The analysis of hormone–receptor interaction. Theoretical and practical aspects

Article *in* Russian Journal of Plant Physiology · January 2008

DOI: 10.1007/s11183-008-2015-y

2 authors:

Sergey N Lomin
Russian Academy of Sciences
44 PUBLICATIONS **1,239** CITATIONS
SEE PROFILE

Georgy A Romanov
Russian Academy of Sciences
172 PUBLICATIONS **3,530** CITATIONS
SEE PROFILE

ISSN 1021-4437, Russian Journal of Plant Physiology, 2008, Vol. 55, No. 2, pp. 259–273. © Pleiades Publishing, Ltd., 2008.
Original Russian Text © S.N. Lomin, G.A. Romanov, 2008, published in Fiziologiya Rastenii, 2008, Vol. 55, No. 2, pp. 283–299.

LECTURES

The Analysis of Hormone–Receptor Interaction. Theoretical and Practical Aspects

S. N. Lomin[a] and G. A. Romanov[a,b]

[a] *Timiryazev Institute of Plant Physiology, Russian Academy of Sciences, Botanicheskaya ul. 35, Moscow, 127276 Russia; fax: 7 (495) 977-8018; e-mail: gar@ippras.ru*

[b] *Institute of Physicochemical Biology, Moscow State University, Moscow, Russia*

Received April 23, 2007

Abstract—Detailed description of modern methodological approaches for the analysis of the hormone–receptor interaction is presented. Concise and comprehensive theoretical bases for such investigations as well as definite practical recommendations for experimental design and performance are given. We cover here questions concerning methodology for the determination of basic characteristics of the interaction between low-molecular weight ligands and their receptors: specific (nonspecific) binding, type of interaction, affinity constants, receptor quantity, and ligand specificity. Numerous examples of theoretical relationships and experimental results are clearly illustrated by a number of graphics. The lecture is intended not only for the experts directly engaged in the research of molecular aspects of hormone perception and signaling, but also for a wide range of readers interested in the topic of hormonal regulation in eukaryotes.

Key words: hormone - ligand - receptor - hormone–receptor interaction - affinity constants - ligand specificity - radioligand method

DOI: 10.1134/S1021443708020155

INTRODUCTION

Each cell of the multicellular organism is surrounded by the multicomponent solution containing micro- and macromolecules. Some compounds at very low concentrations substantially influence the functional state of the cell. These compounds called hormones are organic substances produced in one part of the organism and acting in another part as signals for switching on or off definite metabolic programs. Why do some compounds exert such a strong effect on the cell, whereas others even though similar in structure do not? The point is that the cells are tuned (competent) to perceive defined chemical compounds as signaling molecules. Competent cells perceive a hormonal signal and transmit it to primary intracellular targets. The genes of primary response or enzymes of the metabolism usually function as such targets.

The first step in cell recognition of the hormonal signal is its perception. Specific receptor proteins fulfill this function. Receptors comprise the functional parts, domains; the most important are a ligand-binding domain and also an effector domain responsible for switching on/off the signal transduction (Fig. 1). The association of the hormone with the ligand-binding domain evokes a change of conformation and functional state (activation or, less frequently, inactivation) of the effector region. Thus, receptors are proteins converting an external chemical signal into intracellular biochemical one, i.e., into activities of definite regulatory proteins. The character of the signal transduction and hence the cell response depends to a great extent on the regime of receptor functioning. A prominent feature of the receptor is its capability of recognition and selec-

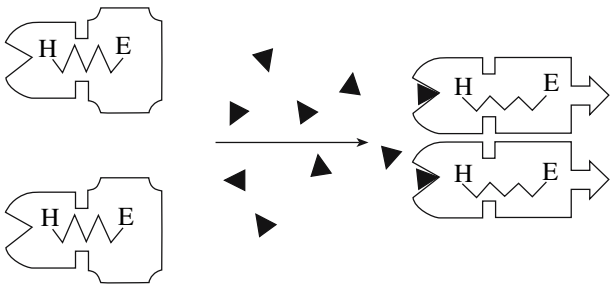

Fig. 1. Scheme of typical hormone receptor action. H—hormone-binding domain; E—effector domain. Dark triangles designate hormones.

Abbreviations and designations: L (U, F)—unbound (free) ligand (hormone); L*—labeled ligand; R—receptor; [L_T], [R_T]—total concentrations of ligand and of receptor-binding sites; LR (B)—ligand–receptor complex (bound hormone); B_S—specific binding; B_T—total binding; B_{NS}—nonspecific binding; IC_{50}—concentration of unlabeled ligand suppressing binding of labeled ligand by 50%; K_D—equilibrium dissociation constant; K_A—equilibrium association constant; K_I—equilibrium constant of inhibition.

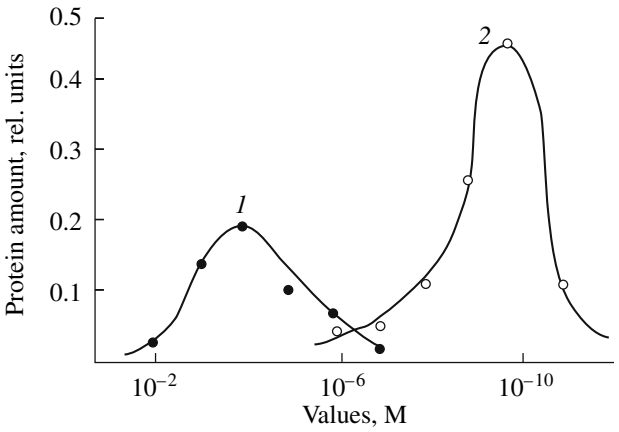

Fig. 2. Difference in the binding constants for enzymatic and receptor interactions (after [11]).
(*1*) Distribution of Michaelis constants (K_M) for enzyme–substrate interaction; (*2*) distribution of dissociation constants (K_D) for hormone–receptor interaction. Along the ordinata axis, the amount of proteins with constants in this range of values.

tive binding of chemical ligands, hormones in particular. Receptor binds hormone with high affinity and usually reversibly, without inducing its chemical changes [1–3]. These specific features are characteristic of hormone binding with receptor, as distinct from its interaction with other proteins, enzymes of metabolism, for example. Receptors can be either located inside the cell or associated with the plasma membrane. After hormone binding, receptors often dimerize, and just such receptor dimers serve as their active forms (Fig. 1) [4, 5].

Receptor studying has a long history. As early as in 1878, while examining the effects of athropine and pilocarpine on the secretion of salivary glands and the effects of nicotine and curare on the contraction of striated muscle, Langley [6] demonstrated the existence of cell mechanisms for recognition of chemical compounds. At this time, a notion arose that the cells contain some substances responsible for this process. At the beginning of the 20th century, Ehrlich and Mechnikov put forward a theory of "lateral chains" rewarded by the Nobel Prize in 1908. They assumed that "lateral chains" on the cell surface recognize chemical compounds and interact with them. Ehrlich designated these "lateral chains" as receptors (from Latin *recipere*, which means recognize) (cited after [7–10]). At the beginning of the 20th century, receptor studying was based on indirect pharmacological data: a dependence of the cell responses on the concentration of the hormone introduced. In 1926, Clark suggested the first quantitative description of this dependence. He noticed that dependencies of cell responses to the inducer concentration resemble the kinetics of enzymatic reaction already known at that time. Clark suggested that the interaction between ligand (L) and receptor (R) could be described as a reversible reaction:

$$L + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftarrows}} LR, \quad (1)$$

where LR is a ligand–receptor complex, k_{+1} is a constant of association, and k_{-1} is a constant of dissociation of this complex. According to the law of mass acting, the constant of dissociation (K_D) at equilibrium is determined as

$$K_D = \frac{1}{K_A} = \frac{k_{-1}}{k_{+1}} = \frac{[L][R]}{[LR]}. \quad (2)$$

Since the stage of ligand interaction with receptor determines in many ways subsequent cell response, the characteristics of this process are of great interest. The equilibrium dissociation constant (K_D) (or a reciprocal value of equilibrium association constant K_A) is one of such important characteristics reflecting receptor affinity for the hormone. In comparison with other hormone-binding proteins, receptors exhibit the highest affinity for their ligands, as K_D ranges from 10^{-7} to 10^{-12} M (Fig. 2 [11]) or, when expressed in terms of K_A, from 10^7 to 10^{12}/M. Such high values of the association constants correspond to great changes in the free energy of interaction, ranging correspondingly from 9.5 to 16.3 kcal/mol (under standard conditions at 25°C). This released chemical energy is spent for changes in the conformation of the receptor protein.

Another very important characteristic of the receptor is its ligand specificity. As a rule, each receptor can bind a very limited number of chemically related ligands; the affinity for the ligand depends on the structure of the latter. The information about the receptor localization and ligand specificity of the receptor makes it possible to evaluate the potential effectiveness of particular form of the hormone or its analogs in a given organ or tissue of the living organism. Also, the knowledge of the parameters of hormone interaction with receptors makes it possible to answer some of the questions arisen during examination of molecular mechanisms of hormone signaling. The fact of a high affinity and high specificity of hormone binding to a definite protein often permits unambiguous identification of a novel receptor protein or confirmation of the receptor function of already known protein, a putative receptor. Quantitative analysis of binding parameters permits us to calculate the proportion of receptor of total cell protein and the number of receptors per cell. Such data are crucial for the correct diagnostic of various hormonal disturbances, in humans in particular. The investigation of the hormone–receptor interaction allows deciphering the mechanism of these interactions (simple, cooperative, or anticooperative), evaluation of the environmental effects on the parameters of hormone association with the receptor, and obtaining other useful data. However, not always such investigations are performed, analyzed, and interpreted correctly.

