14. Estrogen receptor: inhibition of aggregation and dissociation into subunits by chemical perturbation,

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A generally recognized problem in dealing with estrogen receptors, and with steroid hormone receptors at large, is the high tendency of these proteins to aggregate following tissue homogenization. By means of chemical perturbation, we have now succeeded in inhibiting such aggregation and even favor dissociation of receptor into subunits, as shown by (1) absence of large aggregates and indefinite inhibition of their formation in crude uterine low-salt cytosol; no aggregates form even during application of concentration procedures like salting out by $(NH_4)_2SO_4$; (2) sharp peaks of estrogen receptor activity on Sephadex chromatography of crude uterine cytosol in low-salt; the peaks are "included" into Sephadex G-100; (3) appearance of a smaller estrogen binding unit with the following properties: Svedbergs, 2·8; Stokes radius, 28 Å; mol. wt., 32,000; frictional ratio, 1·44. Past and present data are compatible with the hypothesis that the 32,000 mol. wt. species is a subunit which may self-associate to form larger structures. This specific self-association is distinct from aggregation of receptor with other macromolecules of cytosol, a process which is started by homogenization of the tissue and progresses with aging of cytosol. Inhibion of aggregation by chemical perturbation removes an important obstacle in the way of purification of receptor in tangible amounts. (Research supported by the Consiglio Nazionale delle Ricerche, Rome.)

15. Analytical study of gluco- and mineralocorticoid receptors in rat kidney. Dramatic protective effect of ligand on mineralocorticoid receptors,

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Rat kidney cytosol contains both gluco (GC) and mineralo (MC) corticoid receptors labelled respectively by $[^3H]$-dexamethasone (^3H-DM) and $[^3H]$-aldosterone (^3H-A). The purpose of this work was to compare the stability of these receptors in presence or in absence of their ligand. Three different experimental procedures were used: (a) Kidney slices experiments: kidney slices from adrenalectomized rats were incubated 20 min at 20° with ^3H-A or ^3H-DM in presence or in absence of competitors. Slices were homogenized at 4° in 0·25 M sucrose, 1 mM $MgCl_2$ and the cytosol was then prepared. (b) Delayed *in vitro* incubation: kidneys were homogenized in 0·01 M Tris–HCl, 1 mM EDTA, pH 7·4, containing 10% glycerol. The homogenate was centrifuged at $700\,g \times 10$ min and the supernatant centrifuged at $105000\,g \times 1$ h was used for the binding studies. (c) Direct *in vitro* incubation (protected cytosol): the homogenate was centrifuged at $700g \times 10$ min and the supernatant was immediately incubated with ^3H-DM or ^3H-A. Cytosol was then prepared as above. Correction for unspecific binding was systematically performed by adding a 1000-fold excess of non-radioactive steroid. When kidney slices were incubated with 5×10^{-10}M ^3H-A. A inhibited the binding of ^3H-A to its cytosol receptor 8 times more effectively than DM. However, with receptors recovered in the cytosol fraction, A was 2 times less effective than DM in competing for ^3H-A binding sites. This was confirmed by a series of experiments: (1) when cytosol receptors were protected during preparation by adding steroids after homogenization, A was again the best competitor for ^3H-A binding, (2) stability studies showed that after 1 h at 0° unfilled MC receptors were completely lost whereas 50% of the unfilled GC receptors were still present after 5 h. About 30% of A-filled receptors could be detected after 10 h at 0° whereas 80% of DM-filled receptors were recovered, (3) Scatchard plot of ^3H-A showed two slopes on protected cytosol, the steeper slop ($Kd_1 = 6 \times 10^{-9}$M) corresponding to the MC receptor. In unprotected cytosol only the lower slope corresponding to GC receptors ($Kd_2 = 8 \times 10^{-8}$M) was detected. In conclusion: (1) GC and MC binding sites are rapidly destroyed in kidney cytosol unless they are protected by their ligand, (2) GC sites are much more stable than MC sites, (3) it is impossible to study MC receptors in complete *in vitro* experiments in kidney cytosol without protecting them after homogenization.

