A HISTOCHEMICAL APPROACH TO THE STUDY OF HUMAN CHORIONIC GONADOTROPHIN RECEPTORS IN THE RAT TESTIS

By
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ABSTRACT

Standard suspensions of interstitial cells in PBS were exposed to the action of various fixatives, solvents (clearing agents), temperatures and U. V. light, in order to establish the effects of such chemical and physical agents on the HCG receptors. After exposure to the various agents, the interstitial cells were incubated with $[^{125}\text{I}]$HCG for 2 h at 37°C. To check the specificity of the reaction, competitive tests were performed with added excess non-iodinated HCG. Only formaldehyde fixation for short periods of time, preserved satisfactorily the specific binding activity of the receptors. A different degree of thermolability of the receptors was demonstrated, in relation to 37, 45, 54 and 60°C, while freezing in liquid nitrogen had no effect on the receptors binding activity. After the binding reaction, solvents had a significant solubilizing effect on the HCG-receptor complexes. U. V. light had no significant damaging effect on the receptors. The application of the results for a histochemical approach to the study of the HCG receptors is discussed.

The possibility of direct specific binding of gonadotrophins to frozen sections of the rat ovary in experimental conditions approximating to those of the biochemical models has been demonstrated (Midgley 1972, 1973) and that of HCG to frozen sections of rat testis, by our group (Dal Lago et al. 1975). Moreover in order to overcome the complexities of the in vivo experiments,
the method of direct binding of polypeptide hormones to tissue sections of target organs, under strictly controlled conditions, could open new possibilities in the study and analysis of the binding phenomena, from a morphological point of view, not least because it could be applied to sections from human biopsy and surgical specimens.

In biochemical studies, binding of luteinizing hormone (LH) and human chorionic gonadotrophin (HCG) to specific receptors in the cell membrane of testicular interstitial cells, as a first step in the chain of events eventually leading to the secretion of androgens, has recently received substantial evidence (Catt et al. 1972b,c; Catt & Dufau 1973a; De Jong et al. 1974).

Previous morphological attempts of localizing bound gonadotrophins were performed as in vivo experiments after injection of labelled and unlabelled hormones in the live animals (Mancini et al. 1967; De Kretser et al. 1969; Castro et al. 1970, 1972). Unfortunately the interpretation of the in vivo experiments is still subject to the many unknown factors governing the metabolism and fate of the injected hormones and a reasonable doubt still remains whether their subsequent localization is actually the result of a specific binding reaction by receptors in target organs, or merely dependent on other aspecific factors, and also whether some metabolites are actually traced instead of the original hormones.

These considerations prompted us to start a basic research in order to study, in a preliminary way, under what conditions the specific binding activity of polypeptide hormone receptors in target organs may be preserved, in relation to the common techniques at present available to the morphologist at the level of light and electron microscopy.

The results that we have obtained with regard to HCG receptors in the rat interstitial cells are presented in this paper.

**M A T E R I A L S A N D M E T H O D S**

All the experiments were performed at 0–4°C except for the HCG-binding procedure which is specified later.

*Interstitial cells preparation.* – The experiments were conducted in vitro on standard aliquots of suspended interstitial cells prepared according to a modification of a method of Catt et al. (1971, 1972a). Decapsulated testes from adult Wistar rats were immediately placed in an ice-cold Petri dish containing phosphate buffered saline (PBS, Dulbecco “A”, Oxoid Ltd., London), the osmolality of which was 270 mOsm, and thoroughly teased apart by gentle dissection with a needle and a fine tissue forcep. During this procedure a large number of interstitial cells are freed and pass into suspension. With filtration through cotton wool, seminiferous tubules and clumps of interstitial tissue were separated from the interstitial cells suspended in PBS. The filtrate was left overnight to sediment by gravity in one or more test tubes at 0–4°C. Smaller cellular elements, like red cells, cellular fragments and freed nuclei were...
deposited in the upper layers, while larger elements like Leydig cells were found in the lowest layer; this last fraction was used for further processing, after resuspending it in PBS so as to obtain a cellular concentration of 1–2.10^6 elements/mL. Cellular counts were performed in a Bürker counting chamber. With constant stirring, aliquots of 1 ml were then transferred into test tubes 10 x 70 mm and then kept at 0–4°C. When convenient, the procedure could be speeded by centrifuging the filtrate at 50 x g for 5 min in a refrigerated centrifuge, instead of leaving it to sediment spontaneously.

