# NEUROBIOLOGICAL RESEARCH

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Brain Receptor Methodologies, Parts A and B, 1984.

# BRAIN RECEPTOR METHODOLOGIES

## Part A General Methods and Concepts. Amines and Acetylcholine

*Edited by*

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1984

ACADEMIC PRESS, INC.

(Harcourt Brace Jovanovich, Publishers)
Orlando San Diego New York London
Toronto Montreal Sydney Tokyo

ACADEMIC PRESS, INC.
Orlando, Florida 32887

*United Kingdom Edition published by*
ACADEMIC PRESS, INC. (LONDON) LTD.
24/28 Oval Road, London NW1 7DX

Library of Congress Cataloging in Publication Data
Main entry under title:

Brain receptor methodologies.

(Neurobiological research)
Contents: pt. A. General methods and concepts. Amines and acetylcholine.
Includes index.
1. Neurotransmitter receptors--Collected works. 2. Neural receptors--Collected works. 3. Brain chemistry--Collected works. I. Marangos, Paul J. II. Cohen, Robert M. III. Campbell, Iain (Iain C.) IV. Series.
[DNLM: 1. Neuroregulators--Physiology. 2. Brain--Physiology. 3. Receptors, Endogenous substances--Physiology. WL 300 B81365]
QP364.7.B73 1984 599'.0188 83-22479
ISBN 0-12-470350-X (pt. A : alk. paper)
PRINTED IN THE UNITED STATES OF AMERICA

84 85 86 87 9 8 7 6 5 4 3 2 1

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# *General Preface*

Neurobiology or neuroscience is a multidisciplinary subject that has grown out of a common interest in nervous tissue by biochemists, physiologists, and pharmacologists. Researchers in this field therefore require an expertise within their own specialty as well as knowledge of other related areas. The degree of cross-fertilization between the various subdisciplines within neurobiology is extensive, and in most cases is required for the conduct of relevant research in the field.

The Neurobiological Research series provides a comprehensive and current view of various subdisciplines within neurobiology. Each volume will cover a specific area and will present in great detail the methods involved, so that the reader can grasp the general scope of the subdiscipline as well as have sufficient information to actually perform a given methodology. Each subdiscipline will be covered in an extensive manner in order to maximize the probability of finding a given methodology within each volume. This series, therefore, will differ from most existing works in this area, which generally present a few long reviews of selected areas within the realm of neurobiology and do not provide comprehensive coverage of any subdiscipline or the details of methodology.

An additional major emphasis throughout the series will concern how each methodology can be used to address various basic and clinical problems. Critical evaluations of each technique and the meanings of the data obtained from it are intended from each contributor. It is a major goal of the series to facilitate the flow of basic research strategies toward clinical application, and authors have been encouraged to review and to evaluate both past and potential future clinical studies. In this regard the editors are keenly aware of the need for a more rational and critical approach toward clinical neuroscience research.

The Neurobiological Research series should be a unique and valuable addition to the libraries of all neuroscientists. It is hoped that the series will be of equal value for both basic as well as clinical scientists. The first volume (Parts A and B) of the series deal with the area of neurotransmitter and neuromodulator receptors in brain, and future volumes will cover the subdisciplines of neuroanatomy, neurophysiology, brain-specific macromolecules, neurochemistry, and behavioral neurobiology.

# *Preface to Part A*

The area of brain receptors has been chosen as the subject for the first volume[1] of this series because it represents one of the fastest-growing areas in neuroscience. Because the distinction between a receptor and a binding site is of prime importance, we organized this work in a manner that will give readers a perspective to judge for themselves whether or not a site can be termed a receptor.

Section I of Part A opens with general methods and concepts relating to receptor studies. Subjects include a historical review of the receptor concept by Campbell; how one prepares radioactively labeled ligands for use in binding sites by Wan and Hurt; and methods of analyzing receptor data by Munson. A chapter on the peripheral localization of neuropeptides by Polak and Bloom has also been included in order to put in proper perspective the proposed functions of peptides as neuromodulators in brain. The material in Section I, therefore, should be of interest to virtually all those involved in receptor work because the information provided is basic to these studies.

The binding of ligand to receptor represents the first step in a cascade of reactions that lead to the physiological response. Binding studies performed in isolation are totally analogous to studying the binding of a substrate to its enzyme without knowledge of the reaction product or how it fits into the metabolic scheme. Studies of the physiochemistry of the receptor-linked effector mechanisms, therefore, are required to gain insights into the molecular mechanisms that constitute receptor-initiated phenomena. Subsections I,B and C deal with these newly developing areas of study. It is through the rigorous pursuit of such studies that the assignment of true receptor status can be accorded to a binding site. The inclusion of these methodologies in this volume in our opinion is of prime importance because not nearly enough effort has been expended here. This has led to a proliferation of characterized binding sites in the literature for which functions have not been assigned.

Subsection I,B presents a detailed treatment of membrane protein solubilization by Newby and covers two fairly well-worked out systems, the benzodiazepine receptor by Thomas and Tallman and the dopamine receptor by Laduron. Receptor solubilization is necessary for the structural and functional characterization of the molecule. Again, to draw a parallel with enzymology, it is the isolation and structural characterization of each receptor which will likely prove critical in determining the mechanisms involved in receptor-mediated processes.

[1]Published in two separate units, Part A and Part B, which are continuous with each other.

This is complicated by the membrane-associated nature of the receptor molecule and by the necessity to demonstrate that the solubilized receptor molecule has analogous binding properties to that of its membrane-bound precursor. The reviews presented in subsection I,B demonstrate this and present detailed discussions of the methods involved. Another important reason for purifying the receptor molecule is to raise specific antibodies to the molecule that will surely prove to be enormously valuable probes for both functional and anatomical studies.

Additional areas covered in subsection I,B include receptor autoradiography by Herkenham and affinity labeling of receptors by Sokolovsky. Virtually all our knowledge relating to the anatomical distribution of receptors and binding sites has come from the relatively recent advent of autoradiography utilizing thin slide-mounted tissue sections. The anatomical distribution of a binding site is of obvious importance in the functional characterization and physiology of the system. Some of the authors in Section II (which overlaps Parts A and B) include anatomical data. The detailed review of both affinity and photoaffinity labeling of receptors by Sokolovsky is also presented because these procedures offer the ability to irreversibly label receptors and should prove important in studies concerning questions ranging from receptor turnover to purification.

The elucidation and characterization of receptor-linked effector mechanisms have proceeded at a rate much slower than that of defining new binding sites. Subsection I,C deals with some of the proposed mechanisms and includes a treatment of cyclic nucleotides and adenylate cyclase in brain by Stone. Calmodulin-mediated protein phosphorylation is reviewed by DeLorenzo and Goldenring, and methods for purifying the catalytic subunit of cAMP-dependent protein kinase by Flockhart and Corbin. These three chapters exemplify the studies that currently are possible in the area of effector mechanisms involving cAMP and $Ca^{2+}$-calmodulin. The last chapter in this subsection by Crews deals with a recently postulated effector mechanism involving phospholipid methylation in brain and other tissues. This fascinating concept represents an area requiring further study before its physiologic relevance in the brain is established.

Section II presents the actual receptor binding assays reported in the literature, and is divided into three subsections: A, amines and acetylcholine; B, amino acids and neuropeptides, and C, drug-binding sites. (The latter two subsections are in Part B.) In subsection A, reviews of the β-adrenergic (Burgisser and Leftkowitz), α-adrenergic (Perry and U'Prichard), dopamine (Seeman), serotonin (Hamon), and acetylcholine (Ehlert *et al.*) receptors are presented. Although every attempt was made to cover all relevant systems, practical realities unfortunately led to the omission of a few systems such as the VIP binding site and the glutamate binding site. The rapidly developing nature of the field also has made it impossible to include some late procedures. One specific case in point is the calcium antagonist binding site that has recently been described using

[$^3$H]nitrendipine binding. This appears to be a useful label for the voltage-dependent calcium channel that may prove useful in studies concerning calcium-dependent processes in nervous tissue.

In all, there are 20 different binding systems described in Section II, which is a rather substantial proportion of the characterized binding sites currently capable of being studied. Each of the chapters in this section is rather unique in that the methods involved in each procedure are presented in sufficient detail that readers can perform the assay in their own laboratories. Each author also critically reviews the relevant data concerning the properties of the system as well as the actual and potential applications of each binding assay and binding site. In some cases where a complex literature has developed (such as that relating to the dopamine receptor) we have sought to solicit several viewpoints. For example, the chapters concerning the issue of multiple distinct subpopulations of the dopamine receptor by Seeman, by Leff and Creese (Part B), and by Laduron can be compared and contrasted to each other.

# *Contents of Part B*

# *Chapter 1*

# *RECEPTORS: A HISTORICAL PERSPECTIVE*

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## I. INTRODUCTION: THE RECEPTOR CONCEPT

The receptor concept is at least as old as ideas of chemical transmission between neurons. More than 100 years ago, Langley (1878) proposed that drugs act by forming a complex with discrete areas of the cell, and in 1905 he coined the phrase "receptive substance" to describe the nicotine-sensitive areas of the




ISBN 0-12-470350-X

neuromuscular junction (Langley, 1905). Furthermore, it was realized at least 50 years ago that the number of drug receptors present on responsive cells must be exceedingly small (Clark, 1926). At the turn of the century, Elliott (1905) and Dale (1906) established that ergot drugs have different potencies at specific locations, thus raising the possibility of receptor site specificity: this was soon reinforced by the classification of acetylcholine receptors into those which were "muscarine-like" and those which were "nicotine-like" (Dale, 1914).

The validity of the receptor concept was largely derived from indirect evidence obtained in studies of the quantitative aspects of drug antagonism (for review, see Gaddum, 1959). These studies were conceptually facilitated by the fact that enzymological ideas on dose–response curves, stereoisomerism, competitive and noncompetitive inhibition, etc., were already developed. Receptor binding techniques, however, did not develop until the 1960s when, using hormones as ligands, specific binding of compounds such as $^{131}$I-labeled ACTH was demonstrated (Lefkowitz, 1969).

Binding of a ligand to a receptor occurs by various ionic and hydrophobic interactions and by Van der Waals forces. Classically, a receptor fulfills the following criteria: (*a*) Activation elicits a physiological response, (*b*) ligand specificity, (*c*) regional distribution, and (*d*) the receptor is saturable. With the recent development of assays for specific "imipramine binding" (for example) and in other instances when (*a*) has not been shown, it is important to distinguish between a "binding site" and a "receptor."

## A. Law of Mass Action; Log Dose–Response (–Effect) Curve

In 1926 Clark applied mass action principles to his study of cholinergic receptors in the heart and reported that acetylcholine (*a*) produces graded responses over a wide range of concentrations, (*b*) produces different actions in different tissues, and (*c*) has actions that are rapid and reversible. These conclusions are the basis of the so-called *Occupancy assumption*, or *Occupancy theory* (i.e., the amount of receptor–substrate complex present at any one time is related to size of eventual response). Two other constructs (which can easily be disputed) are also present in Clark's papers. These are (*a*) that all receptors of one type are identical, and (*b*) that they do not interact with each other. In essence, by applying the Langmuir Isotherm (which describes binding to a set of identical and independent sites; Langmuir, 1918), Clarke described the dose–response curve, which is usually put on a semilog scale and defined as the log dose–response curve (LDR). The LDR curve has been used extensively over the decades and has certain well-defined parameters, for example, the $ED_{50}$, which is the dose of drug that produces a 50% response. The number of molecules of drug in combination with

the receptor can be estimated from the slope of the curve. It has been shown that the midline slopes (steepest section) of such curves are $0.576n$, where $n$ = drug/receptor combining ratio. Thus, if the drug receptor ratio is 1:1, then the gradient is 0.576; if 1:2, the gradient is 0.288; and if 2:1, it is 1.15. In most instances the LDR midline slope is 0.576 (see Barlow, 1968).

## B. Schild Plot

An extension of the LDR curve is the Schild plot (Schild, 1949). Consider that you wish to assess a series of antagonists in a physiological preparation; in the presence of acetylcholine, administration of a cholinergic antagonist will cause pupillary dilation. If the antagonism obeys the Law of Mass Action, it should be possible to express absolutely the antagonist activity of a particular drug in relation to a particular agonist. However, a 50% reduction in the response to an agonist will be an indication of a smaller antagonist effect when the preparation has a steep dose–response curve than when it has a flat one. It is therefore more accurate to maintain the biological response at a constant level in the middle of the dose–response curve and to assess the antagonistic effect from how much the agonist concentration must be multiplied to restore the response. This value is the *dose ratio* and is not absolute because higher doses of antagonist will raise its value and vice versa. More useful is the $PA_X$ value ($PA_X = -\log B$ by definition, $B$ is the concentration of antagonist, $A$ is the concentration of agonist, and $X$ equals the dose ratio) (Schild, 1949). $PA_X$ is thus the negative logarithm of the molar concentration of antagonist that reduces the effect of a multiple dose of a stimulant drug to that of a single dose. Agonists that act on the same receptors can theoretically be expected to produce the same $PA_X$ with competitive antagonists. Unfortunately, perhaps, the Law of Mass Action does not always apply to receptor–ligand interactions. It has been noted that if the receptors do not have a rate-limiting role in the reaction, then no effect will be seen until a certain fractional occupancy threshold (Fo) is exceeded. This fractional occupancy threshold (below which no drug effect occurs) is defined such that the maximum effect occurs at receptor saturation, that is, where $Fo = 1$. Thus, in studies in which threshold phenomena occur, dose–response curves on a linear scale will not pass through zero (for review, see Ariens and Simonis, 1964).

## C. Occupancy Theory; Spare Receptors; Partial Agonists

Stephenson (1956), also using the LDR curve, observed that when he examined the effects of a series of trimethylammonium compounds on guinea pig ileum, some agonists elicited only a submaximal response even at a high con-

centration. Such compounds he defined as *partial agonists* and further demonstrated that at high concentrations, such agonists were inhibitory on true agonist effects. To explain the phenomenon, Stephenson defined new parameters:

| | Agonist | Antagonist |
|---|---|---|
| Affinity | + | + |
| Efficacy | + | − |

The *efficacy* of a compound is a measure of the concentrations required to produce a graded response, and *affinity* reflects how easily the ligand and its receptor can combine. By definition, partial agonists are compounds with low efficacy (Stephenson, 1956) and thus are compounds that when titrated in the appropriate biological system may never elicit a maximum response. A partial agonist can thus be seen only in relative terms. Similar ideas were presented by Ariens and Van Rossum, 1957. In this model, "potency" is comparable to affinity, and "intrinsic activity" is comparable to efficacy; the latter is measured as the reciprocal of the fractional occupancy required to produce a response equal to 50% of the maximum of which the tissue is capable.

In further studies on the guinea pig ileum, Stephenson showed that histamine-induced contractions could be blocked by the irreversible inhibitor dibenamine, but that on increasing the histamine concentration, he could again demonstrate the effect. When the tissue was washed, the histamine response could not be demonstrated at the original low concentration. These studies led Stephenson to propose that with some pure agonists only a portion of the receptor sites need to be occupied to produce a maximal response and the excess are the so-called spare receptors. The phenomenon has also been named *receptor reserve* (for review, see Ariens and Simonis, 1964). There is physiological support for the proposal (see Furchgott, 1955; Nickerson, 1956), and it should be borne in mind when attempting to compare physiological responses with receptor binding data. Stephenson's ideas are summarized as:

(1) A maximum effect can be produced by an agonist when occupying only a small proportion of the receptors.
(2) The response is not linearly proportional to the number of receptors occupied.
(3) Different drugs may have varying capacities to initiate a response and consequently may occupy different proportions of the receptors when producing equal responses. This property is the efficacy of the drug.

These conclusions are not in agreement with those of Clark (1926), and Stephenson in his paper stated that a major error in Clark's studies was that, unfortunately, the tissues examined (the frog rectus abdominis and ventricle) both contain acetylcholine esterase, and thus the concentration of drug in equi-

librium with the receptors was less than that applied externally. Therefore, a simple dose–response measurement was not possible.

There may be spare capacity in a system beyond the receptor stage, for example, in the cyclic AMP generating system. If there is spare capacity, then there will be no proportionality between the cyclic AMP generated and the effect measured. For example, in the adrenal cortex, various ACTH analogs have different capacities to generate cAMP but show very little difference in their effectiveness in producing corticosterone (Seelig and Sayers, 1973).

## D. Accessory Receptor Sites

Spare receptors should not be confused with accessory receptor sites (Ariens and Simonis, 1964). These have been suggested to exist on the basis of the observations that in several receptor systems there are no apparent structural relationships between agonists and antagonists and in many cases the antagonists have a higher affinity for the receptor than have the agonists.

Another concept that can be mentioned at this point is that of dualism of receptor sites, that is, the idea that the receptor can exist in two forms. This theory is discussed in Section I,F. Certainly, the idea that a receptor can be in two forms has existed for at least 25 years; Katz and Thesleff (1957) used such a model to explain the phenomenon of "desensitization" produced by acetylcholine at the motor end plate. More recently, U'Prichard and his colleagues (1979, 1981) have used it to describe the same phenomena in the $\alpha_2$-adrenoceptor system (for review, see Bylund and U'Prichard, 1983).

## E. Receptor Rate Theory

An alternative to the occupancy theory is the rate theory (for reviews, see Paton, 1961; Rang, 1971). This theory emphasizes the importance of the rate of association of the drug with the receptor in eliciting a response, that is each association provides 1 quantum of excitation. Thus the rate constants of association ($K_1$) and dissociation ($K_2$) are very important. The differences between agonists and antagonists are determined by $K_2$ (i.e., high $K_2 \rightarrow$ agonists; low $K_2 \rightarrow$ antagonists). In this latter case, the drug–receptor complex, once formed, is stable. In rate theory terms, the efficacy of a compound is the rate constant ($K_1$). The magnitude of a response depends on the rate of ligand–receptor formation. Once formed, the combination is considered to be inactive, and dissociation must occur before reactivation can occur. (An analogy of this is the striking of a piano key.) Occupancy and rate theories are unresolved in that the former postulates the existence of graded responses and the latter, quantal effects. However, at equilibrium the rate of receptor occupation is constant, as is the number of

receptors occupied, and thus it is not possible to distinguish between the two theories when dose–response data are examined. Part of the experimental problem is also related to the fact that diffusion in and out of tissues makes it difficult to know ligand concentrations near their site of action, that is, in the "biophase."

## F. Dualism of Receptors; Allosteric Models

The idea that receptors exist in activated and nonactivated states has been discussed by several groups (Monod *et al.*, 1965; Karlin, 1967; Changeux *et al.*, 1967), and basically it is proposed that while at any time there is an equilibrium between activated and nonactivated receptors, the presence of agonists drives this equilibrium toward the activated state and antagonists encourage formation of the nonactivated state. One extension of the dual receptor idea is of course the allosteric model in which agonist and antagonist bind on interdependent sites such that the binding of the agonist to its receptor site changes the affinity of the antagonist for its receptor or vice versa.

## G. Positive and Negative Cooperativity: The Hill Plot

Neither the rate theory nor occupancy theory allow for modulation of response within a very narrow range of agonist or antagonist concentrations. This phenomenon may be rationalized in terms of a model proposing negative cooperativity between clustered receptors, only a fraction of which bind the ligand (De Meyts *et al.*, 1976). These workers describe negative cooperativity as the interaction resulting in a decrease in the apparent affinity of receptors when fractional saturation of the receptors increases, that is, in such a scheme the binding of one hormone molecule to a receptor within a group propagates a conformational change in adjacent receptors so as to reduce affinity for the agonist. Limbird and Lefkowitz (1976) and De Meyts *et al.* (1976) have shown that dissociation of radiolabeled ligand can be accelerated by the presence of unlabeled ligand, and this had been taken as direct evidence in favor of negative cooperativity. Some caution regarding conclusions should perhaps be exercised, since binding studies are fraught with problems of interpretation. Other attempts to explain such findings might be made on the basis of the existence of heterogeneity of receptors, an idea proposed by John Gaddum in 1926. Interestingly, if negative cooperativity exists, then many extra receptors may be involved in maximum responses and therefore the concept of spare receptors could be somewhat weakened. Issues related to cooperativity between sites are often studied using the Hill equation (Hill, 1909). This was originally derived to quantitate the deviations of enzyme–substrate interactions from classic mass action rectangular

hyperbolic behavior (for review, see Rang, 1971). The best-known early application of the Hill equation was to the oxygen saturation of hemoglobin (i.e., the first $O_2$ molecule's binding facilitates the binding of the second oxygen molecule, etc.). This example of positive cooperativity can be described thus:

$$R + L \xrightarrow{K_{D_1}} RL + L \xrightarrow{K_{D_2}} RLL$$

The net ligand binding isotherm is

$$B = \frac{B_{max}\,[L]^n}{[L]^n + K_D}$$

where $n$ = number of binding sites/receptor molecule. The Hill equation is usually expressed thus:

$$\frac{B}{B_{max}} = \frac{L^n}{K_D + L^n}$$

but for convenience in plotting is transformed to a logarithmic form.

$$\text{Log}\,\frac{B}{(B_{max} - B)} = n \log L - \log K_D$$

The Hill Number ($n_H$) is the slope of the plotted line and if near unity, one binding site is present. Hill plots may of course provide information that has no simple physiological significance. However, this is not particularly unusual in receptor binding studies. For example, it is often impossible to determine the significance of the fact that so-called agonist and antagonist sites for one receptor may provide different $B_{max}$ as well as different $K_D$ values.

## H. Receptor Mobility, Aggregation, and Internalization

Much of the work on receptor mobility, receptor aggregation, and receptor internalization has come from studies of peptide hormone responses rather than from neurotransmitter-related experiments (for review, see King and Cuatrecasas, 1981). These studies, using techniques such as the photobleaching recovery method, have demonstrated that following receptor–ligand (agonist) interaction there appears to be an aggregation of the receptors into clusters, which is presumed to occur by lateral movement of the receptor in the membrane. At present the physiological significance of the changes are unknown. However, reversible aggregation or dissociation of receptors had been postulated to be a means by which receptor cooperativity can occur (Singer and Nicholson, 1972). Another process, which has been demonstrated using fluorescent probes, is that

of receptor internalization, which appears to occur by a process of invagination of receptor-rich areas on the cell surface. As the hormones ultimately end up in lysosomes, the process of internalization is likely to be at least partly related to hormone degradation. The process may also be a means of cellular desensitization because the receptor is removed from the cell surface, and, possibly more importantly, may be a means of transporting hormone to intracellular receptor sites. Whether these processes, which have been demonstrated to occur for hormones, may be part of the phenomenology of neurotransmitter receptor action remains unclear at this point. Certainly, it appears unlikely that significant amounts of neurotransmitters enter the postsynaptic neuron.

## II. EFFECTOR MECHANISMS

### A. Cyclic AMP; Adenylate Cyclase

The precise changes that occur in membranes as a result of a ligand–receptor interaction are not fully understood. It now seems likely that there is not one single process but that, for example, a receptor may be relatively closely coupled to the response mechanism (e.g., in the case of the nicotine–acetylcholine receptor and its sodium channel), or alternatively the interaction may involve a cascade of reactions between several discrete units (e.g., in the case of β-adrenoceptors and their coupling to adenylate cyclase). The discovery of cyclic AMP by Rall *et al.*, in 1957, followed by the observation that at least some actions of neurotransmitters are mediated by cyclic AMP acting as a second messenger, represents a milestone in receptor pharmacology (for review, see Sutherland *et al.*, 1965). It has in time led to ideas about separate catalytic and effector subunits that can move independently in the fluid mosaic of the membrane (Singer and Nicholson, 1972; Hanski *et al.*, 1979) and that are brought together by the presence of a receptor agonist (for review see Cuatrecasas and Hollenberg, 1976). In some systems (e.g., dopaminergic) it is still not yet clear whether adenylate cyclase is involved in the functional process, since at least two receptor sites, D1 (adenylate cyclase linked) and D2 (not linked), have been reported (Kebabian and Calne, 1979). Issues related to the dopamine receptor are dealt with in considerable detail in this volume by Laduron (Chapter 7) and Seeman (Chapter 16) and in Part B by Leff and Creese (Chapter 13).

### B. Phospholipid Methylation

The molecular processes that result from receptor–ligand interaction are now widely studied. One interesting concept is that related to methylation of phos-

pholipids. The inception of these studies was by Axelrod and his colleagues (Strittmatter *et al.*, 1977; for review, see Crews, Part A, Chapter 13). In essence, they claim that some ligand–receptor interactions lead to phospholipid methylation and these can then be translocated from the cytoplasm to the outside of cell membranes. This in turn leads to alterations in membrane viscosity with resultant alteration in, for example, the position of receptors and adenylate cyclase and to a cascade of other related events.

## C. Phosphatidyl Inositol Effect

There are numerous recent reports of other membrane events that may be important in the transduction of receptor-mediated signals. The pathways by which $Ca^{2+}$, for example, can enter the cell and thus allow transmitter release from neurons have been examined. In some cases (e.g., the muscarinic–cholinergic receptor) the process is believed to involve changes in phosphatidyl inositol (PI) turnover in the membrane. It has also been demonstrated that $\alpha_1$- (but not $\alpha_2$-) agonists stimulate PI turnover (for review, see Fain and Garcia-Sainz, 1980). The complete pathway is still uncertain, but it would seem that the changes are not related to PI per se but possibly to compounds that are formed as part of the process. For example, Nishizuka and his colleagues (Takai *et al.*, 1982), working mainly with blood platelets, suggest that diacylglycerol (released during stimulated breakdown of inositol) is an important factor in receptor–effector processes. They propose that diacylglycerol and calcium stimulate a C-kinase, which in turn effects biological changes. A second process which might occur is that the diacylglycerol is converted into arachidonic acid (a precursor of prostaglandins), and thus secondary receptor changes might occur. The studies of Michell (1982) suggest that in some systems the influx of $Ca^{2+}$ may also be related to the breakdown of PI. These recent studies represent an important interactive point between students of neurotransmitters and those of lipids though, in fact, perusal of the literature shows that alterations in PI metabolism produced by a neurotransmitter (ACh) were demonstrated some 30 years ago by Hokin and Hokin (1954).

## D. Calmodulin

The demonstration of the existence of the ubiquitous calcium-binding protein calmodulin (Cheung, 1971) can be seen as an important development in studies of receptor effector coupling. While the precise mechanisms of action of calmodulin are unclear, it has been postulated to have a role in the sequestration of intracellular calcium, thereby (together with other factors such as $Ca^{2+}$ ATPase) maintaining low intracellular levels of this ion. Calmodulin has also been shown

to be a potent activator in several enzyme reactions. It has been suggested that the binding of calcium to calmodulin causes it to be conformationally altered and relatively hydrophobic and that it is this hydrophobic state that is important in the activation of membrane enzymes (Tanaka and Hidaka, 1980). A well-documented example is the role of calmodulin in stimulation of cyclic AMP production following β-adrenoceptor activation (see Rodbell, 1980, for review).

### E. Protein Phosphorylation

It has been shown (for reviews, see Greengard, 1978; Rodnight, 1982) that neurons contain protein kinases that are cyclic AMP-dependent, cyclic GMP-dependent, calcium/calmodulin-dependent, calcium phosphatidylserine-dependent, and even those that are cyclic nucleotide and calcium-independent. These kinases are responsible for the phosphorylation of a large variety of proteins (approximately 70 have been identified in the rat brain), and the process can lead to, for example, the activation of enzymes such as tyrosine hydroxylase. Another example of a protein that is phosphorylated is synapsin I (Protein I), which is found in vesicles in neuronal terminals but which at present has no known function.

### F. Electrophysiological Changes Associated with Receptor Occupancy

While biochemical changes such as those mediated by β-adrenoceptor occupancy have contributed greatly to the understanding of receptor-mediated events, electrophysiology has been substantially developed by research in the cholinergic system. The introduction of intracellular microelectrodes (Ling and Gerard, 1949) and their application to the studies of acetylcholine (Fatt and Katz, 1951; Hodgkin and Huxley, 1952) led to the idea that neurotransmitter–receptor interaction causes an alteration in ionic permeability of the membrane: proof for such ideas came some 10 years later with the introduction of the voltage clamp technique (Takeuchi and Takeuchi, 1960). Subsequently, Katz and Miledi (1970) and Anderson and Stevens (1973), again using voltage clamp techniques analyzed fluctuations in membrane potential ("membrane noise") around the average level of depolarization, and from such studies, it became clear that, for example, ions were being transported via channels rather than carriers. It also became possible to measure both the conductance and lifetime of opening of ion channels. More recently, the development of patch clamping (Neher and Sakmann, 1976) has refined these measurements such that single channel currents can be monitored. Recent reports indicate that single channel current rates are higher than were suggested by noise analysis (Cull-Candy and Parker, 1982).

Agonist-activated ionic channels have also been observed in cholinergic systems, where it has been possible to record single opening, multiple opening, and persistent opening events (Sakmann *et al.*, 1980).

Recent studies using *in vivo* and *in vitro* intracellular recording techniques suggest that decreases in $K^+$ conductance (which would lead to depolarization) may be responsible for increases in the neuronal excitability produced by $\alpha_1$- and β-adrenoceptor simulation whereas increases in $K^+$ conductance (which would be hyperpolarizing) might explain the inhibitory effects produced by $\alpha_2$-adrenoceptors (Woodward *et al.*, 1979; Szabadi, 1979; Rogawski and Aghajanian, 1980; Aghajanian and Vandermaelin, 1982; Madison and Nicoll, 1982).

## III. RELATED ISSUES

### A. Presynaptic Receptors

From a reductionist standpoint, we may hope ultimately to define a receptor in physiocochemical terms, but at present, definitions are mainly pharmacological, sometimes physiological, and can be anatomical, for example, pre- or postsynaptic, or extrajunctional. The development of ideas related to presynaptic receptor-mediated control of transmitter release was a milestone in the study of receptors. In the 1950s Brown and Gillespie (1957) showed that phenoxybenzamine (PBZ) increased stimulation-induced overflow of noradrenaline (NA) from the perfused cat spleen, and this was interpreted as being due to PBZ blocking postsynaptic receptors and blocking association by the noradrenaline. However, throughout the 1960s studies of amine re-uptake into presynaptic neurons flourished (e.g., Iversen, 1965) and PBZ was then assumed to block re-uptake. Nevertheless, the fact that complete blockade of uptake by drugs such as desipramine caused little or no increase in overflow (Geffen, 1965) led to the reemergence of the idea that the PBZ effect might be related to blockade of adrenergic receptors (Boullin, 1967) and of course in the early 1970s led to the idea of the presynaptic autoreceptor (for reviews, see Farnebo and Hamberger, 1971; Enero *et al.*, 1972; Langer, 1973, 1977; Starke, 1981). The concept has now been extended to include other systems (e.g., dopaminergic) but ironically, the $\alpha_2$-adrenoceptor originally defined in the periphery as a presynaptic receptor, has in the CNS been shown to exist, in part, at sites postsynaptic to NA neurons.

The process of $\alpha_2$-adrenoceptor–effector coupling has been an area of considerable recent research. It would appear that the $\alpha_2$-agonist–induced decrease in cyclic AMP production is mediated by GTP and a GTP-binding protein (N) which is similar to the GTP-binding protein (N) present in the β-adrenoceptor system. At some stage, the process is also dependent on the availability of

extracellular $Ca^{2+}$ ($\alpha_2$-induced vasoconstriction in the rat is blocked by verapamil; Van Meel *et al.*, 1981), and recently, it has been reported that antidepressant drug-induced decreases in $\alpha_2$-adrenoceptor numbers in the rat cortex were accompanied by an increase in the sensitivity of the noradrenaline release process to extracellular calcium (McKernan and Campbell, 1983).

Currently, there is evidence for a considerable number of other transmitters having some presynaptic receptor sites (e.g., GABA dopamine and acetylcholine).

## B. Endogenous Ligands and Receptor Binding

One conceptual issue that has arisen with the proliferation of receptor binding assays and identification of various receptor populations has been related to the question of physiological role. In some cases, knowledge of the existence of the binding site preceded the identification of the endogenous ligand. This is best known in the case of the opiates, in which receptor binding studies (among other factors) suggested the existence of the endogenous ligands that are now known to be the endorphins and enkephalins (Hughes *et al.*, 1975). The identification of highly specific binding sites for [$^3$H]benzodiazepines on part of the GABA receptor complex (Gavish and Snyder, 1980) has also led to an extensive search for an endogenous ligand that might modulate anxiety. Currently, β-carbolines are candidates: However, the existence of such a binding site does not necessarily signify the existence of an endogenous ligand.

## C. The Process of Receptor Change

There appear to be at least three types of receptor change:

(1) Rapid desensitization, which can occur when receptors are exposed to agonists *in vitro*. This should not be confused with the very rapid receptor–ligand association, which in rate theory terms is inactive.
(2) Circadian changes in the number of receptors in several different receptor populations. This phenomenon is still a source of controversy.
(3) Desensitization/supersensitivity, which occurs following prolonged administration of drugs such as antidepressants or following lesioning with compounds such as 6-hydroxydopamine.

### *1. Rapid densensitization that occurs as a result of agonist being present*

Approximately 25 years ago, Kakiuchi and Rall (1968), using a rabbit cerebellar preparation, demonstrated that incubation with either histamine or nor-

adrenaline transiently increased cyclic AMP production, but that a second exposure to the same agonist produced a diminished response. This rapid apparent densensitization has been demonstrated in blood cells (Remold-O'Donnel, 1974) and in human fetal fibroblasts (Franklin *et al.*, 1975), and in this latter case it was reported that although recovery occurred in 24 hr, it could be blocked by continued exposure to low agonist concentrations. Apart from demonstrating the existence of this important phenomenon, the paper of Kakiuchi and Rall (1968) showed that, if the second exposure was done with the alternative agonist, that is, histamine instead of noradrenaline or vice versa, then the desensitization did not occur, suggesting that either the adenylate cyclase was unique for specific systems or it was mobile and could be utilized by alternative agonists. However, the opposite phenomenon occurs in certain astrocytoma cell lines in which agonist-induced subsensitivity of β-adrenoceptors is accompanied by decreased responsivity of prostaglandin E receptors (Su *et al.*, 1976). The agonist-specific effect has been named *homologous* and the nonspecific one *heterologous densensitization*.

Using erythrocyte membranes, Lefkowitz and his colleagues (1976) showed that agonist-induced rapid densensitization as shown by changes in cyclic AMP production correlated with decreases in β-adrenoceptor number, and a similar correlation between decreases in α-adrenoceptor number and effect ($K^+$ release) was reported by Strittmatter *et al.* (1977) using parotid acinar cells. The mechanism of these rapid densensitizations is still unclear. However, studies with various mutant cell lines lacking adenylate cyclase or cyclic AMP-dependent protein kinase indicate that β-adrenoceptor occupancy must be accompanied by changes in the activity of the related adenylate cyclase before the effect will occur (Shear *et al.*, 1976). Su *et al.* (1979) have proposed that the process involves uncoupling of the receptor from adenylate cyclase. The changes do not involve loss of receptors and are reversible on removal of the agonist. Prolonged association with the agonist may lead to loss of receptor, and the process of recovery requires *de novo* protein synthesis (for reviews, see Levitski and Atlas, 1981; Harden, 1983).

### 2. *Circadian Changes in Receptor Populations*

There are reports of circadian changes in several receptor populations of the rat brain: α- and β-adrenoceptors, dopaminergic, and cholinergic receptors have been shown to alter their numbers throughout 24-hr periods (Wirz-Justice *et al.*, 1982). The changes are in receptor number (as measured by receptor binding assays) and at present do not have specific physiological correlates. The processes are poorly understood at present but since different brain areas show different patterns of change, the processes may be independently mediated. However, the existence of the rapid densensitization process described above provides some conceptual support for the existence of a rapidly cycling system.

*3. Desensitization or supersensitivity that occurs following prolonged administration of drugs (e.g., antidepressants or 6-hydroxydopamine)*

Cannon, in 1939, showed that when, in a series of efferent neurons, a unit is destroyed, an increased sensitivity to chemical agents develops in the isolated structure or structures, the effect being maximal in the part directly denervated. Some 40 years later, antidepressant drug-induced desensitization of β-adrenoceptors, for example, has become an extensively documented phenomenon (e.g., Sulser *et al.*, 1978). The process requires at least 3 days of drug administration and is presumed to be related to the presence of excess neurotransmitter. Similar reports have been produced for α-adrenoceptors (Cohen *et al.*, 1982). Changes in dopamine receptors have also been produced by chronic administration of neuroleptic drugs (Clow *et al.*, 1979; for review, see Hoffmeister and Stille, 1980). The molecular changes that accompany these receptor alterations are as yet unknown. As antidepressant drugs, for example, are known to produce both behavioral and biochemical changes in circadian rhythms (Wirz-Justice and Campbell, 1982) it could be argued that these long-term changes are due to phase shifting of the circadian rhythms.

## D. Receptor Binding Assays

The publication of Michaelis-Menten kinetics in enzymology can be considered a milestone in receptor studies in that it is the extrapolation of these ideas that led to the definitions of $B_{max}$ and $K_D$. The use of the Scatchard Plot to linearly transform binding data can also be seen as an important development, though its publication by Scatchard in 1949 probably had limited impact on classical pharmacologists, who at the time were probably more concerned with issues such as those related to the α and β classification of adrenoceptors (Alquist, 1948), which were contrary to the concept of sympathin E and sympathin I (Cannon and Rosenblueth, 1937). There are, of course, constraints on the use of Scatchard plots (Klotz, 1982; Munson, Chapter 3) and there are other methods for the analysis of binding data. Interestingly, the Woolf plot and the Direct Linear plot (Eisenthal and Cornish Bowden, 1974) have recently been reported to be statistically robust useful alternatives (Curry, 1982). Lest anyone have the misfortune to search for the original Woolf plot reference, it was published by the enzymologist J.B.S. Haldane in 1957 and attributed to work done by his friend Barnett Woolf at a much earlier time (circa 1930). Recently, nonlinear regression techniques for the analysis of binding data have been developed, and these are described in detail in this volume (e.g., Munson; Burgisser and Lefkowitz) and elsewhere (De Lean *et al.*, 1978; Munson and Rodbard, 1980).

## E. Receptor Isolation

The description of α-bungarotoxin (αBGT) as an irreversible curare-like antagonist of the acetylcholine (ACh) receptor (Chang and Lee, 1963) was followed by the isolation of a postulated ACh protein– bungarotoxin complex from the receptor-rich *Torpedo* electric organ (Miledi *et al.*, 1971; for review see Heidemann and Changeux, 1978). Irreversible binding of a radioligand to a receptor has now become a general approach to the task of receptor isolation, especially when coupled to the process of photoaffinity labeling (e.g., Shorr *et al.*, 1982; Venter, 1982; Caron and Lefkowitz, 1983; in this volume: Thomas and Tallman, Chapter 6; Laduron, Chapter 7). Finally, the various techniques that have been developed for the preparation of liposomes and for the insertion of proteins has been of value in the validation of isolated receptor systems.

## F. Future Developments

For the future, it is likely that biochemically, receptor–effector coupling will soon be much more clearly understood, though the physiology in relation to conductance may be more elusive. The demonstration of coexistence of monoaminergic neurotransmitters and peptides in neurons has made the idea of simple wiring diagrams in the brain rather unlikely, and it is probable that studies on the interaction of different receptors will develop. Autoradiography of receptor systems will no doubt become more sophisticated, especially with more widespread use of tritium-sensitive film and computer-assisted microdensitometry. As an extension of this, position emission tomography will allow localization of receptor sites in patients. Finally, the application of the methods of molecular biology such as the use of monoclonal antibodies and techniques for protein sequencing are likely to provide an area of growth in the subject (Noda *et al.*, 1983).

## ACKNOWLEDGMENTS

I would like to thank my colleagues Ruth M. McKernan, John D. Stephenson, and Graham Dunn for their constructive criticism of this manuscript.

