RECEPTOR BIOCHEMISTRY AND METHODOLOGY SERIES

RECEPTOR LOCALIZATION

LABORATORY METHODS AND PROCEDURES

edited by MARJORIE A. ARIANO

RECEPTOR LOCALIZATION

Laboratory Methods and Procedures

Edited by

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SERIES PREFACE

The activation of cell surface receptors serves as the initial step in many important physiological pathways, providing a mechanism for circulating hormones or neurotransmitters to stimulate intracellular signaling pathways. Over the past 10–15 years, we have witnessed a new era in receptor research, arising from the application of molecular biology to the field of receptor pharmacology. Receptors can be classified into families on the basis of similar structural and functional characteristics, with significant sequence homology shared among members of a given receptor family. By recognizing parallels within a receptor family, our understanding of receptor-mediated signaling pathways is moving forward with increasing speed. The application of molecular biological tools to receptor pharmacology now allows us to consider the receptor–ligand interaction from the perspective of the receptor as a complement to the classical approach of probing the binding pocket from the perspective of the ligand.

Against this background, the newly launched Receptor Biochemistry & Methodology series will focus on advances in molecular pharmacology and biochemistry in the receptor field and their application to the elucidation of the mechanism of receptor-mediated cellular processes. The previous version of this series, published in the mid-1980s, focused on the methods used to study membrane-bound receptors at that time. Given the rapid advances in the field over the past decade, the new series will focus broadly on molecular and structural approaches to receptor biology. In this series, we interpret the term "receptor" broadly, covering a large array of signaling molecules including membrane-bound receptors, transporters and ion channels, as well as intracellular steroid receptors. Each volume will focus on one aspect of receptor biochemistry and will contain chapters covering the basic biochemical and pharmacological properties of the various receptors, as well as short reviews covering the theoretical background and strategies underlying the methodology. We hope that the series will provide a valuable overview of the status of the receptor field in the late 1990s, while also providing information that is of practical utility for scientists working at the laboratory bench. Ultimately, it is our hope that this series, by pulling together molecular and biochemical information from a diverse array of receptor fields, will facilitate the integration of structural and functional insights across receptor families and lead to a broader understanding of these physiologically and clinically important proteins.

David R. Sibley
Catherine D. Strader

PREFACE

The detection of receptor locations and the density of distribution within the body, and the nervous system in particular, have received intensive interest. This volume is intended to be a "user-friendly" guide to numerous approaches that have been designed recently to examine various receptor systems. The contributors to this book are leaders in their scientific fields and study a range of receptor subtypes (nicotine, muscarine, tackykinins, dopamine, adenosine, GABA). The standard laboratory "recipes," tricks employed in these detection methods, and advantages and limitations of each procedure are discussed by the contributors and are illustrated photographically. Many of these techniques are amenable to dual localization of different receptor systems or can detect multiple aspects in the biosynthetic pathway of receptors, i.e., mRNA transcripts with protein, protein with binding, and protein and protein.

Individual chapters describe specific scientific questions that can be posed and answered using these approaches and cover a broad spectrum of current neuroscience research. Leading the discussions are chapters on receptor ligand binding methods, detected using irreversible (Sorenson and Chiappinelli) and reversible (Maggio and Mantyh) compounds that are labeled using radioisotopes or fluorescent moieties (Ray and Ariano). These are followed by an extensive presentation of antireceptor antisera technology using synthetic peptides (Petralia and Wenthold) and fusion proteins (Gilmor, Rouse, Heilman, Nash, and Levey) at the cellular (Swanson and Rivkees) and subcellular (Yi and Hersch) resolution levels. Physiological analyses of receptor function and cellular detection in the brain slice (Levine, Cepeda, Colwell, Yu, and Chandler) and cultured neurons (Rayport) describe novel visualization paradigms. The next series of chapters deals with molecular assessments of receptors and describes *in situ* hybridization (Chesselet), reverse transcriptase–PCR (Yan, Vrana, Vrana, Song, and Surmeier), and fluorescent *in situ* transcription (Noblett and Ariano). The final chapter focuses on the use of PET and SPECT to assess *in vivo* receptor distributions in animals and man (Gatley, Gifford, Logan, Volkow, and Fowler).

In closing, I would like to thank all the contributors to this volume for providing lucid descriptions of their methods and providing experimental examples to substantiate what might otherwise be a very staid, descriptive work. I hope this book provides useful information for novice and seasoned investigators in their quest for receptor expression patterns.