**Fixatives tested.** – The following fixatives, reagent or analytical grade, were tested with regard to their effects on the specific binding activity of HCG receptors.

1) Ethanol (ethyl alcohol) absolute, (C₂H₅OH). 2) Acetone pure (CH₃COCH₃). 3) Osmium tetroxide (OsO₄), sol. 1% in PBS (pH 7.4). 4) Potassium permanganate (KMnO₄), sol. 0.6% in PBS (pH 7.4). 5) Potassium dichromate (K₂Cr₂O₇), sol. 2.5% in distilled water. 6) Picric acid (C₆H₅NO₂OH), sat. sol. in distilled water. 7) Acetic acid (CH₃COOH), sol. 6% in distilled water. 8) Mercuric chloride (HgCl₂), sat. sol. in distilled water. 9) Glutaraldehyde (CHO(CH₂)₆CHO), sol. 3% in PBS (pH 7.4). 10) Glutaraldehyde, sol. 1% in PBS (pH 7.4). 11) Newly formed formaldehyde (HCHO) from paraformaldehyde, sol. 4% in PBS (pH 7.4). 12) Formalin (40% formaldehyde), sol. 10% in PBS (pH 7.4). The concentrations of the fixatives were those commonly used in routine fixation methods.

Two ml of each fixative were added to standard aliquots of interstitial cells reduced to pellet form by centrifugation at 120 x g (10 min) and removal of the supernatant. Fixatives and cells were thoroughly mixed by manual shaking. Exposure to the fixatives was performed at 0–4°C for 15 min and, in the case of formalin, also for 30 min, 1, 2, 3 and 4 h. At the end of fixation the tubes were centrifuged at 120 x g (10 min) and the fixed cells were washed for 60 min with three changes of PBS. Controls were subjected to the same procedure, except for the exposure to the fixatives which were substituted with PBS.

**Solvents (clearing agents) tested.** – The following undiluted solvents (or clearing agents) were tested for a possible disrupting or solubilizing effect on the HCG-receptor complexes formed as a result of a binding reaction: xylene (C₈H₁₀); benzene (C₆H₆); propylene oxide (1–2 epoxypropane, CH₃(CH₂)O); dioxane (C₄H₈O₂).

Standard aliquots of interstitial cells, in pellet form, were incubated with [¹₂⁵I]HCG and washed as later described. The cells were then mixed with 2 ml of each solvent for 2 h at 0–4°C. With differential centrifugation at 120 x g for 10 min and at 50 000 x g for 1 h, three fractions were obtained: fraction (I) formed by intact cells and large cellular fragments, fraction (II) possibly containing smaller cellular particles including microsomes, fraction (III) was consisting of the supernatant to fraction (II) possibly containing very small or solubilized particles not precipitated with the second fraction. Controls were exposed to PBS and fractionated in the same way. Counting in a gamma-spectrometer and assessment of the results were performed as for the other experiments. The effects of solvents were tested on HCG-receptor complexes, instead of on unreacted receptors as in the other experiments, as a matter of practical necessity: in fact the latter procedure would have required, before incubation with [¹₂⁵I]HCG, the washing out of the solvents from the cells with other solvents, like alcohol or acetone, which themselves act as fixatives.

**Temperature effects.** – Standard aliquots of interstitial cells suspended in 1 ml PBS were exposed to the following temperatures: 37, 45, 54 and 60°C, for periods of 15 min, 30 min, 1, 2 and 3 h. Exposure was terminated by adding 2 ml of ice-cold PBS and
centrifugation at 120 x g (10 min) in the refrigerated centrifuge. Controls were kept at 0-4°C throughout.

Additional aliquots of interstitial cells in pellet form were frozen with liquid nitrogen and subsequently thawed to 0-4°C. Three methods of freezing were tested: snap freezing by direct contact of liquid nitrogen and cells in the test tubes; slow freezing by partial immersion of the test tubes in liquid nitrogen in which the cells were frozen through the glass wall; step-wise freezing, progressively for 30 min bringing up the test tubes to the surface of liquid nitrogen inside a Dewar flask, before final immersion.