## REFERENCES

Aghajanian, G. K., and Vandermaelin, C. P. (1982). *Science* **215,** 1394–1396.
Ahlquist, R. P. (1948). *Am. J. Physiol.* **153,** 586–600.
Anderson, C. R., and Stevens, C. F. (1973). *J. Physiol. (London)* **235,** 655–691.

Ariens, E. J., and Simonis, A. M. (1964). *J. Pharm. Pharmacol.* **16,** 289–312.
Ariens, E. J., and Van Rossum, J. M. (1957). *Arch. Int. Pharmacodyn. Ther.* **113,** 89.
Barlow, R. B. (1968). "Introduction to Chemical Pharmacology." Methuen, London.
Boullin, D. J., Costa, E., and Brodie, B. B. (1967). *J. Pharmacol. Exp. Ther.* **157,** 125–134.
Brown, G. L., and Gillespie, J. S. (1957). *J. Physiol. (London)* **138,** 81–102.
Bylund, D. B., and U'Prichard, D. C. (1983). *Int. Rev. Neurobiol.* **24,** 344–433.
Cannon, W. B. (1939). *Am. J. Med. Sci.* **198,** 737–750.
Cannon, W. B., and Rosenblueth, A. (1937). *Autonomic Neuro-Effector Systems* MacMillan, New York.
Caron, M. G., and Lefkowitz, R. J. (1983). *Proc. 5th Catecholamine Symp.*, No. 521.
Chang, C. C., and Lee, C. Y. (1963). *Arch. Int. Pharmacodyn. Ther.* **144,** 241.
Changeux, J. P., Thiery, J., Tung, Y., and Kittel, C. (1967). *Proc. Natl. Acad. Sci. U.S.A.* **57,** 335–341.
Cheung, W. Y. (1971). *J. Biol. Chem.* **246,** 2859–2869.
Clark, A. J. (1926). *J. Physiol. (London)* **61,** 530–556.
Clow, A., Jenner, P., Theodorou, A., and Marsden, C. D. (1979). *Nature (London)* **278,** 59–61.
Cohen, R. M., Campbell, I. C., Dolphin, M., Tallman, J. F., and Murphy, D. L. (1982). *Neuropharmacology* **21,** 293–298.
Cuatrecasas, P., and Hollenberg, M. D. (1976). *Adv. Protein Res.* **30,** 251–451.
Cull-Candy, S. G., and Parker, I. (1982). *Nature (London)* **295,** 410–412.
Curry, D. J. (1982). *Biometrics* **38,** 907–919.
Dale, H. H. (1906). *J. Physiol. (London)* **34,** 163–206.
Dale, H. H. (1914). *J. Pharmacol. Exp. Ther.* **6,** 147–160.
De Lean, A., Munson, P. J., and Rodbard, D. (1978). *Am. J. Physiol.* **235,** E97–E102.
De Meyts, P., Branet, A. R., and Roth, J. R. (1976). *J. Biol. Chem.* **251,** 1877–1888.
Eisenthal, R., and Cornish-Bowden, A. (1974). *Biochem. J.* **139,** 715–720.
Elliott, T. R. (1905). *J. Physiol. (London)* **32,** 401–407.
Enero, M. A., Langer, S. Z., Rothlin, R. P., and Stefano, F. J. E. (1972). *Br. J. Pharmacol.* **44,** 672–688.
Fain, J. N., and Garcia-Sainz, J. A. (1980). *Life Sci.* **26,** 1183–1194.
Farnebo, L.-O., and Hamburger, B. (1971). *Br. J. Pharmacol.* **43,** 97–106.
Fatt, P., and Katz, B. (1951). *J. Physiol. (London)* **115,** 320–370.
Franklin, T. J., Morris, W. P., and Twose, P. A. (1975). *Mol. Pharmacol.* **11,** 485–591.
Furchgott, R. F. (1955). *Pharmacol. Rev.* **7,** 183–265.
Gaddum, J. H. (1926). *J. Physiol. (London)* **61,** 141–150.
Gaddum, J. H. (1959). *Pharmacology,* Oxford Univ. Press, London/New York.
Gavish, M., and Snyder, S. H. (1980). *Nature (London)* **287,** 651–652.
Geffen, L. B. (1965). *J. Physiol. (London)* **181,** 69–70P.
Greengard, P. (1978). "Cyclic Nucleotide Phosphorylated Proteins and Neuronal Function." Raven Press, New York.
Haldane, J. B. S. (1957). *Nature (London)* **179,** 832.
Hanski, E., Rimon, G., and Levitski, A. (1979). *Biochemistry* **18,** 846–853.
Harden, T. K. (1983). *Pharmacol. Rev.* **35**(1), 5–32.
Heidemann, T., and Changeux, J. P. (1978). *Annu. Rev. Biochem.* **47,** 317–357.
Hill, A. V. (1909). *J. Physiol. (London)* **41,** 361–373.
Hodgkin, A. L., and Huxley, A. F. (1952). *J. Physiol. (London)* **117,** 500–544.
Hoffmeister, F., and Stille, G. (1980). *Handbook of Experimental Pharmacology* Vol. 55/1, Springer-Verlag, Berlin/New York.
Hokin, M. R., and Hokin, L. E. (1954). *J. Biol. Chem.* **209,** 549–558.

Hughes, J., Smith, T. W., Kosterlitz, H. W., Fothergill, L. A., Morgan, B. A., and Morris, H. R. (1975). *Nature (London),* **258,** 577–579.
Iversen, L. L. (1965). *Adv. Drug Res.* **2,** 5–23.
Kakiuchi, S., and Rall, T. W. (1968). *Mol. Pharmacol.* **4,** 367–378.
Karlin, A. (1967). *J. Theor. Biol.* **16,** 306.
Katz, B., and Miledi, R. (1970). *Nature (London)* **226,** 962.
Katz, B., and Thesleff, S. (1957). *J. Physiol. (London)* **138,** 63–80.
Kebabian, J. W., and Calne, D. B. (1979). *Nature (London)* **277,** 93–96.
King, A. C., and Cuatrecasas, P. (1981). *N. Eng. J. Med.* **305**(2), 77–88.
Korn, E. D. (1966). *Science* **153,** 1491–1498.
Langer, S. Z. (1973). *Frontiers in Catecholamine Research* (E. Usdin and S. H. Snyder, eds.), pp. 543–549. Pergamon, New York.
Langer, S. Z. (1977). *Br. J. Pharmacol.* **60,** 481–497.
Langley, J. N. (1878). *J. Physiol. (London)* **1,** 339–361.
Langley, J. N. (1905). *J. Physiol. (London)* **33,** 374–413.
Langmuir, I. (1918). *J. Am. Chem. Soc.* **40,** 1361.
Lee, C. Y., and Tseng, L. F. (1966). *Toxicon* **3,** 281–290.
Lefkowitz, R. J., Roth, J., Pricer, W., and Pastan, I. (1970). *Proc. Natl. Acad. Sci. (U.S.A.)* **65,** 745–752.
Lefkowitz, R. J., Mullikin, D., and Caron, M. G. (1976). *J. Biol. Chem.* **251,** 4686–4692.
Levitski, A., and Atlas, D. (1981). *Life Sci.* **28,** 661–672.
Levitski, A., Atlas, D., and Steer, M. L. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71**(7), 2773–2776.
Limbird, L. E., and Lefkowitz, R. J. (1976). *J. Biol. Chem.* **251,** 5007–5014.
Ling, G., and Gerard, R. W. (1949). *J. Cell. Comp. Physiol.* **34,** 383–396.
Madison, D. V., and Nicoll, R. A. (1982). *Nature (London)* **299,** 636–638.
McKernan, R. M., and Campbell, I. C. (1983). Proc. 5th Intern. Catecholamine Symposium, Göteborg, Sweden.
Michell, R. H. (1982). *Nature (London)* **296,** 492–493.
Miledi, R., Molinoff, P., and Potter, L. (1971). *Nature (London)* **229,** 554–557.
Monod, J., Wyman, J., and Changeux, J.-P. (1965). *J. Mol. Biol.* **12,** 88.
Munson, P. J., and Rodbard, D. (1980). *Anal. Biochem.* **107,** 220–239.
Neher, E., and Sakmann, B. (1976). *Nature (London)* **260,** 799–802.
Nickerson, M. (1956). *Nature (London)* **178,** 697–698.
Noda, M., Takahashi, H., Tanabe, T. *et al.* (1983). *Nature (London)* **301,** 251–254.
Paton, W. D. M. (1961). *Proc. R. Soc. Med.* **53,** 815–821.
Rall, T. W., Sutherland, E. W., and Berthet, J. (1957). *J. Biol. Chem.* **224,** 463–475.
Rang, H. P. (1971). *Nature (London)* **231,** 91–96.
Remond-O'Donnell, E. (1974). *J. Biol. Chem.* **249,** 3615–3621.
Rodbell, M. (1980). *Nature (London)* **284,** 17–22.
Rodnight, R. A. (1982). *Prog. Brain Res.* **56,** 1–25.
Rogawski, M. A., and Aghajanian, G. K. (1980). *Nature (London)* **287,** 731–734.
Sakmann, B., Patlak, J., and Neher, E. (1980). *Nature (London)* **286,** 71–73.
Scatchard, G. (1949). *Ann. N.Y. Acad. Sci.* **51,** 660–672.
Schild, H. O. (1949). *Br. J. Pharmacol.* **4,** 277–280.
Seelig, S., and Sayers, G. (1973). *Arch. Biochem. Biophys.* **154,** 230.
Shear, M., Insel P. A., Mekmen, K. L., and Coffino, P. (1976). *J. Biol. Chem.* **251,** 7572–7576.
Shorr, R. G. L., Heald, S. L., Jeffs, P. W., Lavin, T. N., Strohsacher, M. W., Lefkowitz, R. J., and Caron, M. G. (1982). *Proc. Natl. Acad. Sci. U.S.A.* **79,** 2778–2782.
Singer, S. J., and Nicholson, G. L. (1972). *Science* **175,** 720.

Starke, K. (1981). *Annu. Rev. Pharmacol.* **21,** 7–30.
Stephenson, R. P. (1956). *Br. J. Pharmacol. Chemother.* **11,** 379–393.
Strittmatter, W. J., Davis, J. N., and Lefkowitz, R. J. (1977). *J. Biol. Chem.* **252,** 5478–5482.
Su, U. F., Johnson, G. L. Cubeddu, L., Leichtling, B. H., Ortman, R., and Perkins, J. P. (1976). *J. Cyclic Nucleotide Res.* **2,** 271–285.
Sulser, F., Vetulani, J., and Mobley, P. L. (1978). *Biochem. Pharmacol.* **27,** 257–261.
Sutherland, E. W., Oye, I., and Butcher, R. W. (1965). *Recent Prog. Horm. Res.* **21,** 623–646.
Szabadi, E. (1979). *Neuropharmacology* **18,** 831–843.
Takai, Y., Minakuchi, R., Kikawa, U., Sanks, K., Kaibuchi, K., Yu, B., Matsubara, T., and Nishizuka, Y. (1982). *Prog. Brain Res.* **56,** 287–299.
Takeuchi, A., and Takeuchi, N. (1960). *J. Physiol. (London)* **154,** 52–67.
Tanaka, T., and Hidaka, H. (1980). *J. Biol. Chem.* **255,** 11078–11080.
U'Prichard, D. C., Bechtel, W. D., Rouot, B. M., and Snyder, S. H. (1979). *Mol. Pharmacol.* **16,** 47–60.
U'Prichard, D. G., Diaguji, M., Tong, C., Mitrius, J. C., and Meltzer, H. (1981). $\alpha_2$-Adrenergic receptors: Comparative biochemistry of neural and nonneural receptors and *in vitro* analysis in psychiatric patients. *In* "Biological Markers in Psychiatry and Neurology" (I. Hanin and E. Usdin, eds.), pp. 205–218. Pergamon, Oxford.
Van Meel, J. C. A., De Tonge, A., Kalman, H. O., Welffert, B., Timmermans, B. M. W. M., and Van Zweiten, P. A. (1981). *Eur. J. Pharmacol.* **69,** 205–208.
Venter, J. C. (1982). *Pharmacol. Rev.* **34**(2), 153–179.
von Euler, U. S., and Gaddum, J. H. (1931). *J. Physiol. (London)* **72,** 74–87.
Williams, L. T., and Lefkowitz, R. J. (1978). "Receptor Binding Studies in Adrenergic Pharmacology." Raven Press, New York.
Wirz-Justice, A., and Campbell, I. C. (1982). *Experientia* **38,** 1301–1309.
Wirz-Justice, A., Kafka, M. S., Naber, D., Campbell, I. C., Marangos, P. J., Tamarkin, L., and Wehr, T. A. (1982). *Brain Res.* **241,** 115–122.
Woodward, D. J., Moises, H. C., Waterhouse, B. D., Hoffer, B. J., and Freedman, R. (1979). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **38**(7), 2109–2116.

# *Chapter 2*

# *PREPARATION OF LABELED RECEPTOR LIGANDS*

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## I. INTRODUCTION

During the past decade, one of the most rapidly expanding areas of neurobiology has been the study of radioligand–receptor binding interactions. The ease of detection of radioactivity has made this an exceedingly popular technique. In addition, a great deal of information concerning the dynamics of the interaction between the ligand and receptor can be ascertained using this technique. However, receptor binding studies are not without pitfalls. Results of an artifactual nature can arise from a variety of sources, including ones attributable to the radioligand. Therefore, the acquisition of an appropriate radioligand is crucial to the success of the binding experiment. The explosive growth of receptor binding studies has only been possible because of the large number of suitable radioligands that have been prepared in the past few years. The purpose of this chapter is to summarize the methods utilized to prepare useful radioligands.

The design of a radioligand is subject to a number of constraints imposed by the nature of the binding experiment. These constraints have been reviewed in detail (Bennett, 1978). Briefly, the main factors one must consider are radioligand specific activity, purity, and stability.

Typically, the receptor sites under study bind ligands with equilibrium dissociation constants in the nanomolar range and are present in very low tissue concentrations. This necessitates the use of a radioligand with a specific activity




ISBN 0-12-470350-X

of at least 10 Ci/mmol, and ideally, greater than 30 Ci/mmol. Tritium and $^{125}I$ are the two radionuclides most commonly used to achieve this specific activity. Accordingly, this chapter will be limited to methods utilized to introduce tritium and $^{125}I$ labels.

Generally, the ligand binding study is carried out so that less than 10% of the total radioligand is bound at equilibrium (Bennett, 1978). As a result, the radiochemical purity of the ligand is of utmost importance; a minimum purity of 98% is usually required. In order to maintain this level of purity for an acceptable period of time, the stability of the radioligand is of concern. Therefore, this chapter will also outline methods of purification and storage of radioligands.

## II. METHODS OF LABELING

### A. Tritiation

#### *1. General Exchange*

Exposure of a ligand to either tritium gas or tritiated water, generally in the presence of a catalyst, achieves a general exchange of the chemically labile hydrogen atoms in the ligand. Exchange reactions with tritiated water are usually carried out under either acidic or basic conditions; tritium gas exchanges commonly employ a transition metal catalyst (e.g., platinum, palladium, or rhodium).

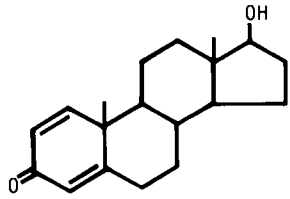
The major advantage of the general exchange method is simplicity. However, specific activity rarely exceeds 10 Ci/mmol; complex product mixtures often result; purification may be difficult and yields are usually low; and seldom is the exchange either stereochemically or site specific. Nevertheless, in limited cases satisfactory results can be obtained. [2,8-$^{3}$H]Cyclic AMP can be prepared by general exchange at a specific activity of 30–50 Ci/mmol. Tetrodotoxin can be tritiated by the Wilzbach method of exposing the compound to an atmosphere of tritium gas (Hafemann, 1972), albeit at low specific activity (0.5–1 Ci/mmol).

#### *2. Multiple Bond Reduction*

*a. Double Bonds.* The catalytic reduction of double bonds with tritium gas has been widely exploited as a means of introducing tritium into a molecule. With proper selection of conditions, specific activities of 30–60 Ci/mmol can be obtained. Highest specific activity is usually achieved when the reaction is carried out in an aprotic solvent such as dioxane or ethyl acetate as opposed to protic solvents, for example, ethanol or acetic acid.

A second consideration is the choice of catalyst. Group VIII transition metals (e.g., Pt, Pd, and Rh) on a variety of heterogeneous supports (activated carbon, alumina, barium carbonate) have been widely used. The resultant specific activity is usually high (30–60 Ci/mmol); the major exception is Adams catalyst ($PtO_2 \cdot H_2O$), which may give unacceptably low specific activity. In recent years, homogeneous catalysts such as Wilkinson's catalyst (tristriphenylphosphine-rhodium bromide) have become increasingly popular. One disadvantage of homogeneous catalysts may be difficulty in removing the catalyst after completion of the reaction.

Depending on the catalyst used, striking differences stereochemical selectivity may occur. For example, reduction of dienone **1** over Pd/C gives testosterone labeled with tritium on the β-face (**2**), whereas reduction using Wilkinson's catalyst introduces tritium almost exclusively on the α-face (**3**).

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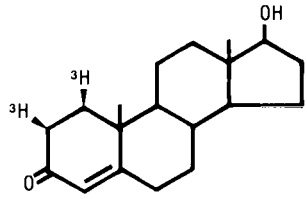


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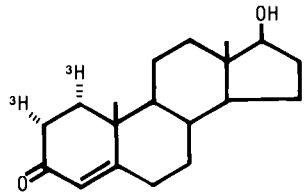
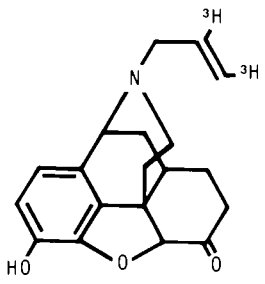



**3**

*b. Triple Bonds.* Carbon–carbon triple bonds are also easily reduced catalytically. By using Lindlar catalyst (Lindlar, 1952), the reduction can be stopped at the olefin stage; this strategy was followed in preparing the tritium-labeled opiate antagonist naloxone, [*N-allyl*-2,3-$^3$H]**4** (Filer *et al.*, 1981). A more active catalyst such as Pd/C will reduce triple bonds to single bonds. Thus, reduction of a propargyl precursor yields the labeled benzodiazepine antagonist propyl β-carboline-3-carboxylate ([*propyl*-2,3-$^3$H]**5**) with a specific activity of >90 Ci/mmol.

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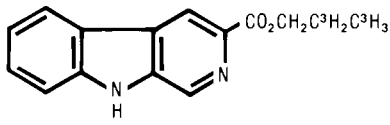
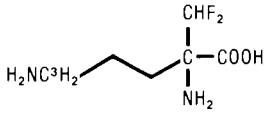



**5**

Reduction of the nitrile triple bond has been utilized to prepare tritiated amines. In many cases the reaction must be run in a protic solvent, so dilution of specific activity may be a problem. The ornithine decarboxylase inhibitor α-difluoromethylornithine (**6**) and the GABA antagonist baclofen (**7**) have both been tritiated via reduction of nitrile precurors.

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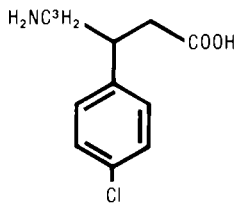
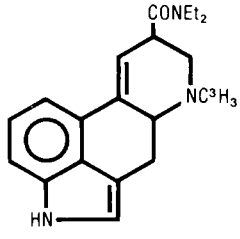



**7**

*3. Incorporation of Labeled Precursors*

*a. [$^3$H]Methyl Iodide and [$^3$H]Methanol.* In the labeling strategies discussed in other sections of this chapter, the source of tritium is either tritiated water or tritium gas. The availability of appropriate halogenated or unsaturated precursors therefore limits the radioligands accessible by these approaches. In order to overcome these limitations, synthetic pathways have been devised in which tritium is incorporated via labeled precursors. So far, the most widely used has been [$^3$H]methyl iodide, which can be prepared at 70–87 Ci/mmol.

A number of useful ligands are either N-methyl substituted amines or amides; their desmethyl precursors can usually be prepared either by N-demethylation or direct synthesis. Methylation with [$^3$H]methyl iodide then yields the desired radioligand; examples include [$^3$H]LSD (**8**) and [$^3$H]flunitrazepam (**9**).

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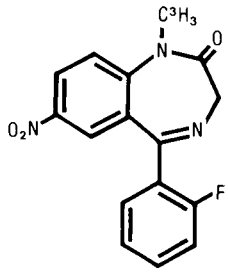
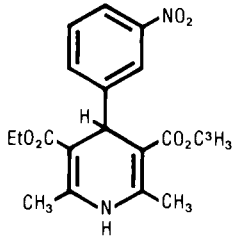



**9**

Tritiated methyl esters can also be prepared from [$^3$H]methyl iodide by alkylation of a salt of the corresponding carboxylic acid. The calcium antagonist nitrendipine (**10**) and the benzodiazepine antagonist methyl β-carboline-3-carboxylate (**11**) have been labeled in this way.

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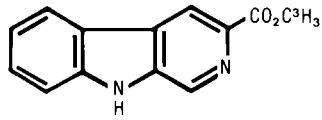
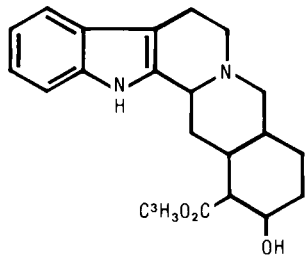


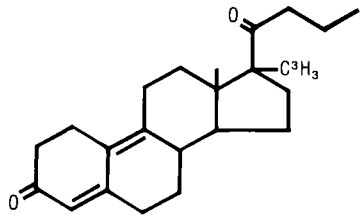

**11**

In the case of yohimbine (**12**), competing alkylation occurs on the nitrogen. This necessitates that esterification be carried out via a carbodiimide coupling of yohimbinic acid with high specific activity [$^3$H]methanol (70–87 Ci/mmol).




**12**

[$^3$H]Methyl iodide is again the source of tritium in the labeled steroid hormones promgestone (**13**), moxesterol (**14**), and methyltrienolone (**15**). [$^3$H]-Promgestone and [$^3$H]moxesterol are obtained by C-alkylation of an enolate and O-alkylation, respectively. [$^3$H]Methyl iodide is converted to [$^3$H]methyl magnesium iodide, which when added to a ketone precursor yields [$^3$H]methyltrienolone.

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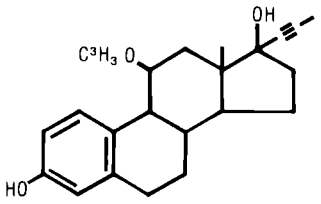


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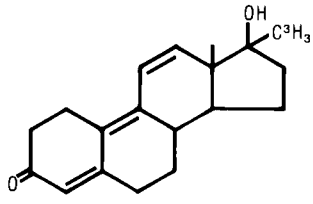
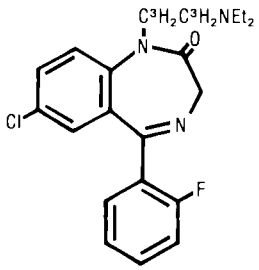



**15**

*b. [$^3$H]Ethylene Dibromide.* Introduction of a labeled two-carbon fragment is possible through the use of [$^3$H]ethylene dibromide. This precursor can be prepared at a specific activity of >80 Ci/mmol and has been utilized in the synthesis of a labeled benzodiazepine, [$^3$H]flurazepam (**16**).

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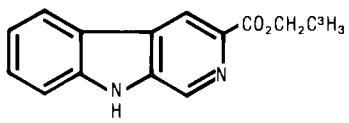
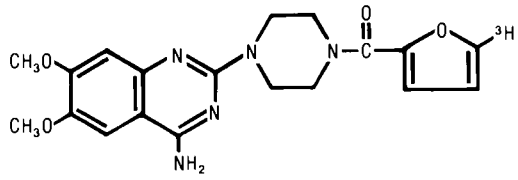



**17**

*c. [$^3$H]Ethyl Iodide.* Radioligands containing a labeled ethyl substituent may be accessible via alkylation with [$^3$H]ethyl iodide. For example, alkylation of the precursor carboxylic acid with [$^3$H]ethyl iodide yields [$^3$H]ethyl β-carboline-3-carboxylate (**17**) at >70 Ci/mmol.

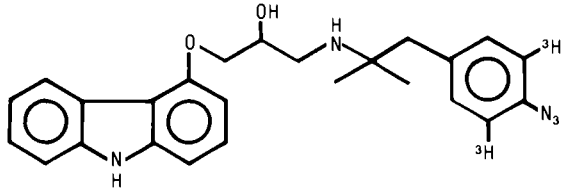
*4. Dehalogenation*

Aryl halides can be catalytically dehalogenated with carrier-free tritium gas to produce tritiated ligands with high specific activity. Prazosin, [*furoyl*-5-$^3$H]**18,** at 10–30 Ci/mmol was prepared by catalytic tritiation of 5-bromoprazosin. Azi-



**18**

dobenzylcarazolol, L-*p*-[*benzyl*-3,5-$^3$H]**19,** at 40–60 Ci/mmol and dopamine, 3,4-[*ring*-2,5,6-$^3$H]**20,** at 40–60 Ci/mmol were prepared from the appropriate brominated precursors.



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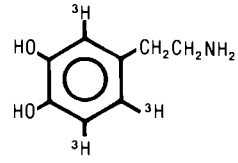
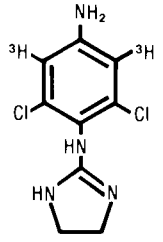


**20**

Tritiated peptides with tyrosine residues are often prepared by this method. The peptide is first converted to its 3,5-diiodotyrosine derivative by reaction with molecular iodine or iodine monochloride. The iodinated peptide is then catalytically dehalogenated in the presence of tritium gas to give the tritium-labeled peptide. Tritiated angiotensin II with a specific activity of 56.3 Ci/mmol was synthesized (Morgat *et al.*, 1970) by this two-step method. Tritiated neurotensin (40–100 Ci/mmol), which contains two tyrosine residues, was also prepared by a similar procedure.

Because of a difference in the relative reactivity of the aryl halides (I > Br > Cl) toward catalytic dehalogenation, selective dehalogenation can sometimes be achieved with polyhalogenated precursors. Thus, *p*-aminoclonidine ([3,5-$^3$H]**21**) at 40–60 Ci/mmol was obtained from the catalytic tritiation of 4-amino-3,5-dibromoclondine and clonidine ([4-$^3$H]**22**) from 4-bromoclonidine.



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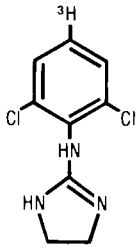
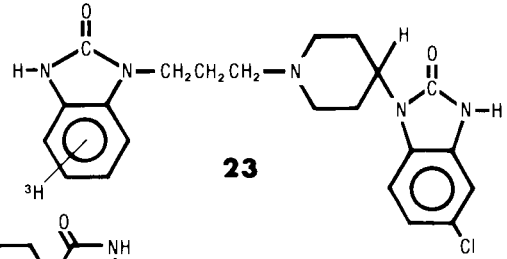


22

Tritiated domperidone (**23**) at 30–60 Ci/mmol and spiroperidol (**24**) at 20–40 Ci/mmol were also prepared by the partial reduction of the appropriate polybrominated derivatives in the presence of tritium gas.



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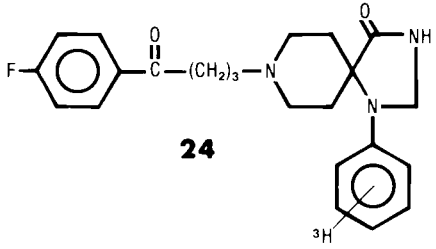
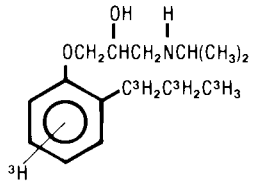


24

Simultaneous catalytic tritiation of a precursor with more than one reducible function can also be achieved. Thus, the catalytic reduction of brominated alprenolol in the presence of tritium gas gives high specific activity (90–120 Ci/mmol) dihydroalprenolol (**25**) with tritium atoms on both the ring and propyl side chain.



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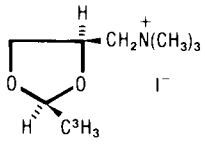
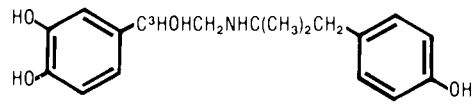


26

Alkyl halides occasionally can be replaced by tritium upon catalytical reduction. L-(+)-*cis*-Dioxolane, [2-*methyl*-$^3$H]**26,** with a specific activity greater than 25 Ci/mmol was obtained by the catalytic reduction of L-(+)-*cis*-trichloromethyldioxolane with tritium gas.

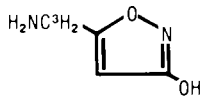
*5. Modification of Carbonyl Groups*

*a. Carbonyl Group Reduction.* Reduction of carbonyl double bonds can be achieved by using high specific activity (>60 Ci/mmol) sodium borotritide to produce tritiated ligands of modest (10–20 Ci/mmol) specific activity. *p*-Hydroxybenzylisoproterenol ([7-$^3$H]**27**) is prepared by this procedure.




**27**

*b. Decarboxylation.* Decarboxylation of ibotenic acid in $^3H_2O$ gives muscimol, ([methylene-$^3$H]**28**), with a specific activity of 5–20 Ci/mmol.




**28**

## B. Iodination

Radioidine-labeled ligands have frequently been used in receptor binding studies. Iodine-125, which has a longer half-life than iodine-131, (60 versus 8 days) is the more commonly used isotope. One advantage of radioiodine labeling is that radioiodine provides a much higher specific activity than tritium (~30 Ci/mmol for $^3$H, ~2200 Ci/mmol for $^{125}$I). However, one must be cautious in utilizing the radioiodine-labeled compound, since the introduction of the relatively large atom(s) into the molecule can sometimes significantly affect the structural orientation and hence the properties of the compound.

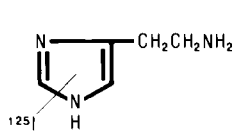
*1. Chloramine-T Method*

The use of elemental radioiodine or radioiodine monochloride as the iodinating agent has been known for many years; however, because of their volatile

nature these agents are rarely used. The most widely used method today is iodination with chloramine-T (Hunter and Greenwood, 1962) and sodium radioiodide. Chloramine-T, acting as an oxidizing agent, oxidizes iodide to iodine *in situ*. The procedure is relatively straightforward and usually gives high efficiency of utilization of the radioactive iodine.

There is a structural requirement for the compound to be iodinated; the precursor needs to possess an activated aromatic ring in the molecule. Peptides containing tyrosine or histidine residues are good candidates for radioiodination. Leucine enkephalin, Tyr-Gly-Gly-Phe-Leu-OH, was iodinated using chloramine-T and sodium iodide-125 to give [monoiodinated-Tyr]leucine enkephalin with a specific activity of ~2200 Ci/mmol. Monoiodinated thyrotropin releasing hormone, pGlu-[$^{125}$I]His-Pro$NH_2$, was obtained with a specific activity ~2000 Ci/mmol by the iodination of thyrotropin releasing hormone.

Radioiodination of nonpeptide ligands has also been accomplished using a similar procedure. [$^{125}$I]Iodohistamine (**29**) was synthesized with a specific activity of ~2200 Ci/mmol; [$^{125}$I]iodohydroxybenzylpindolol (**30**) was prepared with a specific activity of ~2200 Ci/mmol.

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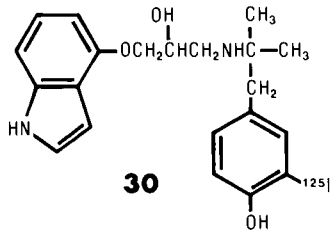
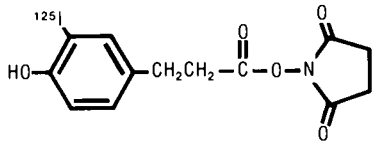



**30**

## 2. *Bolten-Hunter Reagent*

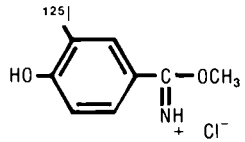
For biologically active peptides not containing tyrosine residues or peptides which become biologically inactive after being iodinated on the tyrosine moiety, radioiodine labeling with Bolten-Hunter reagent is usually a good choice. Bolten-Hunter reagent is the *N*-hydroxysuccinimide ester of iodinated *p*-hydroxy-phenylpropionic acid (**31**). As an active ester, the reagent acylates the free amino groups of the peptide with the iodinated *p*-hydroxyphenylpropionic residue. Miller *et al.* (1981) prepared $N^{\alpha}$-[$^{125}$I-labeled desaminotyrosyl]-CCK-8 and found that the iodinated derivative was identical with CCK-8 in potency and efficacy for stimulation of *in vitro* pancreatic enzyme secretion. Similar procedures were also used to prepare biologically active radioiodinated cholecystokinin for radioreceptor assay and radioimmunoassay (for example, Innis and Snyder, 1980).



**31**

*3. Wood Reagent*

Besides Bolten-Hunter reagent, Wood reagent (**32**), an iodinated *p*-hydroxybenzimidate·HCl, is also used for labeling peptide ligands in a similar fashion. The radioiodinated CCK-8 derivative was prepared at a specific activity of 2000 Ci/mmol (Praissman *et al.*, 1982).



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## III. PURIFICATION AND STORAGE OF RADIOLIGANDS

Purification of radioligands generally is a straightforward task. For radioligands of high specific activity a chromatographic purification is usually the method of choice, since rarely does one have sufficient mass for crystallization or distillation. Thin layer, paper, and high performance liquid chromatography are all routinely used. Comparison of the chromatographic properties of the radioligand with those of authentic unlabeled standard also is essential to verify the identity of the radioligand. In addition, spectroscopic measurements such as UV, tritium NMR, or colorimetric reactions are necessary for initial characterization and specific activity determination.

Proper storage of radiochemicals is necessary to minimize decomposition due to radiation effects. The factors responsible for radiochemical decomposition have been reviewed elsewhere (see, for example, Sheppard, 1972). Experience has shown that radioligands of high specific activity (>10 Ci/mmol) are generally more stable when stored in dilute solution (~1 mCi/ml) than as neat liquids or solids; the principal cause of decomposition under these conditions is interaction of the radioligand with free radicals generated by irradiation of solvent molecules.

Most neurochemicals are relatively polar molecules and for reasons of solubility must be stored in protic solvents. Water by itself is usually a poor choice, since it readily forms free radicals upon irradiation; the addition of free radical scavengers such as 2-mercaptoethanol to aqueous solutions may improve stability. Ethanol is also an effective free radical scavenger, so ethanol or ethanol/water mixtures may be the solvent of choice in many cases.

Storage temperature may also have a dramatic effect on the rate of decomposition. Most radioligands have satisfactory stability when stored at freezer (−20°C) temperature; in some cases ultrafreezer (−80°C) storage may be required. Actual freezing of the solvent should be avoided, since resultant aggregation of solute molecules may promote decomposition.

Finally, individual radioligands may require specialized storage precautions. For example, light-sensitive compounds should be stored in a light-free environment; easily oxidized compounds should be stored under an atmosphere of nitrogen or argon.

In summary, radiation effects usually render radioligands much less stable than their unlabeled counterparts. Care in storage of radioligands should not be underemphasized, since it may make the difference between the success or failure of a binding experiment.

## REFERENCES

Bennett, J. P., Jr. (1978). Methods in binding studies. *In* "Neurotransmitter Receptor Binding" (H. I. Yamamura, S. J. Enna, and M. J. Kuhar, eds.), pp. 58–61. Raven Press, New York.

Filer, C. N., Ahern, D. G., Fazio, R., and Sequin, R. J. (1981). *J. Org. Chem.* **46,** 4968–4970.

Hafemann, D. R. (1972). *Biochim. Biophys. Acta* **266,** 548–556.

Hunter, W. M., and Greenwood, F. C. (1962). *Nature (London)* **194,** 495–496.

Innis, R. B., and Snyder, S. H. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 6917–6921.

Lindlar, H. (1952). *Helv. Chim. Acta* **35,** 446–449.

Miller, L. J., Jamieson, J. D., and Rosenzweig, S. A. (1981). *J. Biol. Chem.* **256,** 12417–12423.

Morgat, J. L., Fromageot, P., and Hung, L. T. (1970). *Biochim. Biophys. Acta* **207,** 374–376.

Praissman, M., Berkowitz, J. M., and Izzo, R. S. (1982). *Anal. Biochem.* **121,** 190–198.

Sheppard, G. (1972). *At. Energy Rev.* **10,** 3–66.

# *Chapter 3*

# *HETEROGENEOUS RECEPTORS AND BINDING CURVE ANALYSIS IN NEUROBIOLOGY*

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## I. INTRODUCTION

The central role of receptors in mediating many physiological events has emerged as one of the basic tenets of modern neurobiology. Since most receptors cannot be observed directly, proof of their existence is largely based on their ability to bind certain ligands (neurotransmitters, hormones, drugs, etc.) selectively and to produce a characteristic action when stimulated. Therefore, use of appropriate means of data analysis for receptor experiments is crucial. New "receptors" are "discovered" almost weekly based on findings of specific



ISBN 0-12-470350-X

binding of radioactive compounds. Established receptor types are subclassified on the basis of only subtle changes in the shape of the binding isotherm. Nevertheless, a certain naiveté persists in this field concerning the need for careful statistical analysis of such data. Casual graphical analysis is fraught with potential pitfalls and can sometimes be misleading. Because of the indirect nature of binding studies, an incorrect or incomplete analysis of the experiment can alter the fundamental biological inferences drawn from the experiment. It is sometimes possible to see more structure in the data (e.g., two classes of sites in a curved Scatchard plot) than is warranted on the basis of a truly objective statistical test. Computerized statistical analysis can aid in the interpretation of the data, but overly simplistic attempts at computerization of the analysis can be less reliable than simple graphical methods. Therefore, the prudent investigator should become familiar with the principles of appropriate statistical analysis of ligand binding data and attempt to apply them.

In this chapter, we give an overview of a set of computerized analysis techniques developed to address the fundamental questions posed in receptor binding studies. Most of the mathematical and statistical details are avoided here but are covered in the references (Munson and Rodbard, 1980; Feldman, 1972; Munson, 1983b, Rodbard *et al.*, 1980).

Owing to the complexity of the mathematical expressions arising from even relatively simple equilibrium binding models, computerized analysis is likely to have increasing importance as an aid to the investigator. Finally, the potential of not just analysis, but of computerized design of experiments, is virtually untapped.

## II. LIGAND BINDING STUDIES: QUESTIONS AND ISSUES

### A. Characterizing Saturable Binding

Since radiolabeled ligands and drugs frequently bind to almost any protein or tissue and even sometimes to inorganic material, the first question asked in a binding study should be: "Is there evidence for saturable binding?" Surprisingly, it is hard to find agreement on a precise objective definition of saturability, especially if nonspecific binding is present. In the plot of Bound ($B$) versus Free ($F$) ligand concentration, saturation may be evidenced by a "plateauing," or leveling, of the curve. Yet, because the plateau must be extrapolated outside the range of the data, convincing evidence of its existence is difficult to obtain in practice. In fact, one investigator claims that only by observing a definite inflection point in the binding curve displayed as [Bound] versus log [Free] can we

legitimately define saturablity (Klotz, 1982). However, even this overly conservative approach can be quite subjective, since recognition of the inflection point can be difficult when the data points are even moderately scattered. Furthermore, this approach may be misleading when dealing with heterogeneous receptors (Munson and Rodbard, 1983).

In the Scatchard plot ($B/F$ versus $B$), data from true saturable binding should fall on a straight line. Therefore, any tendency toward a linear trend ought to signal the presence of saturable binding. Unfortunately, in all but the most clear-cut cases, the distortions of statistical errors resulting from the data transformation and the plotting technique itself make this approach unworkable. The Scatchard plot method may be subject to serious misinterpretation if, in addition to a linear trend, there is a suggestion of curvature in the data.