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CHAPTER 1

AUTORADIOGRAPHY OF IRREVERSIBLE LIGANDS/TOXINS
NICOTINIC ACETYLCHOLINE RECEPTORS

EVA M. SORENSON and VINCENT A. CHIAPPINELLI

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1. INTRODUCTION

1.A. General Principles of Autoradiography

Receptor autoradiography is the localization of radioactive ligands bound to specific receptors in tissue sections. The distribution of the radioligand in the tissue is mapped when the energy emitted from the radioactive molecules collides with nuclear emulsion or film opposed to the tissue section. The autoradiograms thus generated provide a detailed localization of specific receptors or other molecules of interest. The resolution of the binding can be to specific brain regions, individual cells, or even subcellular structures, depending on the techniques used to

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generate the autoradiograms. In addition, autoradiography is very sensitive, allowing the detection of low levels of receptors in specific cells. This chapter presents the use of irreversible ligands in receptor autoradiography based on studies of nicotinic receptor localization with α-bungarotoxin and κ-bungarotoxin (also called *neuronal bungarotoxin* in the literature) as examples.

α-Bungarotoxin has been used extensively in studies of muscle and neuronal nicotinic receptors, including autoradiographic studies (Porter and Barnard, 1975; Fertuck and Salpeter, 1976; Swanson et al., 1983; Clarke et al., 1985; Sorenson and Chiappinelli, 1992). It binds with high affinity to muscle receptors, all of which have α1 nicotinic receptor subunits, and to neuronal receptors containing the α7, α8, and/or α9 subunits. (For a review of neuronal nicotinic receptors, see Sargent [1993].) The α7-containing receptor is currently the focus of much research, since it has been found to be a functional nicotinic receptor with a significant permeability to calcium (Vernino et al., 1992; Mulle et al., 1992; Mollard et al., 1995; Trouslard et al., 1993). However, the physiological role of most α7-containing receptors is still not clear. κ-Bungarotoxin is structurally related to α-bungarotoxin, but has a different pharmacological spectrum. It binds most potently to α3-containing neuronal nicotinic receptors, which are not recognized by α-bungarotoxin. The α3 subunit is part of the receptor mediating nicotinic neurotransmission in autonomic ganglia. κ-Bungarotoxin binds with lower affinity to other nicotinic receptor subtypes (Papke et al., 1993). (For a review of toxins affecting nicotinic receptors, see Chiappinelli, [1993].) Other ligands that have been used for nicotinic receptor autoradiography include ^{3}H-nicotine, ^{3}H-acetylcholine, ^{3}H-methylcarbamylcholine, ^{3}H-cytisine, and ^{3}H-epibatidine (Clarke et al., 1985; Perry and Kellar, 1995; Abood and Grassi, 1986; Rubboli et al., 1994). These ligands bind reversibly and require the use of techniques that minimize receptor dissociation and diffusion from the binding site. They are specific for the high-affinity nicotinic receptors containing α4 or α2 subunits. Although all subtypes of nicotinic receptors bind nicotine by definition, at 2–4 nM ^{3}H-nicotine binds preferentially to the high-affinity sites on α4- and α2-containing nicotinic receptors. These high-affinity nicotine sites do not bind α-bungarotoxin or κ-bungarotoxin. The different classes of nicotinic receptors can therefore be differentially localized depending on the concentration and relative affinities of the ligands used (Fig. 1.1). It is useful to compare the localization of different receptors by comparing the binding of individual ligands in adjacent sections of tissue (Clarke et al., 1985; Watson et al., 1988; Schulz et al., 1991; Sorenson and Chiappinelli, 1992) (Fig. 1.1).

Several principles are important when doing *in vitro* autoradiography. The first are those governing general receptor ligand binding studies. An appropriate radioligand with high specific activity, having saturable binding and pharmacological specificity for the receptor of interest, must be available. It is important to understand the specificity of the radioligand to correctly interpret the autoradiograms. Many ligands bind to more than one site. In this case, the radioligand may still be used if one of the sites can be masked by a cold ligand. This is true for κ-bungarotoxin. It binds to two classes of nicotinic receptor but can be used to specifically localize the α3 site if the α7 site is blocked by cold α-bungarotoxin. The specificity of the radioligand needs to be determined in both biochemical and autoradiographic experiments, as is described below. A ligand

