Receptors and
Recognition

Series B Volume 11

Membrane Receptors

Methods for Purification and Characterization

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S. Jacobs
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Wellcome Research Laboratories,
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Receptors and Recognition

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About the series

Cellular recognition – the process by which cells interact with, and respond to, molecular signals in their environment – plays a crucial role in virtually all important biological functions. These encompass fertilization, infectious interactions, embryonic development, the activity of the nervous system, the regulation of growth and metabolism by hormones and the immune response to foreign antigens. Although our knowledge of these systems has grown rapidly in recent years, it is clear that a full understanding of cellular recognition phenomena will require an integrated and multidisciplinary approach.

This series aims to expedite such an understanding by bringing together accounts by leading researchers of all biochemical, cellular and evolutionary aspects of recognition systems. This series will contain volumes of two types. First, there will be volumes containing about five reviews from different areas of the general subject written at a level suitable for all biologically oriented scientists (Receptors and Recognition, series A). Secondly, there will be more specialized volumes (Receptors and Recognition, series B), each of which will be devoted to just one particularly important area.

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Preface

Hardly a decade ago, membrane receptors were an attractive but largely unproven concept. Since that time enormous progress has been made, and we are now able to consider receptors much more concretely. Their existence has been established, their binding properties have been determined, and in some cases, they have been highly purified and their physical-chemical properties studied. It is now even possible to visualize microscopically some receptors. This progress has resulted largely from the development of highly powerful methods. These methods are the subject of this volume.

Although considerably diverse, different receptors share certain common properties, and common problems are encountered in their study. Consequently, a small number of techniques are particularly useful in studying different types of receptors. Thus, it makes sense to speak about membrane receptor methodology.

A very apparent problem in the study of membrane receptors is their presence in exceedingly small quantities and in a highly impure state. Therefore, very sensitive and specific techniques are required for their detection, characterization and purification. Such sensitivity and specificity is provided by the ability of receptors to bind certain ligands with very high affinity, and it is not surprising that most of the methods described in this volume depend upon this high affinity binding. The antigen-antibody interaction is of comparable sensitivity and specificity. Recently, a number of anti-receptor antibodies have been produced or found to occur spontaneously in auto-immune diseases. Undoubtedly, more will be produced in the future. The development of techniques to produce monoclonal antibodies *in vitro* is likely to enhance this trend. This should make the quantitative analytical immunologic methods described by Bjerrum *et al.* increasingly valuable.

Another aspect common to membrane receptors is that they are integral membrane proteins. This presents two problems. First, in order to fully understand their behavior it is necessary to determine their organization and dynamic arrangement within the membrane. Recent studies using the electron-microscopic and fluorescent techniques, described by Schlessinger and Elson, have clearly emphasized the importance of these factors. Second, in order to purify a receptor and to determine several of its physical chemical properties, it is necessary to extract it from the membrane. The chapter by Reynolds describes the scientific basis of the art of solubilizing receptors and characterizing them in the presence of detergents. Not only is it desirable to take receptors out of the membrane, but one of the ultimate goals of membrane receptor research is to reconstitute active

receptor systems from isolated, purified components. Hebdon, *et al.* describe the tools currently available to accomplish this. Another technique which has been particularly useful in analyzing multicomponent receptor systems, and which shortcuts the need for physical separation of components and reconstituting them involves a genetic approach. Johnson *et al.* describe the use of this technique.

In this volume, no attempt has been made to provide exhaustively referenced review articles, nor have procedural details been presented in cook-book fashion in detail sufficient to allow the laboratory worker to actually carry out the procedures. Such detail is available in the cited literature, and will probably vary with the particular system studied. Rather, an attempt has been made to describe the underlying principles behind the various methods, to indicate in which situations they will be most useful, and to point out their potential limitations and drawbacks. We hope this will be of use to readers interested in understanding recent advances in the receptor field, as well as to researchers who wish to apply these methods to the particular receptor system that they are studying.

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1 Receptor Binding Assays

MORLEY D. HOLLENBERG AND EBBA NEXØ

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1.1 INTRODUCTION

The rapid advance in the understanding of the receptor mediated mechanisms, whereby a variety of drugs, hormones and toxins act at the cell membrane, can be attributed in large part to the development of reliable ligand binding assays. Although the methods employed in receptor related studies do not differ in principle from techniques used to measure ligand binding in other biochemical systems, the high affinity, low capacity and strict chemical specificity of pharmacologic receptors have presented a special challenge. Since the landmark studies of Clark (Clark, 1926a, b; 1933), it has been recognized that membrane receptors for drugs or other agents are present in vanishing small numbers (about 10^5 molecules per cell or fewer) compared to the amounts of other constituents in cell membranes. Thus, although in the past sensitive bioassay methods have yielded some limited quantitative data related to drug receptor interactions, precise measurements require the use of highly radioactive biologically active ligand probes. Further, it has been necessary in studies of ligand binding to distinguish 'specific' or receptor related binding from the 'non-specific' binding of radiolabeled ligand to structures other than the receptor. As summarized in a relatively recent review article (Cuatrecasas and Hollenberg, 1976), there are now available a variety of methods for the preparation of suitable ligand probes; further it has been possible to develop a number of criteria to aid in distinguishing between 'specific' and 'non-specific' binding in receptor studies (Hollenberg and Cuatrecasas, 1979). Thus, for the purposes of this chapter, it will be assumed that a suitable ligand probe may be available for a study of interest and that the distinction of receptor from non-receptor binding will not prove a problem. Attention will be focused on the methods that have proved successful in the study of membrane receptors, both in the particulate and soluble state. Since the methods outlined yield information not only about the receptor, but also about the ligand probe, consideration will also be given to the use of receptor preparations for radioreceptor assays.