Finally, the definition of saturability depends strongly upon how the nonspecific binding is defined and measured. Conventionally, nonspecific binding measured by first saturating the specific binding with excess cold ligand, is subtracted from total binding measurements to obtain "specific binding." However, the subtraction of two measured quantities results in a magnification of statistical errors and may make reliable observation of the plateau in the saturation curve difficult (Chrousos *et al.*, 1982; Munson, 1983b). Failure to add sufficient excess cold ligand when measuring nonspecific binding may also bias results of this type of analysis, since the specific binding site may not be completely saturated.

## B. Identifying the True Receptor

After detecting saturable binding, one should next determine its specificity for different ligands or classes of ligands. Stereospecificity may be evidence of a true receptor, as may its particular pattern of binding specificity for a series of agonist or antagonist hormones or drugs.

Saturable binding in the same tissue may be measured using different labeled probes. To determine if these ligands are binding to the same or to independent classes of receptors, competition studies using the corresponding unlabeled ligands should be performed. Complete cross-displacement should be observed if only a single receptor binding site is involved. Failure of one of the unlabeled ligands to compete or reduction of the slope of the competition curve suggests that two or more independent sites may be involved. Saturation of the same receptor with different labeled ligands should result in the same measured binding capacity. Differences in $B_{max}$ may also indicate multiple receptors.

The relative potency of a series of ligands has been used as an identification criterion, or "signature," of a particular receptor. Thus, we might expect the ordering of $IC_{50}$ values for a set of ligands and even their relative spacing to

remain unchanged for the same receptor molecule located in different tissues or in different species. Conversely, a significant change in the order of potency may signal a new and different receptor. Furthermore, if biological effect is to be mediated by receptor occupancy, then the order of potency for binding ought to correlate with the order of potency for activation determined in pharmacological studies.

We might also reasonably expect that the $K_D$ of a true receptor be in something approaching the physiological range of concentration of the hormone or ligand, say 0.1–1000 n*M*.

### C. Estimating Binding Parameters

Finally, after establishing the identity of a receptor (or binding site) we may then wish to characterize the shape of its binding curve, either in terms of empirical parameters (slope, midpoint location, plateau height, deviation from symmetry, etc.) or under the assumption of a particular binding model with parameters for affinity, $K_D$, and binding capacity, $B_{max}$, and optionally, in terms of departures from a simple homogeneous equilibrium binding model, for example, the presence of positive or negative cooperativity.

Each of these issues—defining and detecting saturable binding, measuring binding specificity, observation of binding heterogeneity, and estimation of $K_D$ and $B_{max}$—depend on accurate statistical analysis of the original binding curve, which is greatly aided by appropriate computerization.

## III. COMPUTERIZED ANALYSIS: CURVE FITTING

Traditionally, binding studies have depended upon graphical analysis. Unfortunately, because of the variety of plotting techniques and of the widespread misinterpretation and misuse of some of them, considerable controversy has been raised about their suitability (Klotz, 1982; Munson and Rodbard, 1983). The substantive issues and questions mentioned above should be addressed in the context of a computerized statistical analysis. Such automated analytic techniques, correctly applied, avoid the subjectivity of traditional graphical approaches and result in more precise results. Unfortunately, some early attempts at computerization of graphical techniques may have been too simplistic or even incorrect; use of such programs may result in unacceptable results. In order to prevent such difficulties, the investigator should become familiar with the statistical principles guiding the analysis.

Computerized analysis (in fact, any precise analytic method) begins with the postulation of a mathematical or biochemical model or framework into which the

experimental data are fitted. The model may take the form of a set of equations or may be defined by the computer program actually used in calculation.

We must also have a method for fitting the model to the experimental data. Usually, this takes the form of adjusting the parameters of the model (e.g., affinity and binding capacity) until the predicted curve closely resembles the observations. After fitting the curve, we must next validate the correctness of the particular mathematical model or reject it in favor of an alternate model. This may be done on the basis of the degree of agreement (goodness-of-fit) of the model with all available experimental data. These ideas have been formalized and given a sound statistical basis in the theory of weighted nonlinear least squares (WNLLS) analysis (Bard, 1973). The technique is entirely general in that any desired model may be specified (e.g., homogeneous receptor, multiple binding sites, etc.) and the resulting parameter estimates will be optimal in the sense that the raw untransformed experimental observations (usually bound counts of radioactivity) are being fitted. This form of analysis is superior to graphical approaches in ligand binding analysis in that (1) results are objective, (2) less bias is introduced than with most other common methods, (3) the variation of the final results due to statistical fluctuation in the data is smaller, and (4) results are easily reproducible by different investigators in different laboratories.

Several points should be observed when applying WNLLS to ligand binding data. First, it is essential that the untransformed raw experimental data be fitted by the model. Unfortunately, some investigators have attempted to fit the data directly in the Scatchard plot ($B/F$ versus $B$) using linear regression, a specialized case of least squares analysis. Owing to the unusual distortion of experimental errors induced by this transformation, the assumptions underlying linear regression (homogeneity of variance, error-free independent variable, etc.) are not met. Use of linear regression here will result in significantly underestimated values for affinity, with corresponding overestimates of capacity. Second, since even the raw experimental data may have unequal precision (e.g., 10,000 counts bound has 1% error, but 100 counts has 10% error according to the laws of Poisson statistics), it is important and sometimes essential to use appropriate statistical weighting. Several methods for determining the correct type of weighting have been developed elsewhere (Munson and Rodbard, 1980; Rodbard *et al.*, 1976). Third, the validity of the model must always be under suspicion. After all, until the ultimate biochemical mechanism for hormone action is explicated, mathematical models can only provide successively finer approximations to the truth. Methods for checking model adequacy include graphical inspection of residuals (differences between observed data and model predictions), nonparametric and parametric statistical tests for patterns (nonrandomness) in the residuals (Belsley *et al.*, 1980), and comparison of the goodness of fit or residual variances (average difference between predicted and observed values) for the current model with that for the next more complex and the next simpler models.

For example, we should compare the fit of a homogeneous receptor (one class of sites) model with that of a model with two classes of sites. If the heterogeneous receptor model fits significantly better, we can discard the homogeneous model in its favor.

Once a computerized analysis program based on WNLLS technique has been set up, we can use it to address the series of issues raised in Section II. The presence or absence of specific binding can now be determined on an objective basis. It is now a question of fitting the mathematical model for receptor binding to the data at hand, then testing (with the t-test or the "extra-sum-of-squares" F-test) (Draper and Smith, 1981) whether the binding capacity, $B_{max}$, is statistically different from zero. A binding capacity which is indistinguishable from zero means there is no objective evidence from the shape of the curve that a saturable binding site exists. Thus, we can avoid the issues of graphical analysis altogether; the results of such statistical test do not depend on particular techniques of graphical display, but simply on our knowledge of the type of random errors in the original, untransformed data. In practice, binding data are adequately described with a Gaussian distribution with a variance increasing with the magnitude of the measured values.

Further, nonspecific binding may be handled as a part of the model, using the same approach. Here, it is no longer necessary to presubtract nonspecific binding measured separately before preparing the saturation curve or defining the inflection point on the $B$ versus log $F$ plot. Rather, to determine if saturable binding is present, one should fit the model, including terms for both specific and nonspecific binding, to the raw data and then again test if the $B_{max}$ value is significantly greater than zero.

## IV. RECEPTOR HETEROGENEITY

Multiple or heterogeneous receptors have been postulated for a large number of neurotransmitters. The α- and β-adrenergic receptor system represents perhaps the first known example. More recently, established receptor types have been further subdivided into $\alpha_1$ and $\alpha_2$, $\beta_1$ and $\beta_2$, etc. Histamine, dopamine, serotonin, and opiate receptors are now each thought to exist in two or more distinct forms. Distinct pharmacological responses to various drug analogs seem to require the existence of multiple receptors to mediate their response. Evidence of heterogeneous pharmacological action has motivated much of the research into multiple receptor types. Variation of autoradiographic patterns in different brain regions also suggests receptor heterogeneity, as do various cross-tolerance and dependency studies in the opiate receptor field.

## A. Evidence from Saturation and Competition Studies

A separate line of evidence for heterogeneity may be obtained from *in vitro* binding studies. Here, the evidence is less direct and may depend on subtle changes in the shape of the binding curve. Binding studies are generally performed in one of two forms, saturation or competition experiments. Saturation studies (addition of increasing amounts of labeled ligand to tissue preparations) may sometimes yield data which are concave upward on the Scatchard plot. This is frequently accepted as indicative of heterogeneity, although other explanations must be considered. Negative cooperativity involving site–site interactions is possibly the most popular alternative explanation, but a host of experimental artifacts can also explain this finding (Munson, 1983a). Imprecise measurement of nonspecific binding, incomplete separation of bound and free ligand, or uncorrected background radioactivity all may influence the results. Conversely, a linear Scatchard plot should not be uncritically accepted as proof of homogeneity. Clearly, two different receptors could bind with nearly identical $K_D$ values, or the concentration range covered in the experiment might be too narrow to include the $K_D$ of the second site. Thus, determination of receptor homogeneity must go beyond statements about the linearity of the Scatchard plot.

Competition studies (in which varying doses of unlabeled ligand compete with labeled species for a finite number of receptor binding sites) may also reveal receptor heterogeneity. Reduced slope of the competition curve or inability to completely displace labeled ligand suggest the presence of multiple classes of receptors.

Interestingly, saturation studies and competition studies do not really offer independent evidence of receptor heterogeneity, since both studies measure binding of varying hormone concentrations to the same receptor preparation. Thus, both studies should be expected to obey the same reaction equilibrium if the same ligands are used. Saturation studies (Scatchard plots) therefore cannot confirm the existence of receptor heterogeneity discovered using competition studies (displacement curves), as is sometimes claimed in the literature.

## B. Analysis of Curved Scatchard Plots

Resolution of curved Scatchard plots into two components is seldom performed correctly, using graphical procedures. Regrettably, use of several incorrect procedures has become widespread. The most common type of error is the attempt to divide the curve into two nearly linear segments, and then estimate $K_D$ and $B_{max}$ from the slope and intercept of each segment using linear regression or a similar technique. However, the slope of the line in a Scatchard plot only

represents the binding affinity if the plot is linear (i.e., if there is a single homogeneous class of binding sites). In the more complex nonlinear case, this rule no longer applies. Incorrect calculation of $K_D$ values can result in substantial, five- to tenfold errors in some cases. Also, the indeterminacy in locating the breakpoint between the two segments contributes to the unreliability of this method. Formulas are available which give the correct $K_D$ and $B_{max}$ from accurate measurements of the slope in the extreme regions of the curve ("limiting slopes" method) (Hunston, 1975; Thakur *et al.*, 1980), but the utility of this method is limited by the need to smooth and extrapolate the data and by the complexity of the formulas themselves.

The optimal solution to this problem is to fit the curve described by the exact mathematical model for two independent binding sites, that is, computerized curve fitting. Again, one should not attempt to fit the curve directly to the Scatchard plot, owing to the bizarre, distorting effect this plot has on random statistical errors but should fit the untransformed experimental observations, usually bound ligand concentration. Of course, once the correct fit is obtained, it may be displayed in the Scatchard or any other coordinate system. Fitting the curve to the bound ligand concentration requires simpler mathematical expressions than does the equivalent curve in the Scatchard plot and will also give statistically more reliable results.

## V. EXPERIMENTS USING SEVERAL LIGANDS

Use of multiple analogs can be very informative when defining two or more classes of receptors. Receptors seem to differ in their requirements for recognizing agonists (or antagonists). Conversely, ligands differ greatly in their abilities to bind and activate the receptors. The ideal situation from the investigator's point of view would be the existence of completely selective ligands for each class of receptors; ligand A binds only to receptor A and ligand B only to receptor B. In this case, there would be no question about the existence of multiple receptors.

The difficult question arises when the ligands are not completely selective, that is, ligand A binds to both receptor A and receptor B, possibly with only a small difference in the affinity. Here, we might observe a Scatchard plot with only a minor degree of curvature, not enough to precisely characterize the two binding sites, or possibly no curvature at all if ligand A is completely unselective.

The problem can be resolved if more selective ligands can be found or if several partially selective ligands can be used simultaneously. Thus, although ligand A alone may not reveal two receptor classes, used together with ligands B,

C,D, etc., in a properly designed experiment, it may help to precisely characterize multiple distinct classes.

The most commonly used design involving two different ligands is the competition or displacement curve. One of the key features of such a curve is its slope. A logit-log slope (slope of the competition curve in the logit-log coordinates) less than one may indicate receptor heterogeneity. Other characteristics of such curves may also be noted. If a sufficiently wide range of concentrations of competitor is used, we should observe both an upper and lower plateau connected by a smooth, usually symmetric S-shaped curve in the plot of [Bound] versus log [Competitor]. The logit-log slope (sometimes incorrectly referred to as the Hill slope) and the midpoint location ($ED_{50}$) can be determined using linear regression on the logit-log plot of the data. In this plot, the ordinate is y = logit($Y'$) = log($Y'/(1-Y')$), where $Y'$ is the response variable normalized within zero to one. The experimentally measured response variable may be bound counts per minute, in which case the normalized response is written $Y' = B/B_0$. The abscissa of the graph is the logarithm of the concentration of competitor.

The plateau values used to normalize the response must be accurately determined before the logit-log transformation can be applied, because the $ED_{50}$ depends on their level. This is equally true of the untransformed data; we cannot determine the midpoint of a curve unless we know its upper and lower limits. In the absence of nonspecific binding, the lower plateau or minimum response should be zero. A baseline greater than zero may result from nonspecific binding or if the competitor cannot fully displace the labeled ligand.

## A. The Four-Parameter Logistic

A useful, simple, and empirical description of the competition or displacement curve is the four-parameter logistic equation (De Lean *et al.*, 1978; Rodbard, 1974);

$$Y = \frac{a - d}{1 + (X/c)^b} + d$$

The parameters of this mathematical model correspond exactly to the qualitative features of the graph. The parameters $a$ and $d$ are the upper and lower plateau levels, respectively. The $ED_{50}$ is represented by the parameter $c$, and $b$ is the logit-log slope. Fitting all four parameters to the competition curve conveniently summarizes the main characteristics of the competitor ligand and its interaction with the binding site. This equation may be applied to a single curve or to families of displacement curves for a series of competitors (De Lean *et al.*, 1978).

Further, we may assess heterogeneity by testing if the slope factor $b$ is compatible with unity, again utilizing a t- or F-test. Ironically, this empirical four-parameter logistic model becomes inapplicable if too much heterogeneity is present. In this case, the curve will break into two or more steps and cannot be described by a smooth, symmetric, sigmoidal shape.

## B. Relating $K_i$ to $ED_{50}$

Very commonly, competition curves are used as the basis for estimating $K_i$, the inhibition constant of the unlabeled ligand. Such estimation procedures are generally only valid in the homogeneous receptor case and should not be applied if the logit-log slope is significantly different from one. Even in the simple one-receptor case, the complete mathematical description of the interaction of labeled ligand, unlabeled ligand, and the receptor involves solving three or four nonlinear equations. Therefore its behavior cannot easily be predicted intuitively, except under special conditions. The exact midpoint location bears a complex relationship to not only the $K_i$ (affinity of the inhibitor), but also to the $K_D$ (affinity of tracer ligand), $R$ (binding site capacity), and $p^*$ (initial concentration of labeled ligand) values as well. Nevertheless, the inhibition constant is commonly assumed equal to te midpoint location, $K_i = ED_{50}$. While many investigators recognize that $K_i$ is not always identical with the $ED_{50}$, they do not realize that the most commonly used correction method (Cheng and Prussoff, 1973) is also an approximation and may result in errors of several hundred percent. The Cheng-Prussoff correction adjusts for the nonneglible tracer ligand concentration:

$$K_i \cong ED_{50}/(1 + L/K_D)$$

where $L$ is the free tracer concentration at the $ED_{50}$. Very often, total rather than free tracer concentration is known, so in practice, the above relation becomes:

$$K_i \cong ED_{50}/(1 + p^*/K_D)$$

If the tracer concentration $P^*$ is less than $0.1 \cdot K_D$, this correction has little effect on the calculated $K_i$. Even with the Cheng-Prussoff correction, however, results can be substantially (>100%) in error if either (1) initial binding, $(B/F)_o$, is higher than 0.8 or (2) if the true $K_i$ is substantially smaller than $K_D$. Under these conditions, the implicit assumption that free concentration equals total concentration for both labeled ligand and competitor is invalid. High values of $(B/F)_o$ are not uncommon when using high-affinity, partially purified receptors, hence further correction of the results may be necessary. The exact relationship of the $K_i$ to the $ED_{50}$ is given in Rodbard and Lewald (1970).

A superior approach, which does not make use of any of the above approximations, is the model-based curve-fitting method. Rather than estimating the $ED_{50}$

values for each curve independently and then converting these to $K_i$ estimates, it is possible, with computerized curve fitting, to estimate the $K_i$ directly from the data. This method has the following advantages:

1. It fits the exact model for equilibrium binding of hormone to receptor.
2. Each curve and each data point are given appropriate weight in the analysis.
3. $K_D$ and $B_{max}$ are estimated along with the $K_i$ from appropriate data, eliminating the need to arbitrarily assume $K_D$ before $K_i$ can be estimated.
4. With computerized curve fitting, it is possible to estimate the precision of the parameters along with their actual value, taking into account the precision of the original experimental measurements. Noisy measurements of the binding isotherm will therefore result in imprecise estimates of $K_D$, and the investigator is informed of the effect of measurement error on his results.
5. With computerized curve fitting, it is easy to generalize the model beyond a single, homogeneous receptor, unlike the cruder $ED_{50}$ approach. Thus, if there is evidence for receptor heterogeneity, it may be difficult if not impossible to get accurate estimates of $ED_{50}$ values for the high- and low-affinity sites and conversion to $K_i$ values cannot be made. However, the mathematical model for two binding sites may be fit to these displacement curves (with appropriate constraints on the parameters) and the best estimates for each of the two $K_i$ values obtained. The computerized curve-fitting approach may be further extended to include three binding sites, nonspecific binding, cooperative binding, or many other phenomena, as necessary, which is not possible with methods based on empirical or graphical descriptions of the curve.

## C. Noncompetitive Inhibition

An additional complication is introduced if the inhibitor does not behave in a true competitive fashion. Modes of inhibition have been classified as classical competitive, noncompetitive, uncompetitive, and mixed, depending on the effect of the inhibitor on the apparent affinity and binding capacity (Wong, 1975). In order to characterize the type of inhibition, we must perform multiple saturation curves at differeing inhibitor concentrations or, equivalently, multiple competition curves at differing tracer ligand concentrations. We may analyze the data initially by monitoring the changes in the $ED_{50}$ values of the displacement curves or by looking at changes in $K_D$ and $B_{max}$ for Scatchard plots. Ideally, both methods should ultimately give the same results. A superior, unified approach is to fit the entire set of data simultaneously using computerized WNLLS analysis. Here, we may compare models for competitive, noncompetitive, or other types of inhibition and choose the one that fits the data best with fewest parameters. Once the appropriate model is determined, we can obtain the estimates of its

parameters. These values will not be influenced by which graphical analysis step was performed initially and are optimal in a statistical sense.

## VI. DIFFERENTIATING MULTIPLE RECEPTORS: THE "N BY M" MODEL

Experiments involving several ligands and possibly multiple classes of receptors are best handled using the "N by M" (*N* ligands, *M* binding sites) model first formulated by Feldman (1972) and expanded and applied by Munson and Rodbard (1979, 1980), De Lean *et al.* (1982), and others. Using this mathematical model, incorporated into an appropriate computer program, one may analyze families of saturation or displacement curves without numerical or graphical approximations. Further, one is not limited to analyzing binding curves in which the concentration of only a single ligand is varied. One can now conceive of experiments wherein several ligand concentrations are varied simultaneously. Thus, instead of dose–response curves, one may analyze dose–response surfaces or even three-dimensional dose–response hypersurfaces.

As particular cases, the "N by M" model can analyze Scatchard plots (or saturation curves) as "1 by 1" or "1 by 2" models. Displacement curves assuming homogeneous receptors are represented as a "2 by 1" model, that is, labeled and unlabeled (two different) ligands with one class of receptors. As distinct from the graphical displacement curve approach, this method can also handle the "2 by 2" case: labeled and unlabeled ligands competing for two different classes of binding sites, as well.

With this type of modeling, Hancock and colleagues (1979) were able to quantify accurately $\beta_1$ versus $\beta_2$ adrenergic receptors in frog myocardium utilizing multiple competition curves. Remarkably, they were able to do this with only a single, completely unselective radioligand ([$^3$H]dihydroalprenolol) by utilizing a series of partially selective competitors. Similar work has been done in the $\alpha$-adrenergic system (Hoffman *et al.*, 1979).

More incisive analysis can be performed when multiple radioligands are available. Here, a complete cross-displacement study may be attempted: Each ligand is run in self- and cross-competition experiments. Results of these experiments can tell us if all the ligands share the same set of binding sites, or if some sites are available only to certain ligands. This approach was used by Clayton *et al.* (1979) in the study of GnRH analogs binding to pituitary membranes. Multiple binding sites were available to native GnRH, and these classes bound all the analogs tested. A similar experimental design was used by Pfeiffer and Herz (1981, 1982) in the search for a third ($\kappa$) opiate receptor. Multiple opiate receptor subtypes had been postulated on the basis of distinctive pharmacological profiles of certain opiate agonists. Martin *et al.* (1976) had suggested there are at least

three types, named for their prototypic agonists; μ [morphine, dihydromorphine (DHM)], σ (SKF10047), and κ [ketocyclazocine, ethylketocyclazocine (EKC)]. Yet another class [δ with prototype ligand Leucine-enkephalin or DAla-DLeu-enkephalin (DADL)] had already been defined on the basis of its preference for binding enkephalins. However, the binding data did not generally fit the picture developed from the pharmacology; no distinctive binding to κ or σ receptors could be found. Pfeiffer used the radiolabeled prototypic ligands and their unlabeled counterparts in a complete cross-displacement study, yielding a complex family of competition curves with a variety of shapes, many with apparent logit-log slopes less than one. Owing to the distinctive, nonsymmetric shape of many of these curves, graphical analysis would clearly have been inadequate. Rather, computerized curve fitting of the "N by M" model and comparison of the fit of two, three, or four binding site models established that exactly three sites were necessary and sufficient to explain the data. Two of these had previously been characterized in binding studies as the μ and δ sites, and the third class showed evidence of being the κ site. These results brought the opiate binding data in line with the pharmacology of the system.

Interestingly, prior to this study, several groups (Harris and Sethy, 1980; Hiller and Simon, 1980; Snyder and Goodman, 1980) had published claims of failure to detect a third opiate binding site; the two sites, μ and δ, appeared adequate. These earlier studies, though similar in design to Pfeiffer's, were hampered in part by the relative insensitivity of standard competition curve analysis techniques. Looking for a distinctive pattern of $ED_{50}$ values in displacement curves using the radiolabeled κ ligand led some investigators to conclude there was no evidence of a separate κ binding site. As it turns out, because [$^3$H]EKC apparently labels both κ and μ (and to a lesser degree, δ) sites with high affinity, its pattern of $ED_{50}$ values was not very different from that for a pure μ label such as DHM. Nevertheless, the shape and location of the various displacement curves was sufficient to distinguish a third site, as Pfeiffer showed.

Kosterlitz *et al.* (1981) and Chang *et al.* (1981) also published evidence of binding to a third opiate receptor class using a modification of the standard binding experiment. Recognizing the lack of κ selectivity of the binding of [$^3$H]EKC or [$^3$H]diprenorphine, these investigators ran the displacement curves in the presence of saturating quantities of highly selective μ and δ compounds, thus preventing further binding to these sites. The resulting displacement curves produced using a variety of opioid ligands showed distinctive $ED_{50}$ patterns and were generaly consistent with binding to a single homogeneous class of receptors, possibly the κ site.

Thus, in this case, the computer modeling approach was able to resolve the third binding site without the use of new, highly selective ligands and without revising the standard experimental design. Also, computer analysis provided a quantitative check that the qualitative assumptions and conclusions were compatible with the experimental data.

## VII. THE LIGAND COMPUTER PROGRAM

The techniques of WNLLS analysis for the "N by M" model has been implemented in the LIGAND computer program system (Munson, 1981). This system includes programs for data preprocessing, curve-fitting, and graphical presentation of results. The program is written in BASIC for the DEC System 10 computer, and has been converted to run on several popular microcomputers (HP-9845, HP-9836, Apple II, IBM PC). The program provides for the analysis of possibly multiple ligand, multiple receptor experiments, including explicit parameters for nonspecific binding, correction, or scaling of different experiments relative to the first of a series. It also incorporates a generalized cooperativity model (Munson and Rodbard, 1984). The program allows for formulation and testing of several alternative hypotheses (or models) so that the most appropriate one may be chosen on the basis of an objective, statistically valid criterion, the "extra sum-of-squares" F-test (Draper and Smith, 1981). Several features are included that take advantage of the interactive environment of small microcomputers: parameter values may be changed dynamically, the curve-fitting procedure may be interrupted at any point and a graph of the intermediate results obtained, and parameters may be forced to share a common value or to hold a fixed, predetermined value. The program is designed to handle virtually any equilibrium experiment involving multiple ligands and receptors. The program may be obtained from the author upon request.

## VIII. CONCLUSION

No discussion of computerized analytic methods is complete without some mention of its limitations. Every analytic method is dependent on certain assumptions. In Equilibrium binding studies, it is frequently assumed that (1) equilibrium has been established, (2) bound and free ligand are perfectly separated and accurately measured, (3) neither ligand nor binding site is degraded, (4) the binding site is in true solution, and (5) the particular model for ligand–receptor interaction applies. These assumptions qualify all results from such experiments, either graphical or computerized. One of the real values of a formal statistical analysis is that it forces one to consider these assumptions explicitly and ultimately to test them experimentally.

Nevertheless, graphical analysis of data should remain an important part of receptor binding studies. The eye–brain system is, in fact, several orders of magnitude more complex than even the most powerful computer, and its method of operation is not well understood. Qualitative, intuitive understanding is undoubtedly aided by graphical presentation. However, these graphical tools are

often the source of unnecessary confusion in interpreting the data. On the other hand, formal statistical tests based on computerized WNLLS analysis can give unambiguous answers to questions such as "Is there saturable binding?", "Is there evidence of nonlinearity in the data?", or in general, "Does model A fit the data better (or worse) than model B?" Application of these tests removes much of the ambiguity inherent in the subjective graphical approach and can often clarify and establish results with certainty. Moreover, computerized analysis facilitates the consideration of more complex and probably more realistic models of ligand–receptor interaction, and avoids several commonly used but seldom validated approximations. (Also, certain "nuisance" parameters, such as nonspecific binding or scaling factors between experiments, can be explicitly included in the model, avoiding the need to remove their effect by "precorrection" of the data.) By considering all the unknown parameters simultaneously, we get a better idea of the precision of their values and may therefore avoid ambiguous or contradictory results.

In analysis of multiple or heterogeneous receptor systems, the need to use multiple ligands, both labeled and unlabeled, is clear. If a specific ligand for each receptor is not available, it is still possible to accurately analyze the system, provided the right combination of partially selective ligands can be found. Here, the use of the "N by M" model is extremely useful if not essential to the analysis of the data, as results in the adrenergic and the opiate receptor systems have demonstrated. Since experiments are frequently designed around the available analytic tools, computerized curve fitting should increase experimental possibilities since it removes many restrictions imposed by traditional graphical techniques. More than one (or two) ligands may be used in a single experiment, with several concentrations being varied simultaneously. Experimental results from different runs can be pooled and analyzed simultaneously. Furthermore, the techniques of computerized optimization of experimental design can lead to new, more economical, and more powerful approaches to the binding experiments themselves.

## REFERENCES

Bard, Y. (1973), "Nonlinear Parameter Estimation." Academic Press, New York.

Belsley, D. A., Kuh, E., and Welsch, R. E. (1980). "Regression Diagnostics." Wiley, New York.

Chang, K. J., Hazum, E., and Cuatrecasas, P. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 4141–4154.

Cheng, Y.-C., and Prusoff, W. H. (1973). *Biochem. Pharmacol.* **22,** 3099–3102.

Chrousos, G. P., Renquist, D., Brandon, D., Eil, C., Pugeat, M., Vigersky, R., Cutler, G. B., Jr., Loriaux, D. L., and Lipsatt, M. B. (1982). *Proc. Natl. Acad. Sci. U.S.A.* **79,** 2036–2040.

Clayton, R. N., Shakespear, R. A., Duncan, J. A., and Marshall, J. C. (1979). *Endocrinology* **105,** 1369–1376.

De Lean, A., Munson, P. J., and Rodbard, D. (1978). *Am. J. Physiol.* **235,** E97–E102.
De Lean, A., Hancock, A. A., and Lefkowitz, R. J. (1982). *Mol. Pharmacol.* **21,** 5–16.
Draper, N., and Smith, H. (1981). "Applied Regression Analysis," 2nd ed. Wiley, New York.
Feldman, H. A. (1972). *Anal. Biochem.* **48,** 317–338.
Hancock, A. A., De Lean, A., and Lefkowitz, R. J. (1979). *Mol. Pharmacol.* **16,** 1–9.
Harris, D. W., and Sethy, V. H. (1980). *Eur. J. Pharmacol.* **66,** 121–123.
Hiller, J. M., and Simon, E. J. (1980). *J. Pharmacol. Exp. Ther.* **214,** 516–519.
Hoffman, B. B., De Lean, A., Wood, C. L., Schocken, D. D., and Lefkowitz, R. J. (1979). *Life Sci.* **24,** 1739–1746.
Hunston, D. L. (1975). *Anal. Biochem.* **63,** 99–109.
Klotz, I. M. (1982). *Science* **217,** 1247–1249.
Kosterlitz, H. W., Paterson, S. J., and Robson, L. E. (1981). *Br. J. Pharmacol.* **73,** 939–949.
Martin, W. R., Eades, C. B., Thompson, J. A., Huppler, R. E., and Gilbert, P. E. (1976). *J. Pharmacol. Exp. Ther.* **197,** 517–522.
Munson, P. J. (1981), "User's Guide to LIGAND. A Program System for Fitting Multiple Ligand, Multiple Binding Site Data." Biomedical Computing Technology Information Center, Nashville, Tennessee.
Munson, P. J. (1983a). *J. Recept. Res.* **3,** 249–259.
Munson, P. J. (1983b), LIGAND: A computerized analysis of ligand binding data. *In* "Methods in Enzymology" (J. J. Langone and H. Van Vunakis, eds.) 92, pp. 543–576. Academic Press, New York.
Munson, P. J., and Rodbard, D. (1979). *Endocrinology* **105,** 1377–1381.
Munson, P. J., and Rodbard, D. (1980). *Anal. Biochem.* **107,** 220–239.
Munson, P. J., and Rodbard, D. (1983). *Science* **220,** 979–981.
Munson, P. J., and Rodbard, D. (1984). Computerized analysis of ligand binding data: Basic principles and recent developments. *In* "Computers and Endocrinology" (D. Rodbard and G. Forti, eds.), pp. 117–146. Raven Press, New York.
Pfeiffer, A., and Herz, A. (1981). *Biochem. Biophys. Res. Commun.* **101,** 38–44.
Pfeiffer, A., and Herz, A. (1982). *Mol. Pharmacol.* **21,** 266–271.
Rodbard, D. (1974). *Clin. Chem. (Winston-Salem, N.C.)* **20,** 1255–1270.
Rodbard, D., and Lewald, J. E. (1970). *Acta Enodocrinol. (Copenhagen)* **64,** Suppl. 147, 79–103.
Rodbard, D., Lenox, R. H., Wray, H. L., and Ramseth, D. (1976). *Clin. Chem. (Winston-Salem, N.C.)* **22,** 350–355.
Rodbard, D., Munson, P. J., and Thakur, A. K. (1980). *Cancer* **46,** 2907–2918.
Synder, S. H., and Goodman, R. R. (1980). *J. Neurochem.* **35,** 5–15.
Thakur, A. K., Jaffe, M. L., and Rodbard, D. (1980). *Anal. Biochem.* **107,** 279–295.
Wong, J.T.-F. (1975). "Kinetics of Enzyme Mechanisms." Academic Press, New York.

# Chapter 4

# THE DISTRIBUTION OF PERIPHERAL REGULATORY PEPTIDES: A DUAL IMMUNOCHEMICAL (IMMUNOCYTOCHEMISTRY AND RADIOIMMUNOASSAY) APPROACH

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ISBN 0-12-470350-X

## I. INTRODUCTION

The recent discovery that active peptides, originally extracted from the brain and/or the gut, are found in most peripheral tissues has revolutionized our ideas of control of bodily functions (Polak and Bloom, 1981a). Regulatory peptides have now been found by immunocytochemistry in typical endocrine cells or in autonomic or sensory nerves (Polak and Bloom, 1982a). Indeed, the two main controlling systems of the body, the neural and endocrine, are now considered to be so closely related by the production and release of the same active peptides that they are combined as the "diffuse neuroendocrine system" (Polak and Bloom, 1982b).

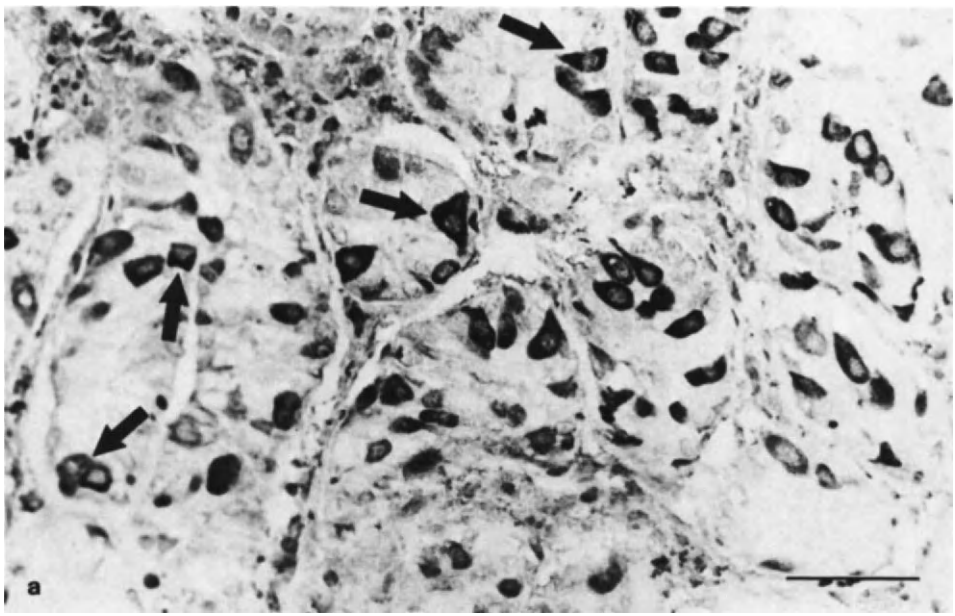


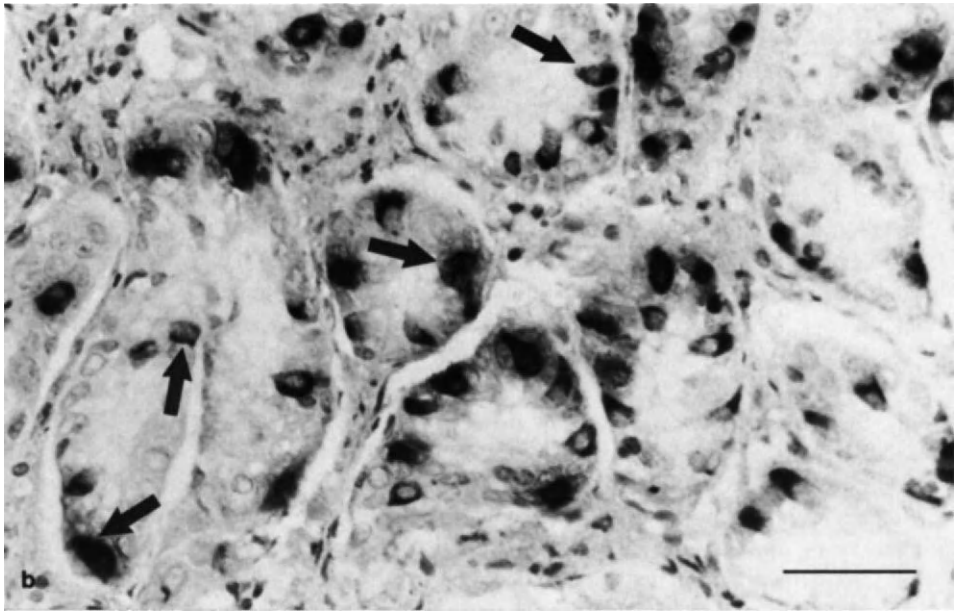

**Fig. 1.** Human antrum fixed in benzoquinone vapor, three in serial sections stained for neuron-specific enolase (a) and for gastrin (b). Long arrows point to endocrine cells containing both peptide and enzyme. Other cells are clearly stained only for neuron-specific enolase, indicating the pres-

## A. Means of Visualizing the Peripheral Regulatory Peptide-Containing System

This system can now be visualized and delineated in its entirety by immunocytochemistry using antibodies to a brain glycolytic enzyme, neuron-specific enolase (Marangos *et al.*, 1982). (For details see chapter by DeFeudis, Part B). Antibodies to neuron-specific enolase (which are now commercially available from Polysciences, Paul Valley Industrial Park, Warrington, Pennsylvania) are capable of detecting all peripheral endocrine and neural cell types, not only those containing known regulatory peptides, amines, or classical neurotransmitters but also other cell types whose product remains to be discovered. Immunocytochemical staining with antibodies to neuron-specific enolase reveals a much larger mass of neuroendocrine elements than any other method or combination of methods (Bishop *et al.*, 1982) (Fig. 1a–b).

## B. Immunochemical Methods for Regulatory Peptides

The distribution and concentration of regulatory peptides can now be successfully measured by the use of two immunochemical methods, immunocytochemistry and radioimmunoassay, used in combination.




ence of endocrine elements containing peptides other than gastrin. P.A.P. method. (×250; bar, 100 μm)

Immunocytochemistry provides information on the precise localization within the tissue of a given regulatory peptide (Polak and Bloom, 1983), that is, its presence in endocrine cells or nerves and, within these categories, its intracellular localization in cytoplasmic secretory granules or even in the endoplasmic reticular system. Radioimmunoassay will tell us the absolute quantities of the peptide and the relative concentration of its molecular form variants. The principles of and specific methods for radioimmunoassay of regulatory peptides are not discussed in this chapter. For the principles of radioimmunoassay the authors refer to other chapters in this and subsequent volumes. Regarding specific methods for the measurements of regulatory peptides see Bloom and Long (1982).

## C. Immunocytochemical Methods

Since the introduction of the immunofluorescence method by Coons *et al.* in 1955, immunocytochemistry has established itself as a discipline in its own right, finding uses in conjunction with many other scientific procedures. The variety of methods that have led to further improvement of its specificity and versatility is so large so to be beyond the scope of this monograph. For details and further references see Polak and Van Noorden (1983). Mention, however, must be made of three new approaches to antigen detection that have been proposed recently.

### *1. Monoclonal Antibodies (or the Hybrid Myeloma Method)*

The development of the hybrid myeloma method of antibody production (Kohler and Milstein, 1975) opened up the possibility of improving further the existing immunocytochemical methods by considerably reducing unwanted background staining. The latter is primarily due to the use of polyclonal antibodies containing a mixed population of antibodies, many of which react with unwanted tissue components. Although only a limited number of monoclonal antibodies to regulatory peptides are now available, their availability will doubtlessly be increased in the near future.