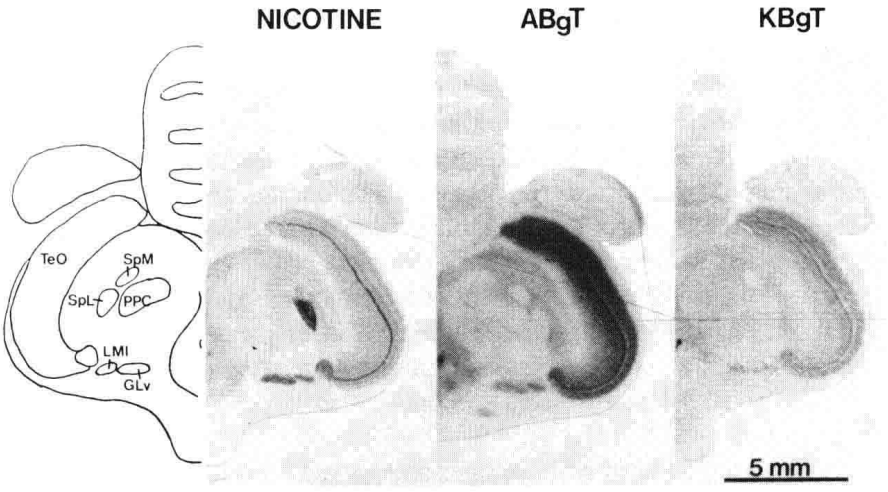


Figure 1.1. An autoradiographic comparison of ^{3}H-nicotine, ^{125}I-α-bungarotoxin (ABgT), and ^{125}I-κ-bungarotoxin (KBgT) binding in adjacent sections of the chicken forebrain. The nucleus spiriformis lateralis was unique because it contained a very high density of ^{3}H-nicotine sites but no ^{125}I-toxin sites. Specific conditions for the incubations were as follows: *^{3}H-nicotine*: Incubations and washes were done at 4°C. All sections were preincubated for 5 minutes in 50 mM Tris-HCl buffer containing 8mM $CaCl_2$. They were then incubated for 30 minutes in buffer containing 2 nM ^{3}H-L-nicotine (*N*-methyl-^{3}H, 70-80 Ci/mmol; New England Nuclear). To assess nonspecific binding, 10 μM L-nicotine bitartrate was included in the incubation solutions for a control group of sections. Following incubation, the sections were washed in buffer three times for 5 seconds and then dipped in distilled water. *^{125}I-α-bungarotoxin, ^{125}I-κ-bungarotoxin*: Sections were preincubated in 50 mM Tris-HCl buffer containing 1mg/ml bovine serum albumin for 30 minutes. They were then incubated for 1 hour in buffer containing 0.5 nM ^{125}I-α-bungarotoxin (430–840 Ci/mmol) or ^{125}I-κ-bungarotoxin (200–650 Ci/mmol). The sections were given three 10 minute washes and rinsed in distilled water. Nonspecific binding of ^{125}I-α-bungarotoxin was determined by adding 1 μM α-bungarotoxin or 1 mM nicotine bitartrate to the preincubation and incubation buffers used for a control group of near-adjacent sections. Similarly, 1 μM α-bungarotoxin, 1 μM κ-bungarotoxin, or 1 mM nicotine bitartrate was added to the preincubation and incubation buffers of a control group of near-adjacent sections treated with ^{125}I-κ-bungarotoxin. GLv, nucleus geniculatus lateralis, pars ventralis; LMl, nucleus lentiformis mesencephali, pars lateralis; PPC, nucleus principalis precommissuralis; SpL, nucleus spiriformis lateralis; SpM, nucleus spiriformis medialis; TeO, tectum opticum. (Reproduced from Sorenson and Chiappinelli [1992], with permission of the publisher.)

also may be a radiolabeled antibody specific for the protein of interest. Antibodies against specific nicotinic receptor subunits have been iodinated and used in autoradiography (Swanson et al., 1987; Watson et al., 1988). If it is a monoclonal antibody, tritium can be incorporated into it by providing a tritiated amino acid, usually lysine, in the hybridoma culture medium (Cuello and Milstein, 1981). Another consideration is the rate of dissociation of the radioligand away from the receptor and its diffusion into the tissue. If the radioligand is allowed to diffuse away from its binding site, a false distribution of receptor sites will be generated. Sections are usually dried with a stream of cool, desiccated air after

incubation with the ligand, and film rather than emulsion is used to detect binding to help prevent dissociation. The use of irreversible ligands eliminates the problem of radioligand diffusion away from its binding site. Specific techniques in dealing with diffusible ligands are addressed in Chapter 2 (this volume).