In this review, we focus on universal quantitative methods for analysis of the hormone–receptor interaction, which are widely applied at present for studying the receptors in animals, plants, and microorganisms. We consider theoretical basics and present concrete practical recommendations for planning, performing, and interpreting the experiments.

THEORETICAL BASIS FOR DETERMINATION OF RECEPTOR AFFINITY AND CONCENTRATION

On the basis of simple equations (1) and (2), we can build theoretical dose dependence curves of the hormone binding to receptor. Under equilibrium conditions and in the presence of the constant concentration of the receptor, hormone binding is described by a hyperbolic function with the asymptote determined by the total amount of receptor (Fig. 3a). Irrespective of the hormone concentration, its specific binding to the fixed amount of the receptor would be limited by the number of hormone-binding sites (R_T). Most frequently, each receptor molecule contains a single hormone-binding region; therefore, the position of the asymptote for the binding curve indicates the total amount of receptors in the sample (Fig. 3a).

Once the position of the asymptote is known, we can estimate the K_D value from the hormone binding curve. K_D corresponds to the hormone concentration saturating 50% of the binding sites and is expressed in the units of the hormone concentration. Figure 3a presents the binding curves of hormones whose affinity for receptor differs by an order of magnitude. It is worth mentioning that presenting such curves in linear coordinates can sometimes be inconvenient because hormones are usually tested at the concentrations differing by several orders of magnitude.

It is more convenient and illustrative to present the concentration dependencies in semi-logarithmic coordinates, where the abscissa axis represents the logarithms of hormone concentrations. In this case, the saturation curves become S-shaped, while the K_D value is positioned in the quasilinear central region and corresponds to the point of the curve bending (Fig. 3b). However, such presentation does not always permit a graphical determination of K_D and R_T values. Linearization of the saturation curve allows the most precise determination of these parameters.

The task of precise determination of principal characteristics of hormone–receptor interactions based of dose dependencies of hormone binding was solved in 1949 by physicochemist Scatchard [12]. By a simple transformation of the equation (2), he derived the following equation:

$$\frac{1}{K_D}[R_T] - \frac{1}{K_D}[LR] = \frac{[LR]}{[L]}. \quad (3)$$

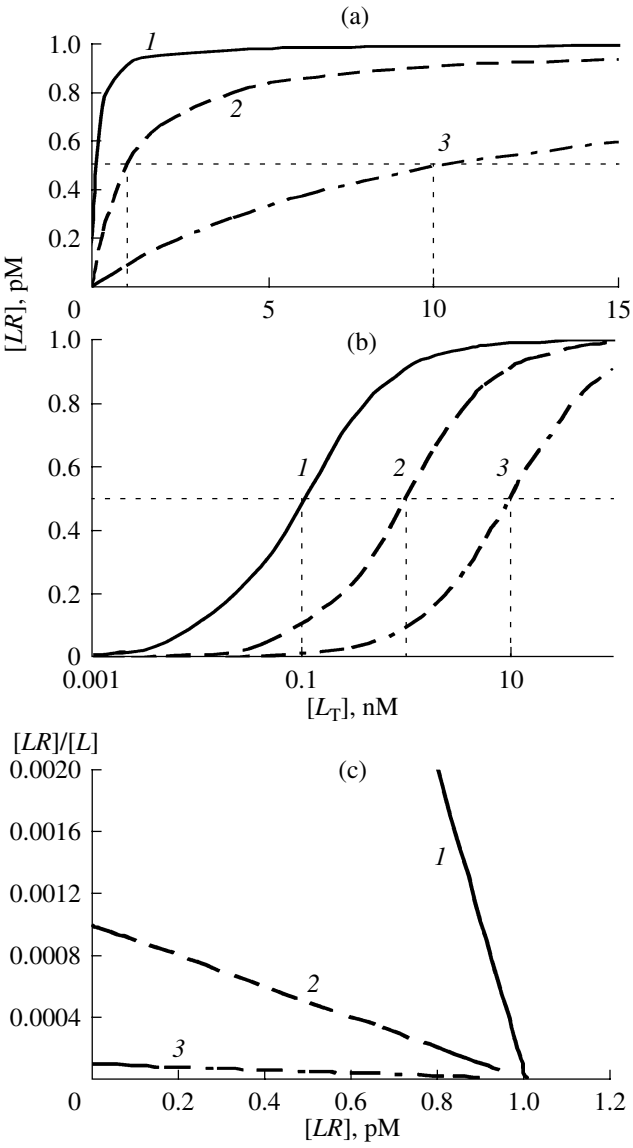

Fig. 3. The modes of representation of results on hormone (ligand) binding to receptor.

The concentration dependence of the formation of hormone–receptor complexes in (a) linear, (b) semi-logarithmic and (c) Scatchard [12] coordinates. The concentration of the hormone–receptor complexes [*LR*] depends on K_D: for curves *1–3*, K_D are equal to 0.1, 1. and 10 nM, respectively. The concentration of the binding sites was accepted to be 1 pM. [L_T] is a total concentration of ligand (hormone). K_D values correspond to the concentration of ligand saturating 50% of binding sites (dotted lines).

Equation (3) is a linear one, $y = ax + b$, where y is the ratio [*LR*]/[*L*] and x is [*LR*]. Namely, a plot of [*LR*]/[*L*] vs [*LR*] (usually called the Scatchard plot) gives a straight line of slope tangent ($-1/K_D$); point of this line crossing the abscissa axis corresponds to the total receptor concentration (free + bound) [R_T] (Fig. 3c). Until now, Scatchard coordinates are widely used for

the calculation of binding parameters and representation of the results of experiments, although numerous specialized computer programs intended for this purpose appeared recently.

In order to correctly estimate the equilibrium characteristics of the hormone–receptor interaction, certain conditions should be met regardless of the calculation system used. These include:

(1) A wide range of ligand concentrations including the concentrations manifold exceeding those of receptors;

(2) Stable equilibrium in samples, where the concentrations of all components (free and bound ligand and receptor) remain unchanged; and

(3) Similar availability of all receptors to ligand.

However, even when all these conditions would be met, it is not easy to obtain in experiments a linear Scatchard dependence. Therefore, in further parts of this review we focus on some of the practical aspects of hormone binding experiments (i.e., their correct planning and performing) as well as on determination of the parameters of hormone–receptor interaction in spite of possible deviations from the "classical" theoretical model.

RADIOLIGAND (RADIORECEPTOR) METHOD

As evident from the equation (3), to determine parameters of the interaction between hormone and receptor, it is necessary to know the concentration of ligand–receptor complex in the sample [*LR*] and the ratio of this concentration to that of free ligand *L* ([*LR*]/[*L*]). To this end, the radioligand method is applied; this method is most frequently used for the analysis of hormone-binding properties of the receptor (sometimes this method is called as the radioreceptor method). It is based on the usage of labeled hormone, which permits monitoring the concentration of ligand–receptor complexes (*LR*) from the amount of label bound. Knowing the amount of labeled hormone used, we can also easily calculate the second variable of the equation (3), i.e., the concentration of free ligand (*L*). The concentration of the ligand (hormone) added to the sample is usually by several orders of magnitude higher than that of receptor; therefore, we can use total concentration of the ligand (L_T) instead of the concentration of free ligand *L*. In radioligand studies, radioactive ligands prepared by various biomedical firms are commonly used. As a rule, ligands are labeled with tritium (^{3}H) or iodine (^{125}I) and should meet certain requirements. In particular, they should (1) be identical to their cold counterpart regarding the parameters of their interaction with receptor; (2) have high specific radioactivity, best if not lower than 750 GBq/mmol or 20 Ci/mmol; (3) display a high affinity for the receptor, i.e., have nanomolar or even lower dissociation constants; (4) display high specificity of binding to the receptor of interest; and (5) have a high degree of purity (especially radiochemical), and the label should not dissociate from the ligand during experiment.

It is possible to use a label other than radioactive (e.g. fluorescent label, which permits quantification of the sample fluorescence) presuming that it does not change ligand biological activity and allows its quantification with a high sensitivity. The advantage of such labels is their safety for the researcher and the environment. However, since radioactive ligands are most frequenly used in practice, we consider the interaction between ligand and receptor through radioligand method usage. It should be emphasized that all main approaches and regularities of the radioligand method are applicable to the cases of differently labeled ligands.

A typical radioligand (radioreceptor) experiment comprises four stages:

(I) Sample incubation with radioactive ligand until equilibrium is reached;

(II) Separation of free and bound radioactivity;

(III) Measurement of the amount of bound radioactivity;

(IV) Mathematical, graphical, or computer analysis of the results.

Let us consider each of these stages in more detail.

Stage I. Traditionally, radioligand studies are performed in vitro on various biological entities or stuff at 0–4, 20–25, or (less frequently) 37°C, which depends on the properties of the receptor under study [2, 13–18]. Soluble nuclear–cytoplasmic receptors in the cytosol or partially purified ones are incubated with hormone in the homogenous solution (sometimes receptors are immobilized on affinity matrices, see below). For membrane receptors, preliminary isolation of corresponding membranes is required. Thereafter, the suspension of isolated membranes (microsomes) is incubated with hormone. When using the suspension, its homogeneity should be provided; otherwise, not all receptors would be similarly accessible for hormones. Fortunately, the kinetic rates of hormone interaction (association and dissociation) with receptors are sufficiently high (equilibrium is usually attained for minutes or tens of minutes); therefore, microsomes do not have an opportunity to aggregate during incubation. Sometimes, living materials, intact cells or tissue sections, are used for analysis.