### *2. Electron Immunocytochemistry*

A considerable number of methods for the ultrastructural localization of regulatory peptides have recently been proposed. Our group has used mainly a variety of immunogold staining procedures (Varndell *et al.*, 1982). These are based on the principle that colloidal gold particles are electron-dense and possess the ability to adsorb macromolecules such as antibodies. They can also be produced in different sizes, allowing for the simultaneous demonstration of multiple

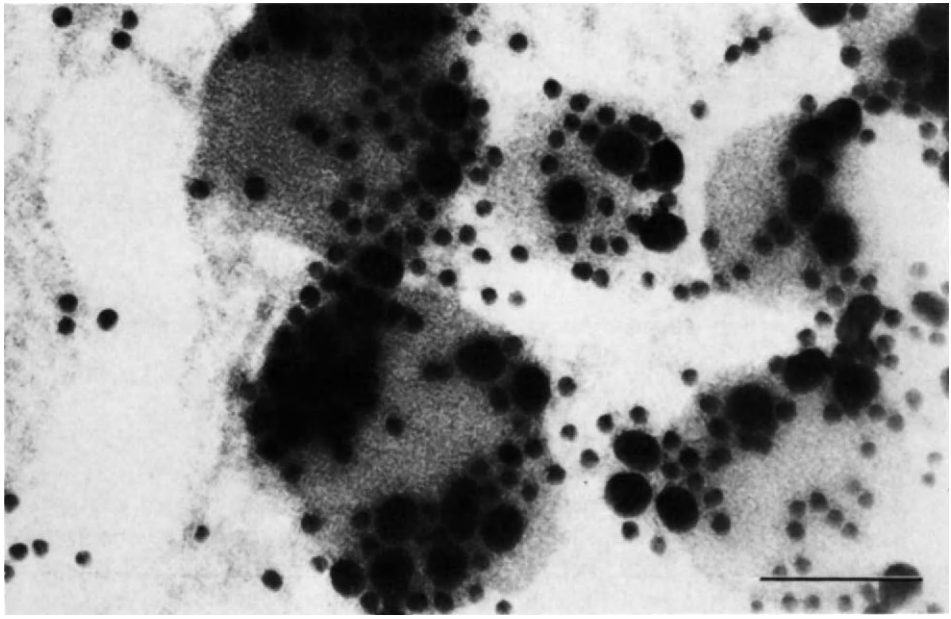


**Fig. 2.** Catecholamine-containing granules from a cat adrenal medulla fixed in glutaraldehyde and embedded in resin for electron microscopy. DBH immunoreactivity is recognized by the 40-nm gold label, whereas enkephalin-like immunoreactivity is labeled by 20-nm gold particles. Both enzyme and enkephalin are localized in the same adrenal medullary granules. (×120,000; bar, 0.25 μm)

antigens in a single tissue section (Tapia *et al.*, 1983) (Fig. 2) (for details of technology see the Appendix to this chapter). By employing the immunogold staining procedures on either frozen thin sections or on resin-embedded material and using a panel of region-specific antibodies recognizing different parts of the peptide and its molecular form variants, it is now possible to visualize the main intracellular events of peptide synthesis, from their first formation in the Golgi area to their storage in secretory granules and release from the cell (Polak *et al.*, 1982).

### *3. Intracellular Demonstration of mRNA, which Controls Peptide Precursor Biosynthesis*

It will soon be possible to show the intracellular localization of the mRNA species that direct the synthesis of peptide precursors. One of these methods is based on the use of recombinant cDNA (hybridization histochemistry) (Hudson *et al.*, 1981). It is, in addition, probable that the structure of the enzymes

responsible for both mRNA and peptide processing will soon be determined, and thus it may be possible to visualize disturbances of their structure and function in disease.

Some of the immunocytochemical methods employed in our unit are given in the Appendix to this chapter.

## II. GENERAL CHARACTERISTICS OF THE MOST ABUNDANT REGULATORY PEPTIDES

In this section we shall describe the main features of the most abundantly distributed regulatory peptides.

### A. Vasoactive Intestinal Polypeptide (VIP)

VIP is a 28-amino-acid peptide originally extracted from the gut and later found in the brain and in a large number of peripheral tissues. Immunocytochemistry localizes VIP to central and peripheral neurons (Polak and Bloom, 1982c). VIP nerves are frequently seen in association with blood vessels, secretory glands, and smooth muscle, in keeping with the reports that VIP is a potent vasodilatory, secretomotor, and muscle-relaxing peptide. The postulate that VIP acts as a neurotransmitter is supported by the finding of VIP in enriched synaptosomal fractions, the release of VIP after nerve stimulation, and the replication or mimicking of a number of nerve-mediated tissue responses after microinjections of VIP into the local circulation (Said, 1982a).

VIP nerves orginate in most peripheral tissues from a system of local cell bodies. This finding may have important implications in terms of autonomous functions. It is likely that the peripheral VIPergic system acts at least in part independently from its central neuroanatomical connections.

### B. Substance P

Substance P is an 11-amino-acid peptide discovered in 1931 when von Euler and Gaddum (1931) noted the release after nerve stimulation of a substance capable of inducing a marked atropine-resistant gut muscle contraction. The noncommittal name of substance P was subsequently chosen. Like VIP, substance P is very widely distributed both in the brain and in most peripheral tissues (Polak and Bloom, 1981b). Immunocytochemistry localizes substance P to autonomic and sensory neurons. The finding of substance P in primary sensory neurons of the dorsal root ganglia and in the dorsal horn of the spinal cord and its subsequent depletion after dorsal rhizotomy led to the postulate of substance P being a sensory neurotransmitter (Otsuka *et al.*, 1975). This postulate still holds

true, and it is supported by a wealth of neurophysiological evidence including the release of substance P after sensory neuron stimulation (Olgart *et al.*, 1977). Specific substance P blockers, based on the development of analogs to the original substance P molecule, have recently been described (Engberg *et al.*, 1981).

## C. Somatostatin

Somatostatin was originally extracted from the brain and characterized as a 14-amino-acid peptide (Brazeau *et al.*, 1973). Recent evidence, however, indicates the presence of larger molecular forms, in particular that of a larger peptide composed of 28 amino acids (Vaysse *et al.*, 1981). In the periphery somatostatin is found in many tissues including the gastrointestinal tract, the pancreas, the thyroid, the lung, the skin, and the urogenital system. Immunocytochemistry localizes somatostatin both to endocrine cells and to autonomic nerves and preliminary evidence indicates that the predominant molecular form of neural somatostatin is a shorter sequence of 14 amino acids, whereas its longer fragment is predominantly localized in endocrine cells (Patel *et al.*, 1981a). Somatostatin displays a wide variety of inhibitory actions, not only on the release of many other regulatory peptides but also on the target tissue (Patel *et al.*, 1981b) (Table I).

## D. Bombesin

Bombesin was originally extracted from the skin of the discglossid frog *Bombina bombina* and characterized as a 14-amino-acid peptide (Erspamer and Melchiorri, 1973). Its powerful stimulatory actions in mammals led to the postulate that a bombesin-like material may be found in mammals. Indeed, this is the case. In mammals, including man, bombesin is found in the brain, the gastrointestinal tract, the pancreas, and the lung. Mammalian bombesin has recently

TABLE I

The Main Inhibitory Actions of Somatostatin

| | |
|---|---|
| Growth Hormone | Gastric acid |
| TSH | Pepsin |
| Insulin | Gastric emptying |
| Glucagon | Pancreatic enzymes |
| PP | Pancreatic bicarbonate |
| Gastrin | Choleresis |
| GIP | Gall bladder contraction |
| Motilin | Motility |
| CCK | Xylose absorption |
| Enteroglucagon | Celiac blood flow |

been characterised as a 27-amino-acid peptide possessing in common with amphibian bombesin the last 7 C-terminal amino acids (McDonald *et al.*, 1978). The potent releasing properties of bombesin contrast with the inhibitory properties of somatostatin. Bombesin stimulates the release of most other regulatory peptides. Immunocytochemistry localizes bombesin both to endocrine cells (e.g., of the lung) and to central and peripheral (autonomic) nerves.

### E. The Enkephalins

The enkephalins are a pair of 5-amino-acid peptides differing by one amino acid (leucine/methionine) at the C-terminus. Contrary to earlier assumptions, the enkephalins seem not to share the same precursor with other chemically related peptides such as the endorphins and lipotropins. However, both Leu- and Met-enkephalin have been reported to derive from a larger common precursor recently isolated from adrenal medulla tissue and characterized (Comb *et al.*, 1982). The enkephalins are found in the brain and in several peripheral tissues including the gastointestinal tract, the pancreas, and the adrenal medulla. Immunocytochemistry localizes the enkephalins to enteric nerves and to adrenal medullary cells. The coexistence of the two enkephalins within the same nerve fiber in the gut and the coexistence of catecholamines and the enkephalins within the same adrenal medullary cell have recently been demonstrated using double immunogold staining procedures.

### F. New Peptides

Tatemoto and Mutt (1980) devised an ingenious method for the chemical detection of new regulatory peptides based on the principle of fragmentation methodology by treating tissue extracts with endopeptidases. The latter are able to split off the characteristic C-terminal amide fragment, which is very common in a large number of regulatory peptides. Using this novel procedure, at least three new regulatory peptides have recently been described and characterized. These include peptide HI (PHI) (histidine isoleucine), PYY (tyrosine at both ends), NPY (neuropeptide with tyrosine). The first one belongs to the glucagon–secretin family and the last two to the pancreatic polypeptide family (Table II).

## III. DISTRIBUTION OF REGULATORY PEPTIDES IN PERIPHERAL TISSUES

In this section we shall describe the presence of regulatory peptides in a number of peripheral tissues in which they occur in significant quantities and in which their distribution has been considerably investigated.

TABLE II

Amino Acid Sequence Homologies of the Pancreatic Polypeptide Family

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| PYY | Tyr | *Pro* | Ala | Lys | *Pro* | Glu | Ala | *Pro* | *Gly* | Glu | *Asp* | *Ala* | Ser | *Pro* | *Glu* | Glu | Leu | Ser | Arg | *Tyr* | Tyr | *Ala* | Ser | *Leu* | *Arg* | His | *Tyr* | Leu | *Asn* | Leu | Val | *Thr* | *Arg* | Gln | *Arg* | *Tyr*$NH_2$ |
| NPY | Tyr | *Pro* | Ser | Lys | *Pro* | Asp | Asn | *Pro* | *Gly* | Glu | *Asp* | *Ala* | Pro | Ala | *Glu* | Asp | Leu | *Ala* | Arg | *Tyr* | Tyr | Ser | Ala | *Leu* | *Arg* | His | *Tyr* | *Ile* | *Asn* | Leu | Ile | *Thr* | *Arg* | Gln | *Arg* | *Tyr*$NH_2$ |
| APP | Gly | *Pro* | Ser | Gln | *Pro* | Thr | Tyr | *Pro* | *Gly* | Asp | *Asp* | *Ala* | Pro | Val | *Glu* | Asp | Leu | Ile | Arg | Phe | Tyr | Asp | Asn | *Leu* | Gln | Gln | *Tyr* | Leu | *Asn* | Val | Val | *Thr* | *Arg* | His | *Arg* | *Tyr*$NH_2$ |
| HPP | Ala | *Pro* | Leu | Glu | *Pro* | Val | Tyr | *Pro* | *Gly* | Asp | Asn | *Ala* | Thr | *Pro* | *Glu* | Gln | Met | *Ala* | Gln | *Tyr* | Ala | *Ala* | Asp | *Leu* | *Arg* | Arg | *Tyr* | *Ile* | *Asn* | Met | Leu | *Thr* | *Arg* | Pro | *Arg* | *Tyr*$NH_2$ |
| PPP | Ala | *Pro* | Leu | Glu | *Pro* | Val | Tyr | *Pro* | *Gly* | Asp | *Asp* | *Ala* | Thr | *Pro* | *Glu* | Gln | Met | *Ala* | Gln | *Tyr* | Ala | *Ala* | Glu | *Leu* | *Arg* | Arg | *Tyr* | *Ile* | *Asn* | Met | Leu | *Thr* | *Arg* | Pro | *Arg* | *Tyr*$NH_2$ |
| BPP | Ala | *Pro* | Leu | Glu | *Pro* | Glu | Tyr | *Pro* | *Gly* | Asp | Asn | *Ala* | Thr | *Pro* | *Glu* | Gln | Met | *Ala* | Gln | *Tyr* | Ala | *Ala* | Glu | *Leu* | *Arg* | Arg | *Tyr* | *Ile* | *Asn* | Met | Leu | *Thr* | *Arg* | Pro | *Arg* | *Tyr*$NH_2$ |

## A. Gastrointestinal Tract

Most, if not all, of the most commonly found regulatory peptides are present in the gastrointestinal tract (Polak and Bloom, 1982b). Some of them are found in typical mucosal endocrine cells (Fig. 3), others in a complex network of enteric nerves (Fig. 4), and some show a dual localization in both endocrine cells and enteric nerves (e.g., somatostatin). Regulatory peptides found in mucosal endocrine cells show a precise distribution in limited anatomical areas of the bowel. In contrast, regulatory peptides in enteric nerves are much more widely distributed and are found in almost every area of the gut (see Tables III and IV). Ultrastructurally, both endocrine cells and enteric nerves are characterized by the presence of electron-dense neurosecretory granules whose morphological features distinguish one neural or endocrine cell type from another. These features reflect the property of producing and releasing separate regulatory peptides, which can be further associated with particular cell or granule types by the use of electron immunocytochemistry (Polak and Bloom, 1983).

Regulatory peptide-containing endocrine cells and enteric nerves have been

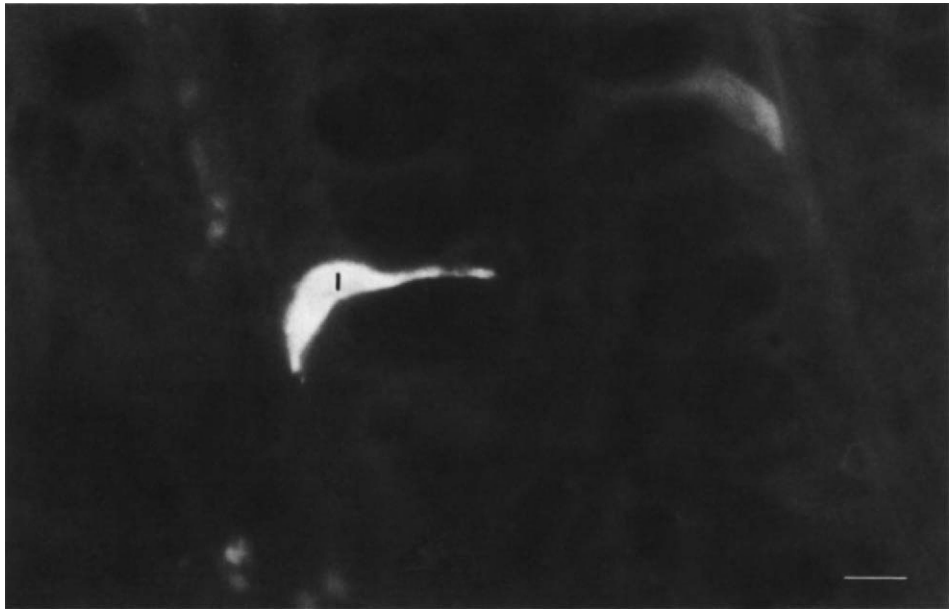



**Fig. 3.** Enteroglucagon-containing cell in a piece of human colon fixed in a solution of benzoquinone. Note the connection with the lumen (l) and the broadly extended base of the cell. Immunofluorescence method (×1150; bar, 10 μm)

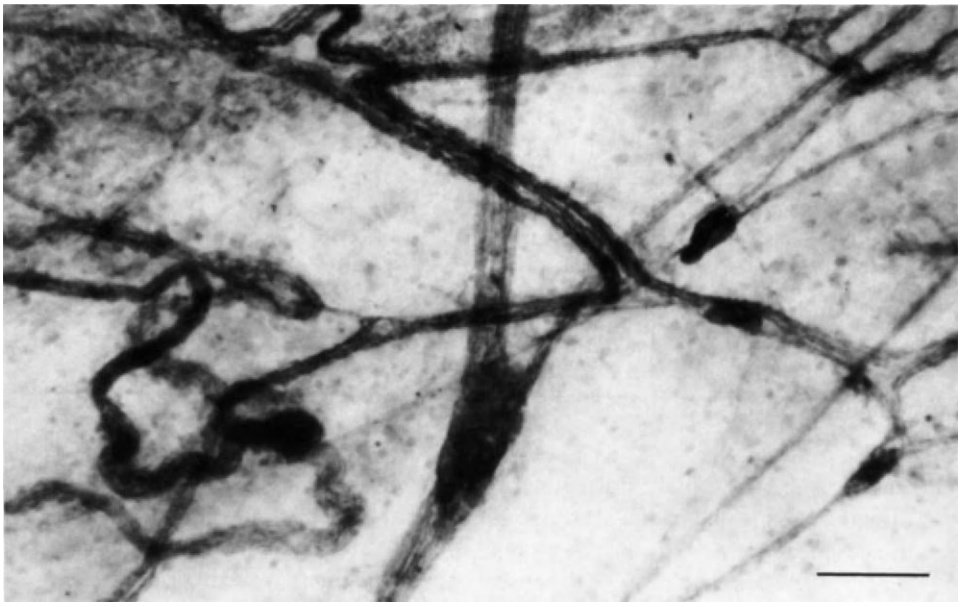


**Fig. 4.** A complex network of VIP-containing enteric nerves is seen in this immunostained stretch preparation from the lamina propria of the human bowel. P.A.P. method. (×210; bar, 100 μm)

TABLE III

Distribution and Characteristics of Regulatory Peptides of the Gut Acting via Circulation

| Function | Peptide | Fund | Ant | Duod | Jej | Ile | Col | MW | Aminoacid no. | Granule size (nm ± SD) |
|---|---|---|---|---|---|---|---|---|---|---|
| Circulating hormone | Gastrin 17 | | ●● | ●● | | | | 2100 | 17 | 360 ± 56 |
| | Gastrin 34 | | ●● | ●● | ●● | | | 3800 | 34 | 175 ± 32 |
| | Secretin | | | ●● | ●● | | | 3073 | 27 | 240 ± 32 |
| | Motilin | | | ●● | ●● | | | 2700 | 32 | 180 ± 24 |
| | CCK | | | ●● | ●● | | | 3883 | 34 | 250 ± 17 |
| | GIP | | ●● | ●● | ●● | | | 5105 | 43 | 350 ± 24 |
| | EG | | | | ●● | ●● | ●● | | | 210 ± 26 |
| | Neurotensin | | | | | ●● | | 1673 | 13 | 300 ± 46 |

TABLE IV

Distribution and Characteristics of Locally Acting Regulatory Peptides of the Gut

| Function | Peptide | Fund | Ant | Duod | Jej | Ile | Col | MW | Aminoacid no. | Granule size (nm ± SD) |
|---|---|---|---|---|---|---|---|---|---|---|
| *Paracrine* | SRIF | ●● | ●● | ●● | ●● | ●● | ●● | 1639 | 14 | 310 ± 46 |
| Neuro- | VIP | ●● | ●● | ●● | ●● | ●● | ●● | 3326 | 28 | 90 ± 11 |
| transmitter/ | Sub P | ●● | ●● | ●● | ●● | ●● | ●● | 1347 | 11 | 82 ± 7 |
| *modulator* | Bombesin | ●● | ●● | ●● | ●● | ●● | ●● | 1620 | 14 | |
| | Leu-Enk | ●● | ●● | ●● | ●● | ●● | ●● | 556 | 5 | 83 ± 8 |
| | Met-Enk | ●● | ●● | ●● | ●● | ●● | ●● | 574 | 5 | 83 ± 8 |
| | SRIF | ●● | ●● | ●● | ●● | ●● | ●● | 1639 | 14 | 92 ± 8 |

shown to be abnormal in a number of gastrointestinal diseases. These include diseases manifested by malabsorption (e.g., celiac disease), in which some of the mucosal endocrine cells have been reported to be severely abnormal (Bloom and Polak, 1980), and diseases of the bowel manifested by marked abnormal motility (e.g., Hirschsprung's disease, Chagas' disease, and Crohn's disease) in which a number of regulatory peptide-containing nerves have been shown to be grossly abnormal (Bishop *et al.*, 1981).

## B. Central Nervous System

### *1. Brain*

Neuropeptides possess a specific localization and projection pattern within the brain (Roberts *et al.*, 1981). Nearly all neuropeptides have been found within cell bodies and fibers in the hypothalamus. Outside the hypothalamus, neuropeptides are most prevalent in the cerebral cortex, striatum, and within regions collectively known as the limbic system. VIP, CCK-8, and somatostatin, for instance, are present within cortical interneurons and within cell bodies of the interneuron type in the hippocampus. Cell bodies and fiber terminals immunoreactive for VIP, CCK, neurotensin, Met-enkephalin, substance P, and somatostatin have been found within discrete nuclei of the amygdala suggesting a complex intra-amygdaloid connectivity. Substance P has a wider distribution, occurring with the highest concentration in the substantia nigra and globus pallidus, and a few cell bodies have also been observed in the raphe nucleus and trigeminal nucleus of the brainstem.

Recent evidence suggests that neuropeptides are contained within major pro-

jection pathways of the brain. The presence of VIP and neurotensin has been confirmed from the amygdala to the hypothalamus via the stria terminalis. Substance P projects from the substantia nigra to the striatum and from the habenular nucleus of the thalamus via the fasciculus retroflexus to the interpreduncular nucleus. A long neurotensin pathway arising from the subiculum and projecting to the anterior cingulate cortex has been discovered more recently.

Despite success in localizing peptides within the brain, little is known of their function. Electrophysiological studies indicate that peptides may modulate the actions of the classical neurotransmitters (monoamines, acetylcholine, and amino acids). The few investigations concerning behavior show that central administration of CCK-8 alters the feeding behavior of rats or may influence the process of memory consolidation. Substance P is capable of lowering the pain threshold, whereas the enkephalins conversely are both analgesic and euphorigenic.

### 2. *Spinal Cord*

During the past decade more than 20 regulatory peptides have been localized within the mammalian spinal cord. All these peptides have a well-defined pattern of distribution in the various laminae of the dorsal and ventral spinal cord, the majority tending to be most concentrated in the upper laminae of the dorsal horn, the substantia gelatinosa.

It is in this region of the spinal cord that the fine afferent fibers terminate, and it represents an area of intense neuronal activity. It is here, within the substantia gelatinosa, that sensory information is processed directly or transcribed and relayed to higher centers in the brain. One of the major sources of these peptides, namely substance P, somatostatin, CCK/gastrin-like peptides, and VIP, in the spinal cord is extrinsic (i.e., from afferent fibers) (Gibson *et al.*, 1981). The peptides are synthesized within the small cells of the dorsal root ganglia (primary sensory neurons) (Fig. 5) and are transported to the central terminations in the substantia gelatinosa. Interference with the afferent pathway thus removes this source of peptide from the dorsal horn (e.g., dorsal rhizotomy, peripheral nerve section, or pharmacological destruction such as treatment neonatally with capsaicin). Immunocytochemical analyses have demonstrated that while these manipulations remove a substantial amount of peptide from the substantia gelatinosa a small amount remains, which must be attributed to an intrinsic source within the spinal cord.

This remaining peptide content and the other peptides which are not present in primary sensory neurons, such as neurotensin, neurophysin, and enkephalin, are therefore derived from systems either descending from or ascending to the brain or locally from cells (interneurons) found in the spinal cord. Recently, the use of axonal transport blockers, such as colchicine, applied directly to the spinal cord

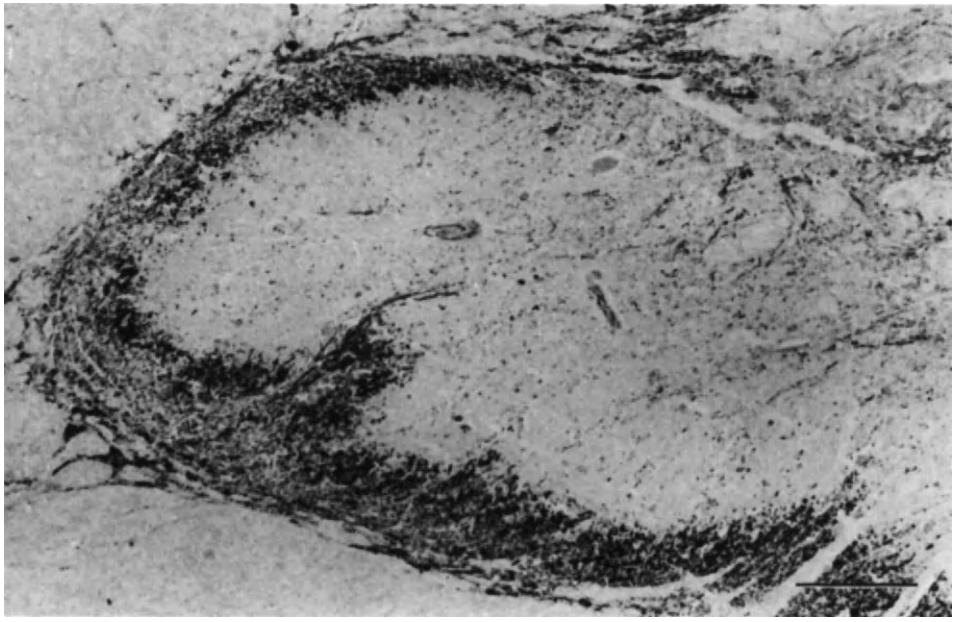


**Fig. 5.** Rat spinal cord fixed by perfusion in benzoquinone and immunostained for substance P. Note the characteristic localization of substance P immunoreactive nerve fibers in the dorsal horn of the spinal cord. P.A.P. method. (×45; bar, 500 μm)

has allowed the demonstration of numerous peptide-containing cell bodies in the upper dorsal horn. This neurotoxin induces a buildup of peptide within the neuronal cell body and facilitates visualization of cells which are not usually visible in the normal animal. A large population of peptide-containing cell bodies has been described in the spinal cord, each having a distinct and well-defined morphology.

In order to determine tha amount of peptide which is derived from the ascending and descending pathways, a series of lesion experiments, that is, spinal cord transections and destruction of specific brainstem nuclei, is necessary. To date, substance P, enkephalin, and 5-HT have been identified within the descending pathways. Following spinal cord transection, there is marked reduction in the content of substance P in the ventral horn accompanied by a smaller decrease in the dorsal horn.

## C. Respiratory Tract

At least six regulatory peptides have been found in the respiratory tract (Polak and Bloom, 1982d,e). They include VIP, substance P, bombesin, CCK,

somatostatin, and calcitonin. Bombesin and, to a lesser extent, calcitonin are present in mucosal endocrine cells, whereas the others are primarily found in autonomic or sensory nerves of the respiratory tract. Bombesin cells are particularly numerous in fetal and neonatal lung (Fig. 6). The bombesin content of the lung declines rapidly after birth. In view of this and the reported findings of significant depletion of bombesin-containing cells in poorly grown lungs from premature babies who develop a hyaline membrane, it has been suggested that bombesin may be a growth-promoting factor to the lung. This is further supported by the finding that bombesin is trophic to the pancreas (Solomon *et al.*, 1979) as well as in tissue culture of lung tumours (Oie, 1984). The finding of bombesin-containing cells in the lung is interesting in view of the repeated series of reports in the literature (e.g., Moody *et al.*, 1981; Sorenson *et al.*, 1982) of a subclass of small cell carcinoma of the lung capable of producing high concentrations of bombesin.

In contrast to bombesin, VIP concentrations are more pronounced after birth. VIP-containing autonomic nerves show a clearly graded pattern of distribution, with the largest number of nerves present in the nasal mucosa and the upper respiratory tract (Fig. 7). VIP-containing nerves in these regions are frequently seen in association with seromucous glands, blood vessels, and smooth muscle.

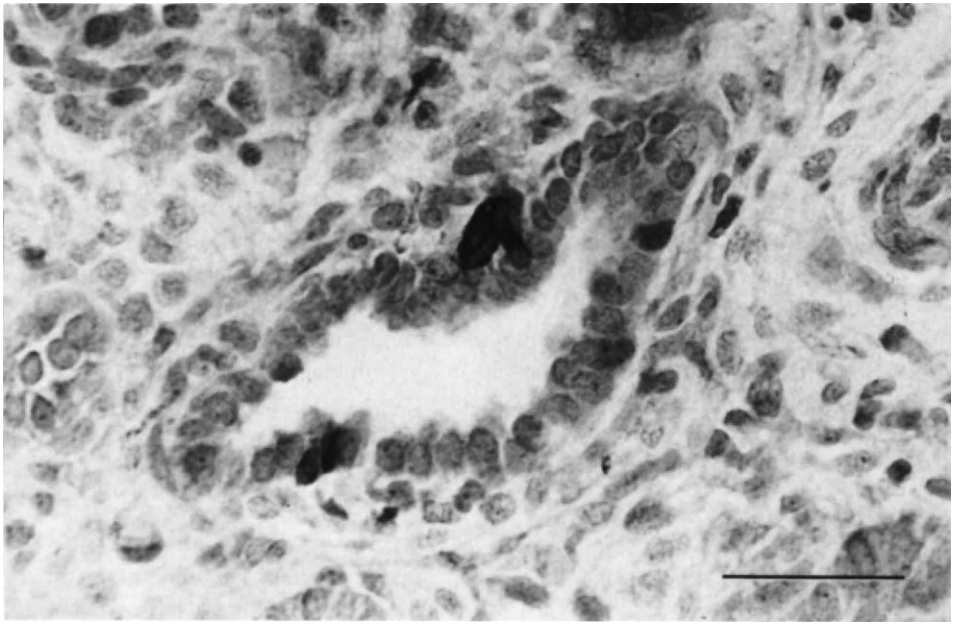


**Fig. 6.** Human fetal lung fixed in benzoquinone vapor and immunostained for bombesin. Note the abundance of bombesin-immunoreactive cells in the bronchial mucosa either singly or in clusters. P.A.P. method (×325; bar, 100 μm)

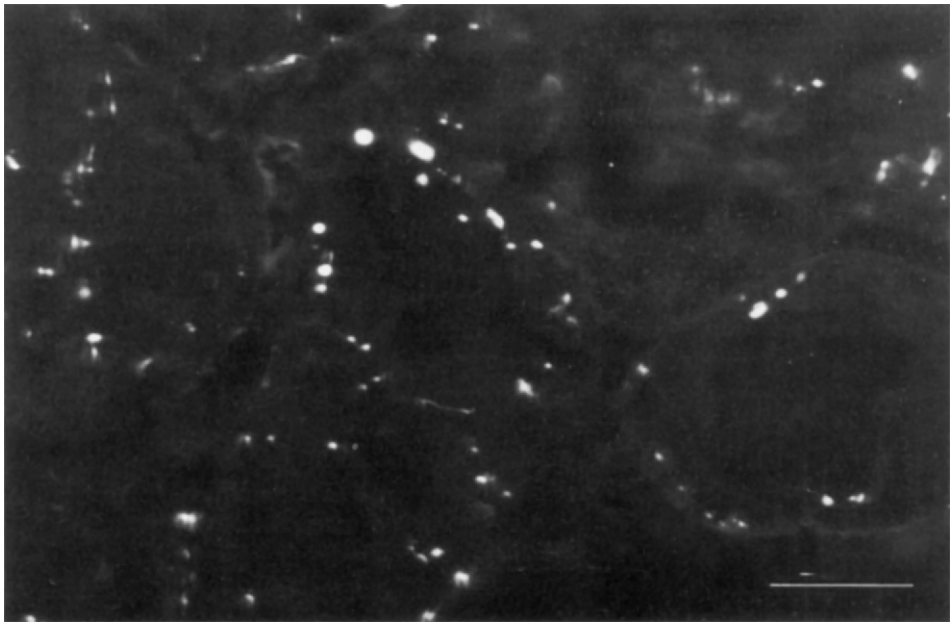


**Fig. 7.** Human nasal mucosa fixed in benzoquinone solution. Numerous VIP-immunoreactive nerves can be seen surrounding blood vessels and seromucous glands. Immunofluorescence method. (×540; bar, 50 μm)

This is in keeping with the reported set of actions of VIP in the lung, that is, modulating watery secretion from seromucous glands and enhancing muscle relaxation and vasodilatation (Said, 1982b).

Substance P is found in sensory nerves which originate from primary sensory neurons of the nodose ganglion, the peripheral branches of which innervate both the lung and the heart. Substance P-containing nerves in the lung are particularly numerous in the bronchial epithelium and around blood vessels.

Somatostatin and CCK are found in many mammals but not in man. Their concentrations are considerably lower than those of bombesin, VIP, and substance P.

## D. Urogenital Tract

The male and female urogenital tracts of many mammals including man contain considerable concentrations of VIP, substance P, and, to a lesser extent, somatostatin. The distribution of VIP and substance P is interesting. In the female genital tract, for instance, VIP-containing nerves are particularly numer-

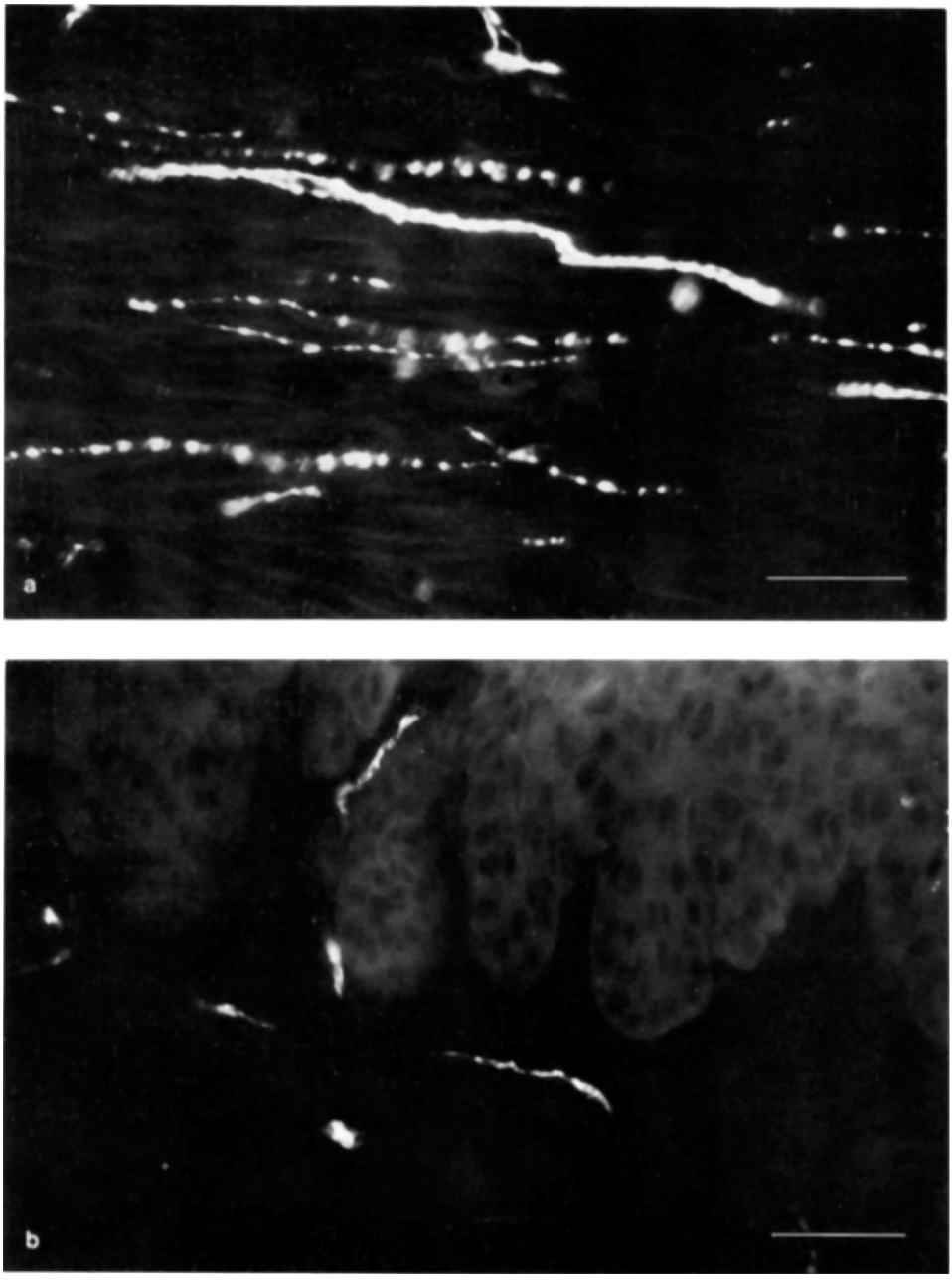



**Fig. 8.** (a) Human female genitalia. Numerous immunoreactive VIP-containing nerves are seen in the muscle layer. Immunofluorescence method. (×540; bar, 50 μm)
(b) Guinea pig female genitalia fixed in benzoquinone solution and stained for substance P. Numerous substance P-immunoreactive nerves are seen in close association to the ectocervix. Immunofluorescence method. (×500; bar, 50 μm)

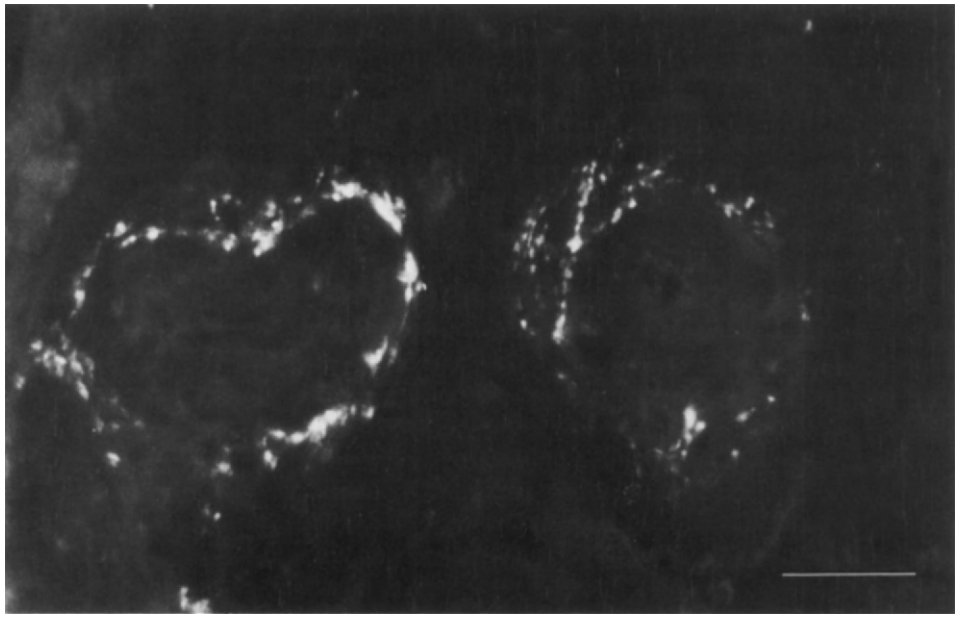


**Fig. 9.** Human penis. VIP-immunoreactive nerves are seen in abundance surrounding blood vessels of the erectile tissue. Immunofluorescence method. (×500; bar, 50 μm)

ous in the uterocervical canal in close association with seromucous glands and smooth muscle (Lynch *et al.*, 1980), whereas substance P-containing nerves are preferentially localized in sensory areas (Fig. 8a–b). Neurophysiological experiments indicated that VIP enhances glandular secretion, vasodilatation, and muscle relaxation in this area of the female genital tract (Ottesen *et al.*, 1982). In the male external genitalia, VIP-containing nerves are very numerous in the erectile tissue (Fig. 9) and are seen around blood vessels (Polak *et al.*, 1981). Substance P, however, shows a much more limited distribution with particular preference for the sensory areas around the sensory corpuscles of the glans penis.

## IV. CONCLUSION

Immunocytochemistry has played a major role in determining the precise distribution of regulatory peptides to neural and/or endocrine elements of the diffuse neuroendocrine system. In addition, electron immunocytochemistry has allowed the accurate demonstration of the intracellular storage sites of these regulatory peptides.