Ultraviolet irradiation. – Standard aliquots of interstitial cells suspended in PBS were exposed to U. V. light, in small open Petri dishes, at a distance of 2 cm from the U. V. source for 16 h at 0-4°C. A long wave U. V. > 3150 Å was used (Blak-Ray UVL-21 of U.V. Products Inc., San Gabriel, Calif., USA). At the end of exposure, the cells were transferred to the test tubes. Controls were subjected to the same procedure except for exposure to U. V. light.

HCG-receptors binding procedure. – The following hormones were used:

Human chorionic gonadotrophin (HCG), highly purified, with a biological activity of 12 000-18 000 IU/mg, mw ~ 47 000, supplied by Serono Immunochemicals, Rome, Italy. Radioiodinated human chorionic gonadotrophin ([125I]HCG), iodinated with the chloramine-T method according to Greenwood et al. (1963), and resulting specific radioactivity of ~ 100 μCi/μg, supplied by Serono Immunochemicals, Rome, Italy.

To each test tube containing in pellet form, a standard aliquot of treated interstitial cells or untreated controls, 0.25 ml of [125I]HCG (1 ng/ml in PBS, pH 7.4) was added and thoroughly mixed. To determine the degree of specificity of the binding reaction, the competitive test was always performed in parallel tubes, adding an excess of non-iodinated HCG (1 μg/ml final concentration).

All the tubes of a given experiment were then transferred into a thermostatic chamber at 37°C for 2 h. Incubation was terminated by adding 2 ml of ice-cold PBS to each tube, followed by centrifugation in the refrigerated centrifuge and two PBS washings each of 15 min. Bound radioactivity was then measured in a gamma-spectrometer. The results were corrected by subtracting the values of the background and blanks (test tubes without interstitial cells where all the procedures of an experiment were carried out). The data obtained were the result of at least triplicate values and were generally expressed as % bound radioactivity in relation to that assessed for controls, or as specified in the text.

Autoradiography. – In several cases, in order to confirm the effects of the fixatives, autoradiography was performed on liquid nitrogen frozen sections of rat testis, cut in the cryostat at −20°C and at 7 μm thickness. The sections were attached to glass slides, covered with 50 μl of [125I]HCG, 1 ng/ml, and incubated at 37°C for 1 h, followed by two PBS washings each of 5 min. Competitive tests, with excess non-iodinated HCG added, were also performed on the frozen sections. The slides were then coated with liquid nuclear emulsion Kodak NTB-2 and exposed at 0-4°C for 2 weeks.

After development the autoradiography reaction was observed and recorded with an automatic Leitz Dialux Photomicroscope. Analysis of the binding was performed by comparison of grain counts of equal tissue areas in the interstitial tissue and in the seminiferous tubules, after subtraction of background values.
Table 1.
Effects of fixatives on HCG receptors of interstitial cells of rat testis.

<table>
<thead>
<tr>
<th>Fixatives</th>
<th>Total uptake of $[^{125}I]$HCG</th>
<th>Competition test by added excess non-iodinated HCG</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CPM % of controls</td>
<td>CPM % inhibition</td>
</tr>
<tr>
<td>Unfixed controls</td>
<td>3050 ± 181 100 %</td>
<td>320 ± 27 90 %</td>
</tr>
<tr>
<td>Ethyl alcohol absolute</td>
<td>545 ± 71 17 %</td>
<td>560 ± 47 -</td>
</tr>
<tr>
<td>Acetone pure</td>
<td>602 ± 38 19 %</td>
<td>594 ± 55 -</td>
</tr>
<tr>
<td>Osmium tetroxide 1 %</td>
<td>4756 ± 260 155 %</td>
<td>5003 ± 317 -</td>
</tr>
<tr>
<td>Potassium permanganate 0.6 %</td>
<td>8477 ± 432 277 %</td>
<td>8369 ± 390 -</td>
</tr>
<tr>
<td>Potassium dichromate 2.5 %</td>
<td>1025 ± 28 33 %</td>
<td>1180 ± 44 -</td>
</tr>
<tr>
<td>Picric acid sat. sol.</td>
<td>1040 ± 56 34 %</td>
<td>895 ± 38 14 %</td>
</tr>
<tr>
<td>Acetic acid 6 %</td>
<td>765 ± 71 25 %</td>
<td>712 ± 72 7 %</td>
</tr>
<tr>
<td>Mercuric chloride sat. sol.</td>
<td>3338 ± 175 109 %</td>
<td>3198 ± 188 5 %</td>
</tr>
<tr>
<td>Glutaraldehyde 3 %</td>
<td>3250 ± 193 106 %</td>
<td>3272 ± 152 -</td>
</tr>
<tr>
<td>Glutaraldehyde 1 %</td>
<td>3184 ± 218 104 %</td>
<td>3156 ± 217 -</td>
</tr>
<tr>
<td>Formaldehyde 4 % (from paraformaldehyde)</td>
<td>2749 ± 144 90 %</td>
<td>433 ± 38 84 %</td>
</tr>
<tr>
<td>Formalin 10 %</td>
<td>2766 ± 155 90 %</td>
<td>416 ± 46 85 %</td>
</tr>
</tbody>
</table>

