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Estrogen receptor in hen oviduct chromatin, digested by micrococcal nuclease

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ABSTRACT

Nuclei from laying hen oviduct were prepared according to Hewish and Burgoyne i.e. in the presence of spermine and spermidine and in the absence of divalent cations and were then moderately digested by micrococcal nuclease. When the resulting chromatin was analysed by ultracentrifugation on a sucrose gradient, a peak of specific estradiol-binding sites was observed, sedimenting slightly faster (13-14 S) than the mononucleosomes (12 S). When the chromatin was centrifuged on a gradient containing heparin (5 µg/ml) the sedimentation coefficient of the estradiol receptor peak shifted to 7-8 S ; it returned to the 13-14 S position in the absence of heparin, when target organ chromatin was also present in the gradient. The preparation of the chromatin is described and the validity of the method to explore receptor localisation is discussed, as is the specificity of the receptor-DNA interaction.

INTRODUCTION

The bulk of eukaryotic chromatin is currently described as being organized into nucleosomes, presumably made up of DNA wound around a core of histones, (1-3) which are separated by short bridges of internucleosomal DNA (4,5). A number of experiments have been undertaken in order to understand the relationship between this configuration and the transcriptional activity of chromatin, some separating "active" and "inactive" chromatin by the combined action of DNAase II and Mg^{++} ions (6), some searching for identifiable genes, after treatment by nucleases of chromatin where these genes are expressed or repressed (7,8). In these studies the role of non-histone proteins has not yet been clarified and it seemed interesting to follow a regulatory protein such as the estrogen receptor during the course of nucleosome preparation, using methods where very mild conditions and minimal handling of the chromatin is required.

Using the method described by Gottesfeld et al (9) to separate transcribing and non-transcribing chromatin, Levy and Baxter have reported that in cultured pituitary (GC) cells inactive chromatin contained a larger percentage of glucocorticoid and thyroid hormone receptor sites (10) than active chromatin, whereas Hemminki (11) has found in chick and hen oviduct an enrichment in estrogen receptor sites associated with the active chromatin.

The studies reported here concern the localisation of endogenous estrogen receptor in the laying hen oviduct (where it is mainly nuclear) in the conditions described by Hewish and Burgoyne (12) to obtain so-called "native" chromatin (12-14). Except during enzyme action the use of divalent cations was avoided (12,15,16), as were critical salt concentrations which are known to cause histone displacement and chromatin rearrangement (17,18).

MATERIAL AND METHODS

Animals and materials

Hens were White Leghorns (France Ponte) in the laying state. Estradiol [2,4,6,7(n)^{3}H] (85-100 C/mmole) was purchased from the Radiochemical Center, Amersham, and was tested regularly by thin layer chromatography. If necessary it was repurified by celite column chromatography. Unlabelled estradiol was obtained from Roussel-Uclaf. Micrococcal nuclease (3.1.4.7.) from staphylococcus aureus was from Worthington, and its activity regularly tested as recommended by the manufacturer. Spermine and spermidine were from Calbiochem, RNAase-free sucrose was from Schwartz-Mann and all other reagents were from Merck, BDH or Prolabo.