Recently, a new method, so-called tissue segment binding method has been developed [19]: small segments (few millimeters in size) of living tissues placed in the complete physiological solution are used for analysis. In this case, receptors function under conditions close to natural ones, and the procedure becomes substantially simpler because there is no need in material fractionation. However, when applying this method, the homogeneity of incubation medium is not always easy to achieve. Still more substantial drawback of the method is the possible occurrence in living cells

during incubation some processes, which might affect hormones, receptors, or their interaction. Among such processes are metabolism and intracellular transport of the labeled ligand, proteolysis and/or internalization of receptors and receptor–hormone complexes, and others. Moreover, several receptor types are usually occur in the cell, and this hampers the analysis of properties of particular receptor. One more disadvantage of homological living systems is the presence of endogenous hormones, which could interfere with labeled ligand during hormone–receptor interaction and affect the results obtained.

To avoid these limitations, we have recently suggested a new way for the analysis of hormone-binding properties of eukaryotic receptors using transgenic bacteria expressing a particular receptor protein [20]. Bacteria have neither analogs of most eukaryotic hormones nor the systems for their synthesis or perception that excludes the effects of endogenous hormone analogs on the outcome of the experiments. Moreover, the transformation of bacteria with a single gene makes it possible to investigate only one particular receptor protein. Eukaryotic receptor inserted in the bacterial membrane usually retains full functional activity, which can be verified on the bacterial test-system [21, 22]. In cases when definite posttranslational modification (glycosylation, phosphorylation, etc.) is required, an attempt could be made to introduce into the bacterium not only the gene encoding receptor but also the gene encoding the enzyme of its modification. It should also be mentioned that the bacterial suspension used in experiments is rather homogenous and can be treated as a true solution, i.e., it can be stirred, poured into other vessels, and aliquoted.

Stage II. The most important part of experiment is the quantification of bound hormone. This can be done using equilibrium dialysis or physical separation of free and bound hormone. Dialysis is performed in the special cell partitioned with a membrane; the sample of receptor is placed in one compartment and labeled hormone, in another. Then incubation is performed at constant cell rotation, and, after attaining the equilibrium, aliquots from both compartments are counted. This is the most equilibrium and mild method; however, it requires a long incubation, specific equipment, and a high precision of radioactivity measurement. Physical separation is more available; however, it requires very careful performing. The following conditions should be met during this procedure:

(1) Separation should be thorough and quantitative, without mutual contamination of fractions; and

(2) System equilibrium should not be disturbed, whenever possible, in order to avoid additional dissociation of ligand–protein complexes.

To separate free and bound hormone, centrifugation or filtration is applied most frequently. The suspension of microsomes, cells, or tissue fragments could be sedimented by rapid centrifugation; in this case, bound labeled hormone would be in the pellet, whereas free one, in the supernatant. Since all initial components remain in the system subjected to separation, the equilibrium changes during centrifugation are the lowest possible. There are some approaches to separate free hormone from that bound to soluble receptor by centrifugation. One of them is sedimentation of proteins, including those bound hormone, by their salting out, for example, with high concentrations of ammonium sulfate [23–26]. Protein sedimentation can be also performed by the adding any protein-binding sorbent to the incubation mixture, for example, hydroxyapatite [23–27]. In all cases, protein-bound hormone sediments together with protein, whereas free hormone remains in the supernatant. An alternative possibility is the application of activated charcoal, which is capable of irreversibly absorption of low-molecular compounds (simple ligands) but not proteins [28–30]. Short-term contact of the incubation mixture with the charcoal suspension results in the absorption of free hormone by coal, while bound hormone remains in the protein solution. Thereafter, charcoal with absorbed hormone is sedimented by centrifugation, and the level of bound hormone is measured in the aliquot of the supernatant.

One more approach used for separation of free from the protein-bound ligand is membrane filtration [14–18]. At present, biomedical firms prepare a wide set of various membrane filters capable of retarding particles of various sizes, from microsomes to entire eukaryotic cells. However, these filters should meet one principal condition: they should not absorb labeled ligand (L^*). Using a special vacuum apparatus, it is possible to filtrate the incubation suspension very rapidly, within few seconds. At such a high filtration rate, hormone–receptor complexes have no chance to markedly dissociate. As a result, bound hormone remains completely on the filter; in addition, after filter drying, its radioactivity could be measured directly in the scintillation counter.

Other suggested methods for the separation of free and bound hormone, such as gel filtration, ion-exchange chromatography, or electrophoresis [29] are not widely applied because of equilibrium disturbance during their application, i.e., a great probability of partial complex dissociation.

Regardless of the method applied, it is important to verify that the separation of the fractions is strictly quantitative. To this end, special control experiments are to be performed, with the usage of inactivated receptor and the excess of unlabeled ligand; it is also necessary to control instrumentally the absence of protein, microsomes, cells, or coal in the supernatants and/or filtrates.

A development of modern technologies of recombinant proteins offered new possibilities for the analysis of hormone–receptor interactions, in particular, for separation of hormone–receptor complexes from unbound ligand. When using this approach, the coding part of the receptor gene is fused by gene-engineering methods

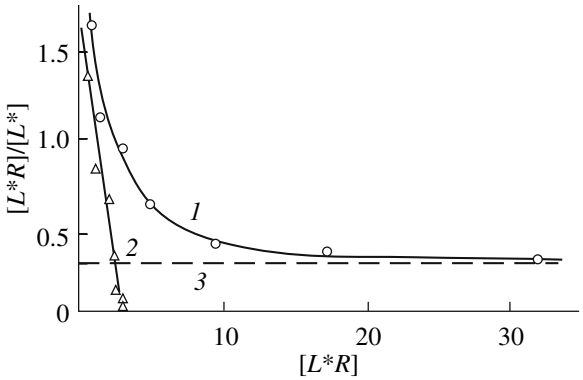

Fig. 4. An example of resolution of the Scatchard curve for total hormone binding into specific and nonspecific components (according to Rosenthal [32]).
(*1*) Total binding of ^{3}H-dexamethasone in the cytosol of rat liver; (*2*) specific binding; (*3*) nonspecific binding. [*L*R*] is the concentration of bound hormone (*B*) expressed in mol/mg of cytosolic protein $\times 10^{-13}$; [*L**] is the concentration of unbound (free) hormone (*U* or *F*) (after [28]).

with the sequence for peptide easily recognizable by a definite sorbent or antibody (affinity tag) [31]. After or during incubation with hormone, such recombinant receptor can be easily immobilized on the affinity matrix or by using antibodies, and the amount of bound hormone can be determined. It is only important that the affinity labels would not affect hormone-binding properties of the receptor tested.

Stage III. Measurement of the radioactivity of bound hormone is performed using the scintillation counter after the addition of scintillation coctail suitable for the sample, or the gamma counter, as in the case of ^{125}I, is used. The efficiency of tritium counting is much higher in the homogenous solution than in insoluble radioactive pellets. Therefore, it is useful to extract the radioactive hormone from the pellets with any eluent, which is well soluble in the scintillation cocktail but does not significantly affect the counting efficiency. For many lipophilic ligands, such a suitable solvent is ethanol [20, 23–27]. Another way to attain homogeneity is the usage of organic pellet solubilizers, specially produced for scintillation counting by some biomedical firms. In fact, when the ligand per se is well soluble in the scintillator, the presence of the insoluble organic matter can markedly retard ligand solubilization and reduce the total counting efficiency. To achieve higher precision, the duration of the counting process must be chosen depending on the average level of the sample radioactivity as well as on the ratio between sample radioactivity and that of the background (scintillator with pure solvent). To ensure that the standard error does not exceed 3–5%, the number of measuring replicates should be high enough. Since all main parameters of hormone–receptor interaction (K_D, [R_T]) are expressed in the units of the concentration, it is better to express the results of counting in dpm (decays per min). Such results can be easily transformed in the values of the ligand concentration, assuming that ligand specific radioactivity is known. When the results of counting are expressed in cpm (counts per min), it is necessary to estimate the counting efficiency under the same experimental conditions.

Stage IV. As was mentioned, the special analysis of counting data is performed to determine parameters of hormone–receptor interaction. To present data in Scatchard coordinates, it is necessary to determine, for each sample, the content of bound hormone and the ratio of bound to free hormone. The concentration of the bound ligand corresponds to the value [*LR*] and the ratio of bound to free hormone, the ratio [*LR*]/[*L*] in equation (3). Therefore, this equation is very often represented as

$$\frac{B}{U} = -\frac{1}{K_D}B + \frac{R_T}{K_D}, \quad (4)$$

where *B* and *U* are concentrations of bound and free (unbound) hormone, respectively (the symbol *F* (free) is often used instead of *U*). The example of such representation is shown in Fig. 4 (curve *1*) [28]. In this experiment, the parameters of highly labeled synthetic glucocorticoid, ^{3}H-dexamethasone, binding to the corticosteroid receptor from rat liver were determined. It is seen that, in Scatchard plot (*B*/*U* vs. *B*), the curve is not linear but bends at some distance from the abscissa axis and further approximates to the asymptote parallel to this axis. Such shape of the curve in Scatchard coordinates usually indicates that the sites for nonspecific binding are present and contribute to the value of measured bound radioactivity.

