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Isolation and preliminary characterization of an oestradiol-macromolecule complex from calf uterus "nuclear" fraction

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Endocrinologia. — *Isolation and preliminary characterization of an oestradiol–macromolecule complex from calf uterus 'nuclear' fraction.* Nota di GIOVANNI ALFREDO PUCA, ERNESTO NOLA e FRANCESCO BRESCIANI, presentata (*) dal Socio L. CALIFANO.

RIASSUNTO. — Sezioni d'utero di vitello incubate *in vitro* con 6,7–^{3}H–estradiolo–17 β concentrano l'ormone con meccanismo specifico. Simili sezioni di intestino non hanno questa proprietà. L'ormone non viene modificato chimicamente dall'utero ed interagisce con il tessuto in modo non–covalente. Circa lo 80% dell'ormone si trova nella frazione nucleare dell'omogenato d'utero. Trattamento della frazione nucleare con una soluzione contenente 10^{-2} M *tris*–ac.cloridrico, pH 8,5, 0,4 M KCl e 10^{-3} M $CaCl_2$ solubilizza un complesso estradiolo–macromolecole con le seguenti proprietà: 1) precipita tra 0,145 e 0,245 gm/ml di $(NH_4)_2SO_4$; 2) sedimenta a 5,4 S in gradiente di saccarosio; 3) è distrutto da proteasi ma non da nucleasi.

INTRODUCTION.

Uterus, vagina, anterior hypophysis and mammary gland are able to concentrate and retain 17β–oestradiol against a large concentration gradient with the blood; the hormone is not chemically transformed and is not covalently bound in these organs [1–3]. Recently it was shown that also organs other than the classic targets for oestrogens specifically retain the hormone, although at much lower levels [3].

At least for uterus [4–7] and mammary gland [8] the ability to concentrate and retain 17β–oestradiol was found to depend upon the presence of macromolecules which bind 17β–oestradiol and oestrogenic molecules in general. Two such molecules have been preliminarily described; they can be differentiated by their sedimentation constant on sucrose gradient centrifugation, and by their location at subcellular level. A component of the 105,000 xg supernatant fraction of rat uterus, which sediments at 9.5S in a sucrose gradient, was isolated; it is at least in part protein and has a high specific affinity for oestrogenic molecules [5,9]. Solubilization of an oestradiol-macromolecule complex from rat uterus 'nuclear' fraction—which contains about 80 % of the hormone in the cell—was also described [6,7]. This 'nuclear' complex sediments at about 5 S and is at least in part protein. Demonstration of a high specific affinity for oestrogens of the macromolecule in the 5S complex was also given [7]. These macromolecules appear to fit the conceptual expectations of receptor molecules, as recently discussed by Bush [10].

Further progress in the chemical as well as physiological characterization of these receptors for oestrogens heavily depends upon their purification.

(*) Nella seduta dell'8 marzo 1969.

This, however, is not an easy task because of their low concentration, even in the uterus which contains the most. On the basis of 17β–oestradiol bound by the mouse uterus [3] a concentration of the order of 1 mg/Kg of wet weight can be estimated for the nuclear receptor, by assuming that it is a protein of 100,000 M. W. with a single binding site. Clearly rodents are not well suited for purification of oestrogen receptors.

Therefore, we have directed our attention to calf uterus. This organ is already know to form a 5S–complex upon incubation *in vitro* with ^{3}H–oestradiol–17β [6]. However, in contrast to rodent tissues [11] there is no study of the kinetics of uptake of 17β–oestradiol by calf uterus *in vitro*; an efficient solubilization and purification of the 5S complex as well as the demonstration of a high specific affinity of the molecular interaction in the 5S complex are also lacking. In addition to a comparative study of ^{3}H–oestradiol–17β uptake *in vitro* by strips of uterus and intestine of immature calves, this report describes a method for rapid and efficient extraction and 40 to 60 fold purification of an oestradiol–macromolecule complex sedimenting at 5.4S from calf uterus ' nuclear ' fraction.

MATERIALS AND METHODS.

Reagents were of analytical grade. Ammonium sulphate was free from heavy metals. 6,7–^{3}H–oestradiol–17β (specific activity 42.4 C/mM) was purchased from NENC (Boston) and was $\geq$ 97 % pure at the time of use. Purity checks were carried out by thin layer chromatography.