Fixation at 0–4°C for 15 min.
Incubation with $[^{125}I]$HCG (1 ng/ml) at 37°C for 2 h.
Competitive tests with added non-iodinated HCG (1 µg/ml).
The results (cpm) are the mean ± sd of at least triplicate values.

RESULTS AND DISCUSSION

Effects of fixatives. – The results are reported in Table 1. With the exception of formalin and newly formed formaldehyde from paraformaldehyde, none of the fixatives tested could satisfactorily preserve the specific binding activity of the interstitial cells receptors for HCG, after the short fixation time (15 min) that was tried.

Specificity of the binding reaction was checked by the competition test, where non-iodinated HCG in excess competed for the binding sites with the $[^{125}I]$HCG. For the unfixed controls we obtained inhibition values of about 90 %, which compares well with the values obtained in the biochemical models (Catt & Dufau 1973a).

Although not immediately relevant to the scope of the present study, of some interest is the wide range of values obtained for the total uptake of radioactivity after the various fixation methods: osmium tetroxide and potassium perman-
ganate, the oxidative action of which especially on the cell membrane is well known, increase very markedly the total uptake of $^{125}\text{I}]\text{HCG}$, while ethanol and acetone, which act mainly as denaturing and precipitating agents for proteins, very considerably decrease such uptake. Decreased uptake of $^{125}\text{I}]\text{HCG}$ was also observed with potassium dichromate, picric acid and acetic acid, while only very slight increases were observed with glutaraldehyde and mercuric chloride fixation.

The comparison of the results after fixation with glutaraldehyde and formaldehyde, mono and bi-functional aldehydes in their monomeric form, might offer some interesting insight into their respective mechanism of action on the HCG receptors: in fact while glutaraldehyde, even at 1% concentration, blocks any specific uptake of $^{125}\text{I}]\text{HCG}$, formaldehyde preserved 84–85% of the specificity of the receptor-hormone binding reaction.

Recent evidence (Dufau et al. 1973; Lee & Ryan 1973), as well as confirming the location of the HCG and LH receptors in the membranes of the target cells in the testis and ovary, has strongly emphasized that the receptors are composed of an essential protein part with an associated and functionally important phospholipid component. Dufau et al. (1973) succeeded in solubilizing the HCG testis receptors with a detergent (Triton X-100) with full retention of their specific binding activity; the molecular weight of such solubilized receptors has been calculated i.e. about 194,000, but their composition and structure are still unknown.

In the context of the present knowledge, we could tentatively postulate that glutaraldehyde, because of its steric configuration and bi-functional reacting groups, very readily cross-links the various protein end-groups with which it is known to react. Formaldehyde, on the other hand, has to react with other compounds containing an active hydrogen atom before forming the cross-linking methylene bridges between protein end-groups, and is much slower in its action (Pearse 1968; Hopwood 1973). Thus, in our experimental conditions, there was probably not sufficient time allowed for formaldehyde blocking the receptors’ end-groups responsible for the specific binding reaction with the hormone.