Immunocytochemistry has, in addition, supported biochemical data that indicate the precise quantities of regulatory peptides in a given piece of tissue and physiological data that delineate their particular sets of actions.

The discovery of regulatory peptides and of this major controlling system is in its very infancy. While new peptides are being discovered, the techniques for the precise intracellular demonstration of the biosynthetic, storage, and releasing sites of regulatory peptides are being further developed, and the possibility of visualizing mRNA species responsible for the biosynthesis of active molecules is becoming a clear reality.

The finding that regulatory peptides are highly involved in a number of human diseases further supports the contention that the massive system containing these regulatory peptides is not a vestigial structure but more likely an active participant in the basic control of bodily functions.

## APPENDIX

### A. Light Microscopy

#### *1. Benzoquinone Solution Fixation (BQS)*

1. Just before use prepare a 0.4% solution of purified benzoquinone in 0.1 *M* phosphate-buffered saline (PBS; pH 7.1–7.4). Allow a tissue to solution ratio of at least 1:10, using specimens no larger than 2 × 2 cm.
2. Agitate the solution until all the benzoquinone crystals are dissolved.
3. NB: If solution is brown, discard it. The solution must be bright yellow when it is used.
4. Fix the tissue by immersion in the solution for the appropriate time period (see list below).
5. Following fixation, transfer the tissue into PBS containing 7% sucrose and 0.01% sodium azide. The solution need not be freshly prepared each time. It can be prepared in bulk and stored at 4°C.
6. Rinse in PBS sucrose at 4°C for several hours.
7. Freeze a block of tissue for cryostat sectioning.

| Fixation times (histological block size only) | |
|---|---|
| Small endoscopic biopsies | 30 min |
| Thin-walled gut (child or animal) | 1 hr |
| Thick-walled gut (adult) | 2 hr |
| Pancreas | 2 hr |
| Tumor | 2 hr |
| Brain | 30 min–4 hr, thick slices |

### 2. *Freeze-drying and vapor (benzoquinone or formaldehyde) fixation*

1. Flatten each piece of tissue (no larger than 5 mm thick) on a PTFE-coated metal spatula at room temperature. Snap freeze by plunging into melting Arcton (Freon; Isceon) 12 or isopentane precooled to a slush in liquid nitrogen.
2. Transfer the blocks, flat surface down, with cooled forceps to the precooled plate of a tissue dryer (−40°C). The apparatus is evacuated and the tissue is freeze-dried overnight or for an appropriate period.
3. After warming to room temperature *in vacuo* the tissue is transferred to the vapor fixation chamber. This is a jar which may be sealed tightly in which there is a small open pot containing a solution of approximately 0.1% *purified* benzoquinone in toluene (AnalaR grade or equivalent). The jar and benzoquinone solution should be placed into an oven at 60°C for at least 30 min to warm up before being used. The jar should be kept closed *and must be opened only in a fume cupboard.* The piece of tissue is suspended over the benzoquinone solution in a rack. The jar is resealed and replaced in the oven for 3 hr. Formaldehyde vapor fixation is achieved in the same way, the benzoquinone solution being replaced by paraformaldehyde powder (*not* solution—water vapor causes diffusion of cell contents).
4. After fixation the tissue is transferred to paraffin wax at 60°C, infiltrated *in vacuo,* and then embedded for sectioning in the normal way. Immunostaining is carried out according to standard procedures.

## B. Electron Microscopy

### 1. *Tissue Processing*

1. Small blocks of tissue (no larger than 1 $mm^3$) should be fixed by immersion in a standard aldehyde-based electron microscope fixative solution for 2 hr at 0–4°C. In our laboratory we use a 2.5% solution of ultrapure glutaraldehyde in 0.1 *M* phosphate buffer (pH 7.2) or a mixture of 1% formaldehyde (prepared from its *para-* polymer) plus 2% glutaraldehyde in 0.075 *M* phosphate buffer (pH 7.3). Cacodylate buffers may also be used.
2. Following fixation the tissue is washed in ice-cold buffer containing 0.1 *M* sucrose for 3 × 30 min.
3. The tissue is dehydrated via an ascending series of ethanols (20%, 50%, 70%, 90%, 3 × absolute—15 min each), cleared in propylene oxide, and infiltrated with resin. Suitable resins include Araldite, Epon, and Spurr. Methacrylate and acrylic polymer resins have not been found to be entirely successful for electron immunocytochemistry.

4. Silver to silver-grey (60–100 nm) sections should be cut and collected on cleaned, uncoated 200- or 300-mesh nickel or gold grids. Allow the sections to dry overnight.

## 2. *Preparation of Colloidal Gold-Conjugated Immunoglobulins*

*a. Preparation of monodisperse colloidal gold sols.* (For references, see Frens, 1973; Geoghegan and Ackerman, 1977; Horisberger, 1979; De Mey *et al.*, 1981.) NB: All water should be absolutely free of organic contaminants. Scrupulously cleaned, siliconized glassware should be used throughout.

(1) 5 nm particles

1. Prepare a saturated solution of white phosphorus in 100% diethyl ether in a closed container. Centrifuge to pellet solids remaining after 2 hr. Dilute 1 part with 4 parts 100% ether.

2. Prepare a 1% aqueous solution of chloroauric acid ($HAuCl_4$). Microfilter through a Millipore (0.22-μm pore size) filter. Add 3 ml of 1% $HAuCl_4$ to 240 ml microfiltered water in an Erlenmeyer flask. Add 5.4 ml 0.2 *N* $K_2CO_3$ to increase the pH to about 9. Add 2 ml of the diluted phosphorus ether to the gold solution by pipetting it below the surface to minimize contact with the air. Mix slowly for 15 min at room temperature. Heat under reflux for 20–30 min to remove the ether. Cool to 4°C. Use within 14 days.

(2) 17–20 nm particles

1. Prepare a 4% stock solution of $HAuCl_4$. Microfilter. Boil separately 200 ml microfiltered water. Add freshly prepared 5 ml 1% aqueous sodium citrate to the boiling water. Add 0.5 ml 4% $HAuCl_4$ to this mixture. Mix vigorously. Reflux for 30 min. Cool to 4°C. Use within 14 days.

(3) 40 nm particles

1. Identical to 17–20 nm particles, use 2.8 ml 1% aqueous sodium citrate.

*b. Preparation of the antibody for adsorption.*

1. Adjust immunoglobulin concentration to 1 mg/ml. Dialyze against 2 m*M* borax–HCl buffer pH 9. It is recommended to keep the affinity-purified antibodies for as short a period of time as possible in this low salt buffer. Immediately before use centrifuge at 100,000 *g* for 1 hr at 0°C to remove microaggregates.

2. Determine the optimal stabilizing protein, which is the amount of antibodies required to coat colloidal gold particles, by adding different amounts of protein to colloidal gold aliquots then adding 10% w/v NaCl to a final concentration of 1%. Flocculation results in a change of color from red to blue (optical density measured at 580 nm). The optimal amount of antibody is thus determined to be the point at which the addition of more protein would cause a color change.

*c. Preparation of the Immunostaining Reagents (IGS)*

1. Increase the optimal stabilizing protein concentration by 10% and any volume (up to several liters) of pH-adjusted gold sol is mixed with the appropriate volume of antibody solution. After 2 min add a 10% microfiltered solution of bovine serum albumin (BSA) (Sigma type V) in distilled water, pH adjusted to 9 with NaOH to make a final concentration of 1% BSA.

*d. Washing the IGS Reagent*

1. Make up 1% BSA in 20 m*M* Tris-buffered slaine (pH 8.2). Microfilter (0.22-μm pore size). Spin down the IGS reagent at 60,000 *g* for 5 nm gold; 14,000 *g* for 20 or 40 nm gold at 4°C for 1 hr. These are $g_{max}$ values. Mobile pool, not solid pellet, results. Aspirate supernatant and resuspend pool in small remaining volume. Refill tube to original volume with 1% BSA buffer. Equilibrate overnight and repeat centrifugation. Aspirate supernatant and repeat.

2. Spin down and resuspend in 1% BSA buffer containing $2 \times 10^{-2} M$ sodium azide to a volume such that when diluted 1:20 in 1% BSA buffer the O.D. at 520 nm will be 0.35 for 40 nm gold, 0.5 for 20 nm, and 0.25 for 5 nm. A 1% BSA buffer is used as the blank. O.D. values represent standardization levels only.

*3. Electron immunocytochemistry*

All incubations are carried out in microtest plates, each well holding 15 μl.

1. "Etch" sections in 10% hydrogen peroxide aqueous solution for 10 min at room temperature. This step is believed to permeabilize the resin, thus aiding antibody penetration.

2. Wash thoroughly in microfiltered (0.45-μm pore size) distilled water.

3. Drain grids and place into droplets of normal goat serum (NGS) (1:30 dilution in antiserum diluent, see step 4) for 30 min at room temperature.

4. Drain NGS from the grids using fiber-free absorbent paper and incubate in droplets of primary antibody diluted to optimal titer. The antiserum diluent used routinely is PBS containing 0.1% BSA and 0.01% sodium azide, pH 7.2. Incubation in primary antiserum is carried out for 2 hr at room temperature to 24–36 hr at 4°C depending upon the dilution, avidity, and affinity of the antiserum.

5. After thorough washing in 50 m*M* Tris buffer (pH 7.2) and 50 m*M* Tris buffer containing 0.2% BSA (Sigma type V) for $3 \times 15$ min with agitation in each case, the grids are placed into droplets of 50 m*M* Tris buffer containing 1% BSA (pH 8.2) for 5 min.

6. Incubate the grids in gold-labeled goat anti-primary species IgG at the optimal titer for 1 hr at room temperature. The gold sol should be diluted with the pH 8.2 Tris–BSA buffer before centrifugation at 2000 *g* for 20 min to remove microaggregates accumulating with storage of the stock gold solution.

7. Wash in copious volumes of Tris–0.2% BSA buffer, Tris buffer, and distilled water ("jet" washing and beaker washing) 3 × 15 min each. Finally rinse the grids in microfiltered distilled water, dry by draining, and counterstain for conventional electron microscopy. Methanolic and ethanolic stains may be employed.

## REFERENCES


Bishop, A. E., Polak, J. M., and Bloom, S. R. (1981). Pathology of gut peptidergic innervation. *In* "Gut Hormones" (S. R. Bloom and J. M. Polak, eds.), 2nd ed., pp. 569–575. Churchill-Livingstone, Edinburgh and London.

Bishop, A. E., Polak, J. M., Facer, P., Ferri, G.-L., Marangos, P. J., and Pearse, A. G. E. (1982). *Gastroenterology* **83,** 902–915.

Bloom, S. R., and Long, R. G., eds. (1982). "Radioimmunoassay of Gut Regulatory Peptides." Saunders, Philadelphia, Pennsylvania.

Bloom, S. R., and Polak, J. M. (1980). *Adv. Clin. Chem.* **21,** 177–244.

Brazeau, P., Vale, W., Burgus, R., Ling, N., Butcher, M., Rivier, J., and Guillemin, R. (1973). *Science* **179,** 77–79.

Comb. M., Herbert, E., and Crea, R. (1982). *Proc. Natl. Acad. Sci. U.S.A.* **79,** 360–364.

Coons, A. N., Leduc, N. N., and Connoly, J. M. (1955). *J. Exp. Med.* **102,** 49–60.

De Mey, J., *et al.* (1981). *Cell Biol. Int. Rep.* **5,** 889.

Engberg, G., Svensson, T. H., Rosell, S., and Folkers, K. (1981). *Nature (London)* **293,** 222–223.

Erspamer, V., and Melchiorri, P. (1973). *Pure Appl. Chem.* **35,** 463–494.

Frens, G. (1973). *Nature (London)* **241,** 20.

Geoghegan, W. D., and Ackerman, G. A. (1977). *J. Histochem. Cytochem.* **25,** 1187.

Gibson, S. J., Polak, J. M., Bloom, S. R., and Wall, P. D. (1981). *J. Comp. Neurol.* **201,** 65–79.

Horisberger, M. (1979). *Biol. Cell.* **36,** 253.

Hudson, P., Penschow, J., Shine, J., Ryan G., Niall, H., and Coghlan, J. (1981). *Endocrinology* **108,** 352–356.

Kohler, G., and Milstein, C. (1975). *Nature (London)* **256,** 495–497.

Lynch, E. M., Wharton, J., Bryant, M. G., Bloom, S. R., Polak, J. M., and Elder, M. G. (1980). *Histochemistry* **67,** 169–177.

McDonald, T. J., Nilsson, G., Vagne, M., Ghatei, M., Bloom, S. R., and Mutt, V. (1978). *Gut* **19,** 767–774.

Marangos, P. J., Polak, J. M., and Pearse, A. G. E. (1982). *Trends NeuroSci. (Pers. Ed., June, 1982),* 193–196.

Moody, T. W., Pert, C. B., Gazdar, A. F., Garney, D. N., and Minna, J. D. (1981). *Science* **214,** 1246–1248.

Oie, H. K. (1984). Defined media for culture and cloning of endocrine tumours of the lung. *In* "Endocrinology of the Lung" (K. Becker and A. F. Gazdar, eds.). Saunders, Philadelphia, Pennsylvania (in press).

Olgart, L., Gazelius, B., Brodin, E., and Nilsson, G. (1977). *Acta Physiol. Scand.* **101,** 510–512.

Otsuka, M., Konishi, S., and Takanashi, T. (1975). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **34,** 1922–1928.

Ottesen, B., Moller, M., Willis, E., and Fahrenkrug, J. (1982). Vasoactive intestinal peptide (VIP) and the urogenital tract. *In* "Systemic Role of Regulatory Peptides" (S. R. Bloom, J. M. Polak, and E. Lindenlaub, eds.), pp. 365–376. Schattauer, Stuttgart, West Germany.

Patel, Y. C., Wheatley, T., and Ning Chin (1981a). *Endocrinology* **109,** 1943–1949.

Patel, Y. C., Zingg, H. H., Fitz-Patrick, D., and Srikant, C. B. (1981b). Somatostatin: Some aspects of its physiology and pathophysiology. *In* "Gut Hormones" (S. R. Bloom and J. M. Polak, eds.), 2nd ed., pp. 339–349. Churchill-Livingstone, Edinburgh and London.

Polak, J. M., and Bloom, S. R. (1981b). *Pept.: Synth., Struct., Funct., Proc. Am. Pept. Symp., 7th, 1981* pp. 577–588.

Polak, J. M., and Bloom, S. R. (1981c). *Peptides* **2,** 133–148.

Polak, J. M., and Bloom, S. R. (1982a). Regulatory peptides: New aspects. *In* "Recent Advances in Endocrinology" (E. D. Williams, ed.), pp. 101–107. Saunders, Philadelphia, Pennsylvania.

Polak, J. M., and Bloom, S. R. (1982b). Localization of regulatory peptides in the gut. *In* "Brain-Gut Peptides" (R. A. Gregory, ed.) (in press).

Polak, J. M., and Bloom, S. R. (1982c). Distribution and tissue localisation of VIP in the central nervous system and in seven peripheral organs. *In* "Vasoactive Intestinal Peptide" (S. I. Said, ed.), pp. 107–120. Raven Press, New York.

Polak, J. M., and Bloom, S. R. (1982d). *Exp. Lung Res.* (in press).

Polak, J. M., and Bloom, S. R. (1982e). Distribution of regulatory peptides in the respiratory tract of man and mammals. *In* "Systemic Role of Regulatory Peptides" (S. R. Bloom, J. M. Polak, and E. Lindenlaub, eds.), pp. 241–269. Schattauer, Stuttgart, West Germany.

Polak, J. M., and Bloom, S. R. (1983). Immunocytochemistry of regulatory peptides. *In* "Immunoctyochemistry: Practical Applications in Pathology and Biology" (J. M. Polak and S. Van Noorden, eds.), Chapter 11. John Wright & Sons, Bristol.

Polak, J. M., and Van Noorden, S., eds. (1983). "Immunocytochemistry: Practical Applications in Pathology and Biology." John Wright & Sons, Bristol.

Polak, J. M., Gu, J., Mina, S., and Bloom, S. R. (1981). *Lancet* **2,** 217–219.

Polak, J. M., Varndell, I. M., Tapia, F. J., Probert, L., and De Mey, J. (1982). *MEM (Mag. Electron Microsc.) Zeiss Inf.* **1,** 39–44.

Roberts, G. W., Crow, T. J., and Polak, J. M. (1981). Neuropeptides in the brain. *In* "Gut Hormones" (S. R. Bloom and J. M. Polak, eds.), 2nd ed., pp. 457–463. Churchill-Livingstone, Edinburgh and London.

Said, S. I., ed. (1982a). "Vasoactive Intestinal Peptide," Raven Press, New York.

Said, S. I. (1982b). Vasoactive intestinal peptide (VIP) and the lung. *In* "Systemic Role of Regulatory Peptides" (S. R. Bloom, J. M. Polak, and E. Lindenlaub, eds.), pp. 293–306. Schattauer, Stuttgart, West Germany.

Solomon, T. E., Petersen, H., Elashoff, J., and Grossman, M. I. (1979). Effects of chemical messenger peptides on pancreas growth in rats. *In* "Gut Peptides" (A. Miyoshi, ed.), pp. 213–219. Kodansha, Tokyo.

Sorenson, G. D., Bloom, S. R., Ghatei, M. A., Del Prete, S. A., Gate, C. C., and Pettengill, O. S. (1982). *Regul. Pept.* **4,** 59–66.

Tapia, F. J., Varndell, I. M., Probert, L., De Mey, J., and Polak, J. M. (1983). *J. Histochem. Cytochem.* **31,** 977–981.

Tatemoto, K., and Mutt, V. (1980). *Nature (London)* **285,** 417–418.

Varndell, I. M., Tapia, F. J., Probert, L., Buchan, A. M. J., Gu, J., De Mey, J., Bloom, S. R., and Polak, J. M. (1982). *Peptides* **3,** 259–272.

Vaysse, N., Pradayrol, L., Susini, C., Chayvialle, J. A., and Ribet, A. (1981). Somatostatin 28: Biological actions. *In* "Gut Hormones" (S. R. Bloom and J. M. Polak, eds.), 2nd ed., pp. 358–361. Churchill-Livingstone, Edinburgh and London.

von Euler, U. S., and Gaddum, J. H. (1931). *J. Physiol. (London)* **192,** 74–87.

# Chapter 5

# THE SOLUBILIZATION OF MEMBRANE PROTEINS

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ISBN 0-12-470350-X

# I. THE PROPERTIES OF MEMBRANE PROTEINS, MEMBRANE LIPIDS, AND DETERGENTS ESSENTIAL FOR UNDERSTANDING SOLUBILIZATION

## A. Membrane Proteins

The unique property of intrinsic membrane proteins is that they possess hydrophobic areas of surface capable of interacting with the hydrophobic portions of membrane lipids (Spatz and Strittmatter, 1971). This distinguishes them from the extrinsic membrane proteins, whose association with the membrane depends on ionic or dipolar interactions. Extrinsic membrane proteins can, therefore, usually be obtained in a water-soluble form by exposure to high ionic strength, chaotropic ions or chelators (Singer, 1974). Intrinsic membrane proteins must be either cleaved from the membrane by proteolysis or solubilized by replacement of membrane lipids with detergents.

Sequences of hydrophobic amino acids are apparent in the primary structure of the few intrinsic membrane proteins that have been purified in sufficient amounts (summarized by Robertson, 1981; Kenny and Maroux, 1982). Hydrophobic domains in other proteins can be demonstrated by physical methods (Tanford and Reynolds, 1976), even with impure preparations (Clarke, 1975; Pollet *et al.*, 1981). A useful qualitative technique is the *charge shift* method of Helenius and Simons (1977), which is based on the ability of micelles of charged detergents to alter the electrophoretic mobility of solubilized proteins by binding to their hydrophobic regions. This procedure distinguishes intrinsic and extrinsic proteins and can be used as a diagnostic test for the autolysis of hydrophobic domains during purification procedures.

Membrane proteins are amphiphilic. They have substantial hydrophilic globular portions of structure as well as hydrophobic domains (Robertson, 1981; Kenny and Maroux, 1982; Williams, 1982). In some cases (e.g., glycophorin) the hydrophobic amino acid sequence is only just sufficient to span the lipid bilayer once, whereas for other membrane proteins (e.g., bacterio-rhodopsin; Henderson and Unwin, 1975) most of the structure is intramembranous.

The majority of membrane proteins so far isolated have proved to be oligomeric. These include homodimers such as 5′-nucleotidase (Bailyes *et al.*, 1982; Naito and Lowenstein, 1981) and the microvillar hydrolases (Kenny and Maroux, 1982) or more complex digomers such as the acetylcholine receptor (Conti-Tronconi and Raftery, 1982), the proton-translocating ATPase (Fillingame, 1980), and the $(Na^+,K^+)$-ATPase (Jorgensen, 1974). Although in many cases the oligomers are held together by secondary forces, interchain disulphide bridges have been detected in proteins, including the insulin (Jacobs *et al.*, 1980) and the

acetylcholine (Conti-Tronconi and Raftery, 1982) receptors. A further interesting structural feature of such oligomers is that not all the constituent polypeptides interact with membrane lipids and are therefore not strictly intrinsic membrane proteins. In the case of the proton-translocating ATPase (Fillingame, 1980), the five subunit hydrophilic portion can be separated from the three intrinsic peptides with mild reagents such as 0.2 m*M* EDTA. Three of the hydrophilic peptides are not directly connected to the membrane at all but are bridged to the intrinsic peptides by the other two extrinsic peptides. In the sucrase–isomaltase complex only the isomaltase subunit interacts with membrane lipids. However, the sucrase and isomaltase subunits are so tightly bound that they can only be separated after chemical modification with citraconic anhydride (Kenny and Maroux, 1982).

The quaternary structure of adenylate cyclase (Ross and Gilman, 1980) reveals a further subtlety. The holoenzyme consists of at least three subunits: the hormone receptor, the catalytic subunit, and a regulatory subunit (or subunits) that binds GTP. However, formation of the holoenzyme may be only a transient consequence of receptor occupation (Martin *et al.*, 1979). This is most dramatically demonstrated by the ability of the receptor from one cell to activate the catalytic unit from a second cell after cell fusion (Orly and Schramm, 1976).

Specific interactions between different functional proteins are also well documented. Spectrin, the major extrinsic protein of the erythrocyte membrane, apparently restricts the mobility of both glycophorin (Marchesi and Furthmayr, 1976) and the anion transporter (Liu and Palek, 1979). Interaction of the cytoskeleton with intrinsic membrane proteins has been proposed in cells other than erythrocytes (Mescher *et al.*, 1981; Pober *et al.*, 1981). Another clear example of interaction between intrinsic and extrinsic membrane proteins is suggested by the ability of clathrin to promote selective internalization of vesicles containing intrinsic membrane proteins (Pearse and Bretscher, 1981). Although the evidence is less clear-cut, it is likely that extrinsic proteins are also attached to intrinsic proteins at the external membrane face (Robertson, 1981). Such interactions may have important consequences for the solubilization process.

## B. Membrane Lipids

Membrane lipids are also amphipathic. The most stable arrangement of their hydrophobic and hydrophilic regions in aqueous solution is an extended lipid bilayer (Helenius and Simons, 1975). There is general agreement that this arrangement is an important structural element in biological membranes (Singer and Nicholson, 1972; Singer, 1974). The physical state of membrane lipids influences the function of membrane proteins, and lipids are in many cases required for their activity (Sanderman, 1978). Selectivity for different lipid classes has often been demonstrated (Warren *et al.*, 1974), although this may fall

short of absolute specificity (Bennett, 1982). Fortunately, detergents of diverse structure seem in most cases to be able to substitute for membrane lipids without loss of activity. This may be because the most tightly bound and essential lipids are not removed (Warren *et al.*, 1974). Solubilization in the presence of mixtures of detergents and exogenous lipid may be necessary and successful in cases in which detergents alone inactivate the protein of interest (Agnew and Raftery, 1979).

## C. Detergents

Detergents form yet another subclass of the amphiphiles. Their unique property is that the balance of hydrophobic and hydrophilic forces renders them most stable in solution as micelles rather than lipid bilayers (Helenius and Simons, 1975). Such micelles are of sufficiently low molecular weight to form true solutions. They form mixed micelles with membrane lipids (Helenius and Simons, 1975), and when added to intrinsic membrane proteins, detergent micelles substitute for membrane lipids at their hydrophobic surfaces (Tanford and Reynolds, 1976). Again the result is to reduce the molecular weight of the complexes sufficiently to render them soluble. Detergents bind much less strongly to the majority of hydrophilic or extrinsic membrane proteins and are, therefore, unable to solubilize protein aggregates formed by predominantly polar interactions (Hjelmeland and Chrambach, 1981). Thus, it is likely that interactions between extrinsic and intrinsic membrane proteins survive attempts at detergent solubilization (Mescher *et al.*, 1981). At high concentrations denaturing detergents such as SDS can penetrate to the hydrophobic core of even hydrophilic proteins (Tanford and Reynolds, 1976). They induce a cooperative transition to a random coil protein–detergent complex in which the hydrophobic interaction is between the hydrophobic tails of the detergent and the peptide backbone. Such detergent–protein complexes are denatured, often irreversibly.

## D. Solubilization by Detergents

The solubilization process may be profitably viewed as a complex thermodynamic equilibrium (Helenius and Simons, 1975; Tanford and Reynolds, 1976; Newby *et al.*, 1982). Thus the end point will depend on the concentration of detergent, membrane proteins, and membrane lipids present and on the strength of protein–protein, protein–lipid, protein–detergent, lipid–lipid, and lipid–detergent interactions. Such an equilibrium would be difficult to analyze precisely even for a homogeneous system, let alone for a heterogeneous mixture of proteins and lipids. Nonetheless, some useful qualitative generalizations can be made. First, each type of interaction is based on hydrophobic bonding be-

tween extended hydrocarbon chains and some small aromatic centers. They are therefore likely to be of similar strength. The exception to this may be protein–protein interactions, which are likely to be based on close interaction between extended hydrophobic surfaces. In the case of specific subunit interactions, they may be very strong (e.g., sucrase–isomaltase; Kenny and Maroux, 1982). However, extremely weak interactions such as those found in adenylate cyclase (Ross and Gilman, 1980) are also observed. Nonspecific protein–protein interaction also occurs between intrinsic membrane proteins and accounts for their aggregation after solubilization and removal of detergent (Spatz and Strittmatter, 1971; Kenny and Maroux, 1982). Nonspecific protein–protein interactions may also take place in the solubilized state, and it has been argued that these forces may account for the polydispersity and intractability to purification of this class of proteins (Homcy *et al.*, 1977; Newby *et al.*, 1978a, 1982).

A second generalization is that detergent–detergent interactions must be weaker than interactions between membrane lipids. This is a necessary consequence of their ability to form micelles rather than extended bilayers. One implication of this is that whereas in the membrane nonspecific protein–protein interactions may be energetically unfavored, they may be promoted in detergent micelles. A second consequence is that a considerable excess of detergent over membrane lipid may be needed to completely disrupt the bilayer and displace all membrane lipid–protein interactions. This may be advantageous if specific lipid requirements need to be met.

A further generalization is that detergents that show a low self-affinity may also show a low affinity for the hydrophobic surfaces of membrane proteins. The self-affinity of pure detergents can be assessed from the concentration at which micelle formation takes place (critical micelle concentration) and from the average number of molecules in each micelle (aggregation number). The consequences of this argument for the ability of different detergents to solubilize the same membrane protein are discussed in Section II,A.

In conclusion the solubilization of membranes by detergents may be seen to progress through several dynamic equilibria as the ratio of added detergent to membrane proteins is increased (Fig. 1). Initially, the membrane may be fragmented into mixed detergent–lipid–protein micelles containing more than one functional protein (Fig. 1b). Detergent disruption may lead to the promotion of nonspecific protein–protein interactions and lead to micelles (Fig. 1c) also containing several proteins. Subsequently, a state in which each micelle bears only a single functional protein may be produced (Fig. 1d). This ideal state may then progress to one in which subunit interactions are disrupted and each micelle contains only a single polypeptide (Fig. 1e). Further addition of detergent, especially at high concentration, may cause cooperative unwinding and denaturation (Fig. 1f). There will be a progressive removal of membrane lipid. Loss of function accompanying these structural changes may be irreversible! The vari-

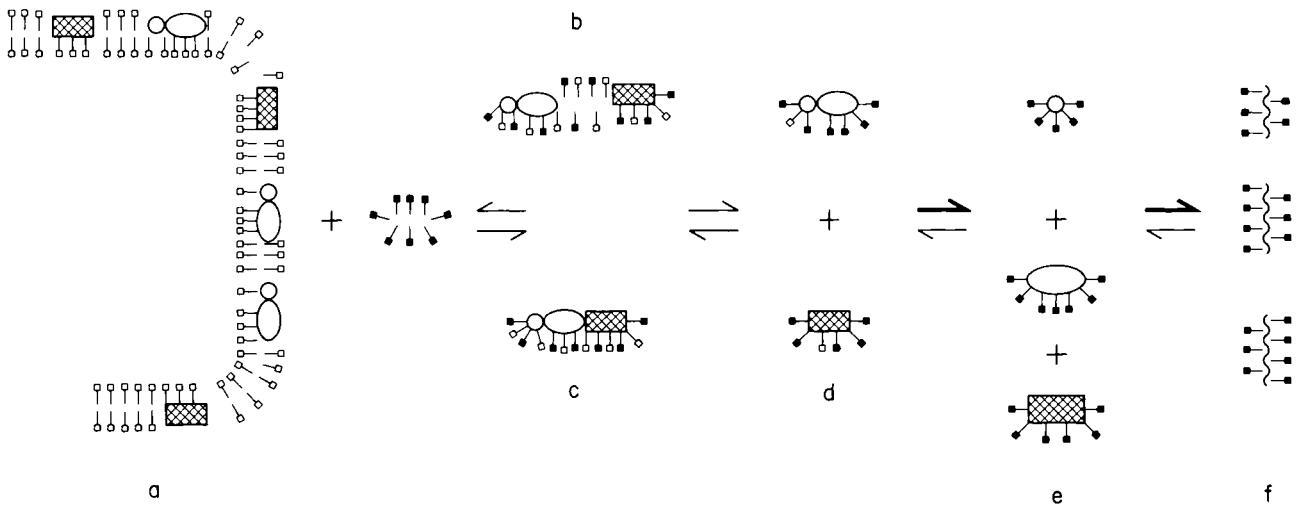


**Fig. 1.** The progress of solubilization. As the proportion of detergent ( ) to membrane lipid ( ) is increased, solubilization may pass through various stages, depicted here as thermodynamic equilibria. a, Membranes; b, mixed micelles with strong lipid–lipid interactions; c, mixed micelles with strong protein–protein interactions; d, micelles with only a single protein; e, micelles with individual polypeptides; f, micelles with denatured polypeptides. Solubilization will be obtimal (d) when each detergent micelle contains only a single functional protein that retains any essential membrane lipid. Solubilization may then proceed to inactive complexes containing individual polypeptide subunits, which may be totally delipidated. Certain detergents may cause a cooperative transition to denatured random coil protein–detergent complexes.

ables that determine whether a large proportion of the active membrane protein of interest can be obtained in its optimally solubilized state (Fig. 1d) will be discussed in Section II.

### E. Solubilization by Proteolysis

A great deal of early success in purifying intrinsic membrane proteins was due to the occurrence of autolyzed or deliberately proteolyzed fragments (Strittmatter *et al.*, 1972; Cresswell *et al.*, 1973; Kenny and Maroux, 1982). Such fragments are useful for partial structural characterization and, moreover, can be used to raise antibodies for use in immunoaffinity purification of the membrane-bound form (Yoshida *et al.*, 1975; Welton *et al.*, 1973; Aharonov *et al.*, 1975; Kenny and Maroux, 1982). The success of this procedure depends on the presence of an exposed susceptible bond between the hydrophobic and hydrophilic protein domains. Unfortunately, not all membrane proteins are tractable to this approach, as exemplified by the brush border alkaline phosphatase (Kenny and Maroux, 1982) and by 5′-nucleotidase (Bailyes, 1982).

## II. VARIABLES THAT AFFECT SOLUBILIZATION

### A. Detergent Structure

Several recent reviews have dealt exhaustively with this subject (Helenius and Simons, 1975; Hjelmeland *et al.*, 1978; Helenius *et al.*, 1979; Hjelmeland and Chrambach, 1981). Helenius and Simons (1975) distinguished two classes of detergents that have been widely used for membrane solubilization. Type A detergents (Table I) have flexible hydrophobic regions and a variety of hydrophilic head groups. These detergents form large, approximately spherical micelles containing 60–180 molecules. The critical micelle concentration is very low for detergents with uncharged head groups (Table I) but relatively high in the case of SDS. Thus, head group repulsion appears to be important in lowering the self-affinity of detergents. This is further supported by the observation that SDS has a lower critical micelle concentration and higher aggregation number at high ionic strength (Helenius and Simons, 1975). A zwitterionic analog of SDS (Gonenne and Ernst, 1978) in which the counterion is "built in" to the structure has a low critical micelle concentration at all ionic strengths. Steric factors also appear important in reducing detergent self-affinity because octylglucoside, in which the hydrophilic headgroup is bulkier than the hydrophobic tail, has a very high critical micelle concentration (Table I).

Type B detergents (the bile acids and conjugated bile acids) have rigid choles-

TABLE I

A Starter Kit for Detergent Solubilization[a]

| Detergent | Example | Type | Charge | CMC (m*M*) | Aggregation number | Micelle molecular weight |
|---|---|---|---|---|---|---|
| Na dodecylsulphate | | A | − | 1.1 | 90 | 26,000 |
| Cetyltrimethylammonium Br | | A | + | | 169 | 62,000 |
| Na *N*-laurylsarcosinate | Sarkosyl | A | − | | | |
| Sulphobetaine | Zwittergent 3-14 | A | +− | 0.6 | 80 | 30,000 |
| Dimethylalkylamine oxide | Ammonyx LO | A | δ + δ − | | | |
| Polyoxyethylene alcohol | Lubrol PX | A | 0 | | 160 | 64,000 |
| Polyoxyethylenenonylphenol | Triton N-101 | A | 0 | 0.075 | 100 | 66,000 |
| Polyoxyethylene *p-t*-octyl Phenol | Triton X-100 | A | 0 | 0.24 | 140 | 90,000 |
| Octylglucoside | | A | 0 | 25 | 27 | 8,000 |
| Digitonin | | A/B | 0 | | 60 | 70,000 |
| Na cholate | | B | − | 13–15 | 2–4 | 900–1,800 |
| Na deoxycholate | | B | − | 4–6 | 4–10 | 1,700–4,200 |
| CHAPS (3[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate) | | B | +− | 4–6 | | |

[a]The above list suggests a selection of detergents of distinct properties for initial studies. More extensive lists with complete chemical structures are given in Helenius and Simons (1975), Hjelmeland *et al.* (1979), Bennett (1982), and Newby *et al.* (1982). Data are taken from Tanford and Reynolds (1976), Hjelmeland (1980), and Rosevear *et al.* (1980). Critical micelle concentrations of ionic detergents depend on ionic strength and are quoted at I = 0.15. Detergents are available from Sigma, Calbiochem-Behring, or, in the case of Ammonyx LO, from Onyx Chemical Co., 190 Warren Street, Jersey City, New Jersey 07032.

terol-based backbones, one face of which is hydrophobic and the other hydrophilic (Helenius and Simons, 1975). This geometry limits their interactions sterically, and they have high critical micelle concentrations and low aggregation numbers (Table I). The mechanism of interaction of Type B detergents with membrane lipids may be quite different from the Type A detergents (Helenius and Simons, 1975), and it is conceptually likely that Type B detergents form a monolayer coat over the hydrophobic zones of membrane proteins. Type A detergents are thought to bind to membrane proteins as complete micelles, although the aggregation number will presumably be altered by the change in surface curvature that incorporation of a membrane protein would involve.

Digitonin bridges the two groups because although it has a rigid hydrophobic zone, its bulky flexible hydrophilic portion allows it to form micelles similar to Type A detergents, albeit with a high critical micelle concentration (Table I).

Detergents with a high critical micelle concentration offer the attractive practical advantage of easy removal by dialysis, and their low self-affinity renders unlikely the formation of large micelles of the type shown in Fig. 1b containing more than one protein. However, if they also display low affinity for the hydrophobic zones of proteins, they may promote aggregation (Fig. 1c). Higher concentrations may be needed to keep proteins in solution with deleterious effects on function, especially if the detergent monomer is the denaturant species. However, detergents with a low critical micelle concentration are presumably better at displacing endogenous lipids from membrane proteins, which again may be harmful in some instances.

Hjelmeland and Chrambach (1981) concluded that no single structural feature conferred either denaturing or nondenaturing character on a detergent. It is the combination of a strongly hydrophobic tail with a head group prone to electrostatic repulsion that appears to be responsible for the denaturant properties of SDS and cetyltrimethylammonium bromide. To combat this tendency, zwitterionic derivatives of both Type A detergents (Gonenne and Ernst, 1978) and Type B detergents (Hjelmeland, 1980) have been synthesized. The history of their use is relatively short but already some notable successes have been recorded (Malpartida and Serrano, 1980; Bailyes *et al.*, 1982; Simonds *et al.*, 1980) as well as some failures (Hjelmeland *et al.*, 1979; Nunez *et al.*, 1981). Zwitterionic and nonionic detergents do not mask the intrinsic charge of membrane proteins, allowing it to be measured and to be exploited in fractionation methods.

Nonionic detergents have been regarded as nondenaturing but less effective solubilizers. Strangely, neither of these conclusions seems entirely well founded. Numerous examples of inactivation by nonionic detergents have been recorded (see Section II,B) although it has not, of course, been suggested that the proteins are cooperatively denatured as in the case of SDS (Hjelmeland and Chrambach, 1981). The idea that nonionic detergents are ineffective solubilizers is based on

the frequent observation of an insoluble residue. However, it is possible that this residue is insoluble as a consequence of polar interactions (Mescher *et al.*, 1981). It is, therefore, significant that a combination of urea and the nonionic detergent NP40 is regarded as an effective solvent for isoelectric focusing of membrane proteins (O'Farrell, 1975). Using adenylate cyclase as a model, we found that addition of ionic detergent did indeed improve the ability of Lubrol PX to disaggregate an aggregated preparation (Newby and Chrambach, 1979), suggesting that ionic groups are useful in preventing nonspecific protein–protein interactions. The ability to disaggregate was correlated with the ability to inactivate the enzyme. Thus, at least in this study, the almost axiomatic belief that the best solubilizers are also the most denaturing detergents was borne out.

To summarize, no available detergent embodies the ideal of a universally effective, universally nondenaturing detergent. Thus, a solubilization strategy should seek as quickly as possible to find the best detergent for the particular protein under study.

## B. The Individual Membrane Protein

In a recent review of the microvillar hydrolases Kenny and Maroux (1982) stated, "Nonionic detergents, such as Triton X-100 or Emulphogen BC720, can solubilize membrane proteins without loss of enzyme activity." This bold statement indicates how universally applicable these detergents are to the microvillar hydrolases. An impressive list of proteins including surface antigents (Williams, 1982), cytochromes (Spatz and Strittmatter, 1971; Welton *et al.*, 1973), the insulin (Jacobs *et al.*, 1980; Harrison and Itin, 1980) and acetylcholine (Conti-Tronconi and Raftery, 1982) receptors, the erythrocyte anion transporter (Findlay, 1974), and some viral coat proteins (Utermann and Simmons, 1974) have been purified in these detergents. Need this article, therefore, give any advice but to dissolve the membranes in Triton X-100? Unfortunately, the following examples show that this is not sufficient. Indeed, the above list may be falsely impressive, since there is a natural tendency for the most tractable problems to be solved first.