1.2 GENERAL CONSIDERATIONS

The approach to the study of a receptor of interest can be relatively straightforward. Nonetheless, for each new ligand studied, a few preliminary considerations are often instructive. Firstly, it is important to know that the radioactive ligand probe chosen for study possesses a specific radioactivity sufficient to detect the expected number of receptor sites. As a rule, target cells possess from 10^4 to 10^5 binding sites per cell; in membrane preparations from receptor-rich organs it is not

unreasonable to find binding capacities in the range of 0.2 to 20 pmol mg^{-1} membrane protein. Thus, for a target cell having 10^4 sites per cell, a ligand possessing a specific activity of 10–20 Ci $mmol^{-1}$ would yield a maximum binding of only a few hundred disintegrations per min for a sample size of 10^6 cells; there is thus considerable advantage in working with ^{125}I or ^{131}I-labeled derivatives, having specific activities in excess of 1000 Ci $mmol^{-1}$.

In choosing a method for study, it is also important to consider the rates of ligand binding. For instance, ligands like insulin, with equilibrium dissociation constants lower than 10^{-9} M exhibit dissociation rate constants (k_{-1}) of about 10^{-3} s^{-1}, corresponding to a half-life in excess of 10 min ($T_{½} = 0.693/k_{-1}$) for the ligand–receptor complex. In contrast, for compounds with receptor affinities lower than that of insulin (e.g. K_d 10^{-8} M), but with similar on-rate constants ($k_1 \simeq 10^7$ $M^{-1}s^{-1}$), a half-life of less than 7 s would be predicted for the receptor–ligand complex. Since the dissociation rate can be prolonged up to ten-fold simply by lowering the temperature, it might prove essential in such cases to 'freeze' the binding reaction at equilibrium by rapidly chilling the reaction mixture and performing all subsequent operations at 4°C. Alternatively, studies of ligands with comparatively low receptor affinities might necessitate the use of methods such as equilibrium dialysis or equilibrium gel filtration (Hummel and Dreyer, 1962).

A third consideration in the design of receptor binding assays relates to the integrity both of the receptor and the ligand. Firstly, for radioactively labeled ligands, it is essential to know the proportion of radioactivity that represents the intact active ligand. Ideally, 100% of the radioactivity should be present as the active ligand; however, with iodinated peptides, storage even at low temperatures (−70°C) over comparatively short time periods (2–3 weeks) may not prevent the formation of inactive iodinated peptide fragments. Despite this difficulty, with suitably high concentrations of either antibody or (preferably) receptor, it is possible to estimate the maximum amount of ligand that can be bound, so as to determine the amount of active labeled compound present in a preparation. For instance, with a radioactive ligand present at a concentration of 10^{-10} M, a receptor exhibiting a K_d of about 10^{-10} M should be capable of binding nearly all of the radioactivity ($\geqslant 99\%$), provided the receptor concentration can be raised to about 10^{-8} M. A good example of the use of receptor binding activity to estimate the proportion of active labeled ligand can be seen in studies with gonadotropic hormones (Catt *et al.*, 1976). In such studies, accurate estimates of receptor affinity can be obtained, despite the use of gonadotropin preparations exhibiting less than the maximum attainable biological activity. Failure to determine the proportion of active radioligand present will lead to an overestimate of the amount of 'free' hormone present and an underestimate of the 'bound' hormone concentration, so as to lead to serious errors in the estimates of ligand affinities by any chosen method of data analysis (e.g. see Chapter 2).

A second problem connected with the integrity of the ligand and its receptor concerns the degradation of both species that can occur during the course of binding

studies. Because of potent proteases that are present in both intact cell and purified membrane–receptor preparations, binding studies performed at 37°C may greatly underestimate both the ligand affinity and the ligand binding capacity. Furthermore, storage of membrane preparations, even at 0°C, can lead to receptor degradation that is not blocked by conventional inhibitors of proteolytic enzymes. The extent of ligand degradation during the binding assay can be assessed either by estimating the ability of unbound ligand to bind to a second aliquot of membranes or by examining other physical characteristics of the unbound ligand (e.g. precipitability with trichloroacetic acid, gel filtration, electrophoretic or chromatographic mobility). The integrity of the receptor may be estimated by measuring the binding of receptor previously incubated in the absence of ligand under the binding assay conditions. Fortunately, in many instances, experiments done at about 24°C appear to minimize ligand and receptor degradation, and permit sufficiently rapid time courses of binding equilibrium.