In a separate experiment the action of formaldehyde on the HCG receptors has been tested for longer periods of time in order to test the possibility that, with longer fixation times, specificity of the binding reaction would decrease. Fig. 1 shows the results obtained after various formaldehyde fixation times, up to 4 h at 0–4°C. It should be noted that while the specific uptake, corresponding to the difference between total uptake and uptake after specific inhibition, remained practically constant in the fixation times tested, the total uptake increased as a function of time. The hypothesis might be put forward that the specific receptors’ end-groups remained unaffected by formaldehyde, at least in the present experimental conditions, but that at the same time modifi-
Fig. 1.
Time dependent effects of formalin fixation on the $[^{125}]$HCG uptake by interstitial cells of rat testis. Each point is the mean of at least triplicate values. The s.d is also indicated.

cations were elicited by formaldehyde on other cellular components which became apt to bind non-specifically the iodinated HCG. Selective blocking or modifications of the various protein end-groups by known histochemical methods (Pearse 1968; Culling 1974) might provide further insight into the action of the fixatives, particularly formaldehyde, on the HCG receptors, and offer some clues as to which end-groups are involved in the specific binding reaction.

Autoradiography on frozen sections was performed, in the course of the present research, to check the results of the binding experiments; fixation with the various fixatives was performed, either before freezing on small pieces of testis tissue for short periods of time (30 min to 2 h), followed by overnight washing in PBS, or directly on the sections for even shorter periods of time (5–10 min), followed by three washes in PBS (15 min each). Incubation with the hormone was then performed as described in Methods. The autoradiographic results in all cases confirmed what was anticipated by the experiments on the PBS-suspended interstitial cells. In Fig. 2 A-B, the autoradiographic results after fixation with formaldehyde and glutaraldehyde are illustrated,
Effects of solvents (clearing agents) on HCG-receptor complexes.

<table>
<thead>
<tr>
<th>Solvents</th>
<th>Total bound radioactivity (cpm)</th>
<th>Fraction I (120 × g) (cpm)</th>
<th>Fraction II (8 × 1000 × g) (cpm)</th>
<th>Fraction III supernatant to fraction II (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>2418 ± 121</td>
<td>2202 ± 108</td>
<td>84 ± 11</td>
<td>75 ± 16</td>
</tr>
<tr>
<td>Xylene</td>
<td>1791 ± 87</td>
<td>30 ± 13</td>
<td>624 ± 40</td>
<td></td>
</tr>
<tr>
<td>Benzene</td>
<td>1893 ± 94</td>
<td>31 ± 7</td>
<td>607 ± 18</td>
<td></td>
</tr>
<tr>
<td>Propylene oxide</td>
<td>1690 ± 90</td>
<td>30 ± 4</td>
<td>688 ± 56</td>
<td></td>
</tr>
<tr>
<td>Dioxane</td>
<td>1776 ± 53</td>
<td>38 ± 7</td>
<td>647 ± 32</td>
<td></td>
</tr>
</tbody>
</table>

Exposure of interstitial cells to solvents for 2 h at 0–4°C.
Three fractions obtained with centrifugation at: (I) 120 × g, (II) 50 000 × g and (III) supernatant to fraction (II). Incubation with \(^{125}\)I-HCG at 37°C for 2 h. The results are the mean ± sd of at least triplicate values.

For comparison between the highly selective localization of the autoradiographic reaction to the interstitial cells after formaldehyde fixation, and the wide non-specific distribution of the same reaction to both interstitial tissue and seminiferous tubules after glutaraldehyde fixation.

Effects of solvents. – The results obtained are summarized in Table 2. It is evident that, while the PBS-treated controls underwent a very slight solubilizing action in fraction (III) and a rather modest disrupting effect in fraction (II), the solvent-treated cells showed a marked solubilizing action on fraction (III), from about 24% in the benzene-treated cells to about 28% in the propylene-oxide-treated cells. The disrupting effect of solvents, represented by the results obtained with fraction (II), is on the other hand practically nil. It is to be noted in this regard that no visible pellet was obtained at 50 000 × g with the solvent-treated cells. We could presume that at higher

Fig. 2 A-B.
Autoradiographic results after incubation with \(^{125}\)I-HCG (1 ng/ml) at 37°C for 1 h, of frozen sections of rat testis.
A) After 10% formalin fixation (10 min at 0–4°C) × 800.
B) After 3% glutaraldehyde fixation (10 min at 0–4°C) × 640.
The specific autoradiographic reaction localized to interstitial cells in (A) and the scattered, non-specific one distributed to both interstitial tissue and seminiferous tubules in (B) are evident.
temperatures, like those routinely used for paraffin or plastic embedding, the solubilizing effect of solvents would be greater, with a severe loss and/or diffusion of the bound radioactivity.