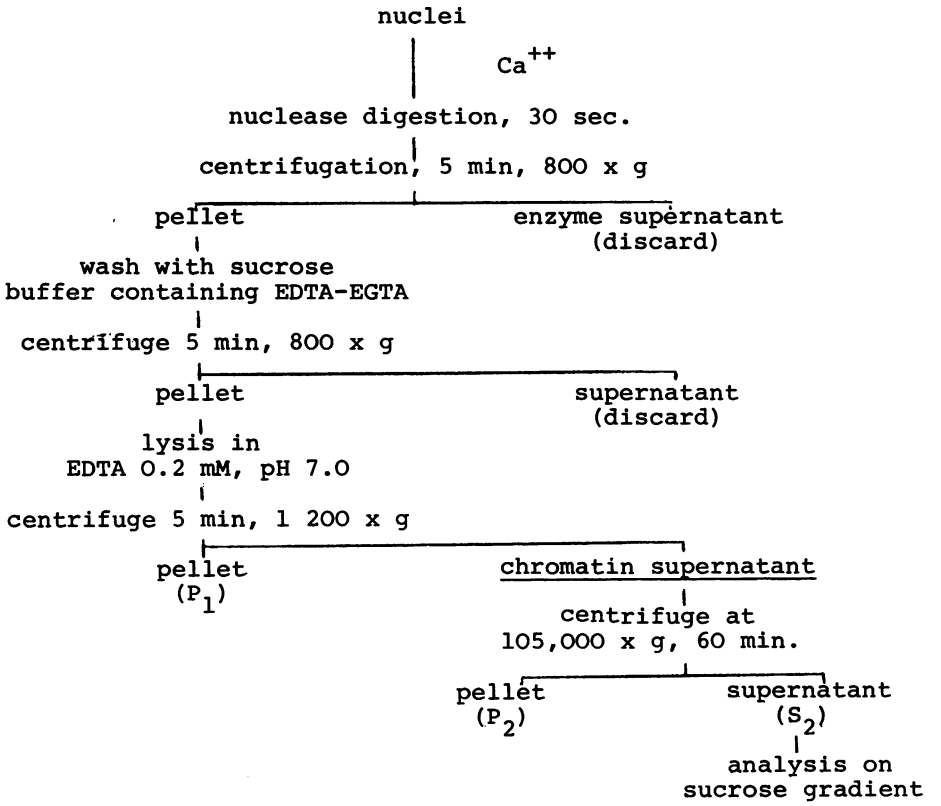
Preparation of nuclei

The magnum portion of the oviduct from a laying hen was excised, minced and homogenized first in a Waring Blender in 7 volumes of buffer A (12) (KCl : 60 mM, NaCl : 15 mM, Tris-HCl : 15 mM, pH 7.4-7.5, Mercaptoethanol : 15 mM, spermidine : 0.5 mM, spermine : 0.15 mM), 0.34 M sucrose, 2 mM EDTA, 0.5 mM EGTA, and then in a Dounce homogenizer (type A), and an aliquot was set aside for DNA measurement. The rest of the preparation followed exactly the method described by Noll et al (14) for rat liver. Once the nuclei had been centrifuged through 2.1 M sucrose, the pellet was resuspended in 0.34 M sucrose in buffer A, without EDTA or EGTA,

and washed twice in this buffer. An aliquot of the last suspension was set aside for DNA determination, in order to calculate the yield of the preparation and the concentration of nuclease necessary for digestion. The washed pellet of nuclei was resuspended in a determined (1 ml/mg DNA) volume of an 0.34 M sucrose-buffer A containing $CaCl_2$ 1 mM. No PMSF* was used in this preparation, because it was observed that this compound interfered with estradiol-receptor binding.

Nuclease digestion and preparation of chromatin

Oviduct chromatin was prepared from purified nuclei following the method of Noll et al (14), as slightly modified :



The nuclear suspension was brought to 37°C in a water bath and the micrococcal nuclease allowed to act for up to 30 sec.

* Phenylmethylsulfonylfluoride.

The reaction was stopped by plunging the tube in ice and adding 20 µl/ml EDTA 0.1 M. The suspension was centrifuged 5 min. at 800 x g to remove the nuclease ("enzyme supernatant"), then the pellet was resuspended in buffer A, 0.34 M sucrose, EDTA, EGTA, and recentrifuged 5 min. at 800 x g. The pellet was resuspended carefully in 1 ml/mg DNA or less of EDTA 0.2 mM, pH 7.0 in order to lyse the nuclei. The lysed nuclei were then centrifuged for 5 min. at 1 200 x g (pellet = P_1) and the chromatin supernatant used as such, or further centrifuged for 60 min at 105,000 x g in a Spinco Ti 50 rotor (pellet = P_2, supernatant = S_2).

Estradiol binding to the chromatin : incubation and determination

The low- or high speed chromatin supernatant was incubated overnight with estradiol-^{3}H 1-10 nM (eventually in the presence of estradiol 1 µM) at 0-2°C ; in most experiments a 90 min incubation at 30°C was added to allow exchange of the endogenous estradiol. A control for denaturation showed that this was negligeable during the chosen period of time, and at the chosen temperature. Estradiol binding was tested in an aliquot by the charcoal-dextran technique (0.25 % charcoal ; 0.025 % dextran) (19), and the rest of the incubated chromatin was analyzed by ultracentrifugation on 5-20 % linear- or 15-27 % convex sucrose gradients or 5-35 % glycerol gradients. Generally, Spinco SW 41 rotors were used, and 0.8-1 ml chromatin was layered on each tube ; at the end of the centrifugation, 3-drop fractions were collected after puncturing the bottom of the tube. When SW 27 rotors were used, up to 3 ml of chromatin could be deposited per tube, and 10-drop fractions were then collected. In each fraction, optical density was measured at 260 nm, and radioactivity then counted after adding 10 ml of Bray's solution (20). Acid-soluble DNA was measured at 260 nm after precipitation of acid-insoluble DNA in 1 M perchloric acid in the presence of NaCl 1 M.