Preparation, incubation and fractionation of tissue. Uterus and pieces of small intestine of immature calves were collected at the local slaughterhouse in a plastic bag and kept in crushed ice during transportation to the laboratory. Unless otherwise specified all the following operations were carried out in a cold room at +4° C. Uterine horns and intestine were slit open and cut into strips with scissors. The strips of tissue were washed in saline and incubated at 37° C in Krebs–Ringer phosphate, pH 7.4 [12] containing 10^{-9} M 6,7–^{3}H–oestradiol–17β and kept in suspension by magnetic stirring. The ratio of tissue to incubation medium (wt/v) was 0.02. After incubation the strips of uterus were rapidly washed with cold saline, suspended in cold TKE medium (10^{-2} M *tris*–hydrochloric acid pH 7.4; 10^{-2} M KCl; 10^{-3} M EDTA) and homogenized in a VIRTIS waring blender (W : V = 1 : 5–10) with the homogenizing cell cooled by a flow of ice-cold water as follows: 3 runs of 30 s at 20–25,000 r.p.m., at intervals of 60 s. The homogenate was spun at 1200xg for 10 min. and the sediment washed 3 times by resuspending the pellet with an Ultraturrax homogenizer (3 runs of 15s at 70–90 V, with 30s intervals) in fresh TKE medium. The final sediment, which is heavily contaminated with myofibrils, is labelled ' nuclear ' fraction. The supernatants were combined and centrifuged at 105,000 xg for 1 hour; a sediment (consisting of a combined mitochondrial and microsomal fraction) and a supernatant (' soluble ' fraction) were thus obtained.

Extraction and thin layer chromatography of radioactivity in the tissue. These methods are described elsewhere [2 , 3].

Solubilization of an oestradiol-macromolecule complex from the 'nuclear' fraction. The 'nuclear' fraction was suspended by the Ultraturrax in TKC medium (10^{-2}M *tris*–hydrochloric acid, pH 8.5; 0.4 M KCl; 10^{-3}M $CaCl_2$), incubated for 1 hour with magnetic stirring and centrifuged for 1 hour at 105,000 xg; the supernatant contained 50–70 % of the radioactivity in the 'nuclear' fraction and was labelled 'nuclear extract'.

Fractional precipitation by salt. Ammonium sulphate was added under continuous stirring in discrete amounts of 0.035 gm/ml. About 15 min after addition of each fraction of salt, an aliquot of the 'nuclear extract' was centrifuged at 10,000 r.p.m. for 15 min and the supernatant essayed for protein and radioactivity. Proteins were determined by a micro-method based on a modification of the biuret reaction [13].

Sucrose gradient centrifugation was carried out according to Martin and Ames [14], using TKE medium pH 8.5 as solvent.

Results and Discussion.

Uptake of ^3H–oestradiol–17β. Fig. 1 shows typical curves of hormone uptake by uterine and intestinal strips suspended in Krebs-Ringer phosphate pH 7.4 and containing 10^{-9}M 6,7–^{3}H–oestradiol–17β; the gas phase was

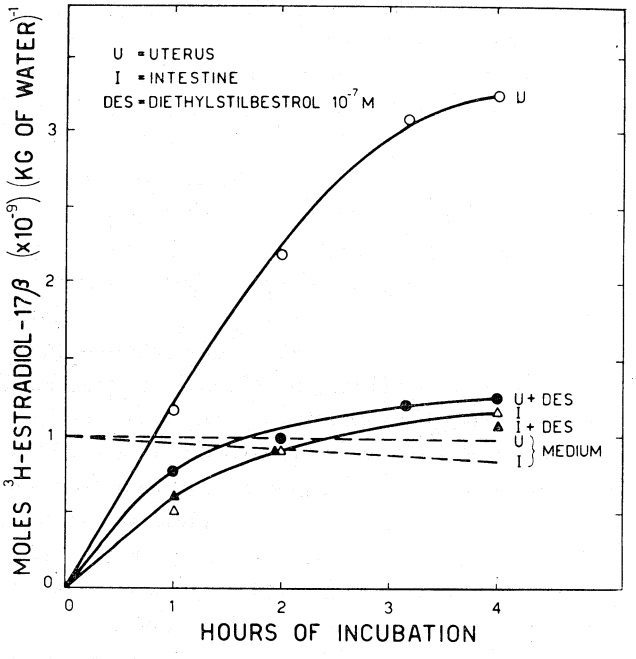

Fig. 1. – Uptake of ^{3}H–oestradiol–17 β by strips of uterus and intestine of calf incubated in Krebs-Ringer phosphate pH 7.4 containing 10^{-9} M 6,7–^{3}H–oestradiol–17β and with/without 10^{-7} M diethylstilbestrol; 37°C; gas phase: air.