Effects of temperature. – The temperatures tested were chosen on the basis of their interest in some of the common morphological techniques of processing tissue or sections.

The results obtained are summarized in Fig. 3. It should be noted that even at 37°C there is a significant decrease in the uptake of [125I]HCG, which is more rapid during the first 60 min of exposure. Similar negative effects in long time exposure to 37°C were observed in homogenates of testis incubated with [125I]HCG and attributed to possible degradation of the labelled hormone (Catt et al. 1972b). In our experimental conditions such degradation should be attributed, at least in part, to degradation of the receptors themselves.

At 45°C the uptake of the [125I]HCG fell to less than 10% of the initial value after 3 h, and this fact should be taken into account when planning to include tissue in plastics requiring such temperatures for polymerization.

The exposure of receptors to 54°C was tested in relation to the possible use of paraffin wax inclusion. Inhibition of the [125I]HCG uptake was almost complete after 90 min, but at 15 min more than 50% was still present. This could be considered of some importance for the freeze-drying methods followed

![Fig. 3.](image)

Time dependent effects of various temperatures on [125I]HCG uptake by interstitial cells of rat testis. Each point is the mean ± sd of at least triplicate values.
by rapid (5 to 10 min) inclusion in paraffin wax of suitable melting point under vacuum conditions (Pearse 1968).

A temperature of 160°C rapidly inhibits the activities of the receptors and is obviously completely inadequate.

As for the freezing methods that were tested with regard to their possible effect on the HCG receptors activity, the results that we obtained indicate that no significant loss of the specific binding activity for $^{[125I]}$HCG occurred in comparison with the controls kept at 0–4°C.

**U. V. light effects.** – Ultraviolet irradiation of interstitial cells was performed in order to ascertain whether this form of energy had any damaging or modifying effect on the HCG receptors, in view of the possible use of U. V. light as a polymerizing agent at low temperatures for water soluble plastics, like glycol methacrylate (Rosenberg et al. 1960; Leduc & Bernhard 1967).

After 16 h exposure at 0–4°C, about 90% of the initial specific binding activity was preserved and this value was not significantly different from that found for controls. Embedding in water soluble plastics at low temperatures could therefore be a promising method for preserving the specific binding activity of the receptors.

**CONCLUDING REMARKS**

The results obtained in the present study indicate that, within restricted limits, a histochemical approach to the study of HCG receptors is possible.

Cryotechniques, at light and electron microscopy level (Burstone 1969; Rebhun 1972), seem to be the first choice, as freezing does not affect the specific binding activity of receptors. Fixation, on the other hand, appears to be a very limiting factor and only a mild fixation with formaldehyde can be envisaged; this however could be very important even in relation to the cryotechniques, to stabilize the structures and prevent possible diffusion artifacts.

The use of aqueous-soluble embedding media (Hayat 1970) that can be polymerized by U. V. light at low temperatures, seems to deserve special interest. Their use is being attempted in our laboratory.

Autoradiography at light microscopy level has been used so far by us as a detector of the hormone-receptor binding reaction. In our opinion a quantitative approach to the study of the binding phenomena in target organs with this technique will be possible under strictly standardized conditions. At electron microscopy level autoradiography could be an important tool in solving some problems of localization at subcellular level.

Other detecting techniques, in which non-radioiodinated hormones are used in the binding reaction, could also find a useful application.

Immunofluorescence has so far scarcely been used to localize hormones in target organs (Nairn 1969), probably because the level of such hormones in these organs it too low in relation to the degree of sensitivity of the method. Saturation of all
hormone binding-sites during the binding reaction (Catt & Dufau 1973b) might provide an answer to this problem.

Immunocytochemistry, in which is use made of some enzymes, especially peroxidase as markers of antibodies (Nakane & Pierce 1967; Avrameas 1968) has recently been substantially improved and has reached a hitherto unsurpassed degree of specificity and sensitivity, at electron microscopy level, in the localization of hormones at the site of origin (Sternberger 1973). Its use as a detecting technique for the hormone-receptor binding reaction in target organs certainly deserves serious attention and might prove to be one of the most promising applications for future work.

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