In many of the studies quoted above, Triton extraction of membrane only solubilized 30–50% of membrane proteins, implying that many membrane proteins can not be solubilized by Triton X-100 alone. Concrete examples are given in Letarte-Muirhead *et al.* (1974), Peterson and Hokin (1980), Vannier *et al.* (1976), and Bailyes *et al.* (1982).

Triton X-100 has been reported to inactivate the vasopressin receptor (Roy *et al.*, 1975), the glucagon receptor (Welton *et al.*, 1977), the β-receptor (Caron and Lefkowitz, 1976), and the opiate receptor (Simonds *et al.*, 1980) when these were unoccupied. The hormone receptor compex was more stable (Roy *et al.*,

1975; Welton *et al.*, 1977; Haga *et al.*, 1977), although in no case have these receptors been extensively purified in this detergent. The β-receptors from frog and turkey erythrocytes have been purified in digitonin (Schorr *et al.*, 1982), although this detergent is ineffective for the β-receptor from mammalian sources (Strauss *et al.*, 1979).

Experience with the adenylate cyclase catalytic subunit has been no better despite the realization that the enzyme is more stable when activated by $F^-$ or pp(NH)pG (Pilkis and Johnson, 1974; Welton *et al.*, 1977). Only one credible extensive purification has been published (Homcy *et al.*, 1977). This scheme relied upon removal of more hydrophobic proteins on an alkyl–Sepharose affinity column. Taken at face value, these experiments support the notion that nonspecific protein–protein interactions are responsible for the polydispersity (Newby *et al.*, 1978a) and tendency to aggregate (Newby and Chrambach, 1979) that have confounded other attempts to purify the enzyme. However, the enzyme isolated by Homcy *et al.* (1977) was apparently hydrophilic, extending the exciting possibility that it was an autolysis fragment. Unfortunately, confirmation of this point is lacking. No reproducible or totally convincing report of a solubilized hormone-sensitive adenylate cyclase has been presented (Ross and Gilman, 1980) and neither has one been reconstituted upon detergent removal. Both the solubilized catalytic unit and the hormone receptor remain associated with a guanyl nucleotide regulatory component (Welton *et al.*, 1977). This subunit has been separated from the enzyme (Pfeuffer, 1977) and purified to homogeneity in sodium cholate (Sternweis *et al.*, 1981). The reasons for the failure to purify hormone-sensitive adenylate cyclase are still unclear. Whereas loss of normonal sensitivity can be explained by disruption of the holoenzyme, the individual subunits also appear to be unstable in detergent solution.

5′-Nucleotidase represents an interesting contrast. The enzyme is active in the presence of a variety of detergents that denature other proteins (Bailyes *et al.*, 1982). However, very little enzyme is removed from rat liver plasma membranes by Triton X-100. The enzyme was purified in good yield in Zwittergent 3–14. The enzyme from rat heart membranes is released by Triton X-100 and can then be purified to a similar extent (Naito and Lowenstein, 1981). Thus, the problem of purifying 5′-nucleotidase may be largely that of efficiently freeing it from the membrane. The basis of the difference between rat liver and rat heart is unknown, but an association between 5′-nucleotidase and the cytoskeleton has been reported (Mescher *et al.*, 1981). It is possible that Zwittergent 3-14 was able to denature an extrinsic protein that normally prevented the solubilization of rat liver 5′-nucleotidase.

Deliberate fractional solubilization of the membrane has been used to purify some mitochondrial membrane proteins (e.g., cytochrome oxidase) (Yu *et al.*, 1975). Evans and Gurd (1972) showed that *N*-laurylsarcosinate solubilized most proteins from mouse liver plasma membranes but left a residue rich in gap

junction protein. A similar strategy is effective for the ($Na^+$, $K^+$)-ATPase (Jorgensen, 1974; Peterson and Hokin, 1980). In this case substrate (ATP) is used to protect the enzyme whereas most proteins are removed with SDS. It is striking that the enzyme in this pellet can subsequently be solubilized with nonionic detergent (Hastings and Reynolds, 1979) entirely contrary to the paradigm that ionic detergents are superior solubilizers. A similar example is provided by the transferrin–transferrin receptor complex (Nunez *et al.*, 1981).

Even proteins that have been successfully purified in Triton X-100 may not be undamaged. Binding stoichiometries of the available preparations of the insulin receptor (Jacobs *et al.*, 1977; Harrison and Itin, 1980) indicate that they may be only 10 to 20% active. Similarly, preparations of the acetylcholine receptor may not be fully reconstitutively active. Separate reports have recently appeared suggesting that the new detergents such as octylglucoside may be useful in isolating more active preparations of both these receptors (Gould *et al.*, 1981; Gonzales-Ros *et al.*, 1981).

Thus, each new protein represents a unique problem for detergent solubilization.

### C. pH, Ionic Strength, and Temperature

Bile acids have pK values in the physiological range and the free acids are poorly soluble. They must, therefore, be used at neutral or alkaline pH. Similarly, all detergents must be used above the critical micelle temperature at which temperature precipitation of crystalline detergent occurs. Fortunately, only SDS among the commonly used detergents precipitates readily at 4°C. High ionic strength decreases the critical micelle concentration and increases the aggregation number of charged detergents. As discussed fully in Section II,A, these changes will alter the ability of the detergents both to solubilize and delipidate proteins. Zwitterionic detergents probably behave at all ionic strengths like their analogous ionic detergent at high ionic strength. One extensive purification of 5′-nucleotidase depended on the use of sodium deoxycholate at high ionic strength (Widnell, 1974).

Ionic strength and pH will affect the solubility of the hydrophilic portions of membrane proteins (Hjelmeland and Chrambach, 1981). This may be a particular problem during isoelectric focusing, in which case detergents would not be expected to prevent or reverse isoelectric precipitation. The solubility of cytochrome *P*-450 shows a dependence on ionic strength even in the presence of sufficient detergent to disrupt hydrophobic interactions (Haugen and Coon, 1976). Membrane proteins may also be "salted-out" in the classical fashion by ammonium sulfate (Yu *et al.*, 1975; Widnell, 1974).

The effect of pH, ionic strength, and temperature on the solubilization process

has been an experimentally neglected area. It is of interest that microvillar alkaline phosphatase was found in several studies to be poorly solubilized by Triton X-100 (Vannier *et al.*, 1976), but simply increasing the pH to 8.5 allowed the enzyme to be so solubilized (Colbeau and Maroux, 1978). Sadly, the structural basis for this phenomenon is unknown. It is well known that hydrophilic proteins may be solubilized under alkaline conditions (Lowry *et al.*, 1951) and many proteins tolerate moderately elevated pH. Such conditions may be generally advantageous for detergent solubilization.

The effect of increased temperature on detergent solubilization is apparent to any Boy Scout who has attempted to wash dishes in cold water! Despite this, remarkably few biochemists have had the courage to explore elevated temperatures in attempts to solubilize active proteins. Our recent purification of 5′-nucleotidase (Bailyes *et al.*, 1982) depended on solubilization and chromatography at ambient temperature. Temperature may have complex effects on the solubilization equilibrium because it may potentially alter any or all of the interactions involved.It is likely that the main effect of increased temperature is to accelerate the attainment of the equilibrium state.

## III. A RATIONAL EXPERIMENTAL STRATEGY FOR RECEPTOR SOLUBILIZATION

### A. Criteria of Solubility

As explained in Section I,D, optimal solubility is obtained when each detergent micelle contains only a single functional protein. Centrifugation at 100,000 *g* for 60 min followed by penetration into gel filtration media or polyacrylamide gels (Wasserman, 1974), together with measurements of ligand binding, allow for preliminary screening. It is then worthwhile to proceed to a determination of the approximate molecular size of the receptor–detergent complex (Neer, 1974; Catt and Dufau, 1977; Haga *et al.*, 1977; Pollet *et al.*, 1981; Venter, 1982). This is particularly useful if this determination can be compared to the size of the receptor determined in the membrane by radiation inactivation (Houslay *et al.*, 1977; Pollet *et al.*, 1982; Venter, 1982). If the two values agree, there is reason to believe that ideal solubilization has been achieved. Unfortunately, for oligomeric proteins the target size in radiation inactivation may be the subunit molecular weight rather than that of the holoprotein (Kempner and Schlegel, 1979). One also has to allow for the contribution of detergent to the molecular weight estimated by hydrodynamic methods (Tanford and Reynolds, 1976). If the values disagree or if there is no previous information on the molecular weight of the receptor, only operational criteria can be applied. We

have suggested that molecular size should be determined under increasingly harsh conditions of solubilization, which lead to substantial inactivation. One can then determine the "smallest and least polydisperse active size" (Newby *et al.*, 1978a, 1982). If more than one aspect of binding can be measured (for example, cooperativity or reversal by guanyl nucleotides) the exciting (but as yet unrealized) possibility exists of defining different minimal sizes for the expression of each aspect of function.

### B. Choice of Protein Source

Purified membrane fractions are convenient for analytical studies because their specific content of receptor is higher, and they can be aliquoted and stored frozen for a series of experiments. Homogenates may be more useful as starting material for preparative work unless membrane fractions can be produced in high yield (Williams and Turtle, 1979; Harrison and Itin, 1980). When applying solubilization conditions from studies on membranes to homogenates, it may be necessary to adjust the concentration of detergent used to achieve the same detergent-to-membrane protein ratio.

### C. Selection of Analytical Methods

Molecular size determination by gel filtration has the advantage that can be conducted at a wide range of ionic strengths and binding assays can be performed in free solution. However, repeated comparative runs are time-consuming and profligate of material. Gel electrophoresis requires less time and material to test each condition. Comparison of several conditions can be performed simultaneously in the same apparatus (Newby *et al.*, 1978b), and the method gives a more sensitive measure of polydispersity as the bandwidth of the activity peak (Newby *et al.*, 1978a). The method is especially suited to analysis of the radiolabeled ligand–receptor complex, although direct binding to receptors eluted from gel slices can be performed (Lang *et al.*, 1980). The application of gel filtration–sedimentation velocity studies and polyacrylamide gel electrophoresis to membrane proteins both involve special considerations that are beyond the scope of this article but have been dealt with elsewhere (Clarke, 1975; Newby *et al.*, 1982). Sedimentation equilibrium studies on impure proteins (Pollet *et al.*, 1979, 1981) offer the possibility of deriving molecular weights and estimating detergent binding without reference to standard proteins, using only 100 μl of impure material. This method has not yet been applied to optimization of detergent solubilization. Particularly, the influence of polydispersity on the results obtained needs to be evaluated.

## D. Removal of Extrinsic Membrane Proteins

Extrinsic membrane proteins have rarely been deliberately removed prior to detergent solubilization of intrinsic proteins, despite the purification likely to result. Uesugi *et al.* (1971) extracted bovine brain microsomes with 1 *M* NaI and achieved a fourfold increase in specific activity before solubilizing the ($Na^+$, $K^+$)-ATPase with Lubrol. Three *M* KCl also removes extrinsic proteins such as cytochrome c from mitochondrial membranes (Singer, 1974). Chaotropic ions such as $ClO_4^-$, $SCN^-$, urea, or guanadinium hydrochloride are also effective (Bennett, 1982), although they are more likely to cause membrane fragmentation (Singer, 1974) and denaturation. Spectrin can be removed from erythrocyte membranes with 5 m*M* EDTA (Reynolds and Trayler, 1971), and the extrinsic portion of the proton–translocating ATPase can be removed by 0.2 m*M* EDTA (Yoshida *et al.*, 1975). Release of receptors during these procedures may result in some cases from activation of endogenous proteases (Meyer and Doberstein, 1980). This fortunate occurrence should be capitalized upon (see next section).

## E. Solubilization with Enzymes

Because of the ease with which hydrophilic fragments of membrane proteins can be purified, this solubilization route should certainly be attempted. The proteases listed below can be tested at various concentrations and incubation times before pelleting the membranes by centrifugation. Loss of the hydrophobic zone of solubilized receptors can be tested by "charge shift" electrophoresis (Helenius and Simons, 1977).

The following proteases may be useful (Bailyes, 1982): Crude pancreatic extract (Type I), bacterial protease (Type IV, *Streptomyces caespitosus*), pronase AS (Type V, *Streptomyces griseous*), pronase P (Type VI, *Streptomyces griseous*), subtilisin (Type VII, *Bacillus amyloliquefaciens*), alkaline protease (Type VIII, *Bacillus subtilis*), proteinase K (Type XI, *Tritrachium album*) and elastase (porcine pancreas, Type 1) from Sigma (London) Chemical Co., Poole, Dorset, England, trypsin (TPCK-treated) and chymotrypsin from Worthington Biochemical Corp., Freehold, New Jersey, papain and neutral protease (*Bacillus polymyxa*) from Boehringer Mannheim, Lewes, Sussex, England, and thermolysin from Calbiochem, C. P. Laboratories Ltd., Bishops Stortford, Hertfordshire, England.

The proteases should be incubated at 37°C for various times at 1 mg/ml with plasma membranes suspended 1–3 mg protein/ml in 10 m*M* Tes [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid], pH 7.5. Papain (a thiol protease) requires 8 m*M* cysteine to activate it.

### F. Solubilization with Detergents and Sedimentation at 100,000 *g*, 60 min

The following procedure has been found to be effective. Quadruplicate lots of membranes (135 μl, containing 3–5 mg/ml) suspended in a suitable buffer are mixed in conical 500 μl plastic tubes (e.g., 72-700, W. Sarstedt Ltd., Leicester, England) with 15 μl of detergent solutions to give a range of concentrations from 0.05 to 2% (w/v). Different temperatures, ionic strengths, and incubation times can be tested. Two tubes from each quadruplicate are then floated in larger water-filled ultracentrifuge tubes and the whole centrifuged at 100,000 *g* for 60 min. Samples of unfractionated mixtures and supernatants from centrifuged samples are then assayed for binding and protein. Detergents are sought that release a large proportion of receptor, preferably selectively, without loss of total activity.

### G. Penetration into Gel Filtration Media and Polyacrylamide Gels

Useful media for gel filtration are Sepharose 6B (Pharmacia) or Ultragel AcA22 (L.K.B.). Polyacrylamide gels containing 4% total monomers and 15% diallyltartardiamide as cross-linking agent (Baumann and Chrambach, 1976) have a similar exclusion limit of $\sim 10^6$ daltons. Penetration into these gels can be taken as an arbitrary definition of solubilization, and detergents should be chosen that yield a large proportion of the binding activity in a single symmetrical peak. Polydispersity is judged from bandwidth at half height of activity profiles. This is more sensitive in the case of gel electrophoresis. Relative estimates of molecular size can be gained from the elution volume in gel filtration, and this may be converted to a value of Stoke's radius if a column calibrated with standard proteins is used (Siegel and Monty, 1966). Relative molecular size can also be judged in gel electrophoresis, provided that electrophoresis is conducted at a series of gel concentrations. A plot of log $R_f$ versus gel concentration is linear, and the slope ($K_r$, retardation coefficient) increases with increasing molecular size (Ferguson, 1964). Again, comparison with standard proteins yields a value for molecular radius (Rodbard and Chrambach, 1971).

### H. Progressive Dissection

Relative molecular size from gel filtration or gel electrophoresis can be determined using harsher disruptive agents. Additions of urea or SDS to the optimal detergents should be suitable for most proteins, although milder ionic detergents such as deoxycholate or *N*-laurylsarcosinate may be sufficient in the case of

readily denatured proteins. Conditions that lead to graded levels of inactivation are sought and estimates of elution volume in gel filtration or retardation coefficient in gel electrophoresis observed (Newby and Chrambach, 1979). Alterations in the contribution of detergents to molecular size will occur during this process but should be small owing to the broadly similar molecular weights of micelles of the Type A detergents (Table I). Conditions should be sought that give rise to the smallest active protein.

## I. Molecular Weight Estimation

Values of molecular weight in the optimal detergent can be obtained from studies of gel filtration–sedimentation velocity (Siegel and Monty, 1966; Clarke, 1975), gel electrophoresis (Rodbard and Chrambach, 1971; Newby *et al.*, 1978a), or from sedimentation equilibria (Pollet *et al.*, 1979, 1981). These values can be compared to those obtained from radiation inactivation (Section III,A) and also act as a benchmark against which to judge more highly purified preparations. This may be valuable in assessing the composition of the holoenzyme in cases such as adenylate cyclase, in which purification of the separate subunits appears to be the only route open (Ross and Gilman, 1980).

## REFERENCES

Agnew, W. S., and Raftery, M. A. (1979). *Biochemistry* **18,** 1912–1918.
Aharonov, A., Kalderson, N., Silman, I., and Fuchs, S. (1975). *Immunochemistry* **12,** 765–771.
Bailyes, E. M. (1982). Ph.D Thesis, University of Cambridge.
Bailyes, E. M., Newby, A. C., Siddle, K., and Luzio, J. P. (1982). *Biochem. J.* **203,** 245–251.
Baumann, G., and Chrambach, A. (1976). *Anal. Biochem.* **70,** 32–38.
Bennett, J. P. (1982). Tech. *Life Sci. Biochem.* **B4**(8), 1–22.
Caron, M. G., and Lefkowitz, R. J. (1976). *J. Biol. Chem.* **251,** 2374–2384.
Catt, K. J., and Dufau, M. L. (1977). *Annu. Rev. Physiol.* **39,** 529–557.
Clarke, S. (1975). *J. Biol. Chem.* **250,** 5459–5469.
Colbeau, A., and Maroux, S. (1978). *Biochim. Biophys. Acta* **511,** 39–51.
Conti-Tronconi, B. M., and Raftery, M. A. (1982). *Annu. Rev. Biochem.* **51,** 491–530.
Cresswell, P., Turner, M. J., and Strominger, J. L. (1973). *Proc. Natl. Acad. Sci. U.S.A.* **70,** 1603–1607.
Evans, W. H., and Gurd, J. W. (1972). *Biochem. J.* **128,** 691–700.
Ferguson, K. A. (1964). *Metab., Clin. Exp.* **13,** 985–1002.
Fillingame, R. H. (1980). *Annu. Rev. Biochem.* **49,** 1079–1113.
Findlay, J. C. (1974). *J. Biol. Chem.* **249,** 4398–4403.
Gonenne, A., and Ernst, R. (1978). *Anal. Biochem.* **87,** 28–38.

Gonzales-Ros, J. M., Paraschos, A., Farach, M. C., and Martinez-Carrion, M. (1981). *Biochim. Biophys. Acta* **643,** 407–420.
Gould, B. J., Ginsberg, B. H., and Spector, A. A. (1981). *Biochemistry* **20,** 6776–6781.
Haga, T., Haga, K., and Gilman, A. G. (1977). *J. Biol. Chem.* **252,** 5776–5782.
Harrison, L. C., and Itin, A. (1980). *J. Biol. Chem.* **255,** 12066–12072.
Hastings, D. F., and Reynolds, J. A. (1979). *Biochemistry* **18,** 817–821.
Haugen, D. A., and Coon, M. J. (1976). *J. Biol. Chem.* **251,** 7929–7939.
Helenius, A., and Simons, K. (1975). *Biochim. Biophys. Acta* **415,** 29–79.
Helenius, A., and Simons, K. (1977). *Proc. Natl. Acad. Sci. U.S.A.* **74,** 529–532.
Helenius, A., McCaslin, D. R., Fries, E., and Tanford, C. (1979). *In* "Methods in Enzymology" (S. Fleischer and L. Packer, eds.), Vol. 56, pp. 734–749. Academic Press, New York.
Henderson, R., and Unwin, P. N. T. (1975). *Nature (London)* **257,** 28–32.
Hjelmeland, L. M. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 6368–6370.
Hjelmeland, L. M., and Chrambach, A. (1981). *Electrophoresis* **2,** 1–11.
Hjelmeland, L. M., Nebert, D. W., and Chrambach, A. (1978). *In* "Electrophoresis 1978" (N. Catsimpoolas, ed.), pp. 29–56. Elsevier/North–Holland, Amsterdam.
Hjelmeland, L. M., Nebert, D. W., and Chrambach, A. (1979). *Anal. Biochem.* **95,** 201–208.
Homcy, C. J., Wrenn, S. M., and Haber, E. (1977). *J. Biol. Chem.* **252,** 8957–8964.
Houslay, M. D., Ellory, J. C., Smith, G. A., Hesketh, T. R., Stein, J. M., Warren, G. B., and Metcalfe, J. C. (1977). *Biochim. Biophys. Acta* **467,** 208–219.
Jacobs, S., Schechter, Y., Bissell, K., and Cuatrecasas, P. (1977). *Biochem. Biophys. Res. Commun.* **77,** 981–988.
Jacobs, S., Hazum, E., and Cuatrecasas, P. (1980). *J. Biol. Chem.* **255,** 6937–6940.
Jorgensen, P. L. (1974). *Biochim. Biophys. Acta* **356,** 36–52.
Kempner, E. S., and Schlegel, W. (1979). *Anal. Biochem.* **92,** 2–10.
Kenny, A. J., and Maroux, S. (1982). *Physiol. Rev.* **62,** 91–128.
Lang, U., Kahn, C. R., and Chrambach, A. (1980). *Endocrinology* **106,** 40–49.
Letarte-Muirhead, M., Acton, R. T., and Williams, A. F. (1974). *Biochem. J.* **143,** 51–61.
Liu, S. C., and Palek, J. (1979). *J. Supramol. Struct.* **10,** 97–109.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). *J. Biol. Chem.* **193,** 265–275.
Malpartida, F., and Serrano, R. (1980). *FEBS Lett.* **111,** 69–72.
Marchesi, V. T., and Furthmayr, H. (1976). *Annu. Rev. Biochem.* **45,** 667–698.
Martin, B. R., Stein, J. M., Kennedy, E. L., Doberska, C. A., and Metcalfe, J. C. (1979). *Biochem. J.* **184,** 253–260.
Mescher, M. F., Jose, M. J. L., and Balk, S. P. (1981). *Nature (London)* **289,** 139–144.
Meyer, D. I., and Doberstein, B. (1980). *J. Cell Biol.* **87,** 498–502.
Naito, Y., and Lowenstein, J. M. (1981). *Biochemistry* **20,** 5188–5194.
Neer, E. J. (1974). *J. Biol. Chem.* **249,** 6527–6531.
Newby, A. C., and Chrambach, A. (1979). *Biochem. J.* **177,** 623–630.
Newby, A. C., Rodbell, M., and Chrambach, A. (1978a). *Arch. Biochem. Biophys.* **190,** 109–117.
Newby, A. C., Matthews, G., and Chrambach, A. (1978b). *Anal. Biochem.* **91,** 473–480.
Newby, A. C., Chrambach, A., and Bailyes, E. M. (1982). *Tech. Life Sci. Biochem.* **B4**(9), 1–22.
Nunez, M. T., Glass, J., and Cole, E. S. (1981). *Biochim. Biophys. Acta* **673,** 137–146.
O'Farrell, P. H. (1975). *J. Biol. Chem.* **250,** 4007–4021.
Orly, J., and Schramm, M. (1976). *Proc. Natl. Acad. Sci. U.S.A.* **73,** 4410–4414.
Pearse, B. M. F., and Bretscher, M. S. (1981). *Annu. Rev. Biochem.* **50,** 85–101.
Peterson, G. L., and Hokin, L. E. (1980). *Biochem. J.* **192,** 107–118.
Pfeuffer, T. (1977). *J. Biol. Chem.* **252,** 7224–7234.
Pilkis, S. J., and Johnson, R. A. (1974). *Biochim. Biophys. Acta* **341,** 388–395.

Pober, J. S., Guild, B. C., Strominger, J. L., and Veatch, W. R. (1981). *Biochemistry* **20,** 5625–5633.

Pollet, R. J., Haase, B. A., and Standaert, M. L. (1979). *J. Biol. Chem.* **254,** 3O–33.

Pollet, R. J., Haase, B. A., and Standaert, M. L. (1981). *J. Biol. Chem.* **256,** 12118–12126.

Pollet, R. J., Kempner, E. S., Standaert, M. L., and Haase, B. A. (1982). *J. Biol. Chem.* **257,** 894–898.

Reynolds, J. A., and Trayler, H. (1971). *J. Biol. Chem.* **246,** 7337–7342.

Robertson, J. D. (1981). *J. Cell Biol.* **91,** 189s–204s.

Rodbard, D., and Chrambach, A. (1971). *Anal. Biochem.* **40,** 95–134.

Rosevear, P., VanAken, T., Baxter, J., and Ferguson-Miller, S. (1980). *Biochemistry* **19,** 4108–4115.

Ross, E. M., and Gilman, A. G. (1980). *Annu. Rev. Biochem.* **49,** 533–564.

Roy, C., Rajerison, R., Bockaert, J., and Jard, S. (1975). *J. Biol. Chem.* **250,** 7885–7893.

Sandermann, H. (1978). *Biochim. Biophys. Acta* **515,** 209–237.

Schorr, R. G. L., Heald, S. H., Jeffs, P. W., Lavin, T. N., Strohsacker, M. W., Lefkowitz, R. J., and Caron, M. G. (1982). *Proc. Natl. Acad. Sci. U.S.A.* **79,** 2778–2782.

Siegel, L. M., and Monty, K. J. (1966). *Biochim. Biophys. Acta* **112,** 346–362.

Simonds, W. F., Koski, G., Streaty, R. A., Hjelmeland, L. M., and Klee, W. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 4623–4627.

Singer, S. J. (1974). *Annu. Rev. Biochem.* **43,** 805–833.

Singer, S. J., and Nicholson, G. L. (1972). *Science* **175,** 720–731.

Spatz, L., and Strittmatter, P. (1971). *Proc. Natl. Acad. Sci. U.S.A.***68,** 1042–1046.

Sternweis, P. C., Northrup, J. K., Smigel, M. D., and Gilman, A. G. (1981). *J. Biol. Chem.* **256,** 11517–11526.

Strauss, W. L., Ghai, G., Fraser, C. M., and Venter, J. G. (1979). *Arch. Biochem. Biophys.* **196,** 566–573.

Strittmatter, P., Rogers, M. J., and Spatz, L. (1972). *J. Biol. Chem.* **247,** 7188–7194.

Tanford, C., and Reynolds, J. A. (1976). *Biochim. Biophys. Acta* **457,** 133–170.

Uesugi, S., Dulak, N. C., Dixon, J. F., Hexum, T. D., Dahl, J. L., Perdue, J. F., and Hokin, L. E. (1971). *J. Biol. Chem.* **246,** 531–543.

Utermann, G., and Simons, K. (1974). *J. Mol. Biol.* **85,** 569–587.

Vannier, C., Louvard, D., Maroux, S., and Desnuelle, P. (1976). *Biochim. Biophys. Acta* **455,** 185–189.

Venter, J. C. (1982). *J. Mol. Cell. Cardiol.* **14,** 687–693.

Warren, G. B., Toon, P. A., Birdsall, N. J. M., Lee, A. G., and Metcalfe, J. C. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71,** 622–626.

Wasserman, A. R. (1974). *In* "Methods in Enzymology" (S. Fleischer and L. Packer, eds.), Vol. 32, pp. 406–422. Academic Press, New York.

Welton, A. F., Pederson, T. C., Buege, J. A., and Aust, S. D. (1973). *Biochem. Biophys. Res. Commun.* **54,** 161–167.

Welton, A. F., Lad, P. M., Newby, A. C., Yamamura, H., Nicosia, S., and Robdell, M. (1977). *J. Biol. Chem.* **252,** 5947–5950.

Widnell, C. C. (1974). *In* "Methods in Enzymology" (S. Fleischer and L. Packer, eds.), Vol. 32, pp. 368–374. Academic Press, New York.

Williams, A. F. (1982). *Biosci. Rep.* **2,** 277–287.

Williams, P. F., and Turtle, J. R. (1979). *Biochim. Biophys. Acta* **579,** 367–374.

Yoshida, M., Sone, N., Hirata, H., and Kagawa, Y. (1975). *J. Biol. Chem.* **250,** 7910–7916.

Yu, C., Yu, L., and King, T. S. (1975). *J. Biol. Chem.* **250,** 1383–1392.

# *Chapter 6*

# *Solubilization and Characterization of Brain Benzodiazepine Binding Sites*

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## I. INTRODUCTION

The clinical effectiveness of the benzodiazepines (BZ) has made them one of the most widely used class of drugs today. They are used as anxiolytics, anticonvulsants, sedative hypnotics, and muscle relaxants. Since the introduction in 1960 of



ISBN 0-12-470350-X

the first benzodiazepine, chlordiazepoxide (Sternbach *et al.*, 1964; Randall *et al.*, 1964), a great deal of research effort has focused on understanding the mechanism of action of these agents. A large body of evidence indicates that the pharmacological actions of the BZ may be due to their interaction with GABAergic neurons (Costa and Guidotti, 1979; Tallman *et al.*, 1980; Haefely *et al.*, 1981). The characterization of specific BZ binding sites has permitted these interactions to be studied directly at the site of action. These studies have provided evidence for a GABA receptor–BZ binding site–ionophore complex. Although these studies have furthered our understanding, many fundamental questions remain. Even if GABA–BZ interactions account for all the pharmacological actions, understanding the molecular mechanism of these interactions will require the dissection and reconstitution of the system's components. Toward this goal the solubilization and characterization of BZ binding sites has been undertaken. This article will attempt to review both the methodology which has been used and the results which have obtained thus far.

## II. SOLUBILIZATION AND ASSAY PROCEDURES

### A. Solubilization Methods

BZ binding sites are membrane-bound proteins and, therefore, either high salt concentrations, organic solvents, chaotropic agents, or detergents are required to release them from neuronal membranes. The protein appears to be an intrinsic membrane protein since solubilization requires the use of detergents; high salt alone is ineffective (Sherman-Gold and Dudai, 1980). Unlike some neuronal receptors, which may require the use of a particular detergent or solubilization conditions, BZ sites have been successfully solubilized by a wide variety of detergents including Triton X-100 (Lang *et al.*, 1979), Lubrol PX (Yousufi *et al.*, 1979), and digitonin (Gavish *et al.*, 1979). We have also obtained soluble binding activity using cholate, deoxycholate, taurodeoxycholate, and CHAPS (Thomas, 1982). Since a variety of detergents are effective, the detergent used can be tailored to the particular needs of the study. The bile salts such as deoxycholate have the advantage of being dialyzable; however, because they are charged their properties may vary with pH and ionic strength. They cannot be used for techniques which require low ionic strength or which depend upon the protein's charge being unaltered by bound detergent. Nonionic detergents such as Triton X-100 and Lubrol PX are less affected by changes in pH and ionic strength; however, their low critical micellar concentration makes them difficult to remove by dialysis. A unique new synthetic detergent, CHAPS (3-[(3-cholamidopropyl)dimethylaminonio]-1-propane sulfonate), can also be used to sol-

ubilize BZ sites (Thomas, 1982). This bile salt analog has a high critical micelle concentration, which makes it dialyzable, and is also zwitterionic so that it does not alter the net charge of solubilized proteins.

## B. Assay Procedures

A wide variety of techniques are available for assaying solubilized binding sites (Olsen, 1980). Solubilized BZ binding sites have been detected by either filtration or column assays. The filtration methods have used either ion-exchange filters (Lang *et al.*, 1979) or glass fiber filters in combination with precipitation by polyethylene glycol (Yousufi *et al.*, 1979) or ammonium sulfate (Gavish *et al.*, 1979). The chromatographic techniques have employed Amberlite XAD-2 ion-exchange resin (Asano and Ogasawara, 1980), gel permeation columns (Massotti *et al.*, 1981), or lectin affinity resins (Lo *et al.*, 1982).

The simplicity and utility of the polyethylene glycol filtration assay (Cuatrecasas, 1972) has made this technique one of the most widely used, and the details for this method follow.

## C. Polyethylene glycol (PEG) Assay Procedures

### *1. Solutions*

1. 0.1% (w/v) bovine γ-globulin in buffer (e.g., 50 m*M* Tris–HCl buffer, pH 7.5 at 5°C).
2. 30% (w/v) polyethylene glycol 8000* in buffer
3. 8% (w/v) polyethylene glycol 8000 in buffer

### *2. Procedure*

Step 1. The washed particulate fraction from a brain homogenate is resuspended in buffer (e.g., 50 m*M* Tris–HCl, pH 7.5 at 5°C). An aliquot of concentrated detergent solution is added. For Lubrol PX the optimal final detergent concentration is approximately 0.5% (w/v) for rat brain membranes suspended in 10 volumes of buffer (original wet wt/v). The membrane suspension is incubated for 30 min at 0°C. The mixture is centrifuged for 1 hr (or longer) at 100,000 *g* (or higher *g* forces) and the supernatant is carefully removed.

Step 2. Aliquots of the supernatant (e.g., 100 μl) are incubated with a tritiated BZ (e.g., [$^3$H]flunitrazepam, 1 n*M* concentration) for 1 hr in an ice water bath.

*Note: PEG 8000 (carbowax 8000) was originally sold as PEG 6000 but re-analysis of the product indicates the actual average MW of the polymer is closer to 8000, so the same product is now marketed as PEG 8000.

The total assay volume is made up to 0.5 ml with buffer; no additional detergent need be included in the buffer and may actually interfere with precipitation.

Step 3. To terminate the binding reaction, 0.5 ml of Solution 1 is quickly added to the sample and the solution is vortexed for a second. Solution 2 (0.5 ml) is quickly added and the sample is vortexed for about 5 sec and then filtered. The filters are washed twice with 5 ml of Solution 3.

Step 4. The dried filters are placed in scintillation vials and the bound radioactivity is counted. This method should give a signal of at least 10,000–20,000 dpm with a signal-to-noise ratio of 10 to 20 when 1n*M* [$^3$H]flunitrazepam is used as the ligand.

Although a wide variety of detergents can be employed for solubilization, that is not to say that all detergents give identical results. Instead, as more of the system's complexities are uncovered, it is likely that only a restricted set of detergents and conditions will be found to be capable of preserving particular regulatory properties such as coupling to other membrane components and regulation by various effector molecules. The choice of detergent may also vary with assay procedures; for example, nonionic detergents such as Triton X-100 have been reported to result in high, variable blanks in the ammonium sulfate precipitation assay, whereas deoxycholate does not interfere with this assay (Sherman-Gold and Dudia, 1980).

## III. PROPERTIES OF BRAIN BENZODIAZEPINE BINDING SITES

### A. General Properties of Soluble Benzodiazepine Sites

Despite the ability to solubilize BZ sites in good yields, it has not yet been possible to solubilize all BZ sites. At best approximately 70% of the sites are obtained in solubilized form (Lang *et al.*, 1979). Although such a result is not uncommon for solubilization of membrane proteins, it is not clear, at least in the case of the BZ binding site, if this is merely a technical problem or if it indicates a hetereogeneity of sites due to significant biological property such as differences in cellular localization (pre- versus postsynaptic, synaptic versus nonsynaptic) or attachment to cytoskeletal elements. For the present, though, it must be kept in mind that most studies of solubilized binding sites have not examined the total BZ binding site population. Variation of solubilization conditions has been used to gain information on cryptic sites, endogenous modulators, cellular localization, and receptor heteogeneity. It has been reported that exposure of rat brain membranes to low concentrations of Triton X-100 (either 0.1 $\mu M$ or 0.1 m*M*) causes significant increases (55 and 40%) in the number of BZ binding sites

(Sabato *et al.*, 1981). These findings may be related to other reports which indicate a population of cryptic sites in brain (Paul and Skolnick, 1978; Gallager *et al.*, 1980; Skolnick *et al.*, 1980). Exposure of rat brain membranes to 0.05% Triton X-100 has been reported to release an endogenous modulator of both BZ and GABA binding sites which has been named GABA-modulin (Guidotti *et al.*, 1978). This factor causes changes in binding affinity with no significant change in the number of sites. The inhibitory modulator of diazepam binding (DIF) is a separate entity from GABA-modulin (Massotti *et al.*, 1981). Other workers have reported that 0.2% Triton results in the loss of 60% of the membrane-bound binding sites in rat brain (Sabato *et al.*, 1981). Since electron microscopy data indicates that 0.2% Triton preferentially solubilizes presynaptic membranes, it has been suggested that the majority of the BZ sites are presynaptic. BZ binding sites have also been subdivided in terms of those solubilized by detergent and those solubilized by detergent plus high salt (Lo *et al.*, 1982). CL-218,812 and Ro 22-7497 were more potent inhibitors of [$^3$H]flunitrazepam binding in the preparation solubilized by detergent alone than in the residual membranes, suggesting that this fraction is enriched in the "type 2" sites, while the pellet is enriched in "type 1" sites.

The BZ binding site's affinity is relatively unchanged by solubilization, and either slightly lower or higher $K_D$ values have been obtained with different conditions. BZ binding activity in the soluble preparations is destroyed by protease activity, suggesting that the site is a protein (Lang *et al.*, 1979). Binding activity is not affected, however, by phospholipase A. Soluble binding activity is also completely abolished by heating (either 5 min at 65°C or 1 min at 90°C) (Lang *et al.*, 1979) or by exposure to SDS (Thomas, 1982). Heating at 50°C clearly distinguishes the membrane-bound and solubilized material, with the solubilized material being more heat-labile (Yousufi *et al.*, 1979). Preparations solubilized by nonionic detergents will bind to DEAE columns at neutral pH, suggesting that the protein is acidic (Gavish and Snyder, 1981; Asano and Ogasawara, 1981). This is consistent with some electrofocusing data indicating an acidic pI for Lubrol-solubilized material (Korneyev, 1982; J. W. Thomas and J. F. Tallman, unpublished data). Solubilized preparations have been assayed using concanavalin A–agarose resins, suggesting that the binding site is a glycoprotein (Lo *et al.*, 1982).

Other factors which may affect solubilization of membrane-bound components include detergent-to-protein ratio, choice of buffer and buffer pH, ionic strength, solubilization time, need for specific factors (e.g., ions, lipids, or sulfhydryl reagents), stability, requirement of proteolytic inhibitors, and need to remove endogenous inhibitors.

Optimal solubilization yields vary with the detergent-to-membrane protein ratio and, therefore, both the detergent concentration and the membrane protein concentration must be controlled to achieve reproducible results. Usually, there

is an optimal range for the detergent-to-protein ratio. At ratios which are too low, little or no solubilization is achieved. At ratios which are too high, denaturation, proteolysis, or detergent interference in the assay procedure may result. Using Lubrol PX, we found that with a 1:10 volume rat forebrain suspension, 0.5% (w/v) detergent gave the best solubilization, which corresponds to a detergent–protein ratio (wt/wt) of about 1:1. With a 1:8 volume rat cortical suspension, 0.7% Triton X-100 has been reported to give optimal yields (Lang *et al.*, 1979). The solubilized preparations are relatively stable and can be used over a period of days. Long-term studies of soluble material require the use of sterile techniques or antibacterial agents; soluble preparations can also be stored frozen. Although good solubilization yields have been obtained in the absence of any exogenous protease inhibitors, two studies have indicated that under optimal detergent conditions solubilization yields can be improved 30–40% by including various protease inhibitors (Lang *et al.*, 1979; Stephenson *et al.*, 1982).

For solubilization of BZ sites buffer choice does not appear to be a critical parameter, and primarily physiological pH and either physiological or lower ionic strength buffers have been used. Membranes are usually incubated with detergent for 30 min to 1 hr before high-speed centrifugation; however, solubilization occurs relatively rapidly and times as short as 5 min have been used (Stephenson *et al.*, 1982). The solubilized preparations have not been reported to have a specific requirement for factors to demonstrate BZ binding activity; however, various compounds are known to regulate binding (see Section IV).