air and the temperature 37° C. Addition of glucose (0.2 %) to the medium and/or incubation in an atmosphere of O_2 does not significantly influence uptake. Uterine strips are able to concentrate radioactivity about 400 % within 4 hours of incubation, while after the same time the level of radioactivity in the intestinal strips is barely higher than that of the medium. Addition of 10^{-7} M Diethylstilbestrol decreases uptake of ^{3}H–oestradiol–17β by uterus to about the level of intestine, but it does not detectably affect uptake by

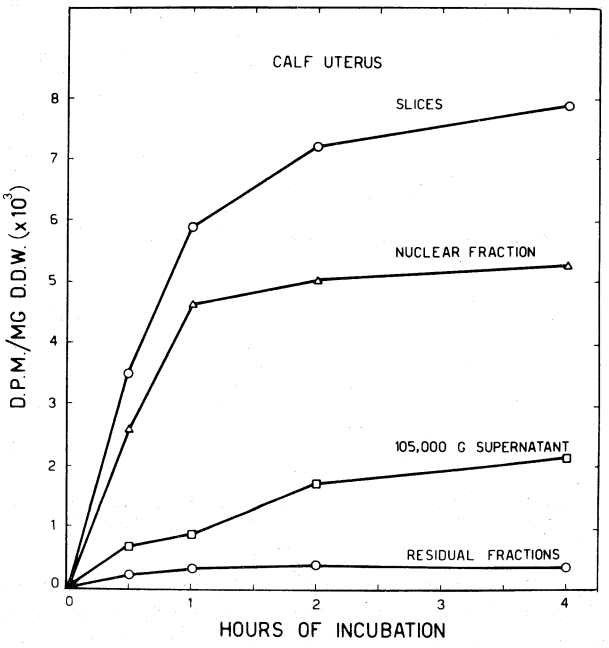

Fig. 2. – Distribution of radioactivity in subcellular fractions of calf uterus strips at different times during incubations in Krebs-Ringer phosphate, pH 7.4, containing 10^{-9} M 6,7–^{3}H–oestradiol–17β; 37° C; gas phase: air. 'Residual fraction' includes mitochondrial and microsomal fractions.

intestine. On the contrary, addition of 10^{-7} M 17α–oestradiol or Cortisol or Deoxycorticosterone has no effect at all on uptake of ^{3}H–oestradiol–17β. Thus, in contrast to intestine, uterine strips *in vitro* possess in high degree an ability to concentrate steroidal and non-steroidal oestrogenic molecules specifically.

Subcellular distribution of radioactivity. Subcellular distribution of radioactivity in the uterus at different times during incubation is presented in fig. 2. According to the duration of incubation, the nuclear fraction contains 67–79 % of the radioactivity in the tissue, the 105,000 xg supernatant 16–28 % and the combined mitochondrial and microsomal fraction only about 5 %.

Analysis of radioactivity in the uterus. Fractionation according to solubility of radioactivity in the uterus shows that there is virtually no water-soluble (conjugate) or insoluble ('protein' bound) radioactivity; all radioactivity

($\leq$ 98 %) is ether-soluble (' free ' steroid), and this fraction was further analysed by thin layer chromatography. The results presented in fig. 3 show that the radioactivity consists of intact ^{3}H–oestradiol–17β. Furthermore, analysis of radioactivity in the medium of incubation of uterine strips shows no metabolic product of the hormone, which could have been released from the tissue. Thus, like the uterus of rodents *in vivo* [1] also calf uterus *in vitro* appears to lack the ability to metabolize 17β–oestradiol.