Miscellaneous

Chromatin DNA was deproteinized and prepared for electrophoresis according to Marmur (21). Slab gel electrophoresis on 2 % agarose was as described by Sugden et al (22). DNA was measured according to Burton (23), proteins according to Lowry (24).

RESULTS

Preparation and characterisation of chromatin

In rat liver, incubation of nuclei with 15-30 enzyme units/mg DNA for 30 sec. at 37°C digested 75 % of the nuclear DNA into a form which did not sediment at 4,000 x g, 2 min. (12). To obtain a similar yield of digestion in hen oviduct, approximately 100 units/mg nuclear DNA were necessary. Above 120 units/mg DNA an increasing amount of chromatin appeared in the S_2 fraction (which did not sediment at 105,000 x g, 60 min.) ; for enzyme concentrations above 400 U/mg DNA, the $O.D._{260\ nm}$ measured in the S_2 fraction remained constant (Fig. 1), showing that digestion was taking place within the shorter strands of chromatin to form nucleosome monomers, dimers and trimers rather than at the expense of heavier chromatin.

Time course of enzyme action showed that after 30 sec. enough monomer was formed to easily appreciate the extent of digestion and that only 10 % maximum of the nuclear DNA was rendered acid-soluble. Longer incubation at 37°C affected nuclear lysis and much lower yields of chromatin were obtained. An incubation time

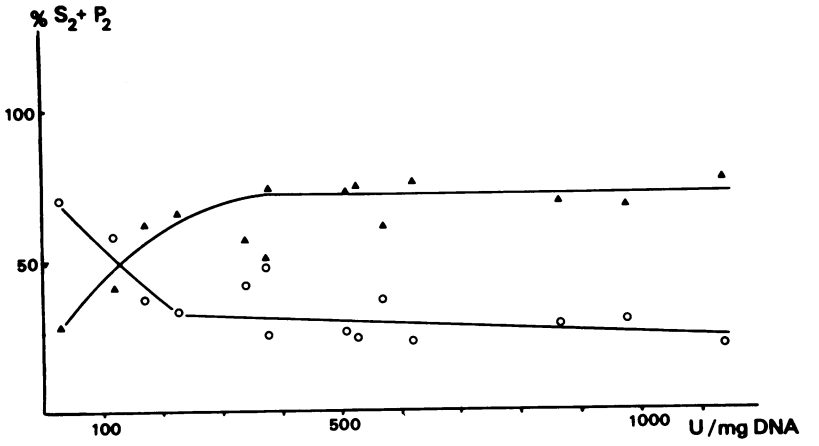


Fig. 1. Distribution of DNA in oviduct chromatin as a function of nuclease activity after a 30 sec. digestion and after a 5 min., 1,200 x g centrifugation followed by a 60 min. 105,000 x g centrifugation.
S_2 : supernatant (▲), P_2 : pellet (o). DNA was measured according to Burton.

of 30 sec. was therefore chosen for most of the experiments reported here.

In the usual conditions used for the present work : 10-20 % of the nuclear DNA was found in the undigested P_1 (see Methods) ; after a high-speed ultracentrifugation, 15-20 % of the nuclear DNA was in P_2 (larger pieces of digested chromatin), and the remainder in S_2 (monomers to tetramers and some unidentified faster sedimenting material). The amount of DNA recovered in P_1 was a function of both nuclease concentration and yield of nuclear lysis, whereas P_2 : S_2 distribution was essentially due to the activity and amount of enzyme used, below 400 U/mg DNA (see above).

Hen liver or kidney chromatin DNA was more sensitive to micrococcal nuclease digestion than oviduct chromatin DNA (see Fig. 3), probably because of a greater decondensation of the chromatin and/or availability of the DNA in the nuclei of these two organs.

