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).

well established method for the assessment of receptor levels in many different target tissues on a routine basis [4]. The application of polyacrylamide-EP still seems to be in an early stage, although this technique is superior with respect to protein resolution. But, while the steroid-binding plasma proteins easily enter the small pores of the gel matrix, the very sensitive receptors tend to aggregate and to denature during this process.

The applicability of sedimentation velocity and gel exclusion procedures is in general limited to the assay of large (8–10S) cytoplasmic receptors. Difficulties arise when extracts contain a mixture of the large (8–10S) and small (3–5S) receptor forms, or exclusively the latter, since the molecular weights of the majority of the steroid binding proteins approximate that of the 3–5S receptor. All forms of the soluble cytoplasmic receptors have a similar net charge and behave electrophoretically as α-globulins, therefore it is possible to distinguish them from interfering contaminants of different mobility. A good example is the separation of androgen receptor and SHBG (β-globulin) by agar electrophoresis [4].

If the affinities for the specific hormone, molecular weights and electrophoretic mobilities of receptors and contaminating plasma proteins are similar – as in the case of CBG and the receptors for progesterone and cortisol – special measures are required.

1. The interference of the contaminant can be abolished by an excess of cold steroid which selectively binds to the plasma protein (e.g. CBG-cold cortisol–receptor-labelled progesterone).
2. Labelled synthetic steroids which bind exclusively to the receptors can be used (e.g. R 5020, D-norgestrel or cyproterone-acetate for gestagen receptor; dexamethasone for corticosteroid receptor).
3. A third possibility – which is only applicable to electrophoresis – consists in a modification of the interfering plasma protein by desialication. Mild neuraminidase treatment of SHBG and CBG considerably decreases their electrophoretic mobility to allow for a distinction from hormone receptor complexes [5].

Basically, by these three approaches all problems arising from the plasma contamination, which impairs an accurate measurement of cytoplasmic receptors in target tissue extracts, can be solved. This will in future allow an expansion of the scope of receptor estimations beyond the range of the estradiol receptor and should lead to a better characterization of tumors deriving from target tissues.

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F. Receptor proteins in tumours, N. BRUCHOVSKY, Department of Medicine, University of Alberta, Edmonton, Alberta, Canada

The growth response of an endocrine target organ such as prostate is divided into three phases, each of which may be influenced directly or indirectly by receptor proteins for steroid-hormones. First, the phase of initiation is induced by the administration of hormone if the number of cells in the organ is below normal; this phase is characterized by a marked stimulation of DNA synthesis and cell proliferation. Second, the phase of negative feedback occurs when the number of cells is restored to normal; DNA synthesis is curtailed and cell proliferation is markedly reduced despite the excess of hormone in the animal. Third, the phase of autophagia commences after hormone is withdrawn, and is distinguished by extensive autolysis of cells and loss of functional performance. It is postulated that each phase is under the control of a separate homeostatic constraint mechanism, and that each mechanism must be intact if growth and maintenance of an endocrine target organ are to remain normal.

Homeostatic constraint mechanisms are probably regulated from within the nucleus by the interaction of steroid-hormone with chromatin, and the production of appropriate controlling signals most likely in the form of messenger RNA. Within certain limits the magnitude of the growth response of a target organ is proportional to the intranuclear concentration of steroid-hormone; it follows that any process which alters the concentration or distribution of steroid-hormone within the nucleus may represent an important focal point of control of organ growth. In this respect cytoplasmic enzymes which metabolize steroid-hormones can raise or lower the concentration of active steroidal compounds in the cell and indirectly affect the amount of active form transported into the nucleus.

The observation that the transfer of steroid-hormone into the nucleus appears to depend on the formation of steroid–receptor complexes in cytoplasm suggests a second way in which the intranuclear concentration of steroid-hormone is regulated. A comparative study of incorporation of testosterone into cells of nine variant lines of the androgen-dependent Shionogi mouse mammary carcinoma has furnished evidence showing that the ability to transport androgens into the nucleus is virtually restricted to lines which possess cytoplasmic receptors; it is concluded that such receptors may regulate the intranuclear concentration of androgens either by acting as carrier molecules or by altering the permeability of the nuclear membrane to enhance the passage of steroids into the nucleus.

Five lines of Shionogi mouse mammary carcinoma are characterized by an absence of cytoplasmic receptor and by an inability to transport testosterone and dihydrotestosterone into the nucleus. Possibly because of these deficiences, growth of the cell lines is not subject to the usual homeostatic constraints, and fluctuations in the concentration of androgen have no effect on cell proliferation. In contrast, two other unresponsive lines of Shionogi mouse mammary carcinoma are characterized by the presence of cytoplasmic receptors and the ability to transfer androgens into the nucleus. Binding of androgens to chromatin is normal and therefore it seems that in these two lines the site on chromatin which controls the initiation phase is bypassed and the cell is permanently switched on to initiate DNA synthesis and cell proliferation.

All tumour lines whether responsive or unresponsive lack the competence to form nuclear receptor. Failure to generate the latter molecule may explain why negative feedback is not evident and cell proliferation continues even in those cell lines which otherwise are dependent on androgens for growth. However, sequential analysis of the concentration of cytoplasmic receptor and nuclear receptor during phases of prostatic involution and growth shows that changes in rates of DNA synthesis and cell proliferation are not attended by coincident changes in receptor concentrations. These observations imply that neither cytoplasmic receptor nor nuclear receptor is directly responsible for switching DNA synthesis and cell proliferation on and off. Alternatively, it remains possible that steroid-hormone alone is required for the initiation

of DNA synthesis and cell proliferation and that the function of nuclear receptor is concerned only with the regulation of the autophagic process. The latter view receives inferential support from observations on the growth behavior of human mammary and prostatic cancers. For example, whereas one tumour may display hormonal dependence, manifested as tumour regression induced by endocrine ablative therapy, another tumour may display hormonal responsiveness, manifested as tumour regression induced by endocrine additive therapy. However, since neither dependence nor responsiveness of mammary tumours is expressed in the absence of receptor proteins for steroid-hormones, it is likely that in both cases tumour regression hinges on the function of a common receptor-dependent mechanism.