Nonspecific binding. The practice shows that, in binding experiments, labeled hormones of diverse structures can bind not only to the receptors (specific binding) but also at some degree of probability to other components of the system: proteins, membranes, cell fragments, other high-polymeric materials, and even tube walls (so-called nonspecific binding). Such nonspecific binding can sometimes be so high that an inexperienced researcher could take it as a true binding to the receptor. However, specific and nonspecific binding differ in some traits, and this permits one to distinguish them explicitly.

The number of sites for hormone specific binding to receptor is limited, and the hormone affinity for receptor is very high. In the case of nonspecific binding, an enormous number of low affinity sites are engaged. When to rewrite equation (4) as $B/U = K_A(R_T - B)$, in the case that the concentration of binding sites greatly exceeds that if the concentration of hormone added (i.e., when $R_T \gg L_T > B$), this equation acquires the following form [13]:

$$\frac{B}{U} = K_A R_T. \quad (5)$$

On the Scatchard plot, dependence (5) of nonspecific binding is expressed as a straight line parallel to

the abscissa axis with $K_A R_T$ being the ordinate axis intercept (here R correspond to nonspecific binding sites).

It is possible to experimentally evaluate the contribution of nonspecific binding; to this end, a great excess of unlabeled hormone (100–10000-fold) should be mixed with radioactive one. Because the number of high affinity sites is relatively small, the excess of cold hormone replaces essentially all labeled hormone from these sites. On the other hand, the number of nonspecific binding sites is enormous, and the added excess (in the range indicated) of unlabeled hormone does not essentially affect the value of label bound nonspecifically. Therefore, only nonspecific binding is measured in experiments where a great excess of cold hormone is added to the same but labeled hormone.

Figure 5 demonstrates the character of total, nonspecific, and specific binding dependencies on the dose of added labeled hormone. Nonspecific binding dependence is linear, whereas total binding curve, although increases as well, clearly deviates from the straight line. Total ligand binding (B_T, curve *1*) is the sum of specific (B_S, curve *2*) and nonspecific (B_{NS}, curve *3*) binding. Therefore, in order to estimate the value of specific binding, nonspecific binding should be subtracted from total binding:

$$B_S = B_T - B_{NS}. \quad (6)$$

The subtraction of B_{NS} from B_T results in a typical hyperbolic curve of receptor saturation with hormone (Fig. 5, curve *2*) characteristic of specific binding. Similarly, when the values of total binding B in Fig. 4 are replaced by those calculated (theoretical) for specific binding B_S, instead of concave curve we obtain a straight line, which is expected theoretically [12], and K_D and R_T could be easily estimated on its basis (Fig. 4, curve *2*). Such transformation of relationships could be performed graphically using the method of Rosenthal [32] or analytically. In experiments, it is better to minimize nonspecific binding in order to achieve more precise estimation of parameters of hormone–receptor interaction. It is desirable that the level of nonspecific binding does not exceed 20–30% of total binding. This could be achieved by reduction of labeled hormone dose because in this case the percent of nonspecific binding of total one decreases (Fig. 5). On the other hand, the fact of significant difference between total and nonspecific binding indicates the presence of the sites for specific binding of the ligand tested. Therefore, in the beginning of the investigation of hormone–receptor interaction, pilot experiments on comparison of total and nonspecific binding in similar samples should be performed to make sure that high affinity sites are present and the approach chosen for their identification is correct.

Recommendation for running the experiment. When pilot experiments detect the presence of sites for specific binding of the ligand, the time required for steady state achievement of the system should be determined. In practice, this time is determined as the time needed for the bound hormone level to attain the plateau. It should be kept in mind that the rate of chemical reaction depends on concentrations of interacting substances; therefore, the time needed to reach equilibrium should be estimated at the lowest ligand concentration. In experiments on dose dependence of binding, it is desirable that the level of the maximum binding (B_S) is between 1000 and 10000 dpm. The lower values might result in an inaccurate counting, whereas at the higher values, excess amounts of radioactive isotopes and/or receptor preparation would be spent. For reliable determination of binding parameters, no less than 5–7 different concentrations of labeled ligand (L^*) are required and no less than four replicates should be used for each concentration: two for total and two for nonspecific binding. Meanwhile, nonspecific binding can be measured at lower number of label concentrations, 3–4 in practice, because it follows linear dose dependence. One of the ligand concentrations should be close to the expected K_D; in this case, the analysis would be most accurate [9, 14]. Other concentrations should produce successive arrays of lower and higher than K_D concentrations, where each next concentration is lower/higher the preceding one by 2–3 times. It is sufficient that the final concentration in each array differed from K_D approximately by 1–1.5 orders of magnitude. The concentration of the receptor is recommended to be at least one order of magnitude lower than K_D [33].

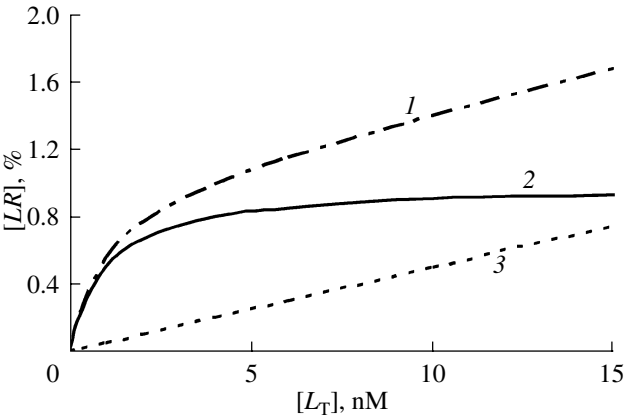

Fig. 5. Concentration dependencies of hormone (*1*) total, (*2*) specific, and (*3*) nonspecific binding.
[LR] is the concentration of bound hormone (B); [L_T] is the total concentration of hormone.

The choice of the method for measuring hormone binding depends to a great extent on the nature of material tested. In saturation experiments, to improve method sensitivity, it is beneficial to follow the verified recommendations [9, 34]. The sensitivity of the radioligand method is determined by the lowest concentration of unlabeled ligand $[L]_{min}$, at which the measured difference between total binding and binding in the presence

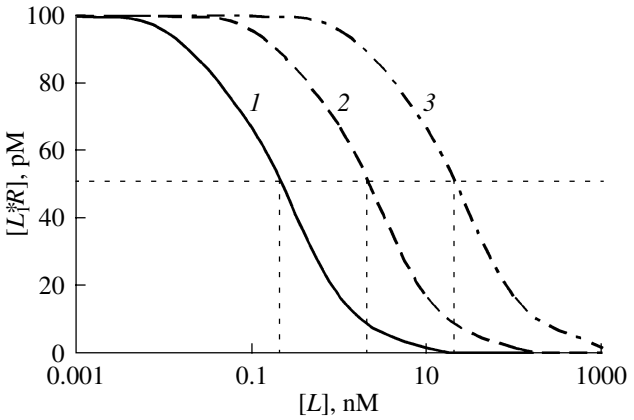

Fig. 6. Displacement of the fixed concentration of labeled hormone with various concentrations of unlabeled ligands differing in their affinity for receptor.

The values of K_D for curves *1–3* correspond to 0.1, 1, and 10 nM. The concentration of ligand-binding sites was taken as 0.01 pM; the concentration of labeled hormone, as 0.1 nM. $[L_1^* R]$ is the concentration of receptor-bound labeled hormone. $[L]$ is the concentration of unlabeled ligand. The proportion of receptors, which bound labeled hormone is on the ordinate axis. Dotted lines designate IC_{50}.

of unlabeled ligand is still statistically significant. Calculations show that the sensitivity increases with (1) a decrease in the error of radioactivity determination; (2) a decrease in the value of $[L^*]/K_D$; thus, radioligand studies should better be performed at relatively low concentrations of labeled hormone; (3) a decrease in the level of nonspecific binding; (4) an increase in the volume of reaction mixture, that limits experiment performing in very small volumes. The sensitivity is also directly proportional to the affinity of unlabeled ligand for the receptor. Therefore, it is desirable to perform the assay under conditions optimal for ligand binding.

INTERACTION OF SEVERAL LIGANDS WITH A SINGLE SITE. COMPETITIVE BINDING

Earlier, we have noted that ligand specificity of binding is one of the most important characteristics of the receptor. The analysis of ligand specificity permits one to understand which of close natural compounds fulfills function of the hormone in a definite system of signal perception. Certainly, we could label with isotope each of the compounds tested and perform binding experiment as described above. However, such approach would be very costly in respect to both money spent and ecology. Besides, not all ligands are available as labeled molecules. Currently, another strategy is applied, i.e., the analysis of radiolabeled hormone displacement from its complex with receptor by unlabeled ligand.

Let us suppose that the test system contains two ligands, one of which is labeled. When these ligands compete for one and the same binding site and the number of ligands is greater than that of the receptors, the level of labeled ligand binding is lower in the presence of the second ligand because the second ligand replaces the first one in the complex with receptor. A dose dependence of label displacement can be obtained when the binding experiment is performed at the fixed concentration of the first ligand in the presence of various concentrations of unlabeled ligand (Fig. 6). It seems evident that the higher the affinity of unlabeled ligand for receptor the lower concentration would be required for the labeled ligand displacement. Thus, the curves of displacement for more affine ligands would be shifted to the region of lower concentrations of the competitor as compared with similar curves for low-affinity ligands. Experiments of this type are called competitive ones; they can be used to determine the affinity for the receptor of various unlabeled ligands on the basis of the concentration dependence of a labeled hormone displacement they cause.