When the S_2 fraction was analysed on 5-20 % RNAase-free sucrose gradients, the classical optical density profile at 260 nm was observed (Fig. 2). Sedimentation coefficients and electrophoretic mobilities (results not shown) of monomers (12-13 S), dimers (17-18 S) and trimers (22-23 S) corresponded to the data in the litterature (16). When the appearance of monomers, dimers and trimers (as monitored by ultracentrifugation) was plotted against increasing nuclease concentration (Fig. 2, inset), monomer, dimer and trimer increased in parallel until approximately 200 units nuclease/mg DNA, but above this concentration the appearance of the monomer was favoured, and the dimer and trimer remained at a plateau level.

Chromatin obtained after weak digestion (120 U/mg DNA) of oviduct nuclei was very sensitive to a second digestion by nuclease. In these re-digestion conditions (5,9), and in the presence of Ca^{++} ions (3 mM), the same monomer/dimer ratio was obtained with 100 units of nuclease for 30 sec. as after a 600 unit digestion of nuclei. The sedimentation coefficients of monomer and dimer were slightly larger, however, the monomer sedimenting at 14-15 S instead of 12 S, the dimer at 20-21 S instead of 17-18 S etc... (as observed by Gottesfeld and Butler in the Mg^{++}-soluble fraction of DNAase II treated chromatin (25). A minimum concentration of 0.3 mM Ca^{++} was necessary to obtain digestion

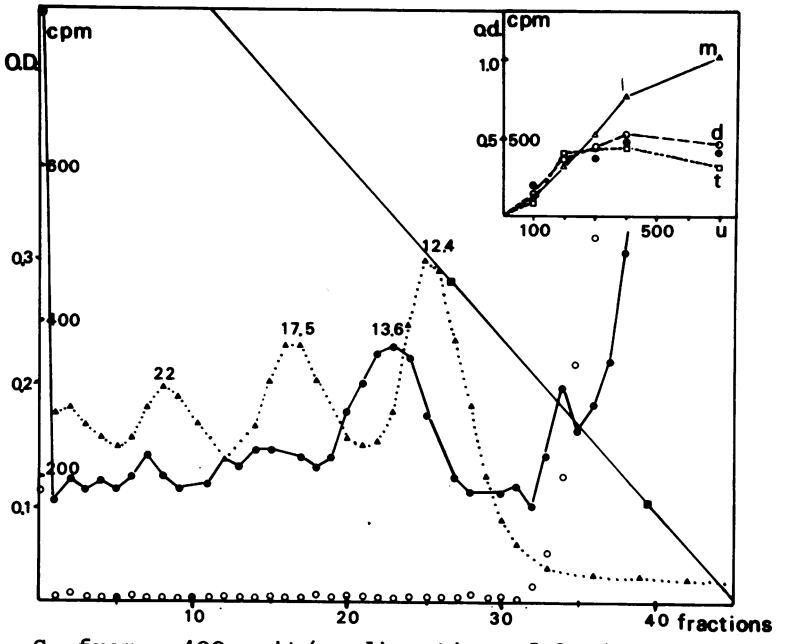


Fig. 2. S_2 from a 400 unit/mg digestion of laying hen oviduct nuclei was incubated 20 h at 0°C + 1 h 30 at 30°C with ^{3}H-estradiol 2 nM in the absence or presence of estradiol 1 µM, and centrifuged 17 h at 29,500 rpm in an SW 41 rotor, on a 5-20 % sucrose gradient. Optical density at 260 nm was read and the entire fraction counted. Numbers at the top of the peaks indicate S values.
Δ---Δ O.D.$_{260\ nm}$; o——o cpm in the absence of non radioactive estradiol ; oo cpm in the presence of estradiol 1 µM ; markers : catalase (11.6 S) and bovine serum albumin (4.2 S).
Inset : equal aliquots of oviduct nuclei were digested with varying concentrations nuclease and centrifuged 17 h 30 at 26,000 rpm in an SW 27 rotor. Open symbols : O.D.$_{260\ nm}$ of monomer (m), dimer (d) and trimer (t). Closed circles : cpm in 13-14 S peak.

of chromatin, however full efficiency of the enzyme was already observed with 0.4 mM Ca^{++}.

Parameters of estradiol binding in oviduct chromatin

Binding of estradiol was measured in chromatin S_2 fraction. A K_D = 1 nM was determined by Scatchard analysis and a number of sites/cell of the order of 2,000 was found. It was remarkable that non-specific binding of estradiol (measured in the presence of estradiol 1 µM) was equal to charcoal background values, in other words no non-specific binding was found in this fraction.