16. Androgen receptor assay with a specific ligand, $[^3H]$-methyltrienolone

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A new methodology for the measurement of androgen binding sites has been developed on animal models with a view to its subsequent application to normal and pathological human tissues. This methodology meets two basic requirements: firstly, the need to distinguish between tissue and plasma binding and secondly, the need to take binding of the endogenous hormone into account. Although binding to the intracellular receptor and to sex steroid plasma binding protein (SBP) may be distinguished by separating the proteins, by agar-gel electrophoresis for instance, the number of binding sites involved can only be estimated with difficulty by these techniques. The use of the synthetic androgen, methyltrienolone (R 1881), labelled with high specific activity (58·2 Ci/mmol), as a radioligand, in a Dextran-coated charcoal method, precludes the necessity of protein separation since this compound is not bound by human plasma (i.e. SBP) but is bound with a higher affinity than androstanolone (DHT) by intracellular receptors ($1/Ka_{R\,1881} = 0{\cdot}6 \pm 0{\cdot}1$ nM, $1/Ka_{DHT} = 1{\cdot}4 \pm 0{\cdot}2$ nM on rat prostate cytosol). The total number of binding sites is determined by exchanging the bound endogenous hormone with radioligand under conditions where the natural hormone–receptor complex is dissociated, but where neither loss of binding sites nor ligand metabolism occur. These conditions are fulfilled by the use of tritiated methyltrienolone since, unlike androstanolone, methyltrienolone is not metabolized by the 3-ketosteroid hydrogenases in cytosol.

17. A comparative study of the biological activity and affinity to cytosol receptor of rabbit uterus of optical, structural and isomeric analogs of estradiol,

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Competitive binding between analogs of estradiol with estradiol receptors of cytosol from rabbit uterus has been studied. The following compounds have been used: D-estradiol-17β and its optical isomers (D,L-,L-estradiol-17β); D-3-deoxyestradiol-17β; D-17-deoxyestradiol; D-3,17-bis-deoxyestradiol; D,L-8-isoestradiol-17β; D,L-D-homoestradiol-17αβ; D,L-3-deoxy-D-homoestradiol-17αβ; D,L-8-iso-D-homoestradiol-17αβ; D-estrone. The Allen–Doisy assay

was employed to determine the biological activity of these compounds. It is established that, for the bonding with the receptor to be effective the steric agreement is indispensable between the steroid molecule and the binding site of the receptor. L-estradiol-17β which has the same 3–17 distance as natural estradiol, but an opposite configuration of the asymmetric centres, was found to have a low affinity for the receptor. The affinity of D,L-estradiol-17β (racemic mixture of D- and L-estradiol-17β) is only 50%. The ability of the optical isomers to bind with the receptor correlates with their biological activity. Alternation in the configuration of asymmetric centre in the molecule of estradiol decreases the binding and the biological activity. The binding and biological activity also decrease if the D-ring becomes six-membered. It is shown that removal of any of the hydroxyl groups decreases the affinity for the cyto-receptor and the estrogenic activity. The binding disappears if both hydroxyl groups are removed, indicating that 3–17 hydroxyl groups are indispensable for the specific binding with the receptor and manifestation of the biological activity. The concept is put forward that the 3–17 distance and the conformation of substituents in the molecule of estrogen is of major importance for the specific binding with the rabbit cytosol receptor to take place and for the biological activity to be displayed.

E. **Critical evaluation of receptor assays in relation to tumors,** R. K. WAGNER, Max-Planck-Institut für Zellbiologie, D 294 Wilhelmshaven, Postfach 1009, Germany

The assessment of steroid hormone receptors in tumors deriving from secondary sexual organs is considered as a useful indicator for hormone dependency. Numerous attempts were made to develop methods which accurately measure receptor concentrations in such target tissues. Since human tumors in particular are available only in small quantities and are also rich in connective tissue, the preparation of sufficient amounts of clean and intact nuclei for the determination of specific nuclear steroid-binding sites [1] or even for the extraction of nuclear receptor is generally not practicable. Therefore the evaluation of receptors in tumors is usually restricted to the measurement of cytoplasmic receptors, which are readily extractable both from fresh and frozen tissue.