To quantify the affinity, the curves of labeled ligand displacement with unlabeled one are analyzed. On the basis of these curves, the concentration of unlabeled hormone displacing 50% of labeled hormone (IC_{50}, Fig. 6) is estimated. To precisely evaluate this concentration, it is necessary that at least three concentration points of unlabeled ligand are located on the quasilinear central region of the competition curve (the interval from ~25 to ~75% of displacement). IC_{50} could be easily determined at logit-transformation of the ordinate axis; in this case, larger part of the competition curve is transformed into the straight line. The concentration of radioactive ligand is chosen in such a way that it is approximately equal to or lower than its K_D with receptor, but it should anyway provide for a significant label counting. In competition experiments, the samples for nonspecific binding should be obligatory included in order to cut off the background of bound radioactivity not related to interaction with receptor. All operations (incubation until equilibrium establishment, separation of bound and free hormones, and measurement of the bound radioactivity) are performed similarly as in the saturation experiments. The inhibition constant K_I of unlabeled ligand corresponding to K_D of its interaction with receptor is estimated after the formula [35]:

$$K_I = K_D = \frac{IC_{50}}{1 + \frac{[L^*]_0}{K_D^*}}, \quad (7)$$

where $[L^*]_0$ and K_D^* are the fixed concentration of labeled ligand and its affinity for receptor, respectively. When $[L^*]_0$ is much lower than K_D^*, K_I becomes approximately equal to IC_{50}, and it is often called as the *apparent* constant of inhibition or dissociation (apparent binding constant). When $[L^*]_0$ is precisely equal to K_D^*, the value of K_I is twice lower than IC_{50}. To correctly calculate K_I after the formula (7), it is of importance that the competition analysis is performed under

conditions (medium composition, temperature, duration) similar to those used for K_D^* determination.

Still easier way for K_I determination is based on the fact that the ratio between IC_{50} (or IC_N generally) for various ligands corresponds to the ratio between the constants of their affinity for receptor. In this connection, it is beneficial to build the displacement curve for unlabeled analog of labeled ligand in competitive experiments. As a rule, the insertion of the radioactive label into the hormone molecule does not affect substantially the parameters of its interaction with receptor. Therefore, when the value of K_D^* is known beforehand from saturation experiments, K_D for every competitive ligand can be calculated from the ratio of its IC_{50} to IC_{50} for unlabeled analog of radioligand. However, at such experiment design, the knowledge of even initial value of K_D^* is not obligatory because it could be determined directly from the displacement curve of labeled hormone by its unlabeled analog. Such binding is analyzed in Scatchard plot. The value of B/U on the ordinate axis (B_S/U, to be more precise) is determined on the basis of this ratio for labeled ligand after nonspecific binding subtraction. In this case, to know the total amount of ligand in the incubation mixture is not required because the ratio of labeled B/U is equal to the ratio of B/U for total amount of the same ligand. However, to determine the concentration of the bound ligand B_S (abscissa axis), it is necessary to calculate just its total amount, i.e., the sum of labeled and unlabeled ligand. The amount of specifically bound label can serve as an orienteer; this value should be multiplied by the coefficient equal to the ratio of unlabeled to labeled hormone in the sample.

For correct calculation of constants from the ratio between IC_{50} values, the level of bound radioactivity should be relatively low (no more than 20% in the absence of the competitor) relative to total label [36]. At correct experimental design and adequate calculations in Scatchard coordinates, we obtain a straight line with parameters close to those established in experiments on radioligand saturation.

Some other ways to estimate the characteristics of unlabeled ligand binding on the basis of labeled ligand displacement from its complex with receptor have also been suggested [7–9, 37], but, being more complex, they are not widely applied.

We have previously considered the case when all ligands interact with one and the same site of a particular receptor protein, as it is most typical case. However, in principle and reality, various ligands can interact with different receptor regions. Therefore, three possible types of binding of the pair of ligands are distinguished [9]: (1) competitive binding, when both ligands bind to the same site on the receptor; (2) noncompetitive binding, when ligands bind to different sites on the same protein; and (3) anti-competitive binding, when the ligand of the second type interacts not with the receptor itself but with the complex produced by the ligand of the first type and the receptor (the most rare type).

To identify binding types for various ligands and to prove, for example, competitive character of binding in the test-system studied, equilibrium experiments with labeled and unlabeled ligands are performed. For labeled ligand, several curves are constructed, one of which is a usual saturation curve and in other cases a definite fixed concentration of unlabeled ligand is present in solution. These constant concentrations applied should markedly (by 25–80%) but not completely displace labeled ligand. Binding is analyzed in double reversed coordinates $1/[LR]$ and $1/[L_T]$.

Let us consider the principle of analysis in the case of competitive binding. Under equilibrium conditions and in the absence of the competitor, the concentration of ligand-receptor complexes is described by the equation:

$$[LR] = \frac{[L_T][R_T]}{[L_T] + K_D}. \quad (8)$$

From equation (8), it is easy to build the equation for $1/[LR]$, which is described as follows:

$$\frac{1}{[LR]} = \frac{1}{[R_T]} + \frac{1}{[L_T]}\frac{K_D}{[R_T]}. \quad (9)$$

It is evident that this dependence is linear in the $1/[LR]$ and $1/[L]$ coordinates. In the presence of unlabeled ligand, the amount of ligand–receptor complexes with labeled hormone is described by the following equation:

$$[L^*R] = \frac{[L^*][R_T]}{[L_T^*] + K_D^*\left(\frac{1 + [L]_0}{K_D}\right)}, \quad (10)$$

where L_T is the concentration of unlabeled hormone and K_D is its constant of dissociation. The value $1/[L^*R]$ is described by the equation:

$$\frac{1}{[L^*R]} = \frac{1}{[R_T]} + \frac{1}{[L_T^*]}\frac{K_D^*\left(1 + \frac{[L]_0}{K_D}\right)}{[R_T]}, \quad (11)$$

where $1 + [L]_0/K_D$ is a constant. In the absence of unlabeled hormone, this value is 1, and equation (11) becomes identical to equation (9). Thus, in the presence of unlabeled hormone, a dependence in reversed coordinates is described also by a straight line, but the tangent of its slope $(K_D^*(1 + [L]_0/K_D)/[R_T])$ is greater than that in the absence of unlabeled hormone $(K_D/[R_T])$.

It is evident from equation (11) that, when $1/[L_T^*] = 0$, we obtain $1/[L^*R] = 1/[R_T]$. Thus, competition straight lines obtained in the presence or absence of tested unlabeled ligand should intercept in one point on

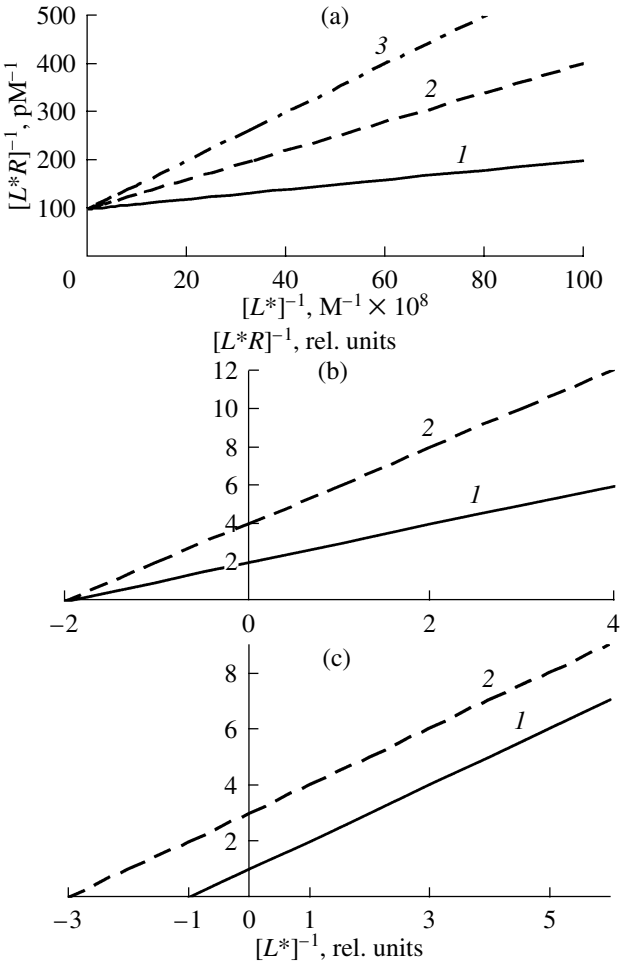

Fig. 7. Examples of binding assays with labeled hormone in the presence of unlabeled hormone of different structure, results are represented in double reversed coordinates.

(a) Competitive binding. The concentrations of unlabeled hormone are (*1*) 0, (*2*) 0.2, and (*3*) 0.4 nM. K_D of labeled hormone–receptor complex is 0.1 nM; concentration of ligand-binding sites is 0.01 pM; (b) noncompetitive binding without change in affinity (*1*) in the absence and (*2*) in the presence of unlabeled hormone; (c) anti-competitive binding (*1*) in the absence and (*2*) in the presence of unlabeled hormone. $[L_1^* R]^{-1}$ and $[L^*]^{-1}$ are the values reverse to the concentrations of receptor-bound and unbound labeled hormone.