With the present level of understanding, the control of proliferative growth by steroid-hormone is best visualized in terms of a model consisting of three regulatory elements assumed to be components of the cellular genome. It is proposed that, first, an initiator gene is responsible for switching on DNA synthesis and cell proliferation in the presence of an adequate concentration of steroid-hormone; second, a nullifier gene is responsible for switching off DNA synthesis and cell proliferation when the organ reaches a normal size, and accounts for negative feedback; and third, an autophage gene programs a cell for its own eventual destruction by capacitating the autophagic mechanism, perhaps through the formation of an inactive steroid–receptor complex. A fall in the concentration of hormone below levels required for the maintenance of a differentiated cell stimulates autolysis and removal of cells; it is conceivable that this effect depends on the function of a receptor molecule while is transformed from an inactive to an active state by declining hormonal concentration.

Whether endocrine therapy will result in carcinostatic or carcinocidal effects can probably be predicted by determining both the concentration of steroid-hormone and of receptor protein within the nucleus of the tumour cell. However, even on this basis some autonomous tumours will be indistinguishable from dependent tumours, and to improve the response rate to therapy, other methods which do not rely on either steroid-hormone or receptor measurements are needed to identify such resistant tumours.

A large number of autonomous tumours do not contain cytoplasmic receptor proteins and fail to transport steroid-hormones across the nuclear membrane. In respect to this group of neoplasms, a matter of practical importance that requires clarification is whether there is any potential for controlling cell proliferation with methods that would promote the entry of steroid-hormones into the nucleus of the autonomous cell to activate homeostatic processes such as those which suppress DNA synthesis or stimulate cellular autolysis. Clinical experience derived from the treatment of human mammary and prostatic cancers suggests that in selected cases the application of high doses of steroid may be sufficient for this purpose, but compounds which increase the permeability of the nuclear membrane to the passage of steroid-hormones should be sought.

In summary, growth of a normal hormone responsive organ appears to be ordered by the function of three constraint mechanisms which are sensitive to the intranuclear concentration of steroid-hormone. For the complete expression of these constraint mechanisms several properties underlying hormonal responsiveness must be manifested by the cell, including the presence of cytoplasmic receptor, the ability to transfer steroid-hormone into the nucleus, the competence to form nuclear receptor, and the fidelity of the interaction between steroid-hormone and chromatin.

Cytoplasmic receptor is not an exclusive indication of hormonal dependence or hormonal responsiveness *in vivo*, but its presence is associated with enhanced ability of the cell to incorporate steroid-hormone into the nucleus. Steroid-hormone is required for the initiation of DNA synthesis and cell proliferation, and nuclear receptor may not be required for these responses. On the other hand, it is possible that the function of the latter molecule is concerned with negative feedback or cellular autolysis.

18. Hormone-responsive mammary tumours in GR-mice
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Continuous treatment of castrated female GR-mice with estrone and progesterone leads to the appearance of mammary tumours within 3–4 months. Such tumours usually are hormone dependent, i.e. they are only transplantable in castrated mice if these animals are given estrone and progesterone. During serial transplantations in hormone-treated animals the tumours progressively lose their responsiveness towards estone and progesterone, and finally they become autonomous. We have obtained evidence from estrogen receptor assays that the hormone-responsive mammary tumours are mixed populations of hormone-dependent cells (which contain estrogen receptor) and autonomous cells (which are practically devoid of estrogen receptor). The hormone-dependent tumour cells do not multiply in the absence of estrone and progesterone, but the autonomous cells multiply in the absence of these hormones. The finding that the mammary tumours lose their hormone responsiveness after repeated serial transplantations appears to be due to the faster multiplication of the autonomous cells in the tumour as compared to the hormone-dependent cells. We have extended these studies to characterize these two types of mammary tumour cells in more detail. The investigations include comparative morphological studies of hormone-responsive and autonomous tumours, assays of various steroid receptors, assays of virions and antigens of the mammary tumour virus, and assays of peroxidase in the tumour cells. A pilot study has been started to determine whether the GR-mouse system can be used as a model to investigate optimal conditions for combined hormonal and chemotherapy.

19. Growth pattern and estrogen receptor levels of dimethylbenzanthracene-induced tumor during pregnancy and lactation,
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Hormonal influences on tumor growth and estrogen receptor (ER) in breast cancer of rats induced by dimethylbenzanthracene were studied during pregnancy and lactation to elucidate the mechanism of prolactin-estrogen action and interaction. During pregnancy, palpable tumors were stimulated to grow rapidly and new active sites were initiated. Just prior to or immediately after delivery, rapid regression of tumor was observed. Some regressed tumors were reactivated, some continued to regress, and some remained static during the latter part of lactation. When cytoplasmic ER of tumors from pregnant rats was examined by the Dextran-charcoal assay, only one of the 23 identified adenocarcinomas did not contain measurable levels of ER. Significant reduction of ER occurred just prior to delivery and during early lactation. Among the 30 regressed tumors from early lactating rats, 19 had very low ER levels ($<1{\cdot}5$ fmol/mg protein) while the rest had significant but lower ER levels