Along with the principles of ligand binding, autoradiography requires an understanding of radioisotopes, nuclear emulsion or film, and quantitation techniques. The resolution of the autoradiographs obtained depends on the choice of radioligand, the proximity of the film or emulsion to the tissue section, and the size of the silver halide crystals in the film or emulsion. Higher resolution will result when tritiated ligands are used with an emulsion of fine silver halide crystals. Quantitation of the binding from the autoradiographs requires the appropriate use of radioactive standards, optical densitometry, and/or counting of silver grains.

Two commonly used radioisotopes are ^{125}I and ^{3}H. Tritium produces low-energy β particles that do not penetrate further than 5 μm in tissue. Therefore, the uniform thickness of the sections above 5 μm is not crucial since only the labeling on the very surface of the section will be recorded on the film. The resolution is high since the particles do not travel far and will collide with the film or emulsion while still close to their source. The lower energy emissions have two disadvantages: The exposure times needed are longer, and they are differentially absorbed, or quenched, by cellular gray tissue and myelinated white matter of the nervous system. The regional differences in quenching can make it more difficult to quantitate the binding, and either defatting of the sections can be tried or a quenching factor can be determined and used in the quantitation. With the higher energy emissions from ^{125}I, ^{14}C, and ^{35}S, quenching is not a problem. The thickness of the section, on the other hand, must be very uniform to prevent errors in quantitation. Thinner sections are preferable with ^{125}I-ligands because the high-energy γ-rays they emit penetrate the tissue much further from the source, reducing the resolution of the autoradiograms. The advantage of the higher energy emitting isotopes is that the exposure times are on the order of hours to days as opposed to weeks to months.

The proximity of the nuclear film or emulsion determines the resolution of the autoradiograms. The easiest method of producing autoradiograms is to tightly oppose the tissue sections next to autoradiographic film in cassettes. Films designed for use with ^{3}H do not have a protective anti-scratch coating, since this would prevent the low-energy particles from reaching the silver halide crystals. The use of film gives a regional localization of the binding and does not present problems of ligand diffusion. To obtain resolution at the single-cell level, emulsion techniques need to be used. For irreversible ligands, slide-mounted sections can be dipped in liquid emulsion. When *in vivo* autoradiographic studies are performed, the sections can be directly thaw-mounted onto emulsion-coated slides or film. The size of the silver halide crystals in the emulsion impacts on the resolution. Larger crystals need shorter exposure times but will give less resolution, while smaller crystals produce a higher resolution but require longer exposure times. Commercially available films and emulsions state the type of radioisotopes to be used. Quantitation of autoradiographs is discussed in Section 4.A.

1.B. Advantages and Disadvantages of Autoradiography

Autoradiography gives very detailed information about the distribution of specific receptors and proteins within a tissue that is not possible to obtain with data from tissue homogenates. Receptor distribution also can be determined using immunohistochemical methods when appropriate antibodies are available. The interested reader is referred to Chapter 4 (this volume). When antireceptor antisera are not available, autoradiography may be the localization technique of choice. In addition to localization, pharmacological studies may use film autoradiography to compare the binding characteristics of sites in different regions of the same section to examine the possibility that subclasses of receptors are distributed differently.

Autoradiography determines levels of a receptor protein rather than the levels of receptor mRNA within a cell as with *in situ* hybridization studies (see Chapter 10, this volume). Changes in receptor levels can be quantitated between normal and pathological or experimental conditions, as has been found for the regulation of the nicotinic receptor subunits (Peng et al., 1994). When autoradiograms are exposed for long periods of time, the film saturates in areas of high receptor density, but those areas having very low densities of receptors will be detected, making autoradiography a technique with great sensitivity. In combination with lesioning studies, autoradiography can determine whether the protein is localized to distant axon terminals as well as to the cell body. For example, several nicotinic receptor subunit transcripts are expressed in the dopaminergic neurons of substantia nigra pars compacta (Boulter et al., 1987, 1990; Wada et al., 1989). Autoradiographic studies have shown that lesioning dopaminergic neurons of the nigrostriatal pathway produces significant decreases in the nicotinic receptor-binding levels in the striatum, indicating that some portion of the nicotinic receptor proteins synthesized in the nigral dopaminergic neurons are transported to their axon terminals (Clarke and Pert, 1985).