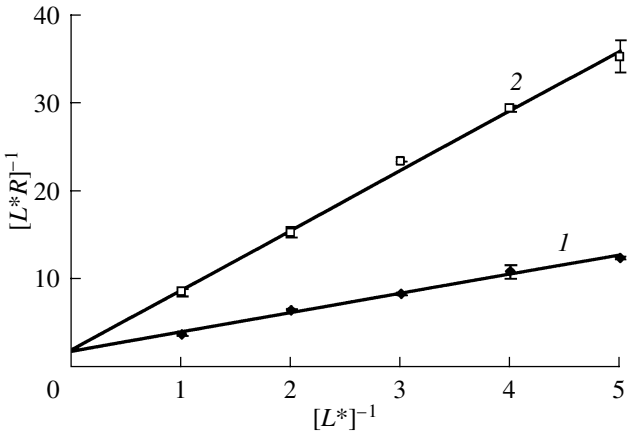

Fig. 8. Concentration dependence for binding of tritium-labeled *trans*-zeatin to the cytokinin receptor AHK4 plotted in double reversed coordinates.

(*1*) In the absence and (*2*) in the presence of 26 nM thidiazuron. $[L^* R]^{-1}$ and $[L^*]^{-1}$ are the values reverse to the concentration of bound and unbound labeled *trans*-zeatin, respectively (here *L* corresponds to tZ) (after [38]).

the ordinate axis (Fig. 7a). When these lines do not cross the ordinate axis in a single point, the total number of available sites for labeled ligand in the presence of unlabeled one seems to be reduced and thus binding is not competitive. In this case, various types of strait line behavior could be observed. When binding of the second ligand affects the affinity of the first one, the curves intercept the abscissa axis in different points, when not, they intercept at the same point on the abscissa axis (Fig. 7b). When binding is anti-competitive, the lines run in parallel (Fig. 7c) [9]. In principle, in the system of reversed coordinates, it is possible to evaluate the receptor affinity and the number of hormone binding sites. However, this system is infrequently applied for these purposes because of the higher standard error for the calculation of reverse values.

Now, we consider an example for the use of reversed coordinates. Thidiazuron is a synthetic cytokinin differing markedly in its structure from natural phytohormones of this class (zeatin and others). To elucidate the types of hormone–receptor interaction for this pair of ligands, experiments were performed on binding of labeled *trans*-zeatin by the cytokinin receptors in the (*1*) absence or (*2*) presence of thidiazuron (Fig. 8, [38]). The plot in reversed coordinates shows that straight lines intercept on the ordinate axis indicating that *trans*-zeatin and thidiazuron compete for the same site on receptor.

MORE COMPLEX TYPES OF HORMONE INTERACTION WITH RECEPTORS

Sometimes, a dose dependence of labeled ligand binding in Scatchard plot is nonlinear in spite of perfect experiment design, performance, and analysis. The cause for such deviations might be a complex process of hormone–receptor interaction. When the curve on Scatchard plot bends down, this can signify that receptor has two or more sites for hormone binding differing in their affinity or the presence of two and more receptors. Theoretically, one can build several appropriate asymptotes to such curve, correspondingly to the number of binding sites [9]. In Fig. 9a, two asymptotes correspond to two binding sites. The tangent of the slope angle of the asymptote to the left part of the curve is

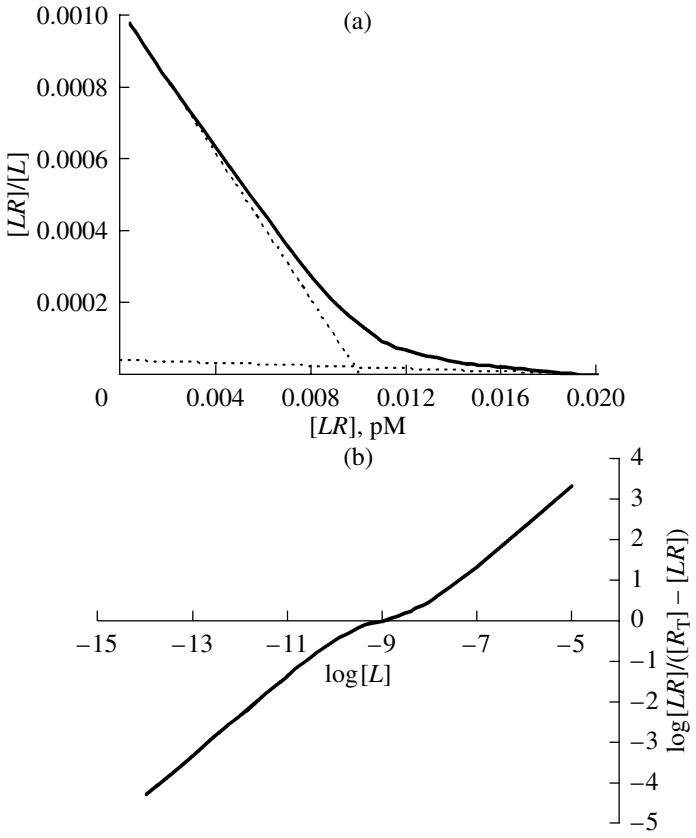

Fig. 9. Example of hormone binding by two receptor sites differing in affinity (K_{D1} = 0.1 nM, K_{D2} = 5 nM).
(a) Binding data expressed in Scatchard plot. [*LR*] ([*B*]) and [*L*] ([*F*]) are the concentrations of bound and unbound hormone, respectively. Dotted lines designate asymptotes for graph branches; (b) binding data expressed in Hill coordinates [39]. [LR] ([B]) is the concentration of bound hormone; [R_T] is the concentration of ligand-binding sites.

equal to $-1/K_{D1} - 1/K_{D2}$, whereas the abscissa intercept corresponds to $[R_{T1}]$. The tangent of the slope angle of the asymptote to the right part of the curve is equal to $-1/K_{D2}$, whereas the abscissa intercept corresponds to $[R_{T1}] + [R_{T2}]$. Using the method of Rosenthal [32] or computer programs, it is possible to determine parameters for each interaction more precisely.

However, such phenomenon could have also other origin. Let us imagine that polyvalent receptor attaches successively ligands according to the following scheme:

$$L + R \rightleftharpoons LR; \quad L + LR \rightleftharpoons L_2R; \text{ and so on.}$$

Initial binding of ligands with receptor could increase (positive cooperativity) or reduce (negative cooperativity) the affinity of interaction with successive ligands. In the case of negative cooperativity, Scatchard curve would bend downward (like in the presence of two sites for binding), and in the case of positive cooperativity, the curve would bend upward. The problem arises how to discriminate the cases of negative cooperativity and of several types of binding sites. To solve this problem, there are special methods of the analysis.

The first one is the treatment of binding data in Hill coordinates [39], which permit estimation of the cooperativity sign and degree. The degree of cooperativity shows how much the affinity constant increases (or decreases) for each following ligand in comparison with initial binding. Considering the system with pronounced positive cooperativity, Hill expressed the interaction of polyvalent receptor with ligand as follows:

$$nL + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} L_nR. \tag{12}$$

In this case, the equation for K_D is described as follows:

$$K_D = \frac{[L]_n([R_T] - [L_nR])}{[L_nR]}. \tag{13}$$

After finding logarithm of equation (13), we obtain the following dependence:

$$\log\frac{[L_nR]}{[R_T] - [L_nR]} = n\log[L] - \log K_D. \tag{14}$$

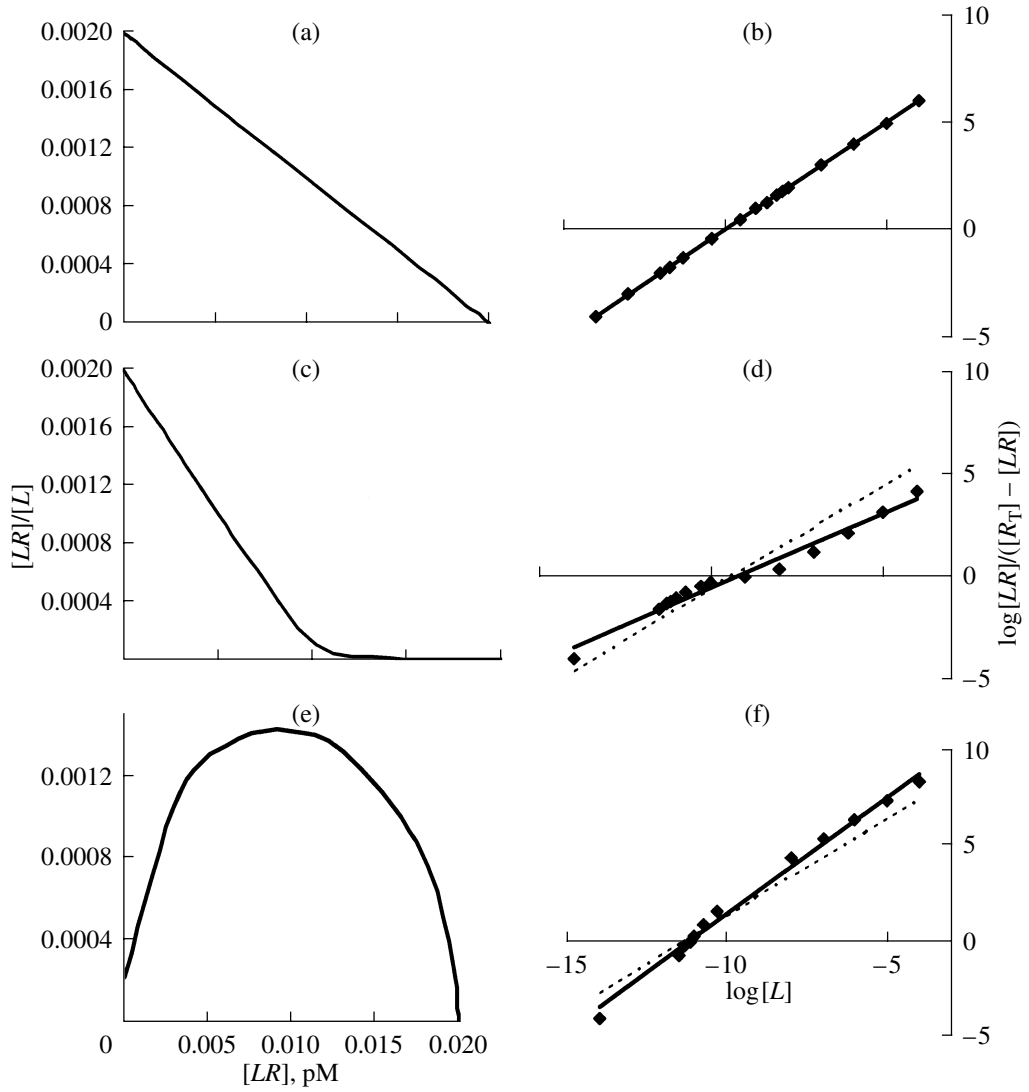

Fig. 10. Effect of cooperativity on hormone binding by receptor.
(a, b) No cooperativity; (c, d) negative cooperativity ($K_{D1} = 0.1$ nM, $K_{D2} = 7$ nM); (e, f) positive cooperativity ($K_{D1} = 0.1$ nM, $K_{D2} = 0.5$ pM). (a, c, e) Scatchard coordinates; (b, d, f) Hill coordinates. Designation as in Fig. 9. Dotted lines in (d) and (f) correspond to the absence of cooperativity.

The graph in coordinates $\log([L_nR]/[R_T] - [L_nR])$ and $\log[L]$ (equation (14)) represents a straight line, and the tangent of its slope angle (n) reflects the degree of apparent cooperativity (γ) (Fig. 10). The values $\gamma > 1$ indicate a positive cooperativity and $\gamma < 1$, negative one [9]. When $\gamma \approx 1$, there is no noticeable cooperativity. The abscissa intercept is $\log K_D$. When two binding sites occur, the line has some bend near the abscissa axis (Fig. 9b). Figure 11 presents data on *trans*-zeatin binding by cytokinin receptor in a Hill plot. It can be seen that $\gamma \approx 1$, indicating the absence of any noticeable cooperativity in this interaction.

To choose between negative cooperativity and the presence of several binding sites differing in their affinity for ligand, coordinates could be transformed by the method of Björrum [9]. Similarly to the previous case, the ligand saturation experiment are taken to analysis. Under equilibrium condition in the system, the concentration of ligand–receptor complex is described by equation (8), hence

$$\frac{[LR]}{[R_T]} = \frac{[L_T]}{K_D + [L_T]} = y \text{ and } K_D = \frac{[L_T](1-y)}{y}.$$

Finding the logarithm of this equality, we deduce the following equation:

$$-\log[L_T] = -\log K_D + \log\frac{1-y}{y}. \qquad (15)$$

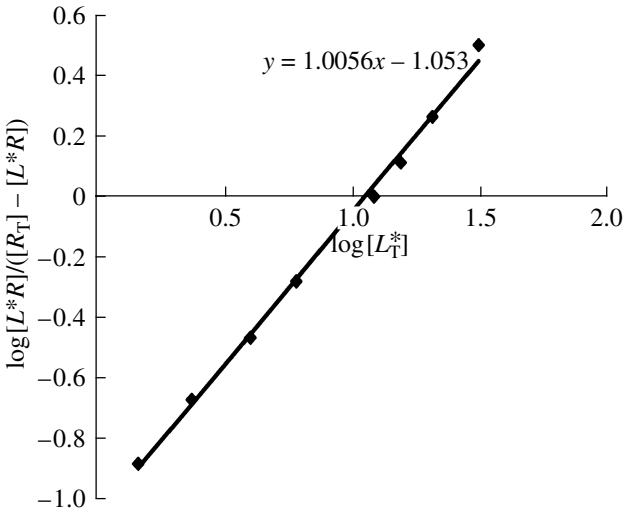

Fig. 11. Data on labeled *trans*-zeatin binding to the cytokinin receptor AHK4 plotted in Hill coordinates.
[*L*R*] is the concentration of bound hormone; $[R_T]$ is the concentration of ligand-binding sites, $[L^*_T]$ is total hormone concentration. The mathematical function is presented, which approximates this dependence ($r = 0.9965$).

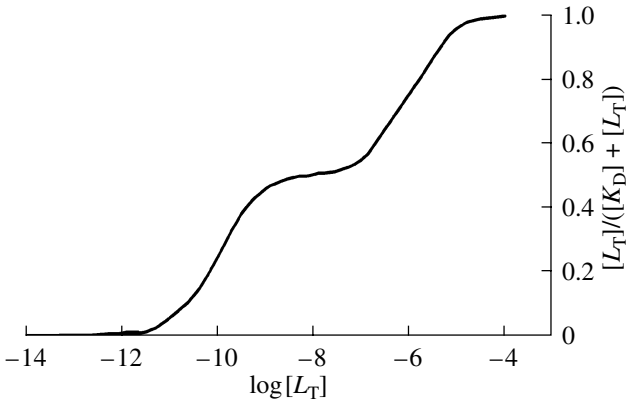

Fig. 12. Example of hormone binding by two receptor sites differing in affinity ($K_{D1} = 0.1$ nM, $K_{D2} = 1$ μM) plotted in Björrum coordinates [9].
$[L_T]$ is a total hormone concentration.

The graph is built in coordinates y, $\log[L_T]$. This is nonlinear dependence, and the number of the curve bends in Björrum coordinates corresponds to the number of types of binding sites in the system (Fig. 12). The abscissa of the point of bending corresponds to $\log K_D$. This method permits identification of two and more sites of ligand binding to receptor, however, only in the case when constants differ sufficiently (more than 500 times) and the differences in the binding site concentrations are in contrast small (less than order of magnitude).

CONCLUSION

Modern methodology for the analysis of hormone–receptor interaction is well developed in both theoretical and practical aspects [1, 2, 7–9, 11–18, 32–37, 39–42], that is very promising for the studying of hormonal signal perception by the receptors in eukaryotic cells. This brief methodological review presents theoretical basics and practical recommendations for determination of principal characteristics of interaction between ligands and receptors, such as specific binding, interaction type, affinity constant, the number of receptors, ligand specificity, etc.

The approximate algorithm for a basic study could be as follows. Pilot experiment is performed to ascertain whether specific binding occurs in the system. In the case it does, attempts are made to optimize its testing by both the variation of the conditions for hormone–receptor interaction as well as separation of formed hormone–receptor complexes. Thereafter, kinetic experiment is performed to determine the time necessary to reach equilibrium in the system. Then, under equilibrium conditions, the curve of receptor saturation with hormone is built and the results are analyzed in Scatchard plot. When dependence is found to be close to linear, a simple "ligand–a single binding site" mechanism is suggested. This makes it possible the direct calculation of K_D and the number of binding sites. When dependence is not linear, the analysis in Hill and Björrum coordinates is fulfilled. In the case when the binding curve in the Scatchard plot deviates downward and the tangent of the slope angle in Hill plot is equal to 1, the conclusion can be made that two or more binding sites differing in affinity are present. When the difference in site affinity is big enough, the curve in Björrum coordinates would have a bend(s). When the curve on the Scatchard plot deviates downward and the tangent of the slope angle in Hill coordinates is less than 1, the conclusion on negative cooperativity can be made. Upward curve deviation in the Scatchard plot together with the slope angle tangent in the Hill plot exceeding 1 are unambiguously indicative of positive cooperativity. After determining the type of receptor interaction with labeled ligand, experiments with its unlabeled analogs, structural and/or functional, could be performed in order to evaluate ligand specificity of the receptor. The analysis of labeled ligand displacement with its unlabeled homolog permits confirmation or more precise determination of the affinity constant, which was obtained previously in saturation experiments. The pattern of displacement curves and data from reverse coordinate plot would provide information required for determination of the type of interaction between labeled and unlabeled ligands on receptor. In general, results of all experimental series described above permit complete and precise characterization of the interaction between the receptor under study and its ligands.

At present, this field of knowledge is so immense that it is not possible to embrace it completely in a single journal paper. Therefore, we did not mention deliberately, for example, kinetic investigations; more complex models of ligand–receptor interaction; other ways to transform and analyze the binding data; the influence of some effectors like G-proteins; some particular types of interactions between agonists and antagonists, and other questions. These particular issues are analyzed in specialized literature cited in the Reference section of this review [1, 2, 7–9, 11–18, 32–37, 39–42]. In addition, new physical methods for analysis of interacting macro- and micromolecules, such as positron emission tomography, high-throughput assay with using of biochips (microarrays), surface plasmon resonance, and others are being actively developed. We hope that, in the nearest future, these methods would serve as a good supplement and more safe and convenient replacement for the experimental techniques described above. However, regardless of the further progress in testing methodology, basic principles and regularities of hormone–receptor interaction that we exemplified using the radioligand method, retain their significance in future studies.

ACKNOWLEDGMENTS

The authors are grateful to Prof. A.N. Smirnov for constructive analysis of the manuscript and valuable remarks.

This work was supported by the Russian Foundation for Basic Research, project nos. 04-04-49120, 07-04-00331, and 07-04-91211-JaF.