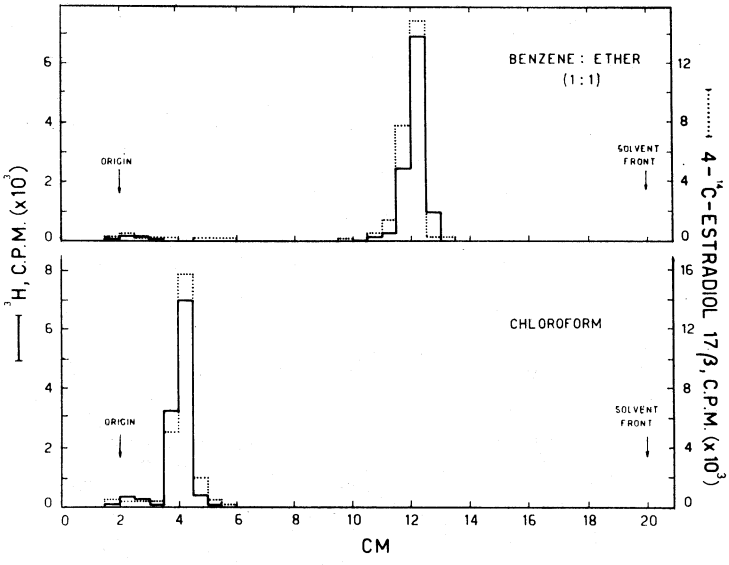

Fig. 3. – Thin layer chromatography of ether-soluble radioactivity in calf uterus strips after 4 hours of incubation in Krebs-Ringer phosphate, pH 7.4, containing 6,7–^{3}H–oestradiol–17β; 37° C; gas phase: air. 4–^{14}C–oestradiol–17β (dotted line) was added to tissue radioactivity as an internal marker. In both systems of solvents, virtually all radioactivity migrates as 17β–oestradiol.

Solubilization of an oestradiol-macromolecule complex from the ' nuclear ' fraction. Little or no 17β–oestradiol is released by the nuclear fraction when suspended in solutions like the TKE, with a low KCl concentration (10^{-2}M) and a pH near neutrality (pH 7.4); increasing the concentration of KCl to 0.4 M and bringing the pH to 8.5, as in the TKC solution, results in solubilization of 50–70 % of the hormone associated with the ' nuclear ' fraction, of which about 70 % is bound to macromolecules. With respect to the solution previously applied to extraction of rat uterus ' nuclear ' fraction [7], TKC contains 10^{-3}M $CaCl_2$ instead of 10^{-3}M EDTA; this change was introduced because the presence of the cation was found to decrease formation of aggregates. The use of buffers at pH 8.5 is a significant improvement of the extraction procedure, as compared to buffers at pH 7.5 [6]. At pH 8.5 solubilization is virtually completed within 1 hour of incubation, while at pH 7.5 long and repeated extraction of the nuclear fraction is required, and the yield of bound hormone is low.

Fractional precipitation by salt. Fractional precipitation by ammonium sulphate of the ' nuclear ' extract is shown in fig. 4. Virtually all radioactivity precipitates between 0.145 and 0.245 gm/ml of salt concentration. The precipitate was redissolved in a small volume of TKC (0.1 M in *tris*–hydrochloric acid) and cleared by centrifugation for 20 min at 15.000xg. After fractional precipitation, the specific activity of different preparations generally varied between 40,000 and 60,000 DPM/mg of protein; as compared to the 'nuclear'

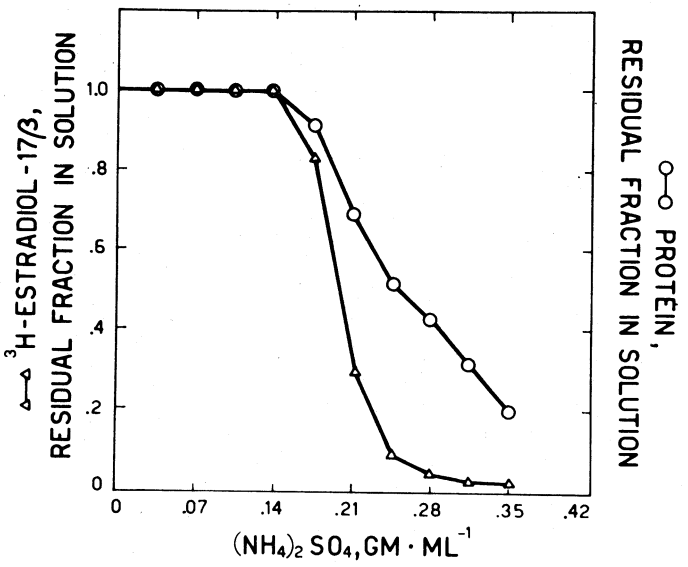

Fig. 4. – A typical fractional precipitation of nuclear extract by ammonium sulphate. The macromolecular complex containing ^{3}H–oestradiol–17β precipitates in salt concentrations between 0.145 and 0.245 gm/ml. After this step, the concentration of bound hormone is 40,000–60,000 dpm/mg protein, which corresponds to a 40 to 60 fold purification.

bound hormone in the original homogenate (~1000 DPM/mg of protein), this corresponds to 40 to 60 fold purification. Then a thousand DPM correspond to 10.6×10^{-8} μmoles of 17β–oestradiol; assuming that the macromolecule binding the hormone has 100,000 MW and a single binding site, one can calculate that in this purified preparation there are still only 42.4 to 63.6 mμg of the macromolecule of interest per mg of protein, that is a concentration of $4–6 \cdot 10^{-5}$.