When S_2, previously incubated overnight at 0-2°C with ^{3}H-estradiol 1-10 nM was analyzed by ultracentrifugation on sucrose gradient (5-20 % linear, or 15-27 % convex) or glycerol gradient

(5-35 %), a peak of estradiol binding bearing a sedimentation coefficient slightly heavier (12.7-13.8 S) than the monomer peak was always found (Fig. 2). The same pattern was seen whether the chromatin was maintained at 0-2°C or was incubated 90 min. at 30°C, the only difference residing in the height of the radioactive peak which increased after hormone exchange. It was checked that this peak represented specific binding since it was not found when the chromatin was incubated in the presence of estradiol 1 µM.

The increase in 13-14 S estrogen receptor sites (Fig. 2, inset) did not follow that of the monomer but rather resembled dimer or trimer kinetics, suggesting that the material sedimenting at 13-14 S represented an intermediate and not a final product of digestion such as the monomer. This observation could give some indication as to the location of these receptor sites in the chromatin.

Preliminary data show that the dimer is also accompanied by a peak of slightly heavier receptor sites, which disappears very rapidly when nuclease digestion lasts longer than 20 sec.

When weakly digested chromatin, which contained the usual concentration of receptor (see above), was digested a second time in the conditions of re-digestion, i.e. in the presence of Ca^{++} 3 mM, no estrogen binding sites were found associated with the faster sedimenting portion of the monomer peak. This could be an effect of Ca^{++} ions on the chromatin proteins, since it appeared that the addition of even very small quantities of $CaCl_2$ (0,225 mM) to the chromatin supernatant produced a decrease in the concentration of estradiol binding sites, (as measured by the charcoal-dextran technique), proportional to the concentration of Ca^{++} added and reaching zero binding for 3 mM Ca^{++}. These results may be compared to those of Shyamala-Harris (26), who noticed that the addition of Mg^{++} inhibited the "extraction" of mouse uterine nuclear receptor by DNAase I, and attributed this inhibition to aggregation of the receptor.

In non-target tissues such as the kidney, the 13-14 S peak of radioactivity was not apparent (Fig. 3), even in conditions where the $O.D._{260\ nm}$ profile was similar in shape and size as that of the oviduct. In the laying hen liver (which is a target organ for estrogens (27)) a similar O.D. profile was also observed

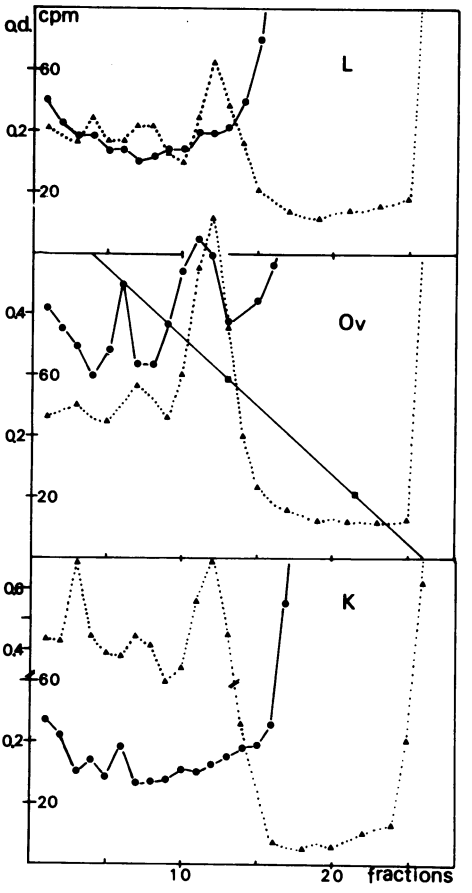


Fig. 3. Liver (L), Oviduct (Ov) and Kidney (K) nuclei from a laying hen were digested with respectively 300,440 and 230 units nuclease/mg DNA, and the S_2 fraction incubated with ^{3}H-estradiol 10 nM and centrifuged 4 h 30 at 58,000 rpm in an SW 60 rotor. Symbols are the same as in Fig. 2.