1.C. General Procedure and Equipment Needed

The general procedure is outlined in Table 1.1. Tissue sections are obtained by decapitating an animal under appropriate anesthesia and quickly removing its brain. Alternatively, the animal may be perfused through the heart with cold saline with or without low concentrations of fixative, prior to removal of the brain. However, fixation may alter binding of the radioligand, and loss of antigenicity of nicotinic receptors to some antibodies has been reported with concentrations of formaldehyde above 2% (Bravo and Karten, 1992). The dissected brain is frozen on dry ice or cold isopentane. Frozen tissue sections, usually 5–20 μm in thickness, are cut on a cryostat. The temperature of the cryostat should be adjusted to the thickness of section being cut to prevent chattering the blade across the section. It is important to have good sections since this will determine the quality of the results. The sections are thaw-mounted onto gelatin subbed slides, allowed to dry at room temperature, and then stored in slide boxes put inside of zipper freezer bags at –20°C or below. If possible, avoid

TABLE 1.1. Procedure for Developing an *In Vitro* Receptor Autoradiography Protocol

Prepare tissue sections
Incubate tissue sections with appropriate ligands
Count radioactivity on sections
Generate initial autoradiograms
Maximize specific binding—wash, preincubation and incubations times
Quantitate autoradiograms

storage in frost-free freezers, as these have brief heating cycles that will destroy the tissue. Nicotinic receptor binding is stable for weeks but appears to deteriorate after approximately 6 months. Repeated freezing and thawing should be avoided since this degrades the tissues.

The binding of the radioligand to duplicate tissue sections is accomplished by incubation of the slides in coplin staining jars with a concentration of the radioligand near the K_d (i.e., total binding). Nonspecific binding of irreversible radioligands is determined by preincubating in the appropriate cold ligand followed by incubation with cold and radiolabeled ligands together. Each section used for total binding of ligand should have a matching adjacent or near-adjacent section in which nonspecific binding is assessed. If the availability of the ligands is limited, the incubation solution can be applied in a large drop on top of individual sections. The sections are washed in cold buffer, rinsed briefly in distilled water, and dried with a stream of cool, desiccated air. One set of the sections is scraped from the slides into scintillation vials, dissolved, and counted in a scintillation counter or a gamma counter, depending on the isotope used in the analysis. If the results indicate that there is sufficient specific binding, the remaining sections are stored overnight in a desiccator under vacuum, the sections are loaded into film cassettes along with standards for quantitation, and put in a safe, undisturbed place for exposure. Alternatively, the slides can be dipped in emulsion and stored in slide boxes with desiccant packets. The film or emulsion should be developed according to the manufacturer's directions initially, after which the parameters can be adjusted as needed. After development the film is stored in protective plastic sleeves.

The equipment needed for autoradiography can be easily obtained and includes

Subbed slides: Acid–cleaned slides are dipped in a 0.5% gelatin and 0.05% chrome alum solution. (The solution is heated to dissolve the gelatin, filtered, and cooled to room temperature prior to dipping the slides.)

Slide boxes for storage of sections

Coplin jars and ice bath

Desiccant for drying air stream

Scintillation vials and solution
Tissue solubilizer
Desiccator with vacuum cock
Film cassettes and black slide boxes
Nuclear film or emulsion
Photographic developer and fixer
Isotope standards

In addition, access to the following major equipment is needed:

Cryostat
Freezer
Darkroom
Scintillation and gamma counters
Imaging system for determining optical density
Microscope