Sucrose gradient analysis. Fig. 5 shows a typical sucrose gradient analysis of the ammonium sulphate precipitate redissolved in TKC and cleared by centrifugation for 20 min at 15,000xg; bovine serum albumin was centrifuged in parallel. Most of the radioactivity sediments at 5.4 S – or slightly less in other preparations – but faster components are also evident. Occasionally the radioactive components faster than 5S may represent a larger fraction of total bound radioactivity, while in other cases they may be virtually lacking. Tendency to aggregation is facilitated by lack of Ca^{2+} in the extraction medium.

Fig. 6 shows a sucrose gradient analysis of ammonium sulphate precipitate of ' nuclear ' extract prepared with Ca^{2+} lacking solutions; the amount of components sedimenting faster than 5 S is clearly much larger than in the preparation of fig. 5, obtained with the routine method described in this paper.

Enzyme sensitivity of the 5 S complex. The results in Table I show that the 5 S complex is destroyed by proteases but not by nucleases. This indicates that the macromolecule is protein, or at least the moiety binding the hormone is protein.

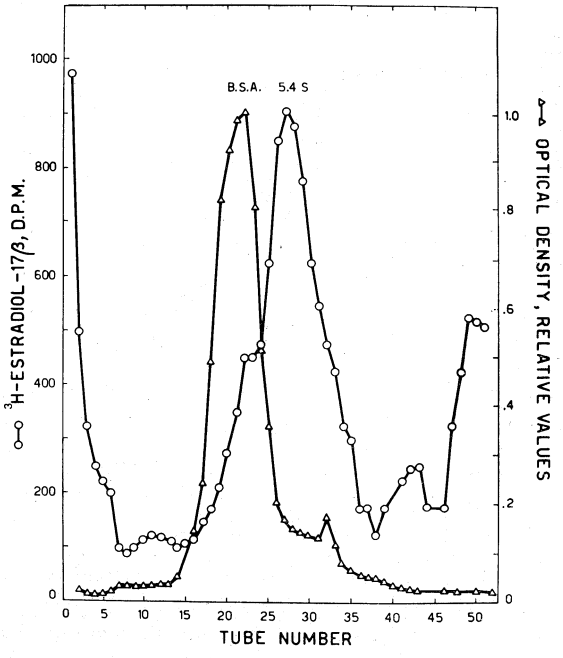

Fig. 5. – Sucrose gradient analysis in TKE solution pH 8.5 of ammonium sulphate precipitate of nuclear extract. Bovine serum albumin was centrifuged in parallel. Most bound hormone sediments at 5.4 S which corresponds to a 92,000 M.W. as compared to albumin. Most likely, faster sedimenting components are aggregates.

Summary and Conclusions.

1) Immature calf uterus strips incubated in Krebs-Ringer phosphate, pH 7.4, containing 10^{-9}M 6,7-^{3}H–oestradiol–17β are able to concentrate radioactivity about 400 % within 4 hours. Similar strips from intestine barely show an increased content of radioactivity with respect to the level in the incubation medium.

2) Addition of 10^{-7}M diethylstilboestrol to the medium decreases uptake of radioactivity by the uterus to about the level of intestine, while it does not detectably affect uptake by intestine. Addition of 10^{-7} M 17α–oestradiol, or Cortisol, or Deoxycorticosterone has no effect on uptake of radioactivity by the tissues. Thus the mechanism of uterus, by which 17β–oestradiol is concentrated, appears to be oestrogen specific.