(Fig. 3) ; there was a small peak of bound estradiol in the 13-14 S position, only 1/10th the size of that seen in the oviduct (110 cpm in the liver vs. 940 cpm in the oviduct), however, for the same O.D. values of the monomer and dimer peaks (not shown)

As can be seen in Table I both oviduct and liver chromatin contain specific estradiol binding sites, although liver somewhat less than oviduct, whereas kidney chromatin does not. No specific binding sites are "solubilized" by enzyme digestion in either of the three organs.

Oviduct S_2 fraction was centrifuged on 5-20 % sucrose gra-

TABLE I - ORGAN SPECIFICITY OF BINDING OF ^{3}H-ESTRADIOL IN CHROMATIN.

Bound dpm*/mg nuclear DNA			
Fraction	Oviduct	Liver	Kidney
Enzyme supernatant	S 764	S 0	S 2165
	NS 2610	NS 2500	NS 2070
Chromatin (low-speed sup.	S + 46,050	S + 15,400	S 0
	NS 3850	NS 2073	NS 1440

S : Bound dpm after incubation of the extract with ^{3}H-estradiol 10 nM.

NS : Bound dpm after incubation with ^{3}H-estradiol 10 nM + estradiol 1 µM.

* as determined by the charcoal-dextran technique.

dients at a high speed (SW 60 rotor, 50,000 rpm, 17 h.) in order to see if any free 4-7 S estrogen receptor could be found in these preparations. Digestion of nuclei whith up to 1100 U nuclease/mg DNA showed only trace amounts of 4-7 S estradiol binding sites.

Filtration of chromatin S_2 fraction on columns containing various types of gels (Sephadex G-25, Biogel P-2, Ultrogel ACA 34 or 44) resulted in aggregation of the estradiol-receptor complex, accompanied by dissociation of the hormone-receptor binding.

Effect of heparin on estradiol-receptor-DNA interactions

The polyanion heparin is known to have a decondensing effect on chromatin (28,29) and has been used to "solubilise" and detach it from the nuclear membrane (30). It also prevents aggregation of proteins such as the estrogen receptors of rat uterus (31) and of chick oviduct (32).

In the preparations of moderately digested chromatin used here, heparin, present in the sucrose layers of the ultracentrifugation gradient, displaced the estrogen binding sites from their 13-14 S position to a 7-8 S position (Fig. 4b).

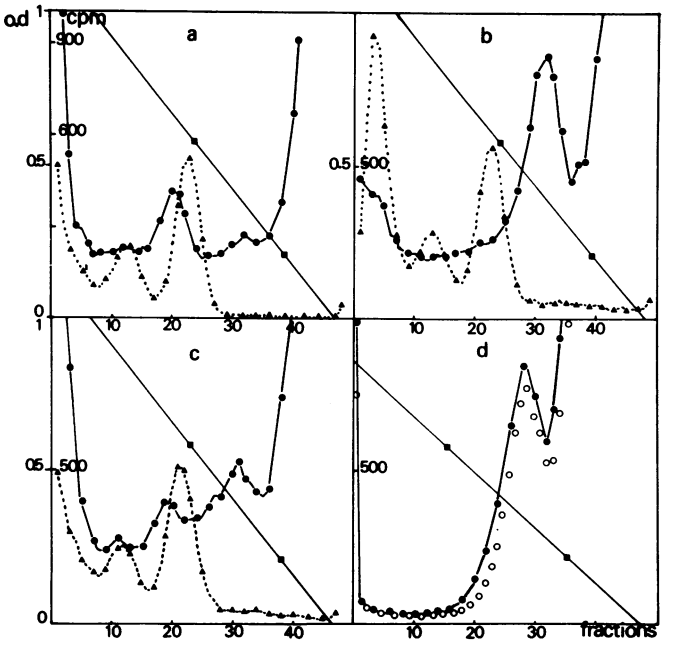


Fig. 4. a-c : nuclei from a laying hen oviduct were digested with 525 U/mg nuclease and the S_2 fraction incubated overnight with ^{3}H-estradiol and b-c : in the presence of 5 µg/ml heparin ; the sucrose gradient in (a) and (c) contained no heparin ; gradient in (b) contained 5 µg/ml heparin. Centrifugation was 33,000 rpm for 17 h in an SW 41 rotor. Symbols are as in Fig. 2. d : the 7-8 S radioactive peak of a gradient similar to (b) was collected and dialysed in the presence of ^{3}H-estradiol 5 nM and rerun for 17 h at 38,000 rpm in an SW 41 rotor. ●——● : cpm, no heparin in the gradient ; oo : cpm, gradient contained 5 µg/ml heparin.

