneous sites, disclosing high affinity and low capacity, as evidenced by a saturation analysis according to Scatchard [11], and after correction for non-specific binding. An apparent positive cooperative effect was observed at low ligand concentration in this work, as in others [14, 21, 22]. However, by increasing the incubation time at 22°C, this apparent cooperative effect was less marked, suggesting that it was at least partly due to incomplete equilibrium between the ligand and the receptor. Some indications of a cooperative effect for estradiol binding to immature rat uterus was obtained *in vivo*, in long term infusion

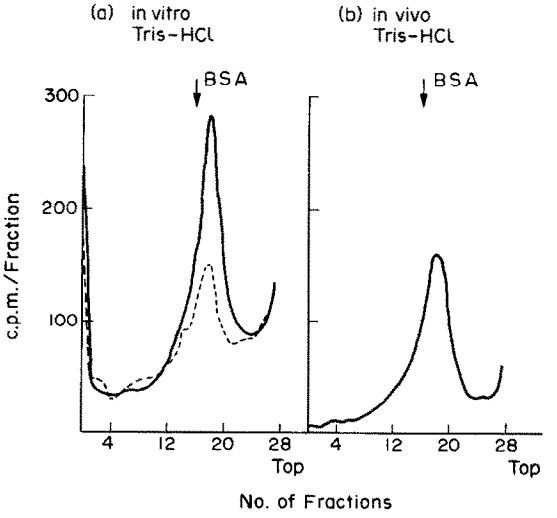

Fig. 7 (a). Sedimentation profiles of cytosol prepared after 1 h incubation of whole uteri at 4°C (upper curve), or 22°C (lower curve), in the presence of 3×10^{-9}M $E_2{}^3H$ on linear 5–20% sucrose density gradients in Tris–HCl buffer containing 0·3 M KCl. (b) Sedimentation profile of cytosol after *in vivo* infusion of $E_2{}^3H$ (see Materials and Methods) on linear 5–20% sucrose density gradient in Tris–HCl buffer containing 0·3 M KCl.

of $E_2{}^3H$. [8]. Here again, lack of equilibrium could be evoked as an interfering cause of this apparent cooperative effect.

Observations made *in vivo* on adult rats [8] or *in vitro*, using uterine cell suspensions or whole tissue [23] converge in describing the binding of estradiol to the uterus receptors as the interaction between the ligand and one set of independent binding sites.

Cytosol prepared in Tris–EDTA or in Tris–NaCl buffers allow the same conclusion. Apparent dissociation constants were the same in both buffers ($1{\cdot}5 \times 10^{-10}$M), and fitted with values reported by others [3]. However, the number of sites appeared to be reduced when Tris–NaCl or Tricine–NaCl buffers were used for homogenizing the uteri.

This reduction was variable, ranging from 15 to 70% when parallel experiments were run with two or more buffers (unpublished, see also Fig. 1).

The heterogeneity of the cytosol receptors prepared in different buffers appeared in the study of the dissociation kinetics. The latter was biphasic in accordance with previous reports [14, 16, 22, 24]. The dissociation curve could be resolved into two first-order components whose dissociation velocity could be separately calculated. The values (k_1D: $1{-}2 \times 10^{-4}$ s^{-1}; k_2D: $1{-}2 \times 10^{-5}$ s^{-1} at 22°C) fit with values reported by others [14, 21, 22]. There was no striking difference in the kD obtained in any of the four buffers used (Tris–EDTA, Tris–HCl, Tris–NaCl, Tricine–NaCl). However, the quantitative importance of the two components was different, depending on the presence of NaCl in the buffer. Hence, the rapidly dissociating component was 3–4 times more important than the slowly dissociating component in the absence of NaCl, whereas in the presence of salt, the relative importance was inversed, the slowly dissociating component amounting to about twice the rapidly dissociating one.