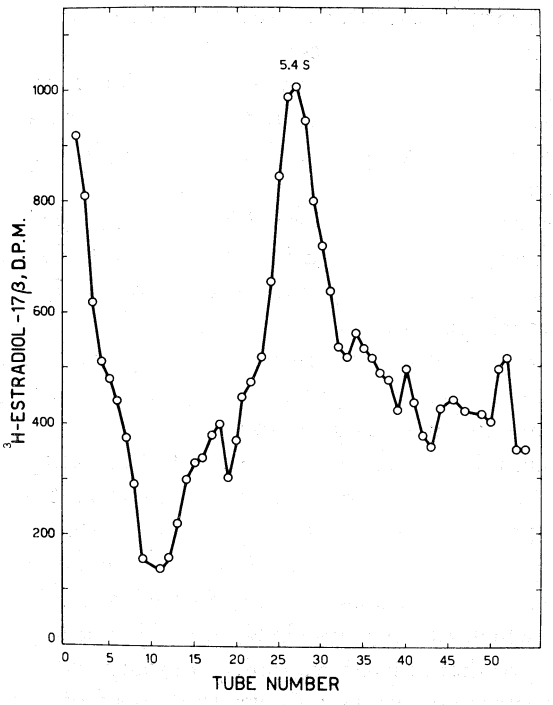

Fig. 6. – Sucrose gradient centrifugation in TKE solution pH 8.5 of ammonium sulphate precipitate of nuclear extract obtained using an extraction medium without Ca^{2+}. The amount of components sedimenting faster than 5.4 S is a larger fraction of total radioactivity, with respect to preparation of fig. 5 obtained using an extraction medium with Ca^{2+}. Note the heterogeneity of these faster components, suggesting that these are aggregates of the 5.4 S 17β-oestradiol-binding macromolecule with other proteins in the preparation.

TABLE I.

Effect of enzymes on the native oestradiol-macromolecule complex from calf uterus 'nuclear' fraction.

ENZYME TESTED	Residual ^{3}H-oestradiol-17 β bound after 30 min at 35° C with enzyme (% of control)
PRONASE (25 μg/ml)	2
TRYPSIN (100 μg/ml)	8
DNase (25 μg/ml)	104
RNase (25 μg/ml)	99

Bound ^{3}H-oestradiol-17β was separated from free by chromatography on columns of Sephadex G-25, under standard conditions at + 4° C. During the 30 min incubation time at + 35° C, the bound tritiated hormone decreased in the control from 48×10^3 to 26×10^3 dpm/mg of protein. Protein concentration: 1 mg/ml in TKE solution.

3) Radioactivity in the uterine strips consists of intact ^{3}H–oestradiol–17β, totally extractable by organic solvent (' free ' steroid).

4) Seventy to 80 % of hormone is associated with the ' nuclear ' fraction, and about 15–25 % with the 105,000 xg supernatant of uterus homogenate. Mitochondrial and microsomal fractions contain the balance of radioactivity in the tissue.

5) Little or no hormone is released by the ' nuclear ' fraction incubated in TKE (10^{-2} M *tris*–hydrochloric acid, pH 7.4; 10^{-2} M KCl; 10^{-3} M EDTA) but increasing pH to 8.5 and KCl concentration to 0.4 M, as in TKC solution (10^{-2} M *tris*–hydrochloric acid, pH 8.5; 0.4 M KCl; 10^{-3} M $CaCl_2$) results in rapid release into solution of 50–70 % of the hormone in the ' nuclear ' fraction. Chromatography on Sephadex G–25 shows that about 70 % of the solubilized hormone is macromolecule-bound.

6) The bound hormone precipitates between 0.145 and 0.245 gm/ml of ammonium sulphate. The precipitate contain about 40,000–60,000 dpm of ^{3}H–oestradiol–17β per mg of protein and represents a 40 to 60 fold purification with respect to the ' nuclear ' bound hormone in the homogenate.

7) Treatment with proteases but not with nucleases destroys the macromolecule to which the hormone is bound. This indicates that the macromolecule is protein, or that at least the moiety binding 17β–oestradiol is protein.

8) Sucrose gradient analysis shows that most of the bound hormone sediments at 5.4 S—or slightly less—but faster components are present especially in the absence of Ca^{2+} in the extraction medium. As compared to bovine serum albumin, 5.4 S corresponds to 92,000 MW. Most likely the faster sedimenting components which do not have specific sedimentation rate but cover the whole range from 5.4 S to the bottom of the gradient are heterogeneous protein aggregates including the specific 5 S component.

9) In conclusion, the macromolecule which is at least in part protein and interacts with 17β–oestradiol in the complex sedimenting at $\sim$ 5 S may be a ' receptor ' for the hormone. The concept of ' receptor ' as recently discussed by Bush [10] requires that the receptor be present in an extremely small amount in the target cell and have a molecular specificity for the hormone. The results presented in this report show that the 5 S macromolecule is actually present in a very small amount, representing about 1 molecule every million protein molecules in the cell. However, the molecular specificity remains to be demonstrated.

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