### B. Thermodynamic Analysis of Benzodiazepine Binding

The binding of benzodiazepines is strongly temperature-dependent. Due to the hydrophobic nature of the benzodiazepines, a hydrophobic association accompanied by a substantial increase in entropy might have been expected. However, this would predict that benzodiazepines would bind with higher affinity at higher temperatures, whereas just the opposite is observed for most benzodiazepines (two exceptions are triazolam and desmethyl-medazepam), (see Kochman and Hirsch, 1982). On the contrary, the binding of most benzodiazepines is primarily enthalpy-driven with a smaller (or in some cases) minor contribution due to entropy (see Table I). In the case of the β-adrenergic receptor, it has been demonstrated that antagonist binding is largely entropy-driven, whereas binding of agonists is enthalpy-driven with a decrease in entropy (Weiland *et al.*, 1980). However, this does not seem to be true for the benzodiazepines, as antagonist binding examined so far is also primarily enthalpy-driven (see Table I). It is of interest, though, that two benzodiazepines which can photolabel benzodiazepine binding sites both have a break in their van Hoff plots consistent with the

TABLE I

Thermodynamic Parameters for Ligands Binding to Brain Benzodiazepine Sites

| Ligand | Temp (°C) | $\Delta G$ (kcal/mol) | $\Delta H$ (kcal/mol) | $\Delta S$ (cal/mol K) | References[a] |
|---|---|---|---|---|---|
| [$^3$H]Clonazepam | 0 | −11.34 | −10.34 | +3.65 | 1 |
| | 37 | −11.20 | −14.98 | −12.20 | 1 |
| [$^3$H]Ro 15-1788 | 0 | −11.34 | −9.87 | −5.3 | 1 |
| | 37 | −11.46 | −9.87 | +5.12 | 1 |
| [$^3$H]Diazepam | 0 | −10.9 | −8.2 | +9.8 | 2 |
| | 0 | — | −9.8 | +4.1 | 3 |
| | 37 | −10.8 | −9.8 | +1.2 | 3 |
| [$^3$H]Propyl β-carboline-3-carboxylate | 0 | −11.9 | −8.3 | +13.3 | 2 |
| [$^3$H]Flunitrazepam | 0 | — | −3.1 | +29.6 | 3 |
| | 37 | — | −12.7 | −4.3 | 3 |

[a]References: 1, Möhler and Richards (1981); 2, Kochman and Hirsch (1982); 3, Quast *et al.* (1982).

speculation that this behavior is related to these ligands' ability to form a covalent bond with the binding site (Quast *et al.*, 1982). The thermodynamic parameters for diazepam binding are also altered by GABA. At 0°C the increase in affinity is primarily due to an increase in the entropic term, while at 37°C the increase is primarily due to an increase in the enthalpic term (Kochman and Hirsch, 1982). Although extensive studies of the thermodynamic properties of soluble binding sites have not yet appeared, binding to soluble sites is known to be highly temperature-dependent with a higher affinity at 4°C than at 25°C (Yousufi *et al.*, 1979).

## C. Physicochemical Characterization

The molecular weight of benzodiazepine binding sites has been investigated by a variety of techniques including gel permeation chromatography, sucrose-gradient centrifugation, polyacrylamide gel electrophoresis, and radiation inactivation. Using a Sephadex G-200 column eluted with Lubrol PX–containing buffer, the [$^3$H]diazepam binding component of a Lubrol extract of rat brain membranes eluted with a Stokes radius of 56 Å, corresponding to an apparent molecular weight of 220,000 ± 10,000 (assuming the binding site is a globular protein whose shape is similar to the shape of the calibration proteins) (Yousufi *et al.*, 1979). A similar value (240,000) has been obtained for [$^3$H]diazepam binding activity in Triton X-100 extracts of rat brain run on an Ultrogel Ac22

column eluted with Triton-containing buffer (Martini *et al.*, 1982). A similar value was also obtained for the [$^3$H]flunitrazepam photolabeled (206,000) component of rat brain membranes when a Lubrol PX extract was run on a Sephacryl S-300 column eluted with Lubrol PX–containing buffer (Thomas, 1982).

Considerably different values have been obtained by other workers using different chromatographic materials and detergents. Solubilization of binding sites by deoxycholate followed by chromatography on columns eluted with Triton X-100 or Lubrol PX–containing buffer seems to yield considerably higher size estimates. The [$^3$H]flunitrazepam binding activity in deoxycholate extracts of rat brain run on Sepharose 6B columns eluted with Lubrol PX–containing buffer had an apparent molecular weight of 670,000 (Asano and Ogasawara, 1981), while the [$^3$H]flunitrazepam binding activity in deoxycholate extracts of bovine complex run on Sepharose 6B columns eluted with Triton X-100–containing buffer had a Stokes radius of 68 Å (Stephenson *et al.*, 1982). Smaller size estimates (100,000 and 116,000) were obtained for the [$^3$H]flunitrazepam-photolabeled component of cerebellar and hipocampal rat brain membranes when Triton X-100 extracts were run on Ultragel AcA-34 columns eluted with Triton X-100–containing buffer (Braestrup *et al.*, 1981). A value of 61,000 ± 5,000 (Stokes radius of 21 ± 3 Å) has been reported for the [$^3$H]diazepam binding activity in Lubrol PX extracts of rat brain run on Sephadex G-200 columns eluted with Lubrol PX–containing buffer (Davis and Ticku, 1981).

The size of solubilized benzodiazepine binding sites has also been estimated by sucrose density gradient centrifugation. Despite differences in detergents and extraction procedures, relatively similar sedimentation values have been reported by most laboratories [12.8 S (Lang *et al.*, 1979), 11 S (Sherman-Gold and Dudai, 1980), 11.3 S (Asano and Ogasawara, 1981), and 12.5 S (Stephenson *et al.*, 1982)] although a smaller value was obtained in one study [8.25 (Davis and Ticku, 1981)]. If the benzodiazepine binding site is assumed to be a globular protein similar in shape and partial specific volume to the standard proteins used, the corresponding apparent molecular weight range is approximately 230,000–300,000 (Halsall, 1967).

Size estimates of detergent-solubilized binding sites obtained by either gel permeation chromatography or sucrose density gradient centrifugation alone normally suffer from at least two problems. First, the methods do not allow both the size and shape of protein to be determined, and second, the contribution of bound detergent cannot be determined. Therefore, size estimates are usually based upon the assumption that the protein is roughly spherical and the apparent molecular weights include an unknown amount of bound detergent. These two limitations can, in principle, be overcome by determining and comparing the hydrodynamic properties of the same detergent-solubilized preparation by gel filtration and sucrose density centrifugation in $H_2O$ and $D_2O$. One such study (Stephenson *et al.*, 1982) has been published for [$^3$H]flunitrazepam binding activity solubilized

with deoxycholate but analyzed by or after chromatography on gel permeation columns eluted with Triton X-100–containing buffer. From the sedimentation data in sucrose gradients in $H_2O$ and $D_2O$ a partial specific volume ($\nu$) of 0.73 ml $g^{-1}$ and a sedimentation coefficient ($S_{20,w}$) of 12.5 S were obtained. From gel permeation chromatography a Stokes radius of 68.1 Å was obtained, and from these values a molecular weight of 355,000 and a frictional ratio of 1.46 were calculated. The high frictional ratio may be due to an asymmetric shape or bound detergent or a combination of both. However, the partial specific volume, which does not differ significantly from the value for globular proteins ($\nu$ = 0.73 ml $g^{-1}$), would appear to rule out the possibility that much Triton X-100 is bound to the binding site (Triton X-100 has a $\nu$ = 0.99 ml $g^{-1}$). This suggests that the bound detergent which is solubilizing the binding site may be deoxycholate ($\nu$ = 0.76 ml $g^{-1}$) and that the amount cannot be determined due to the similarity of the $\nu$ values for the protein and this detergent. These results may be compared with those obtained for the acetylcholine receptor solubilized with Triton X-100: $\nu$ = 0.78 ml $g^{-1}$; $S_{20,w}$ = 12.5 S; Stokes radius = 73 Å; frictional ratio = 1.39; total apparent molecular weight = 470,000; apparent molecular weight of acetylcholine receptor excluding bound Triton = 360,000 (Meunier *et al.*, 1972).

Size estimates for the benzodiazepine binding site have been obtained for the membrane-bound complex using radiation inactivation; however, the molecular weights reported by various studies have varied by nearly fourfold. Using a lyophilized bovine cerebral cortex membrane preparation, a value of 216,000 ± 2,500 was obtained for the [$^3$H]flunitrazepam binding activity using β-galactosidase and yeast alcohol dehydrogenase as reference proteins (Chang *et al.*, 1981). This value is very similar to molecular weight estimates obtained by gel permeation chromatography; however, this may merely be coincidental since the values obtained by gel permeation chromatography were not corrected for bound detergent or for the shape of the solubilized complex. Using a frozen rat cerebral cortical membrane preparation, a value of 57,000 ± 2500 was obtained for the [$^3$H]diazepam binding activity (Paul *et al.*, 1981). This value is similar to values obtained for BZ subunits by polyacrylamide gel SDS electrophoresis (see next paragraph). A third study using lyophilized rat brain membranes obtained a value of 89,000 ± 15,000 for [$^3$H]flunitrazepam binding activity, a value which was decreased to 63,000 ± 7000 if the membranes were treated with $10^{-4}$ *M* GABA prior to lyophilization (Doble and Iversen, 1981). This second value is in good agreement with the 57,000 value obtained for rat cortical membranes. It is not clear why various groups have obtained such widely varying molecular weight estimates using a similar technique.

The molecular weight of photolabeled benzodiazepine binding sites has been determined using polyacrylamide gel electrophoresis under dissociating conditions using a discontinuous buffer system. Under these conditions protein complexes are usually dissociated completely and subunit molecular weights are

obtained. The subunit molecular weight obtained by various groups for the major photolabeled component are all in good agreement (~50,000) (Möhler *et al.*, 1980; Thomas and Tallman, 1981; Sieghart and Karobath, 1980; Gavish and Snyder, 1981). In addition, several groups have reported additional higher molecular weight species. Electrophoresis of partially purified Triton X-100 extract of calf brain gave two bands with apparent molecular weights of 55,000 and 62,000 (Gavish and Snyder, 1981). Also, using unpurified hippocampal and striatal rat brain or mouse membranes additional higher molecular weight species (53,000; 55,000; 59,000) have been reported (Sieghart and Karobath, 1980), and the pattern of these higher molecular weight species changes during development (Sieghart and Mayer, 1982). The same photolabeled bands are obtained when [$^3$H]clonazepam is used as a photolabel instead of [$^3$H]flunitrazepam (Sieghart and Mohler, 1982). These higher molecular weight bands have not been detected in another study (Thomas and Tallman, 1981), but the reason for the discrepancy is unclear. However, since the higher molecular weight bands are normally fainter bands located very close to the main radiolabeled band, the ability or failure to detect these additional components may be due to technical variations affecting the high resolution of this technique.

## D. Chemical Modification Studies

Several studies have appeared in which chemical modification of functional groups in brain membranes or detergent extracts of brain membranes has been undertaken to identify the amino acid residues which are involved in the binding of benzodiazepines. Diethyl pyrocarbonate treatment of rat brain membranes decreases diazepam binding by 40% (Burch and Ticku, 1981), suggesting that a histidine residue may be critical at a portion of the binding sites. Although this reagent is reasonably specific for histidine at the pH used for the reaction, the possibility remains that modification of other nucleophilic sites may be responsible for the decrease in binding.

Tetranitromethane treatment of both membranes and solubilized calf brain preparations results in an 80–100% decrease in [$^3$H]flunitrazepam binding, suggesting that tyrosine is also critically involved in the binding of benzodiazepines (Sherman-Gold and Dudai, 1981). Treatment with *N*-acetylimidazole, which acetylates phenolic groups, decreases [$^3$H]flunitrazepam binding to bovine brain membranes (Martini and Lucacchini, 1982). Also, 0.1 *M* iodoacetamide treatment at 37°C for 30 min has been reported to decrease [$^3$H]diazepam binding to calf brain membranes by 80%, and this decrease can be partially reversed by hydroxylamine, a deacetylation agent. These results strengthen the conclusion that tyrosine modification is responsible for the observed decreases in benzodiazepine binding.

Several studies have investigated the effects of sulfydryl reagents on benzodiazepine binding sites. Both 0.1 *M* dithiothreitol and 0.1 *M* iodoacetic acid have been reported to have a less than 20% effect on [$^3$H]diazepam binding to Lubrol extracts of rat brain membranes (Yousufi *et al.*, 1979). Also 1 m*M* *p*-chloromericuriphenyl sulfonic acid and 5 m*M* *n*-ethylmaleimide treatments have been found to cause no decrease in [$^3$H]flunitrazepam binding to calf cerebral cortex membranes (Sherman-Gold and Dudai, 1981). These results suggest that exposed cysteine residues are not involved in binding. However, other studies have indicated that 0.1 m*M* *N*-ethylmaleamide treatment at 37°C for 3 min results in a 55% decrease in [$^3$H]diazepam binding to bovine brain membranes (Martini and Lucacchini, 1982). Also, 0.1 *M* iodoacetamide treatment at 37°C for 30 min has been reported to decrease [$^3$H]diazepam binding to calf brain membranes by 60% (Gavish and Snyder, 1981). The discrepancy in these results is probably due to differences in the reaction conditions, for example, temperature. Further work will be required to determine the role of cysteinyl residues in benzodiazepine binding in brain.

In summary, studies employing chemical modification of functional groups has suggested that tyrosine residues are critically involved in the binding to benzodiazepines in both membrane and detergent-solubilized preparations. Some data have also been obtained which indicates that both histidine and cysteine residues may be involved in binding at a portion of the benzodiazepine binding sites. Further work will be required to determine the role of these and other amino acid residues in the molecular mechanism of action of these drugs.

## E. Affinity Techniques

### *1. Affinity Labeling*

The selectivity of affinity techniques has made these methods a favorite choice for the study of low-abundance membrane-bound proteins. Affinity labeling, photoaffinity labeling, and affinity chromatography have all been applied to the characterization of benzodiazepine binding sites. Two alkylating benzodiazepine derivatives have been reported, and both are capable of inhibiting *in vitro* [$^3$H]diazepam binding to rat brain membranes in a noncompetitive fashion consistent with covalent attachment (Rice *et al.*, 1979; Williams *et al.*, 1980). When injected into the cerebral ventricle, both of these affinity labels exert long-lasting anticonvulsant effects which are consistent with covalent attachment to the binding site (Williams *et al.*, 1981). Affinity probes such as these are potentially useful for isolation, localization, and characterization of benzodiazepine binding sites.

### 2. *Photoaffinity Labeling*

Several benzodiazepines have proven useful as photoaffinity probes. Benzodiazepines containing a nitro group in position 7 appear capable of undergoing a photochemical reaction when exposed to UV light which results in covalent attachment to the binding site. Flunitrazepam, clonazepam, nitrazepam, and 3-aminoclonazepam are all capable of noncompetitive inhibition of benzodiazepine binding when incubated with brain membranes and exposed to UV light (Thomas, 1982). At least one of these compounds, flunitrazepam, is commercially available in tritium-labeled form and has been used extensively to prepare radiolabeled benzodiazepine binding sites. The photolabeling reaction can be carried out using both membrane-bound and detergent-solubilized binding sites (Thomas and Tallman, 1981). The photolabeling technique has already yielded valuable information concerning the molecular size (Möhler *et al.*, 1980), localization (Battersby *et al.*, 1979), membrane configuration (Mohler *et al.*, 1980; Thomas and Tallman, 1981), and possible agonist–antagonist interactions of the binding site (Möhler, 1982; Thomas and Tallman, 1983).

### 3. *Affinity Chromatography*

Several different affinity gel resins have been prepared for purification of benzodiazepine binding sites, using benzodiazepine analogs containing a primary or secondary amine group. A flurazepam analog, Ro 7-1986, has been coupled to cyanogen bromide– activated agarose through a seven-carbon side arm (Gavish and Snyder, 1981) and to carbodiimide-activated agarose through a seven-atom side arm (Tallman and Gallager, 1979). In early work using these affinity resins it was not possible to achieve affinity elution; however, using an acidic pH step (pH 3.5) elution of 30 to 40% of the [$^3$H]flunitrazepam binding activity with a 133-fold enhancement in specific binding activity has been reported (Gavish and Snyder, 1981). Recently, a Ro 7-1986–agarose affinity resin has been prepared using an adipic acid dihydrazide spacer arm, and affinity elution was achieved using 6 m*M* chlorazepate (Siegel *et al.*, 1982). This affinity method resulted in approximately a 700-fold purification with a 14–20% yield. A lorazepam analog, Ro 5-3027, has been coupled to cyanogen bromide–activated agarose through an adipic acid dihydrazide spacer arm (Martini *et al.*, 1982). Using this agarose–benzodiazepine absorbent, specific elution with 6 m*M* chlorazepate resulted in recovery of 45% of [$^3$H]diazepam binding activity with over a 700-fold purification. Following a concentration step using polyethylene glycol 20,000, a 5200-fold overall purification is achieved, resulting in a final specific activity which is near the expected theoretical limit (assuming a molecular weight of 240,000 for the binding site).

## IV. REGULATION OF BENZODIAZEPINE BINDING

One potential type of information to be gained from studies on purified or partially purified systems is the determination of the system's components and the role each component plays in the overall system function. The benzodiazepine binding site has been proposed to be a part of a complex which also contains a GABA receptor and an anion ionophore (Tallman *et al.*, 1980). A host of additional drugs, ions, and other modulators may also act on various components of the complex. Solubilization and purification of the complex and its components is one means of testing this model.

Solutilization in itself potentially may yield information about the nature of the complex, for example, which components are contained on the same polypeptide chain. However, there is no *a priori* way of knowing if solubilized proteins are present as a single protein with bound detergent or as a complex of two or more proteins with bound detergent or as a mixture of both. Partial purification may clarify the situation, but complete purification may be necessary before definitive conclusions can be reached. In the case of the benzodiazepine binding site, preparation of pure protein requires about 5,000- to 20,000-fold enrichment, so that material purified 1000-fold may still contain 80–95% contaminating protein. With these provisos in mind, what has been learned so far about BZ binding sites from solubilization and purification studies?

One issue which has been addressed by many reports is whether or not benzodiazepine binding sites are a part of the same protein which contains a GABA receptor. Initial reports from studies on solubilized preparations were conflicting, with some studies reporting that GABAergic agents affected BZ binding (Gavish and Snyder, 1980), while others reported that GABA had no effect on binding (Yousufi *et al.*, 1979). Some of these initial differences were probably due to the release of GABA upon solubilization. Apparently, even well-washed membrane preparations contain or can generate GABA which is released during solubilization, since dialysis of detergent extracts results in both soluble muscimol binding activity and at least partial restoration of GABAergic modulation of soluble BZ binding activity in preparations which exhibit neither property before dialysis (Thomas, 1982). Also, two studies using partially purified preparations have found that muscimol and BZ binding activities co-purify and that the BZ binding activity continues to be enhanced by GABAergic agents (Gavish and Snyder, 1981; Asano and Ogasawara, 1981). These results suggest that both of the binding sites are contained within the same protein or a complex of proteins which remains associated. However, since these preparations are impure, additional purification may resolve these two binding activities.

Other studies have suggested that the two components are resolvable. Depend-

ing upon the solubilization conditions, it has been reported that BZ sites alone, GABA sites alone, or both can be solubilized (Massotti *et al.*, 1981). Two separate studies have indicated that Triton X-100 selectively solubilizes BZ binding sites, leaving GABA sites in the particulate fraction (Chiu and Rosenberg, 1979; Massotti *et al.*, 1981). Also, autoradiographic studies (Young and Kuhar, 1979) suggest that GABA binding sites and BZ binding sites have a different pattern of distribution in brain, and ontogenetic studies (Braestrup and Nielsen, 1978; Palacios *et al.*, 1979) have suggested that BZ and GABA binding sites develop at different rates. On the basis of this evidence, it would appear unlikely that GABA and BZ binding sites always exist as a complex of fixed stoichiometry. Where GABA sites are associated with BZ sites, it is still unclear if a complex of proteins or a single protein is present. Recent work using Triton extracts of rat brain has shown that under some conditions heat can inactivate solubilized GABA binding activity without inactivating solubilized BZ binding activity (Lo and Snyder, 1982). Furthermore, the heat treatment decreases the apparent Stokes radius of the soluble BZ binding component, suggesting the dissociation or cleavage of a portion of the original complex; however, additional work is required to test this interpretation.

BZ binding is modulated by a number of other compounds and ions, and the effects of some of these modulators have also been examined in solubilized preparations. Pentobarbital (0.5 m*M*) increases [$^3$H]flunitrazepam binding 30% in deoxycholate extracts of bovine cortex; however, enhancement was not observed reproducibly after gel filtration on a column eluted with Triton X-100 (Stephenson *et al.*, 1982). Avermectin B1a (7 m*M*) increases [$^3$H]flunitrazepam binding 40% in digitonin extracts of rat brain (Pong *et al.*, 1981). Potassium iodide does not enhance [$^3$H]diazepam Lubrol extracts of rat forebrain (Tallman *et al.*, 1980), while ethanol enhances [$^3$H]flunitrazepam binding to Lubrol extracts of rat brain (Ticku and Davis, 1981). The interpretation of these results will be easier when more information becomes available about the composition of the soluble BZ binding sites in these detergent extracts.

## V. SUMMARY

A great deal of biochemical data has already been accumulated concerning the BZ binding site (see Table II). Although no one model can accurately embody all of the available data, this type of simplification provides a convenient means for summarizing some of this information. Currently, the BZ binding site is thought to be a membrane-bound protein which may contain a carbohydrate component, at least on some of its subunits. The protein is not known to contain an intrinsic lipid, nucleic acid, or cofactor component. If specific lipid requirements exist,

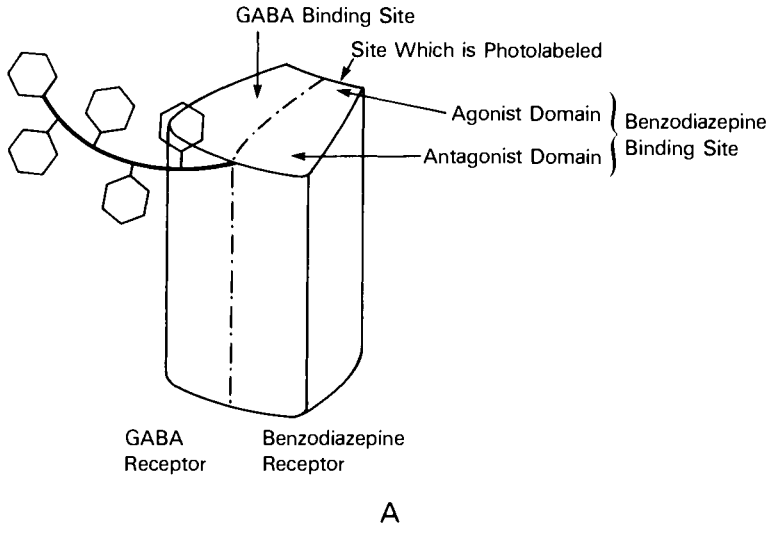
TABLE II

Physiocochemical Properties of Brain Benzodiazepine Binding Sites

| Parameter | Value | Method | References[a] |
|---|---|---|---|
| Molecular weight complex, detergent-solubilized | ~200,000–350,000 | Gel permeation chromatography | 1,2 |
| Subunit | ~45,000–60,000 | SDS–PAGE and photoaffinity labeling | 1,3–5 |
| Subunit composition | Unknown (but data suggest complex contains 4 subunits) | Photoaffinity labeling and radiation inactivation | 6–8 |
| Stokes radius | 56,68 | Gel permeation chromatography | 1,9 |
| Sedimentation coefficient | 11–12.8 S | Sucrose density gradient centrifugation | 1,3–5 |
| Partial specific volume | 0.73 ml/g | Sucrose density gradient centrifugation in $H_2O$ and $D_2O$ | 1 |
| Frictional ratio | 1.46 | Gel permeation chromatography and sucrose density centrifugation | 1 |
| Isoelectric point | Acidic, probably between 5.0 and 6.0 | Ion-exchange chromatograph and isoelectric focusing | 5,10–12 |
| Carbohydrate composition | Unknown, but sites bind to concanavalin A–agarose resin | Lectin affinity resin | 13 |
| Amino acids at binding site | Tyrosine, possibly cysteine and histidine | Chemical modification | 14–16 |

[a]References 1, Stephenson *et al.* (1982); 2, Yousufi *et al.* (1979); 3, Lang *et al.* (1979); 4, Sherman-Gold and Dudai, (1980); 5, Asano and Ogasawara (1981); 6, Möhler *et al.* (1980); 7, Thomas and Tallman, (1981); 8, Nielsen *et al.*, (1983); 9, Thomas, (1982); 10, Gavish and Snyder, (1981); 11, J. W. Thomas and J. F. Tallman, unpublished data; 12, Korneyev, 1982; 13, Lo *et al.*, (1982); 14, Sherman-Gold and Dudai, (1981); 15, Martini and Lucacchini, (1982); 16, Burch and Ticku, (1981)

they have not yet been identified. The basic subunits have a molecular weight of 50,000, and alternate forms of the binding site with molecular weights as high as 60,000 have been reported. The binding site is thought to exist in the membrane as a complex which contains at least one and probably more GABA receptors, several BZ binding sites (possibly four), and at least one anion ionophore. It is not yet known if the GABA receptor and the BZ binding site are located on the same protein or on two separate but closely associated proteins. It is also not



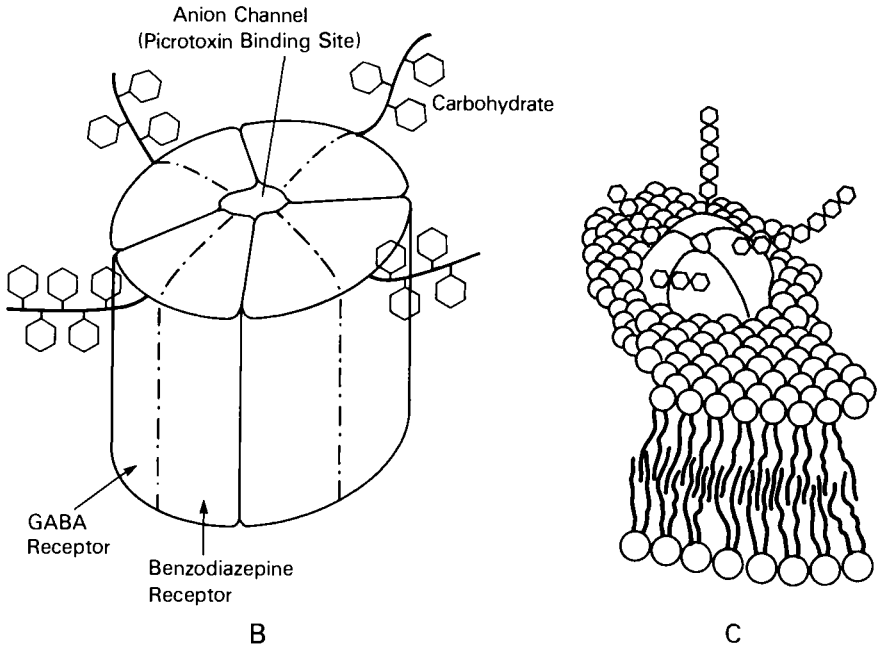




**Fig. 1.** Proposed model of the benzodiazepine binding site–GABA receptor–anion ionophore complex. (A) A single subunit. (B) The four-subunit complex. (C) The membrane-bound complex.

known if the anion ionophore is a separate protein or if the GABA receptor–BZ binding site subunits form the walls for the anion ionophore; however, the later possibility seems quite likely by analogy to model systems (Jain, 1972; Fox and Richards, 1982) and to other known chemically gated ionophores (Giraudat and Changeux, 1980).

Figure 1 shows three models for the BZ binding site. Panel A shows a single subunit which contains a GABA receptor and a BZ binding site. The BZ site is subdivided into an agonist and an antagonist domain, and a site for photochemical attachment of nitro-containing BZ is also indicated. The placement of the agonist domain closer to the GABA receptor and the photolabeling site is consistent with current data on GABA regulation and photolabeling.

Panel B shows the four-subunit complex. Four BZ sites have been included, which is consistent with current data from photolabeling (Möhler *et al.*, 1980; Thomas and Tallman, 1981) and radiation inactivation experiments (Nielsen *et al.*, 1983). Although it seems likely that at least two GABA receptors occur in each complex, the actual number is unknown. For convenience, we have included four GABA receptors. The subunits have been shown with carbohydrate moieties, as might be expected from the lectin binding data (Lo *et al.*, 1982) and by analogy to many other membrane-bound proteins. Panel C illustrates the membrane-bound complex. The model assumes that the receptor–binding site subunits form the walls of the single anion ionophore which forms an open channel conformation more readily in the presence of GABA, especially if the BZ sites are occupied. The accuracy of this model remains to be proven. The availability of useful affinity techniques promises to yield valuable information on the composition of this complex and should eventually lead to better understanding of the molecular mechanism by which this important group of drugs alters neuronal function.

## REFERENCES

Asano, T., and Ogasawara, N. (1980). *Life Sci.* **26,** 607–613.

Asano, T., and Ogasawara, N. (1981). *Life Sci.* **29,** 193–200.

Battersby, M. K., Richards, J. G., and Möhler, H. (1979). *Eur. J. Pharmacol.* **57,** 277–278.

Braestrup, C., and Nielsen, M. (1978). *Brain Res.* **147,** 170–173.

Braestrup, C., Nielsen, M., Skorbjerg, H., and Gredal, O. (1981). *Adv. Biochem. Psychopharmacol.* **26,** 147–155.

Burch, T. P., and Ticku, M. K. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 3945–3949.

Chang, L. R., Barnard, E. A., Lo, M. M., and Dolly, J. O. (1981). *FEBS Lett.* **126,** 309–312.

Chiu, T. H., and Rosenberg, H. (1979). *Eur. J. Pharmacol.* **58,** 335–338.

Costa, E., and Guidotti, G. (1979). *Annu. Rev. Pharmacol. Toxicol.* **19,** 531–545.

Cuatrecasas, P. (1972). *Proc. Natl. Acad. Sci. U.S.A.* **69,** 318–322.

Davis, W. C., and Ticku, M. K. (1981). *J. Neurosci.* **1,** 1036–1042.
Doble, A., and Iversen, L. L. (1982). *Nature (London)* **295,** 522–523.
Fox. R. O., and Richards, F. M. (1982). *Nature (London)* **300,** 325–330.
Gallager, D. W., Mallorga, P., and Tallman, J. F. (1980). *Brain Res.* **189,** 209–220.
Gavish, M., and Snyder, S. H. (1980). *Nature (London)* **287,** 651–652.
Gavish, M., and Snyder, S. H. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 1939–1942.
Gavish, M., Chang, R. S., and Snyder S. H. (1979). *Life Sci.* **25,** 783–789.
Giraudat, J., and Changeux, J.-P. (1980). *Trends Pharmacol. Sci.* **1,** 198–202.
Guidotti, A., Toffano, G., and Costa, E. (1978). *Nature (London)* **285,** 553–555.
Haefely, W., Pieri, L., Polc, P., and Schaffner, R. (1981). *Handb. Exp. Pharmacol.* **55**(II), 13–362.
Halsall, H. B. (1967). *Nature (London)* **215,** 880–881.
Jain, M. K. (1972). "Membranes: A System." Van Nostrand-Reinhold, Princeton, New Jersey.
Kochman, R. L., and Hirsch, J. D. (1982). *Mol. Pharmacol.* **22,** 335–341.
Korneyev, A. (1982). *Neuropharmacology* **21,** 1355–1358.
Lang, B., Barnard, E. A., Chang, L.-R., and Dolly, J. O. (1979). *FEBS Lett.* **104,** 149–153.
Lo, M. M. S., and Snyder, S. H. (1982). *Soc. Neurosci. Abstr.* **8,** 572.
Lo, M. M. S., Strittmatter, S. M., and Snyder, S. H. (1982). *Proc. Natl. Acad. Sci. U.S.A.* **79,** 680–684.
Martini, C., and Lucacchini, A. (1982). *J. Neurochem.* **38,** 1768–1770.
Martini, C., Lucacchini, A., Ronca, G., Hrelia, S., and Rossi, C. A. (1982). *J. Neurochem.* **38,** 15–19.
Massotti, M., Guidotti, A., and Costa, E. (1981). *J. Neurosci.* **1,** 409–418.
Meunier, J. C., Olsen, R. W., and Changeux, J.-P. (1972). *FEBS Lett.* **24,** 63–68.
Möhler, H. (1982). *Eur. J. Pharmacol.* **80,** 435–436.
Möhler, H., and Richards, J. G. (1981). *Nature (London)* **294,** 763–765.
Möhler, H., Battersby, M. K., and Richard J. G. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 1666–1670.
Nielsen, M., Honore, T., and Braestrup, C. (1983). *Biochem. Pharmacol.* **32,** 177–180.
Olsen, R. W. (1980). *In* "Receptor Binding Techniques" (D. B. Bylund, ed.), pp. 206–217. Soc. Neurosci., Bethesda, Maryland.
Palacios, J. M., Niehoff, D. L., and Kuhar, M. J. (1979). *Brain Res.* **179,** 390–395.
Paul, S. M., and Skolnick, P. (1978). *Science* **202,** 892–894.
Paul, S. M., Kempner, E. A., and Skolnick, P. (1981). *Eur. J. Pharmacol.* **76,** 465–466.
Pong, S. S., Dehaven, R., and Wang, C. C. (1981). *Biochim. Biophys. Acta* **646,** 143–150.
Quast, V., Mohlmann, H., and Vollmer, K. O. (1982). *Mol. Pharmacol.* **22,** 20–25.
Randall, L. O., Schallek, W., Sternbach, L. H., and Ning, R. Y. (1964). *Med. Chem. Ser.* **3**(1), 175–281.
Rice, K. C., Brossi, A., Tallman, J., Paul, S. M., and Skolnick, P. (1979). *Nature (London)* **278,** 854–855.
Sabato, U. C., Aguilar, J. S., and De Robertis, E. (1981). *J. Recpt. Res.* **2,** 119–133.
Sherman-Gold, R., and Dudai, Y. (1980). *Brain Res.* **198,** 485–490.
Sherman-Gold, R., and Dudai, Y. (1981). *FEBS Lett.* **131,** 313–316.
Sieghart, W., and Karobath, M. (1980). *Nature (London)* **286,** 285–287.
Sieghart, W., and Mayer, A. (1982). *Neurosci. Lett.* **31,** 71–74.
Sieghart, W., and Möhler, H. (1982). *Eur. J. Pharmacol.* **81,** 171–173.
Sigel, E., Mamalaki, C., and Barnard, E. A. (1982). *FEBS Lett.* **147,** 45–48.
Skolnick, P., Lock, K. L., Paugh, B., Marangos, P., Windsor, R., and Paul, S. M. (1980). *Pharmacol., Biochem. Behav.* **12,** 685–689.
Stephenson, F. A., Watkins, A. E., and Olsen, R. W. (1982). *Eur. J. Biochem.* **123,** 291–298.

Sternbach, L. H., Randall, L. O., and Gustafson, S. R. (1964). *Med. Chem. Ser.* **1,** 137.
Tallman, J. F., and Gallager, D. W. (1979). *Pharmacol., Biochem. Behav.* **10,** 809–813.
Tallman, J. F., Paul, S. M., Skolnick, P., and Gallager, D. W. (1980). *Science* **207,** 274–281.
Thomas, J. W. (1982). Ph.D. Dissertation, George Washington University, Washington, D.C.
Thomas, J. W., and Tallman, J. F. (1981). *J. Biol. Chem.* **256,** 9838–9842.
Thomas, J. W., and Tallman, J. F. (1983). *J. Neurosci.* **3,** 433–440.
Ticku, M. K., and Davis, W. C. (1981). *Eur. J. Pharmacol.* **71,** 521–522.
Weiland, G. A., Minneman, K. P., and Molinoff, P. B. (1980). *Mol. Pharmacol.* **18,** 341–347.
Williams E. F., Rice, K. C., Paul, S. M., and Skolnick, P. (1980). *J. Neurochem.* **35,** 591–597.
Williams, E. F., Rice, K. C., Mattson, M., Paul, S. M., and Skolnick, P. (1981). *Pharmacol., Biochem. Behav.* **14,** 487–491.
Young, W. S., and Kuhar, M. J. (1979). *Nature (London)* **280,** 393–395.
Yousufi, A. K. M., Thomas, J. W., and Tallman, J. F. (1979). *Life Sci.* **25,** 463–469.

# Chapter 7

# SOLUBILIZATION OF THE DOPAMINE RECEPTOR

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## I. INTRODUCTION

The solubilization of a receptor consists of converting it from a complex membrane system into a relatively simpler state that can only be characterized by using methods that cannot be applied to the intact membrane. This is not a goal in itself but, like its purification, both are prerequisite steps toward unlocking the molecular events that ensure that certain substances act as agonists and others as antagonists. We will examine the criteria that must be fulfilled to ascertain that membrane-bound dopamine receptors are truly converted into a molecularly dispersed system.

*In vitro* binding studies have considerably facilitated a better understanding of the brain dopamine receptor and provided a basis for the elucidation of drug action mechanisms. However, probably because of the ease with which *in vitro* binding can be performed, some people have gone so far as to consider a binding site as a receptor site.

Two postulates must be kept in mind when one starts any binding study: First, *a binding site does not necessarily mean a receptor site*. Second, the *postulate of receptor homogeneity* supposes that the receptor sites of a given neurotransmitter are homogeneous, thus having the same molecular structure in all parts of the




ISBN 0-12-470350-X

body. This is, evidently, a working hypothesis but quite fruitful because it forces us to examine more carefully the reason for some intriguing results rather than to propose immediately the occurrence of a receptor subtype. In contrast, the validity of the first postulate is largely supported by the increasing number of studies wherein the chosen [$^3$H]ligand only reveals binding sites but not receptor sites. Like many other receptors, the dopamine receptor has not been able to resist the lure of multiple sites unrelated to physiological receptors.

## II. HOW TO DEFINE THE DOPAMINE RECEPTOR

The term *receptor* is used with so many different meanings that it is practically impossible to cover in one definition the entire set of biological processes in which the receptor has been involved. However, it is beyond doubt that the term has been misused in numerous cases, especially in *in vitro* binding studies.

It is impossible to define a receptor, especially when it is involved in the process of neurotransmission, without taking into account the physiological response elicited by the neurotransmitter itself. In my opinion, the definition proposed by John Newport Langley (1906) in the early twentieth century still remains valid today: "The mutual antagonism of nicotine and curare on muscle can only satisfactorily be explained by supposing that both combine with the receptive substance. It receives the stimulus (agonist) and by transmitting it causes contraction."

Thus, a receptor is a site of competition for agonist and antagonist and the stimulus produced by agonists but blocked by antagonists leads to a physiological response. If one adapts Langley's definition to the dopaminergic system, it is obvious that only the $D_2$ site may be called a receptor site; all the physiological effects of dopamine and of dopamine agonists (emesis, stereotypy, hypermotility, turning and climbing behavior, decrease of prolactin secretion, stomach relaxation, antiparkinson drug, psychosis . . .) are antagonized by neuroleptic and antiemetic drugs (Niemegeers and Janssen, 1979; Costall and Naylor, 1981) and all are mediated through the $D_2$ receptor site (Laduron, 1981, 1982). This $D_2$ subtype (we prefer to call it the dopamine receptor) is the binding site labeled by dopamine antagonists like haloperidol and spiperone at nanomolar concentrations and by dopamine agonists at micromolar concentrations (Seeman, 1980); it is not coupled to adenylate cyclase (Kebabian and Calne, 1979). As reported elsewhere there are more than 17 pharmacological, behavioral, and biochemical parameters related to the effects of dopamine agonists and antagonists that correlate nicely with the *in vitro* binding on the $D_2$ receptor (Laduron, 1980, 1981). In contrast, such a correlation was never found for the $D_1$, $D_3$, and $D_4$ subtypes; the dopamine-sensitive adenylate cyclase defined as the $D_1$ site

(Kebabian and Calne, 1979) is an enzyme; there is no evidence that it is directly involved in neurotransmission of a dopaminergic nature; the only physiological effect that was believed to be mediated through the $D_1$ site is parathyroid hormone secretion (Brown *et al.*, 1977). Recently, it has been found that the parathyroid gland in humans is unresponsive to dopamine (Bansal *et al.*, 1982), a fact that is quite compatible with the lack of change in the parathyroid hormone secretion in patients treated with neuroleptics, even those like the thioxanthene or phenothiazine derivatives, which have potent effects on the dopamine-sensitive adenylate cyclase.