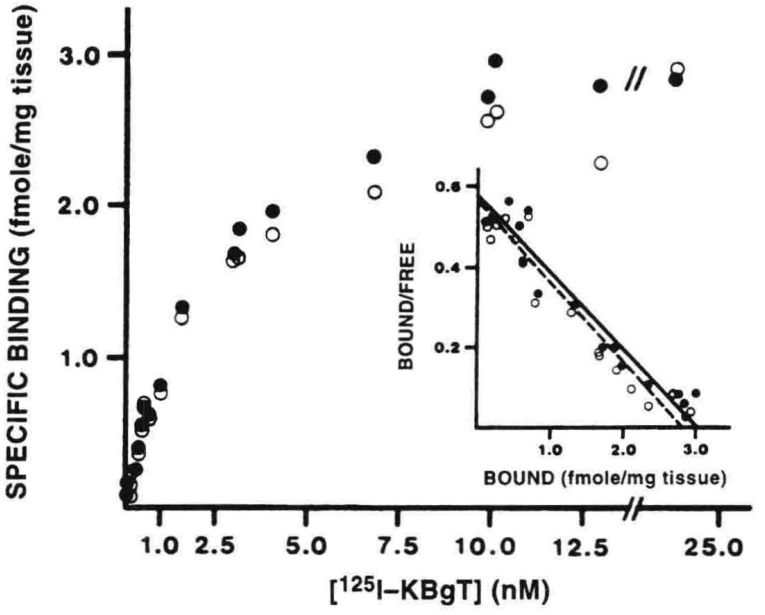
2. DETERMINING AN INCUBATION PROTOCOL

2.A. Preliminary Binding Studies in Homogenates

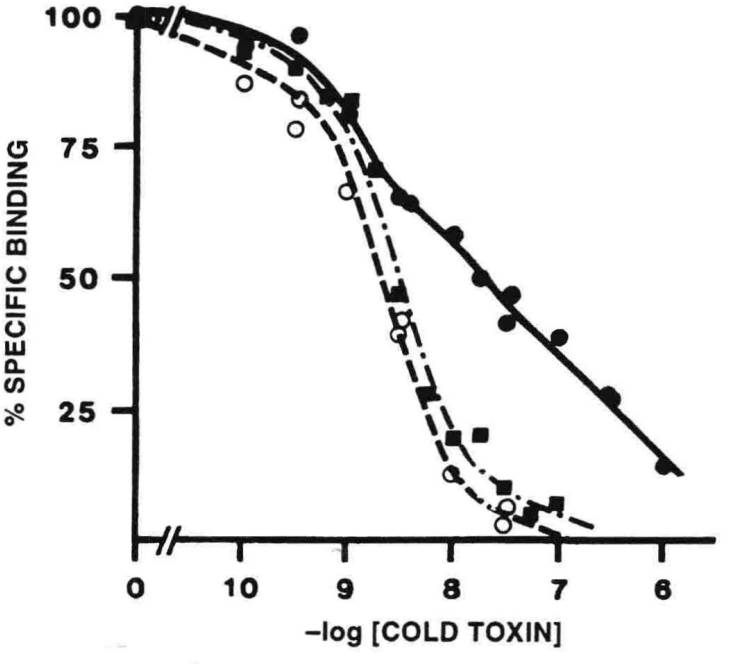
Prior to using a new radioligand in autoradiographic experiments, one should examine radioligand binding in homogenates of the tissue of interest. The K_d and B_{max} for the binding sites is calculated and nonspecific binding evaluated. The binding studies should begin with incubation time studies and a range of radiolabeled ligand concentrations to determine binding equilibrium.

Radioligands with slow association and dissociation kinetics pose particular problems in these assays. In this category are large peptides such as snake toxins that bind to receptors with high affinity ($K_d = 10^{-9}$–10^{-12} M) and antibodies that have even higher affinity for their target peptide sequences. Such ligands are termed *irreversible* ligands, though this is not correct unless the binding is covalent, such as that between lophotoxin and the nicotinic receptor. Since an assay lasts several hours and washout periods last for minutes, these ligands may be considered nearly irreversible. The long association times require prolonged incubations to attain equilibrium binding (Wolf et al., 1988). In the case of ^{125}I-α-bungarotoxin and ^{125}I-κ-bungarotoxin, 4 hour incubations at room temperature are required to obtain a reasonable estimate of K_d (Fig. 1.2A) in contrast to the 5–20 minute incubations that suffice for ligands such as ^{3}H-nicotine.

Nonspecific binding assays must be preincubated with cold ligand prior to adding the radiolabel to allow the cold ligand sufficient time to occupy all of the specific sites. In the case of the snake toxins, a 30 minute preincubation at 1 μM cold toxin concentration is sufficient when followed by a 4 hour incubation with radioligand. The cold ligand concentration must remain at 1 μM throughout the incubation. Preincubations also are required for irreversible ligands to inhibit radioligand binding. In Figure 1.2B, a 2 hour preincubation with



(*a*)



(*b*)

Figure 1.2. A: Specific binding of ^{125}I-κ-bungarotoxin to optic lobe, calculated by two different methods. In these brain sections, all specific binding of ^{125}I-κ-bungarotoxin was to α-bungarotoxin–sensitive sites. Aliquots (16 μl) of optic lobe homogenate were incubated with various concentrations (0.09–24 nM) of ^{125}I-κ-bungarotoxin for 4 hours. Three sets of duplicate tubes were set up at each concentration of ^{125}I-κ-bungarotoxin. Nonspecific binding was determined at each concentration in tubes preincubated for 30 minutes with either 1 μM α-bungarotoxin or 1μM κ-bungarotoxin. Symbols indicate method used in calculating specific binding: •, total binding minus binding in the pres-