The assay of the cytoplasmic receptors can be markedly influenced by inaccuracies arising from tissue processing and storage, homogenization, assay procedures etc., in addition to unavoidable intrinsic disturbances, such as the level of endogenous steroids and the degree of serum contamination. The latter two factors in particular require the application of very sensitive methods with a high degree of resolution.

Cytoplasmic receptor concentrations are inversely related to their respective steroid hormone blood levels [3]. It is therefore a general experience that the cytoplasmic receptor capacity of target tissues during maturity is much lower than before puberty and after the menopause. The loss of cytoplasmic receptor caused by endogenous steroids is irrecoverable, since the steroid–receptor-complexes formed are very quickly removed from the cytoplasm. The assay of cytoplasmic receptor is therefore entirely dependent on the remaining unoccupied "spare" receptor and all attempts to enhance the assay sensitivity by exchange experiments with excess amounts of labelled steroid (at elevated temperatures) will be more or less ineffective. Consequently, the extremely small receptor levels found e.g. in mammary carcinomas of pre-menopausal women and in prostate carcinomas of men – where a high androgen production persists into senility – can still indicate a high hormone dependency. This phenomenon complicates the handling of "borderline cases" and might explain the responsiveness of certain "receptor negative" tumors to endocrine treatment.

Apart from the difficulties due to high levels of endogenous steroids, the receptor assay can be substantially disturbed by the contamination of the target tissue extracts with plasma or lymph proteins. The albumin concentration measured by immunodiffusion e.g. in extracts of human mammary carcinomas was on average 26% of the total protein content corresponding to an even higher plasma protein contamination. Target tissue extracts contain besides the high capacity – low affinity – binders albumin and α_1-acid globulin (AAG) high affinity steroid binding proteins like the corticosteroid binding globulin (CBG), the sex hormone binding globulin (SHBG) etc. (see Table). Characteristic species differences are seen in the occurrence of these specific plasma proteins. In rat and mouse plasma for example, SHBG is not present, but in these species another distinct extracellular androgen binding protein (ABP) was recently detected in epididymal extracts [2]. Since all these specific plasma proteins bind steroids with similar affinity and specificity, receptor assays like the charcoal adsorption procedure or Sephadex chromatography (G 100 and lower), which simply separate firmly bound from weakly bound and excess free hormone, can (disregarding exceptional cases) no longer be considered appropriate for an accurate receptor measurement. Consequently, methods should be employed, which not only separate steroid receptor complexes from excess free hormone, but also distinguish the intracellular receptors from the interfering extracellular steroid binding proteins.

Of the most frequently used procedures, density gradient (DG) centrifugation and gel exclusion chromatography (Sephadex >100, Sepharose 6B) take advantage of differences in molecular weights and shapes. Electrophoretic (EP) techniques rely on characteristic mobilities. Compared to DG-analysis and gel-exclusion methods, EP-procedures (agar- and polyacrylamide electrophoresis) are easier, much quicker and can analyze more samples simultaneously. Agar electrophoresis at low temperature, which operates with gels of large pore size, is already a

Protein	Mol. Wt.	Mobility	Steroids bound
Albumin	69,000		estradiol > progesterone > testosterone
AAG	41,000	α_1	progesterone > testosterone
CBG	50,000	α_1	cortisol = progesterone >> testosterone
SHBG	100,000	β	dihydrotestosterone > testosterone > estradiol
AFP	65,000	α	estradiol = estrone
ABP	60, – 70,000	α	dihydrotestosterone > testosterone
Steroid binding Immunoglobulins	160,000	γ	

AAG = α_1-acid globulin; CBG = corticosteroid binding globulin; SHBG = sex hormine binding globulin; AFP = α-fetoprotein; ABP = androgen binding protein (rat, epididymis).