The change observed was a shift of the entire receptor peak from the monomer region to smaller S values ; no radioactivity remained in the 13-14 S region. For a given quantity of chromatin and for a given period of time (the duration of the centrifugation run), this effect of heparin was a function of the heparin concentration in the gradient (Fig. 5) ; in the experiment shown, a minimum concentration of 2.5 µg/ml was necessary to completely "extract" the receptor sites. An optimum concentration of heparin was determined to extract the receptor : 1 µg heparin/ml for 1 $O.D._{260}$ unit.

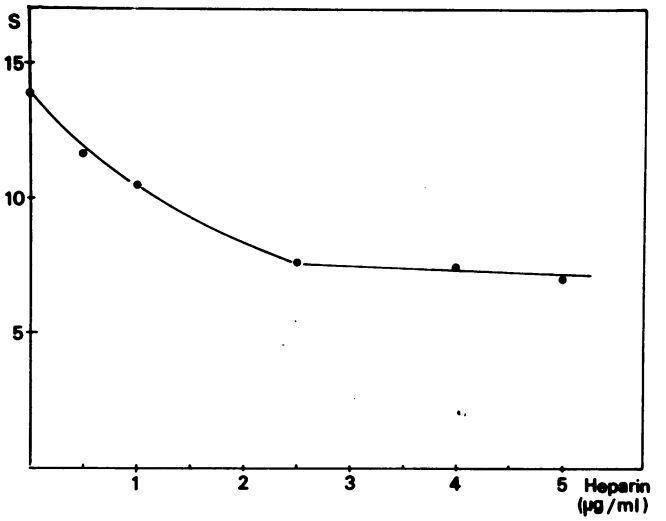


Fig. 5. Sedimentation coefficient (S) of the receptor peak is plotted against the concentration of heparin present in the sucrose gradient.

When the total S_2 fraction, incubated overnight with estradiol-^3H and heparin at the adequate concentration required for receptor extraction, was then centrifuged on a sucrose gradient containing no heparin, partial reversal of the 7-8 S receptor to the 13-14 S position adjacent to the monosome was observed, showing that the latter position was not random (Fig. 4c). In these conditions, part of the extracted receptor also reverted to higher S values, which probably correspond to heavier chromatin fractions of unknown structure. It was checked that 7-8 S receptor, separated by sucrose gradient centrifugation from the other chromatin components and re-run alone on a sucrose gradient in the presence or absence of heparin, retained its 7-8 S sedimentation coefficient (Fig. 4d). Reversal to a higher S value is therefore a process dependent on the presence of other chromatin components and is not due to simple aggregation of the receptor protein.

An experiment was designed to see whether heparin-dissociated receptor returned to its 13-14 S position in the presence of non-target organ chromatin. On one hand weakly digested oviduct chromatin (containing no monomers) was treated with heparin to produce 7-8 S receptor (Fig. 6a), and on the other hand kidney nuclei