A concern in membrane receptor studies that is encountered only infrequently in studies with enzymes relates to the absolute concentration of receptor present in a test system. Because of the high ligand affinity of many receptors ($K_d \simeq 10^{-9}$ M), it is possible, in certain instances, to achieve receptor concentrations equal to or even greater than the ligand equilibrium dissociation constant. In such instances, receptor concentration must be taken into account for design of experiments and for the analysis of binding data. For enzyme kinetics, an analogous situation is seen in the so-called 'zone behaviour' of enzymes in the presence of inhibitors (Straus and Goldstein, 1943).

Provided the ligand–receptor interaction obeys a simple mass action relationship (see, however, Chapter 2), the equilibrium dissociation constant, K_d, will be given by the equation:

$$K_d = \frac{[L] \times [R]}{[RL]},$$

wherein $[L]$, $[R]$, and $[RL]$ represent the concentrations of *free* ligand, the unoccupied receptor concentration and the ligand–receptor complex concentration respectively. Provided the concentration of *free* ligand and of ligand–receptor complex can be measured accurately, the K_d can be determined according to a number of rearrangements of the above equilibrium equation. For instance, a plot of *free* ligand concentration versus the concentration of bound ligand yields the K_d as the abscissa value for which binding is half-maximal. The K_d can be estimated directly from such binding isotherms.

Alternatively, as is frequently done, the data can be analysed according to the method of Scatchard (1949) (Fig. 1.1). Hypothetical binding curves based on the above equations are illustrated in Fig. 1.1, along with the corresponding Scatchard plots of the data. Although in principle the concentration of receptor should not affect the values obtained by this plot of the data, practically, the receptor concentration will have a bearing on experiments designed to obtain data from

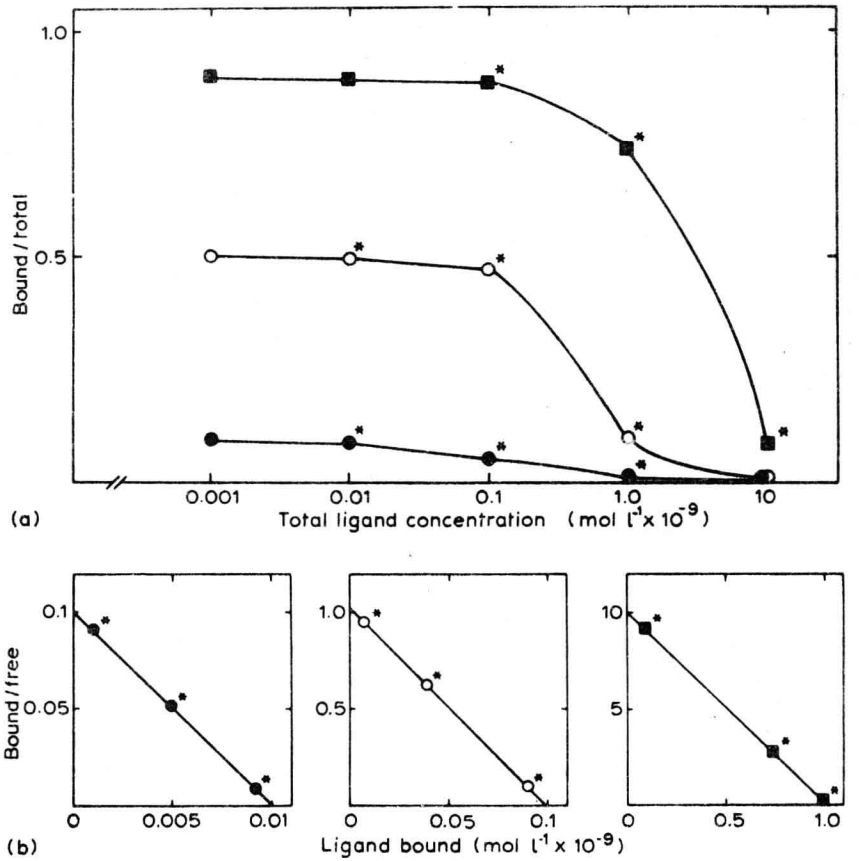

Fig. 1.1 Theoretical binding data and Scatchard Plots for studies with receptor concentrations ranging from 1/10 to 10 times the K_d. (a) Fraction of radioligand bound (ordinate) at ligand concentrations ranging from 0.001 to 10 nM (abscissa, logarithmic scale). A receptor K_d of 0.1 nM is assumed and data are calculated from the mass action equation for receptor concentrations of : 1.0×10^{-9} mol^{-1} (■–■); 0.1×10^{-9} mol^{-1} (○–○); 0.01×10^{-9} mol^{-1} (●–●). (b) Data points indicated by asterisks (*) were used to plot the data according to Scatchard (1949).

Scatchard plots. Ideally, to obtain the K_d for a ligand interaction, data should be obtained over a range of total ligand concentrations sufficient to saturate between about 10% and 90% of the receptor. As a rule of thumb this concentration range corresponds to values from 1/10 to 10 times the magnitude of *either* the K_d *or* the receptor concentration, depending on whichever value is the larger; the consequence of increasing receptor concentration, in relation to the increased total ligand