REFERENCES

1. Cuatrecasas, P., Criteria for and Pitfalls in the Identification of Receptors, *Adv. Cyclic Nucl. Res.*, 1975, vol. 5, pp. 79–89.
2. *Vzaimodeistvie gormonov s retseptorami* (Interaction of Phytohormones with Their Receptors), Levi, D.S., Ed., Moscow: Mir, 1979.
3. Romanov, G.A., Hormone-Binding Proteins of Plants and the Problem of Perception of Phytohormone, *Sov. Plant Physiol.*, 1989, vol. 36, pp. 136–146.
4. Romanov, G.A., The Phytohormone Receptors, *Russ. J. Plant Physiol*, 2002, vol. 49, pp. 552–560.
5. Smirnov, A.N., *Elementy endokrinnoi regulyatsii* (Elements of Endocrine Regulation), Moscow: GEOTAR-Media, 2006.
6. Langley, J.N., On the Physiology of the Salivary Secretion. II. On the Mutual Antagonism of Atropine and Pilocarpin, Having Especial Reference to Their Relations in the Sub-Maximally Gland of the Cat, *J. Physiol.*, 1878, vol. 1, pp. 339–369.
7. Galaktionov, S.G., Golubovich, V.P., Shenderovich, M.D., and Akhrem, A.A., *Vvedenie v teoriyu retseptorov* (Introduction to the Receptor Theory), Minsk: Nauka i Tekhnika, 1986.
8. Sergeev, P.V. and Shimanovskii, N.L., *Retseptory fiziologicheski aktivnykh veshchestv* (Receptors for Physiologically Active Compounds), Moscow: Meditsina, 1987.
9. Varfolomeev, S.D. and Gurevich, K.G., *Biokinetika: Prakticheskii kurs* (Biokinetics: Practical Course), Moscow: FAIR-PRESS, 1999.
10. Gomperts, B.D., Kramer, I.M., and Tatham, P.E.R., *Signal Transduction*, San Diego: Academic, 2003.
11. Varfolomeev, S.D. and Zaitsev, S.V., *Kineticheskie metody v biokhimicheskikh issledovaniyakh* (Kinetic Methods in Biochemical Investigations), Moscow: Mosk. Gos. Univ., 1982.
12. Scatchard, G., The Attraction of Protein for Small Molecules and Ions, *Ann. New York Acad. Sci.*, 1949, vol. 51, pp. 660–672.
13. Rozen, V.B. and Smirnov, A.N., *Retseptory i steroidnye gormony* (Receptors and Steroid Hormones), Moscow: Mosk. Gos. Univ., 1981.
14. Bylund, D.B. and Yamamura, K., Methods for Receptor Binding, *Methods in Neurotransmitter Receptor Analysis*, Yamamura, K., et al., Eds., New York: Raven, 1990, pp. 1–35.
15. *Receptor Biochemistry – a Practical Approach*, Hulme, E.C., Ed., Oxford: IRL Press, 1990.
16. Bylund, D.B. and Toews, M.L., Radioligand Binding Methods – Practical Guide and Tips, *Am. J. Physiol.*, 1993, vol. 265, pp. L421–L429.
17. McKinney, J., Practical Aspects of Radioligand Binding, *Current Protocols in Pharmacology*, Enna, S.J. and Williams, M., Eds., New York: John Wiley and Sons, 1998, pp. 1.3.1–1.3.33.
18. Kenakin, T.P., *Pharmacologic Analysis of Drug-Receptor Interaction*, New York: Raven, 1993.
19. Muramatsu, I., Tanaka, T., Suzuki, F., Li, Z., Hiraizumi-Hiraoka, Y., Anisuzzaman, A.S.M., Yamamoto, H., Horinouchi, T., and Morishima, S., Quantifying Receptor Properties: The Tissue Segment Binding Method – a Powerful Tool for the Pharmacome Analysis of Native Receptors, *J. Pharmacol.*, 2005, vol. 98, pp. 331–339.
20. Romanov, G.A., Spíchal, L., Lomin, S., Strnad, M., and Schmülling, T., A Live Cell Hormone-Binding Assay on Transgenic Bacteria Expressing a Eukaryotic Receptor Protein, *Anal. Biochem.*, 2005, vol. 347, pp. 129–134.
21. Yamada, H., Suzuki, T., Terada, K., Takei, K., Ishikawa, K., Miwa, K., Yamashino, T., and Mizuno, T., The Arabidopsis AHK4 Histidine Kinase Is a Cytokinin-Binding Receptor That Transduces Cytokinin Signals across the Membrane, *Plant Cell Physiol.*, 2001, vol. 42, pp. 1017–1023.
22. Spíchal, L., Rakova, N.Y., Riefler, M., Mizuno, T., Romanov, G.A., Strnad, M., and Schmülling, T., Two Cytokinin Receptors of *Arabidopsis thaliana*, CRE1/AHK4 and AHK3, Differ in Their Ligand Specificity in a Bacterial Assay, *Plant Cell Physiol.*, 2004, vol. 45, pp. 1299–1305.
23. Romanova, N.A., Romanov, G.A., Rozen, V.B., and Vanyushin, B.F., Characteristics of Interaction of Dexamethazone–Receptor Complexes from Rat Liver with DNA, *Biochemistry* (Moscow), 1979, vol. 44, pp. 419–430.
24. Romanov, G.A. and Vanyushin, B.F., Cytosol Induces Apparent Selectivity of Glucocorticoid Receptor Bind-

ing to Nucleic Acids of Different Secondary Structure, *Biochim. Biophys. Acta*, 1982, vol. 699, pp. 53–59.

25. Romanov, G.A. and Gorskova, V.A., Purification and Comparative Study of the Properties of Glucocorticoid– and Estrogen–Receptor Complexes in Rat Liver, *Biochemistry* (Moscow), 1984, vol. 49, pp. 1–8.
26. Romanov, G.A., Taran, V.Ya., and Venis, M.A., Cytokinin-Binding Protein from Maize Shoots, *J. Plant Physiol.*, 1990, vol. 136, pp. 208–212.
27. Romanov, G.A., Romanova, N.A., Rozen, V.B., and Vanyushin, B.F., Ratliver Glucocorticoid–Receptor Complexes. I. Kinetic and Equilibrium Parameters of Their Interaction with DNA: Influence of Ionic Strength and Temperature, *Mol. Biol. (Moscow)*, 1981, vol. 15, pp. 601–612.
28. Romanov, G.A., Sokolova, N.A., Rozen, V.B., and Vanyushin, B.F., Interaction of Dexamethazone–Receptor Complexes with Nuclei from Rat Liver and DNA, *Biochemistry* (Moscow), 1976, vol. 41, pp. 1733–1741.
29. Chard, T., *An Introduction to Radioimmunoassay and Related Techniques,* Amsterdam: Elsevier, 1979.
30. *Bol'shoi praktikum po fiziologii cheloveka i zhivotnykh* (Manual of Human and Animal Physiology), Kudryashov, B.A., Ed., Moscow: Vysshaya Shkola, 1984.
31. Patrushev, L.I., *Iskusstvennye geneticheskie sistemy* (Artificial Genetic Systems), Moscow: Nauka, 2004, vol. 1.
32. Rosenthal, H.E., A Graphic Method for the Determination and Presentation of Binding Parameters in Complex Systems, *Anal. Biochem.*, 1967, vol. 20, pp. 525–532.
33. Chang, K.J., Jacobs, S., and Cuatrecasas, P., Quantitative Aspects of Hormone–Receptor Interactions of High Affinity. Effect of Receptor Concentration and Measurement of Dissociation Constants of Labeled and Unlabeled Hormones, *Biochim. Biophys. Acta*, 1975, vol. 406, pp. 294–303.
34. Zaitsev, S.V., Sergeeva, M.G., and Varfolomeev, S.D., Radioreceptor Analysis: Theoretical Basics of the Method, *Bioorg. Khim.*, 1985, vol. 11, pp. 370–379.
35. Cheng, Y.C. and Prusoff, W.H., Relationship between the Inhibitor Constant (K_i) and the Concentration of Inhibitor Which Causes 50% Inhibition of an Enzymatic Reaction, *Biochem. Pharmacol. Med.*, 1973, vol. 22, pp. 3099–3108.
36. Blondeau, J.-P., Rocher, P., and Robel, P., Competitive Inhibition of Specific Steroid–Protein Binding: Practical Use of Relative Ratios for the Derivation of Equilibrium Inhibition Constants, *Steroids*, 1978, vol. 32, pp. 563–575.
37. Dixon, M., The Determination of the Enzyme Inhibitor Constants, *Biochem. J.*, 1957, vol. 55, pp. 170–171.
38. Romanov, G.A., Lomin, S.N., and Schmülling, T., Biochemical Characteristics and Ligand-Binding Properties of *Arabidopsis* Cytokinin Receptor AHK3 Compared to CRE1/AHK4 as Revealed by a Direct Binding Assay, *J. Exp. Bot.*, 2006, vol. 57, pp. 4051–4058.
39. Hill, A.V., The Possible Effects of the Aggregation of the Molecules of Hemoglobin on Its Oxygen Dissociation Curve, *J. Physiol.*, 1910, vol. 40, pp. IV–VII.
40. *Receptor Classification*, Trist, D.G., Humphrey, P.P.A., Leff, P., and Shankley, N.P., Eds., New York: New York Acad. Sci., 1997.
41. Gurevich, K.G., Determination of Equilibrium and Kinetic Constants at Changes of Ligand Concentration during Ligand–Receptor Interaction, *Biokhimiya*, 1997, vol. 62, pp. 1221–1224.
42. Bylund, D.B. and Murrin, L.C., Radioligand Saturation Binding Experiments over Large Concentration Ranges, *Life Sci.*, 2000, vol. 67, pp. 2897–2911.