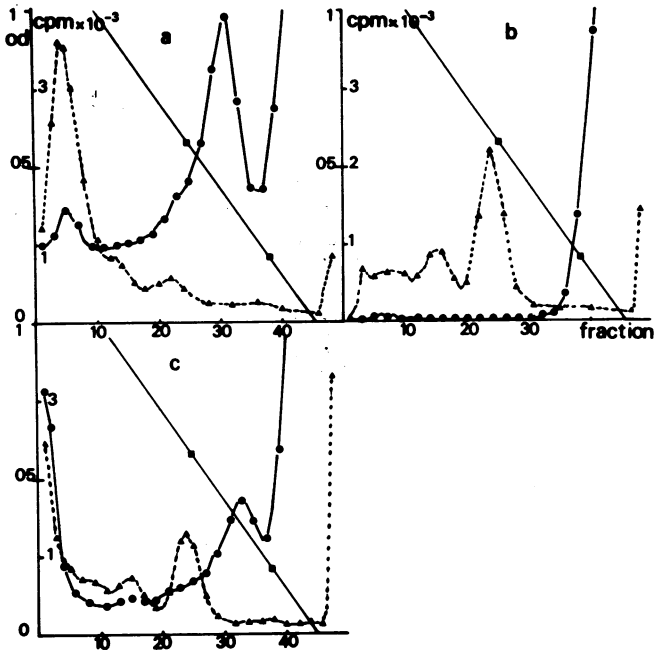


Fig. 6. a) laying hen oviduct nuclei, were digested with 100 U/mg nuclease and S_2 was incubated with ^{3}H-estradiol 5 nM and 10 µg/ml heparin and centrifuged on a sucrose gradient containing 10 µg/ml heparin.
b) kidney nuclei from the same hen were digested with 600 U/mg and the S_2 incubated with ^{3}H-estradiol and 10 µg/ml heparin and centrifuged on a gradient without heparin.
c) 0.5 ml oviduct S_2 (a) + 0.5 ml kidney S_2 (b) were mixed and centrifuged on a sucrose gradient without heparin.

were well digested in order to obtain chromatin containing monomers, dimers and trimers (Fig. 6b). The two extracts were mixed and centrifuged on a sucrose gradient in the absence of heparin, conditions under which, in oviduct chromatin, receptor regains its 13-14 S position (see Fig. 4c). A negligeable amount of oviduct receptor shifted to the 13-14 S position in the presence of kidney chromatin (Fig. 6c), showing that this reversal takes place only in the presence of oviduct chromatin and appears therefore target organ specific. Experiments are under way to find which components are necessary for this reversal and to isolate them, and to check the nature, specificity and affinity of the association which produces this change in receptor sedimentation behaviour.

DISCUSSION

Chromatin from laying hen oviduct was prepared in the very mild conditions described by Hewish and Burgoyne (12). Controlled digestion by micrococcal nuclease was then performed and estrogen binding sites were measured after ultracentrifugation of the chromatin on sucrose gradients. It was found that apart from the estrogen binding sites found associated with unresolved heavy chromatin (> 30 S), a reproducible peak of receptor sites was always found in the 12.7-13.8 S position, slightly heavier than the mononucleosome peak. When following the kinetics of appearance of monomer, dimer or trimer chromatin subfractions and of 13-14 S receptor sites as a function of increasing nuclease concentration, one observed that receptor kinetics follow dimer more closely than monomer kinetics, especially at nuclease concentrations larger than 200 U/mg DNA. With larger quantities of enzyme, monomer concentration increased sharply and 13-14 S receptor-remained at a plateau value. This is compatible with the idea that the receptor is located on a support containing DNA, which has a transient existence, as do nucleosome dimers and trimers, and then is ultimately hydrolysed by the nuclease. This could be internucleosomal DNA, and the 13-14 S receptor could be receptor attached to this piece of DNA as it is still associated with the mononucleosome ; further digestion by higher concentrations of nuclease would destroy this small "tail" of DNA and this could explain the diverging kinetics of appearance of 12 S monomer and of 13-14 S receptor. Similar data obtained for the 17.5 S dimer and the 18.5 S receptor peak are in accordance with this hypothesis.

In the laying hen, the 13-14 S receptor pattern could only be demonstrated in target tissues. Hen liver, which is known to have a fairly large number of nuclear estrogen binding sites (27), showed a small peak of bound ^{3}H-estradiol in the 13-14 S region, while hen kidney showed none.

Estrogen receptor sites could be dissociated from their chromatin "acceptor" by the action of the polyanion heparin. Their sedimentation coefficient would then shift from 13-14 S to 7-8 S. When re-centrifuged alone, the receptor retained its 7-8 S sedimentation coefficient, in the presence or absence of heparin, showing that it did not aggregate in these conditions In the presen-

ce of oviduct chromatin and without heparin the receptor again sedimented in the 13-14 S region, as well as further (> 30 S) in the gradient ; in the presence of kidney chromatin little or no 13-14 receptor was observed, showing that this critical position was target organ specific.

We suggest that chromatin from target organ contains specific sites capable of binding estrogen receptor molecules, that this binding is released by the action of polyanions probably by interaction with the histones and that these sites are in a position exposed to digestion by micrococcal nuclease, and not on the nucleosome core.

Gottesfeld and Butler (25) have recently published a diagram showing that newly transcribed RNA from HeLa cell nuclei digested with micrococcal nuclease, sediments at 13.7 S on sucrose gradient, slightly faster than the mononucleosome, a figure which closely resembles Fig. 2 in this paper. It would be very interesting to demonstrate that the 13-14 S peak does represent a hormone-receptor-newly formed RNA-DNA complex, and experiments are unde way to identify the components of this material and eventually isolate them.

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