This apparent change in the dissociation kinetics by the presence of 0·15 M NaCl in the homogenizing buffer could merely express a loss of the rapidly dissociating component in the NaCl containing cytosol. The total number of sites was indeed always reduced in cytosol prepared with NaCl containing buffer as observed above. The reduction appeared to be most important, for the rapidly dissociating component (see Fig. 1). Another possibility is a change in the kinetics, due to a transformation of the rapidly into the slowly exchangeable component in the presence of NaCl. Such influence of salt should then be rapidly realized and produce a new stable situation. Indeed, the kinetics of dissociation were quantitatively and qualitatively the same whether the exchange with unlabeled estradiol was initiated immediately or only 2 h after the cytosol, prepared in salt containing buffer and labeled with $E_2{}^3H$ at 4°C for 1 h, was incubated at 22°C (see Fig. 3).

Another observation which would support the hypothesis of the influence of salt on the dissociation kinetics was made at 0–4°C, in the presence of Dextran-coated charcoal. The estradiol receptor complex is very stable at 4 C [22]; this was confirmed in the presence of charcoal for up to 28 h, for cytosol prepared in either Tris–HCl or Tris–EDTA buffers. After 28 h, more than 50% of the estradiol remained bound to the receptor. In buffers containing 0·15 M NaCl, up to 50% of the bound estradiol, were removed by the charcoal in the first 4 h, instead of 10% in the cytosol prepared in salt-free buffers. After 4 h, the dissociation of the remaining bound estradiol was much slower and parallel to that seen in the salt-free cytosol.

Finally, analysis on sucrose density gradients of the cytosols prepared in different buffers also showed a characteristic effect of NaCl. Indeed, large amounts of heavy aggregates were always found at the bottom of the centrifuge tubes in gradients from salt containing cytosols (Tris–NaCl, Tricine–NaCl). These aggregates were usually absent in salt-free cytosols (prepared in Tris–EDTA, or Tris–HCl). These aggregates subsisted in the presence of 0·3 M KCl in the gradients. Besides these aggregates, 4 and 5S peaks were observed in all the cases after cell-free incubation of cytosol with $E_2{}^3H$ at 22° C. The presence of "5S" peak corresponds to the temperature activated form of the receptor [25, 26]. Both 4 and 5S bound $E_2{}^3H$ could be exchanged with unlabeled estradiol. Exchangeability of the aggregate-bound $E_2{}^3H$ was less evident, although it could not be excluded.

Binding to the cytosol receptor prepared in salt containing buffer remained ligand specific, being competitive for estradiol and estriol, but not for testosterone (unpublished data).

The observations discussed above should tend to show that experimental procedures may have a preponderant influence on some characteristics of the cytosol receptor, when analyzed in a 'cell-free' system.

Comparison with more physiological situations are needed to ascertain the significance of some of the "cell-free" observations. When incubated *in vitro*, in the presence of $E_2{}^3H$, uteri from immature rats yielded cytosols containing either 4S $E_2{}^3H$–receptor complex, alone, or a mixture of 4 and 5S complexes, depending on whether the uteri were incubated at 4 C or at 22°C. Similar data have been reported for *in vitro* incubation of bovine uterine tissue at different temperatures [27]. Under *in vivo* conditions, when $E_2{}^3H$ was infused continuously over a period of 4 h into the jugular vein, $E_2{}^3H$ was taken up by the uterus and appeared in the cytosol as a homogeneous "4S" peak on 0·3 M KCl-sucrose density gradient [9].

The experimentally induced modifications of the cytosol estradiol-receptor, which appear to be more complex in "cell-free" systems as compared to *in vivo* observations, may have further impact on the study of translocation of this complex to the nucleus. Further work was then devoted to the comparison between "cell-free", *in vitro* and *in vivo* nuclear translocation of the cytosol estradiol–receptor complex [26].

Acknowledgements—The financial support of the Fonds National de la Recherche Scientifique and of the Fonds de la Recherche Scientifique Médicale are gratefully acknowledged.

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