The $D_3$ subtype, which was defined as a dopaminergic site sensitive to nanomolar concentrations of dopamine but micromolar concentrations of neuroleptics (Seeman, 1980), is neither a receptor nor a dopaminergic site; it is a recognition site for catechol derivatives (Laduron, 1982) including norepinephrine (Seeman, 1980). Therefore, one has to conclude that there is only one dopamine receptor, since the dopamine-sensitive adenylate cyclase is not implicated in neuronal transmission but could be responsible for long-term metabolic effects.

## III. SOLUBILIZATION OF DOPAMINE RECEPTOR

The first attempts to obtain dopamine receptors in solution were not very encouraging; [$^3$H]spiperone binding sites solubilized from rat striatum (Gorissen and Laduron, 1978) and from calf striatum (Tam and Seeman, 1978) by means of 1% digitonin had lost the high-affinity properties (except for spiperone) normally found on membrane preparations. Now the reasons of such a failure are known: The solubilized dopamine receptor sites were masked by a large excess of nonspecific but displaceable [$^3$H]spiperone binding sites (spirodecanone sites; see below). The choice of another animal species, the dog, was quite decisive (Gorissen and Laduron, 1979).

### A. Solubilization Procedures

Originally, a microsomal P fraction was prepared and used as starting material for the solubilization because of its high content of dopamine receptors (about 65% of the total homogenate) (Laduron *et al.*, 1978). Dog striata are homogenized in 10 volumes of 0.25 *M* sucrose with a Duall homogenizer and centrifuged at 1100 *g* for 10 min. The pellet obtained (nuclear fraction) is rehomogenized in 10 volumes and again centrifuged at the same speed. Both supernatants are pooled and centrifuged at 30,900 *g* for 10 min to obtain the M + L fraction. The supernatant is further centrifuged at 120,000 *g* for 60 min, and the pellet ob-

tained, the P or microsomal fraction, is suspended in 2 volumes of distilled water and kept at −16°C.

To 2.5 ml of the membrane preparation, 2.5 ml of 2% digitonin (Serva) suspended in a medium containing 0.5 m*M* sucrose, 2 m*M* EDTA, 20 m*M* sodium phosphate buffer (pH 7.2) and 0.02 $NaN_3$, is added so that the final concentration of digitonin is 1%. The mixture is gently agitated for 15 min at 0°C and then centrifuged at 120,000 *g* for 60 min (SW 65 Ti Spinco Rotor). The supernatant, which is considered as the soluble preparation, is removed very carefully without disturbing the pellet.

Although the digitonin procedure is currently used for canine (Gorissen and Laduron, 1979), rat (Gorissen *et al.*, 1980), and human brains (Davis *et al.*, 1981), other mild detergents can be used, such as 0.5% Lubrol PX (Withy *et al.*, 1981), 10 m*M* CHAPS (Lew *et al.*, 1981), and 0.1% (Withy *et al.*, 1981) or 0.25% (Ilien *et al.*, 1982) lysophosphatidylcholine. In some cases 0.1 m*M* phenylmethylsulfonylfluoride (PMSF) was added to prevent proteolytic degradation, although this does not change the yield of solubilized receptors. In contrast, the presence of sucrose obviously improves the solubilization of dopamine receptors.

## B. Methods for Measuring Solubilized Dopamine Receptors

Solubilized receptors can only be measured by using methods that cannot be applied to intact membranes. The original method (Gorissen *et al.*, 1979) was as follows: An aliquot of soluble extract from dog striata was incubated at 0°C for 16 hr in the presence of $2 \times 10^{-9}$ *M* [$^3$H]spiperone (specific activity 23.6 or 53.4 Ci/mmol, NEN) in 0.5 ml total volume. Stereospecific binding is defined as the difference between the binding in the presence of $2 \times 10^{-7}$ *M* (−)-butaclamol and $2 \times 10^{-6}$ *M* (+)-butaclamol.

Aliquots of 0.1 ml of the incubation mixture are layered on the top of a Sephadex G-50 Medium column (13 × 0.5 cm). Elution is carried out at 2°C with 10 m*M* sodium phosphate (pH 7.2) containing 0.01% $NaN_3$. Four-drop fractions are collected in scintillation vials and then counted for radioactivity. The first peak represents the bound [$^3$H]spiperone macromolecular complex, the second one being the free drug. Three major improvements were brought to the original method that enabled us also to characterize solubilized dopamine receptors from rat striatum (Gorissan *et al.*, 1980). First, by incubating at 20°C for 15 min instead at 0°C for 16 hr, the nonspecific binding is markedly reduced. Moreover, the addition of R 5260 ($10^{-5}$*M*) can prevent binding to the spirodecanone sites (cf. below). Finally, the charcoal assay used to separate receptor-bound and free ligands should be preferred to the time-consuming and laborious

gel filtration technique. Thus, the most convenient procedure is as follows: 0.4 ml of soluble extract is incubated in presence of 1 n*M* [$^3$H]spiperone at 25°C for 15 min in the presence and absence of $10^{-6}$*M* (+)-butaclamol. After incubation 50 μl of BSA-coated charcoal (10% charcoal with 2% BSA in water) is added to 0.4 ml of the incubation medium and centrifuged at 14,000 rpm for 3 min in a microfuge. A 0.2 ml aliquot of the supernatant, containing [$^3$H]spiperone bound to soluble receptor, is taken for determination of radioactivity. Although other binding assay procedures have also been proposed such as polyethyleneglycol (Chan *et al.*, 1981; Lew *et al.*, 1981) or ammonium sulfate precipitation (Gorissen *et al.*, 1980) and equilibrium dialysis (Gorissen *et al.*, 1980), the adsorption on BSA-coated charcoal remains the most sensitive and the most appropriate technique for measuring solubilized dopamine receptors.

## IV. RECEPTOR SPECIFICITY

One of the greatest difficulties in the interpretation of *in vitro* binding studies is the problem of making distinction between specific (thus related to a receptor site) and nonspecific binding, which can also be displaceable. This is the reason that various criteria must be fulfilled to ascertain that a binding site really corresponds to a receptor site that is relevant physiologically. These criteria are summarized in Table I.

As already emphasized (Laduron and Ilien, 1982), drug displacement is one of the most, if not the most, important criterion. This must be carefully examined even before performing a Scatchard analysis. To rule out the possibility that the binding sites are only recognition sites for compounds having the same structural moiety (e.g., [$^3$H]cimetidine, [$^3$H]ranitidine, [$^3$H]desipramine, [$^3$H]imipramine,

TABLE I

Criteria of Receptor Specificity

1. Drug displacement (agonists and antagonists belonging to different chemical and pharmacological classes)
2. Correlation between drug affinity *in vitro* and pharmacological potency *in vivo*
3. Correlation between drug affinity in solubilized and membrane preparations
4. Regional distribution
5. High affinity
6. Saturability
7. Reversibility
8. Stereospecificity

most of the biogenic amines, etc.), one needs to test drugs belonging to different chemical series but having the same pharmacological properties. A good example of this is given by the spirodecanone site: When [$^3$H]spiperone binding sites were solubilized for the first time from rat striata (Gorissen and Laduron, 1978), they did not reveal the high-affinity properties of the dopaminergic receptors normally found in membrane preparations.

Figure 1 shows the $IC_{50}$ values for various drugs in membrane preparations and in solubilized preparations from dog (soluble DA receptor) and rat (soluble spirodecanone site) striata. Obviously, there was a good correlation between the first two but not with the soluble extract from rat striata. For the latter, only spiperone and R 5260, two drugs having the same chemical moiety (spirodecanone moiety, Fig. 2) displayed high-affinity properties while benperidol, a butyrophenone as spiperone that lacks the spirodecanone moiety was 3800 times less active in soluble preparations from rat striatum than in membrane preparations. The same was true for haloperidol, (+)-butaclamol, and flupenthixol. Therefore, at that time the [$^3$H]spiperone binding sites solubilized from rat striata corresponded to spirodecanone sites (Howlett *et al.*, 1979) but did not reveal dopaminergic properties.

The lack of these spirodecanone sites in dog striatum enabled us to solubilize dopamine receptors that retained the high-affinity properties of the membranes (Gorissen and Laduron, 1979; Gorissen *et al.*, 1979). Thereafter, the use of the charcoal method and of R 5260 to diminish the proportion of nonspecific binding allowed us also to solubilize high-affinity dopamine receptors from rat striata (Gorissen *et al.*, 1980). From such an example, one cannot stress enough the

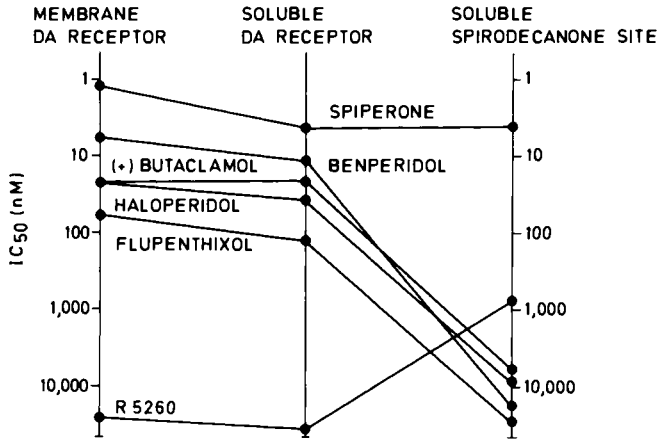


**Fig. 1.** $IC_{50}$ values of various drugs in [$^3$H]spiperone binding in membrane preparations and in digitonin-solubilized extracts from dog striatum (soluble DA receptor) and from rat striatum (soluble spirodecanone site) (cf. Gorissen and Laduron, 1978, 1979).

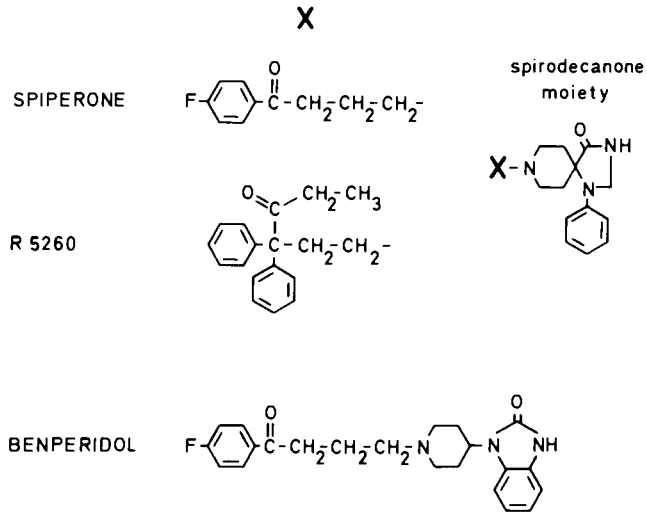


**Fig. 2.** Chemical structure of two butyrophenones, spiperone and benperidol, and R 5260, which is a potent analgesic. Note that R 5260 has the same spirodecanone moiety as spiperone.

need to use displacer other than the ligand itself for measuring nonspecific binding (blank); this represents the first and probably the most important rule when setting up an *in vitro* binding assay.

Since spiperone is known to label dopamine receptors as well as serotonin $S_2$ receptors (Leysen *et al.*, 1978), one has to examine whether the solubilized sites labeled with [$^3$H]spiperone are really of dopaminergic and not of serotonergic nature. Table II gives the $IC_{50}$ values for numerous drugs (agonists and antagonists) in digitonin extracts from rat striatum and in lysolecithin extracts from rat frontal cortex (Ilien *et al.*, 1980, 1982). Dopamine agonists, especially the tetralin derivatives, and dopamine antagonists are much more active in the striatum than in the frontal cortex, while the reverse is true for serotonin, bufotenin, and the serotonin antagonists such as ketanserin, pipamperone, methysergide, mianserin, and pirenperone. This clearly demonstrates that the [$^3$H]spiperone binding sites solubilized by digitonin from striatal tissues are of dopaminergic nature and those from frontal cortex of serotonergic nature. Even if [$^3$H]spiperone can label membrane-bound serotonergic receptors (10–20%) in the striatum, it is without consequence for the solubilization because serotonergic receptors are not solubilized by digitonin (Ilien *et al.*, 1982).

Another important criterion listed in Table I is the correlation between binding in solubilized preparations and the activity in pharmacological tests. Figure 3 shows a good correlation ($r = .89$) between [$^3$H]spiperone binding in solubilized extracts from dog striatum and the antagonism of apomorphine-induced emesis in dog; note the large concentration range of drugs belonging to different chem-

TABLE II

$IC_{50}$ Values for [$^3$H]Spiperone Binding in Soluble Extracts from Rat Striatum (Dopamine) and Frontal Cortex (Serotonin $S_2$)[a]

| | $IC_{50}$ (n*M*) | | |
|---|---|---|---|
| | Striatum (A) | Frontal cortex (B) | A/B |
| Benperidol | 2.2 | 14.1 | 0.15 |
| Spiperone | 6.3 | 6.3 | 1 |
| (+)-Butaclamol | 12.3 | 316 | 0.04 |
| Haloperidol | 37 | — | — |
| Chlorpromazine | 141 | 224 | 0.63 |
| Amino-5,6-dihydroxytetralin | 178 | 158,000 | 0.001 |
| Pirenperone | 280 | 3.2 | 87 |
| Pizotifen | 631 | 52.5 | 12 |
| Cyproheptadine | 891 | 39.8 | 22 |
| Pipamperone | 1260 | 11.8 | 107 |
| LSD | 1410 | 56.2 | 25 |
| Methysergide | 1580 | 25.1 | 63 |
| Ketanserin | 6310 | 10 | 631 |
| Mianserin | 6310 | 66 | 95 |
| (−)-Butaclamol | 8910 | 41,700 | 0.21 |
| Dopamine | 28,200 | 251,000 | 0.11 |
| Bufotenin | 35,500 | 1860 | 19 |
| Serotonin | 178,000 | 3160 | 56 |

[a]Naloxone, tubocurarine, alprenolol, flunitrazepam, mepyramine, GABA, prazosin, clonidine, citalopram >10,000.

ical series. Furthermore, an excellent correlation ($r$ = .94) was also found between the $IC_{50}$ values obtained in membrane and solubilized preparations (Laduron, 1980).

For the dopamine receptor, the regional distribution appears to be a quite important criterion of receptor identity, since certain brain regions such as the cerebellum are practically devoid of receptor sites and thus can be used as blank tissue.

When the first four criteria listed in Table I are fulfilled, one can be practically certain that the binding site is relevant physiologically, which is not the case for the last four (high affinity, saturability, reversibility, and stereospecificity). Although these latter criteria are required, they are far from being decisive. They will certainly be fulfilled if the first ones are established. This is the reason we now present these criteria of receptor specificity in another order than we did previously (Laduron and Ilien, 1982). This gives more emphasis to what we consider to be the most important criteria: drug displacement, correlations be-

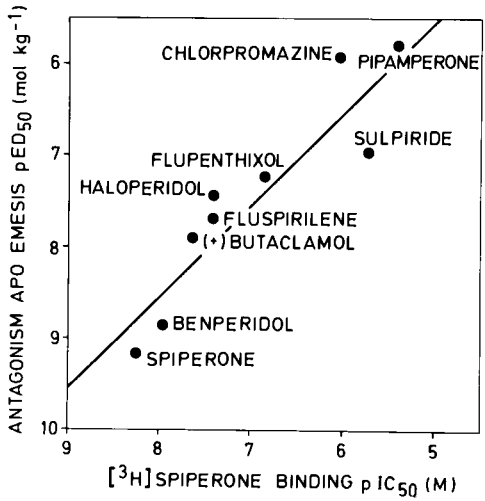


**Fig. 3.** Correlation between [$^3$H]spiperone binding in solubilized preparations from dog striatum and antagonism of apomorphine-induced emesis in the dog.

tween pharmacological activities and binding in membrane preparations, and regional distribution.

## V. CRITERIA OF SOLUBILIZATION

In addition to the criteria of receptor specificity, one must establish that the dopamine receptor is really solubilized. Here the criteria are mostly operational, which means that they are essentially based on the use of biochemical and morphological techniques. These solubilization criteria are listed in Table III. For the dopamine receptor solubilized by means of digitonin, all these criteria have been fulfilled (Gorissen and Laduron, 1979; Gorissen *et al.*, 1979, 1980).

The first criterion needs some comments. The lack of sedimentation may represent an important, if not decisive, test to assess that a receptor has been really solubilized. This is true only if the medium that contains the receptor sites has a lower density than the membranes themselves. If high salt concentrations are used as solubilizing agents, membrane elements are not spun down and remain floating. This problem has been discussed in detail elsewhere (Laduron and Ilien, 1982). Clement-Cormier and Kendrick (1980) claimed to have solubilized dopamine receptors using a high salt concentration (KCl 50%). If this was true, it should mean that the dopamine receptor is an extrinsic receptor. However, it is now well established that high concentrations of KCl cannot

TABLE III

Operational Criteria to Assess Receptor Solubilization

1. Lack of sedimentation in low-density media (100,000 *g*, 60 min)
2. Lower sedimentation coefficient than membranes
3. No retention on small pore size filters (millipore 0.22 μm)
4. Higher retention on gel filtration than membranes
5. Disappearance of lamellar membrane structure
6. Decrease of thermostability

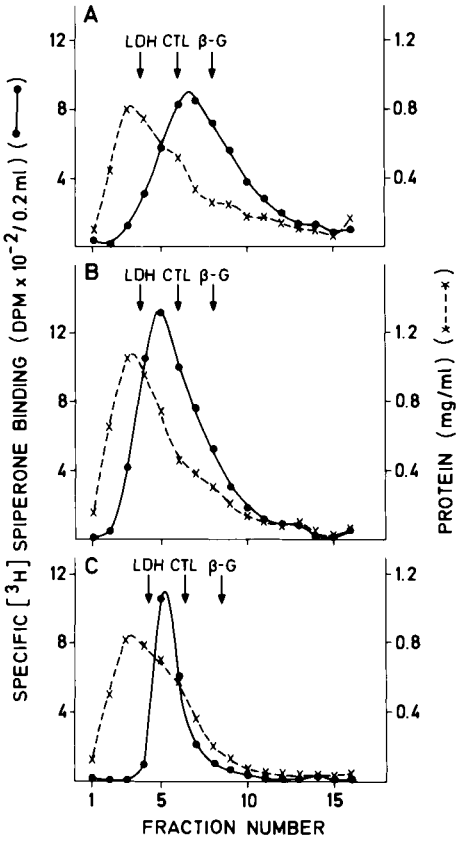


**Fig. 4.** Sedimentation profile of dopamine receptors from dog striatum solubilized with 0.25% lysolecithin (A and B) and with 1% digitonin (C). One ml of soluble extract was layered on a 11.3 ml sucrose gradient (10–30%) buffered with 10 m*M* phosphate buffer (pH 7.2) containing 1 m*M* EDTA and centrifuged at 35,000 rpm for 17 hr at 2°C (SW-40 Ti Spinco Rotor). In (B), 0.4 *M* KCl was added in the gradient. After the run, [$^3$H]spiperone binding was performed in the different fractions using the charcoal method (Gorissen *et al.*, 1980). Marker enzymes: LDH, lactate dehydrogenase; CTL, catalase; β-G, β-galactosidase.

solubilize dopamine receptors; the use of this procedure only allows selection of membrane-bound dopamine receptors of low density. In fact, the excessively high density of the medium did not allow the sedimentation criterion to be validated.

In my opinion, the criterion of sedimentation in sucrose gradients represents probably the most severe test of receptor solubilization because it allows one to compare the sedimentation coefficient of membrane-bound and solubilized receptors, the latter having a much lower sedimentation coefficient. Moreover, this technique gives an idea of the molecular weight of the receptor. However, this must be interpreted with caution; indeed, Fig. 4 shows that the sedimentation coefficient is higher (12.6 S) when the dopamine receptor is solubilized with lysolecithin than with digitonin (9 S). However, it is noteworthy that the addition of 0.4 *M* KCl in the sucrose gradient markedly reduces the Svedberg coefficient to an approximate value of 9 S when dopamine receptors are solubilized with lysolecithin but not with digitonin. This clearly indicates that the obtained S value is dependent on the nature of the detergent, which presumably determines micellar complexes of different nature. It is not impossible that the use of other detergents will give rise to sedimentation coefficients still lower than 9 S, as was demonstrated for the muscarinic receptor (cf. Laduron and Ilien, 1982).

All the other solubilization criteria listed in Table III are also fulfilled for soluble dopamine receptors: no retention on millipore filters, absence of lamellar membrane in electron microscopy, and higher thermolability.

Thus, the spiperone binding site solubilized by digitonin treatment not only retains the characteristics of a dopamine receptor but also was really obtained in a molecularly dispersed form. The dopamine receptor may be considered as an intrinsic protein.

## REFERENCES

Bansal, S., Woolf, P. D., Fischer, J. A., and Caro, J. F. (1982). *J. Clin. Endocrinol. Metab.* **54,** 651–652.

Brown, E. M., Carroll, R. J., and Aurbach, G. D. (1977). *Proc. Natl. Acad. Sci. U.S.A.* **74,** 4210–4213.

Chan, B., Madras, B. K., Davis, A., and Seeman, P. (1981). *Eur. J. Pharmacol.* **74,** 53–59.

Clement-Cormier, Y. C., and Kendrick, P. E. (1980). *Biochem. Pharmacol.* **29,** 897–903.

Davis, A., Madras, B., and Seeman, P. (1981). *Eur. J. Pharmacol.* **70,** 321–329.

Gorissen, H., and Laduron, P. M. (1978). *Life Sci.* **23,** 575–580.

Gorissen, H., and Laduron, P. (1979). *Nature (London)* **279,** 72–74.

Gorissen, H., Aerts, G., and Laduron, P. (1979). *FEBS Lett.* **100,** 281–285.

Gorissen, H., Ilien, B., Aerts, G., and Laduron, P. (1980). *FEBS Lett.* **121,** 133–138.

Howlett, D. R., Morris, H., and Nahorski, S. R. (1979). *Mol. Pharmacol.* **15,** 506–514.

Ilien, B., Gorissen, H., and Laduron, P. (1980). *Biochem. Pharmacol.* **29,** 3341–3344.

Ilien, B., Gorissen, H., and Laduron, P. (1982). *Mol. Pharmacol.*, **22,** 243–249.

Kebabian, J. W., and Calne, D. B. (1979). *Nature (London)* **277,** 93–96.

Laduron, P. M. (1980). *Trends Pharmacol. Sci.* **1,** 471–474.
Laduron, P. M. (1981). In "Apomorphine and Other Dopaminomimetics" (G. L. Gessa, and G. U. Corsini, eds.), Vol. 1, pp. 95–103. Raven Press, New York.
Laduron, P. M. (1982). *Adv. Biosci.* **37,** 71–82.
Laduron, P. M., and Ilien, B. (1982). *Biochem. Pharmacol.* **31,** 2145–2152.
Laduron, P. M., Janssen, P. F. M., and Leysen, J. E. (1978). *Biochem. Pharmacol.* **27,** 323–328.
Langley, J. N. (1906). *Proc. Soc. London, Ser. B* **78,** 170–194.
Lew, J. Y., Fong, J. C., and Goldstein, M. (1981). *Eur. J. Pharmacol.* **72,** 403–405.
Leysen, J. E., Niemegeers, C. J. E., Tollenaere, J. P., and Laduron, P. M. (1978). *Nature (London)* **272,** 168–171.
Niemegeers, C. J. E., and Janssen, P. A. J. (1979). *Life Sci.* **24,** 2201–2216.
Seeman, P. (1980). *Pharmacol. Rev.* **32,** 229–313.
Tam, S., and Seeman, P. (1978). *Eur. J. Pharmacol.* **52,** 151–152.
Withy, R. M., Wheatly, M., Frankham, P. A., and Strange, P. D. (1981). *Biochem. Soc. Trans.* **9,** 416.

# *Chapter 8*

# *AUTORADIOGRAPHIC DEMONSTRATION OF RECEPTOR DISTRIBUTIONS*

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## I. INTRODUCTION

The pharmacological and behavioral effects of all neurotransmitters and most drugs originate with the binding of these substances (ligands) to specific receptor sites embedded in the cell surface membrane. The union of ligand and receptor initiates a particular series of events, dictated by the type of cell (e.g., nerve cell, muscle cell) in which the receptor is found and by the cell's location (e.g., in the wall of the intestine, in a pain pathway, in cerebral cortex). Once a certain receptor has been demonstrated to exist, we proceed to determine *where* it exists. The autoradiographic localization of specific neuronal receptor sites advances


ISBN 0-12-470350-X

our understanding of brain function by showing the sites of action of a variety of drugs and neurotransmitters.

Early studies demonstrated the existence of opiate receptors in nervous tissue (Pert and Snyder, 1973; Simon *et al.*, 1973; Terenius, 1973). Numerous studies since have demonstrated the specific binding to unique brain receptors of many other major classes of drugs, neurotransmitters, neuroactive peptides, and hormones (e.g., Snyder, 1978). Kinetic criteria and the use of ligand analogs in tests of structure–activity relationships have confirmed the extrapolative value of binding in the test tube; the binding of ligands to brain homogenates duplicates the *in vivo* binding of substances, identical to or related to the ligand, which produce behavioral effects in animals.

The first attempts to localize the brain sites of action of certain drugs were modeled after earlier methods used to visualize steroid receptors (Pfaff, 1968; Stumpf, 1968). In studies of cholinergic receptors (Kuhar and Yamamura, 1975) and opiate receptors (Pert *et al.*, 1976b), radiolabeled ligands were injected *in vivo* and allowed to circulate just long enough to maximize the proportion of ligand discriminately bound to its receptor. Animals were sacrificed by decapitation. Their brains were quickly removed and immediately frozen, then cryostat-cut into thin sections for autoradiography.

Efforts to avoid the diffusion of reversibly (i.e., noncovalently) bound ligands account for much of the gradual technical evolution in receptor binding autoradiography (Stumpf, 1976). The use of reversibly bound ligands in the *in vivo* binding method described above precluded the use of aqueous media in subsequent autoradiographic procedures. Dry autoradiography was accomplished in the darkroom, by attaching sections to slides previously coated with nuclear track emulsion (Stumpf, 1976; Rogers, 1979). Alternatively, thin layers of dry or nearly dry emulsion could be applied to labeled tissue sections by the *loop* technique (Caro and Van Tubergen, 1962) or by the *flat substrate* method (Salpeter and McHenry, 1973).

*In vivo* binding provided a promising first glimpse of receptor distributions. Tests of binding saturability and stereospecificity, appropriate pharmacology of blocking agents, and a mode of drug delivery identical to that used in behavioral studies validated the technique. Though *in vivo* binding established the possibility of autoradiographic receptor localization, it has certain disadvantages. Large quantities of radiolabeled ligand are required to ensure that a sufficient number of receptors are marked for visualization. Unfortunately, much unbound and nonspecifically bound ligand remains in the tissue and cannot be washed out, resulting in artifactual or high background labeling. The selection of ligands appropriate for *in vivo* binding is limited to those which cross the blood–brain barrier and form high-affinity bonds with receptors. Furthermore, after systemic injection, the ligand may be metabolized to an active form other than that intended for study, or degraded to an inactive form, rendering binding results invalid or unobtainable.

An early study of nicotinic cholinergic receptor localization used an irreversibly bound ligand in an innovative binding technique (Polz-Tejera *et al.*, 1975). In an *in vitro* method conceptually similar to that used on homogenates in test tubes, radiolabeled ligand was introduced to tissue after sacrifice. "Virgin" brains from animals given no prior drug were removed fresh, then frozen and cryostat-cut. Sections were attached to gelatin-coated slides and incubated in a solution containing [$^{125}$I]α-bungarotoxin, a substance that does not cross the blood–brain barrier. Radiolabeled sections were washed after incubation to remove nonspecifically bound label, then fixed in ethanol and dried. Slides were dipped in liquid nuclear track emulsion (Kodak NTB), according to standard wet autoradiography procedures developed for localization of intra-axonally transported marker proteins (Cowan *et al.*, 1972).

The *in vitro* binding of ligand to slide-mounted sections prevented the use of slides precoated with emulsion for the dry autoradiography of diffusible and nondiffusible ligands. Since wet autoradiography had been made possible by the use of a nondiffusible ligand, the early *in vitro* technique was severely limited. However, the *in vitro* approach to receptor binding and autoradiographic localization had many advantages that were not appreciated until 4 years after its conception. In 1979, Young and Kuhar (1979a) refined the *in vitro* method, adapting it for use with diffusible as well as nondiffusible substances. In a variation on dry autoradiographic technique, they placed predried, emulsion-coated coverslips over slide-mounted tissue sections that had been incubated in opiate ligands, washed, and dried. This method revealed a distribution of opiate receptors identical to that found in earlier *in vivo* studies.

Tritium-sensitive film may be used in place of emulsion-coated coverslips as a dry emulsion medium. Series of incubated slides are arranged in X-ray cassettes and covered with film to produce autoradiographs. Receptor distributions represented on these film images are easily quantified by microdensitometry (Palacios *et al.*, 1981e; Penney *et al.*, 1981; Biegon *et al.*, 1982; Rainbow *et al.*, 1982b). Regional receptor densities can be evaluated with computer assistance (Quirion *et al.*, 1981) or by comparison with autoradiographic standards of known radioactivity and protein content (Unnerstall *et al.*, 1982).

A wet *in vitro* autoradiographic method, developed by Herkenham and Pert (1980, 1982) to be used with reversibly bound ligands, preserves tissue quality and produces high-resolution images. Slide-mounted sections are incubated, washed, dried, and fixed with hot formaldehyde vapors, which preserve tissue quality and prevent the diffusion of a number of reversibly bound ligands in aqueous solutions. Fixed sections are *defatted* and then dipped in liquid nuclear track emulsion by standard wet autoradiographic procedures.

Following *in vitro* binding, either wet or dry autoradiographic procedures may be used to localize the label. The advantages of the *in vitro* binding and autoradiography methods have been presented before (Young and Kuhar, 1979a; Herkenham and Pert, 1982), and are discussed briefly below.

1. *In vitro* binding is suitable for the study of human brain tissue.
2. Adjacent tissue sections may be incubated in different ligands to permit the comparative study of several receptor types in a single brain. They may be given individualized treatment to permit study of the concordance between receptor patterns, features of brain morphology (e.g., cells, fibers, and enzymes), and/or afferent and efferent connections marked by intra-axonally transported substances.
3. Substances that are rapidly metabolized *in vivo* (e.g., peptides) or do not ordinarily cross the blood–brain barrier may be used as ligands.
4. It is possible to perform biochemical and pharmacological manipulations to selectively alter binding conditions; content (e.g., of ions, allosteric effectors, ligand concentration, competitive ligand analogs), duration, and temperature of incubating bath may be varied.
5. Sections may be washed to reduce artifactual and nonspecific binding.
6. Identically prepared sections are used for both the characterization of kinetics and pharmacology and for receptor localization by autoradiography.

A wide variety of receptors and binding sites have been localized by autoradiography. In many cases, a variety of ligands have been used to label a particular receptor. Table I summarizes much of the receptor autoradiography performed to date. *In vivo* studies are indicated with a superscript a.

TABLE I

Partial List of Autoradiographic Studies Localizing Binding of Ligands to Receptors or Binding Sites in Tissue Membranes

| Receptor/binding site | Ligand | References |
|---|---|---|
| Adenosine | [$^3$H]CHA | Lewis *et al.*, 1981; Goodman and Snyder, 1982a; Goodman *et al.*, 1983; Lee *et al.*, 1983 |
| | [$^3$H]NECA | Buckley and Burnstock, 1983 |
| Adrenergic | | |
| $\alpha_1$ | [$^3$H]Prazosin | Dashwood, 1983 |
| $\alpha_1$ and $\alpha_2$ | [$^3$H]WB4101 and *p*-[$^3$H]Amino-clonidine | Young and Kuhar, 1980a |
| $\beta$ | [$^3$H]Dihydroalprenolol | Palacios and Kuhar, 1980a; Barnes *et al.*, 1982 |
| Angiotensin | [$^{125}$I]Ile$^5$-angiotensin II | van Houten *et al.*, 1980 |
| Benzodiazepine | [$^3$H]Flunitrazepam | Young and Kuhar, 1979b, 1980b, Hösli *et al.*, 1980; Palacios and Kuhar, 1981b; Young *et al.*, 1981; Palacios *et al.*, 1981b; |

TABLE I (*Continued*)

| Receptor/binding site | Ligand | References |
|---|---|---|
| | | Unnerstall *et al.*, 1981, 1982; Schlumpf *et al.*, 1983 |
| | [$^3$H]Ro 15-1788 | Schlumpf *et al.*, 1983 |
| Calcitonin | [$^{125}$I]Calcitonin | Olgiati *et al.*, 1983 |
| Calcium channel | [$^3$H]Nitrendipine | Murphy *et al.*, 1982; Quirion, 1983 |
| Cholecystokinin | [$^{125}$I]CCK-33 | Zarbin *et al.*, 1983 |
| Cholinergic | | |
| muscarinic | [$^3$H]PrBCM | Rotter *et al.*, 1979a,b; Kuhar *et al.*, 1981 |
| | [$^3$H]QNB | Yamamura *et al.*, 1974[a]; Kuhar and Yamamura, 1975[a]; Hartzell, 1980; Unnerstall *et al.*, 1982; Barnes *et al.*, 1983 |
| | [$^3$H]NMS, [$^3$H]NMS + carbachol | Wamsley *et al.*, 1980; Palacios, 1982 |
| nicotinic | [$^{125}$]α-BTX | Polz-Tejera *et al.*, 1975; Hunt and Schmidt, 1978; Arimatsu *et al.*, 1978, 1981 |
| Dopamine | [$^3$H]Spiperone | Kuhar *et al.*, 1978[a]; Klemm *et al.*, 1979; Murrin and Kuhar, 1979; Murrin *et al.*, 1979; Palacios *et al.*, 1981a |
| GABA | [$^3$H]GABA | Hösli *et al.*, 1980; Wilkin *et al.*, 1981 |
| | [$^3$H]Muscimol | Chan-Palay, 1978; Chan-Palay *et al.*, 1978; Hösli *et al.*, 1980; Palacios and Kuhar 1980b, 1981b; Palacios *et al.*, 1980, 1981b,c; Penney *et al.*, 1981; Unnerstall *et al.*, 1981; Pan *et al.*, 1983 |
| $GABA_B$ | [$^3$H]Baclofen | Wilkin *et al.*, 1981 |
| | [$^3$H]Bicuculline Methiodide | Hösli *et al.*, 1980 |
| Glutamate | [$^3$H]Glutamate | Greenamyre *et al.*, 1983; Halpain *et al.*, 1983 |
| | [$^3$H]AMPA | Hösli *et al.*, 1983 |
| | *d*-[$^3$H]Aspartate | Parsons and Rainbow, 1983 |
| Histamine | [$^3$H]Mepyramine | Palacios *et al.*, 1979, 1981b,d |
| Imipramine | [$^3$H]Imipramine | Biegon and Rainbow, 1983a; Fuxe *et al.*, 1983; Grabowsky *et al.*, 1983 |
| | [$^3$H]Desimipramine | Slater *et al.*, 1982 |
| | [$^3$H]Desmethylimipramine | Biegon and Rainbow, 1982, 1983b |

(*continued*)

TABLE I (*Continued*)

| Receptor/binding site | Ligand | References |
|---|---|---|
| | [$^3$H]Nitroimipramine | Rainbow and Biegon, 1983 |
| LSD | [$^3$H]LSD | Meibach *et al.*, 1980 |
| Serotonin | [$^3$H]5-HT, [$^3$H]LSD | Young and Kuhar, 1980c |
| Insulin | [$^{125}$I]Insulin | van Houten and Posner, 1979[a] |
| Kainic acid | [$^3$H]Kainic acid | Monaghan and Cotman, 1982; Unnerstall and Wamsley, 1983 |
| Neurotensin | [$^3$H]Neurotensin | Minkovic *et al.*, 1981; Palacios and Kuhar, 1981a; Young and Kuhar, 1981; Quirion *et al.*, 1982a,b |
| Opiates | | |
| μ subtype | | |
| alkaloids | ($^3$H-Labeled dihydromorphine, diprenorphine, etorphine, naloxone) | Pert *et al.*, 1975[a], 1976b[a]; Atweh and Kuhar, 1977a[a],b[a];c[a]; Atweh *et al.*, 1978; Young and Kuhar, 1979a; Herkenham and Pert, 1980, 1981, 1982; Meibach and Maayani, 1980; Pearson *et al.*, 1980; Young *et al.*, 1980; Bowen *et al.*, 1981; Duka *et al.*, 1981; Minkovic *et al.*, 1981; Wamsley *et al.*, 1981b; Hammer *et al.*, 1982; Kent *et al.*, 1982; Lewis *et al.*, 1982, 1983; Moon Edley *et al.*, 1982; Wamsley *et al.*, 1982[a]; Wise and Herkenham, 1982; Geary and Wooten, 1983; Herkenham *et al.*, 1984; Unnerstall *et al.*, 1983 |
| peptides | [$^{125}$I]FK33-824 enkephalin | Beaudet *et al.*, 1979; Goodman *et al.*, 1980; Young *et al.*, 1980 |
| δ subtype | | |
| peptides | D-[$^3$H]Ala-DLeu-enkephalin | Bowen *et al.*, 1981; Duka *et al.*, 1981; Lewis *et al.*, 1983 |
| | D-[$^{125}$I]Ala-DLeu-enkephalin | Goodman *et al.*, 1980 |
| κ subtype | [$^3$H]EKC, [$^3$H]Bremazocine | Goodman and Snyder, 1982b |
| | [$^3$H]EKC | Quirion *et al.*, 1983a |
| Phencyclidine | [$^3$H]PCP | Quirion *et al.*, 1981; Hammer *et al.*, 1982 |
| Substance P | [$^3$H]Substance P | Quirion *et al.*, 1983b |
| Vasopressin | [$^3$H] [Arg$^8$]Vasopressin | Baskin *et al.*, 1983 |

[a]*In vivo* studies.

## II. METHODS FOR *IN VITRO* BINDING AND AUTORADIOGRAPHY

### A. The Importance of Clean Glassware

The exceptional cleanliness necessary for successful autoradiography cannot be overemphasized; a prefatory note is in order. It is advisable to reserve a complete set of clean glassware for use in autoradiography only; contaminants carried over into liquid emulsion can be disastrous. Tissue sections should be mounted only on specially prepared slides. Even precleaned microscope slides might not be clean enough for use in the preparation of sections for emulsion autoradiography. Small dirt particles on slides can interfere with receptor visualization directly (by appearing as bright spots under darkfield illumination) or indirectly (by contaminating liquid emulsion and causing elevation of background labeling). Furthermore, sections tend to fall off grimy slides during incubation. Since optimal adherence of the sections is crucial to all phases of this procedure, each slide should be rigorously cleaned in detergent, alcohol, and distilled water and then gelatin-coated with a *subbing* solution (Hendrickson and Edwards, 1978; Rogers, 1979). Glassware used in the incubation process should also be rigorously cleaned, because hydrophobic ligands "stick" to glass surfaces.

### B. Preparation of Tissue

#### *1. Brain Removal and Freezing*

Animals are sacrificed by decapitation. Small animals are not anesthetized; larger animals are anesthetized, after anesthetic effects on the receptor in question have been determined. Most membrane-bound receptors are stable enough at room temperature to allow time for careful removal of the brain. The head should be submerged in ice-cold isotonic saline several times during this procedure. After removal, small brains are frozen by immersion in isopentane at $-30°C$. This freezing method preserves the shape of the brain; however, care must be taken to transfer the specimen from isopentane to crushed dry ice within 30 sec, to avoid expansion and/or cracking. Larger brains are immersed in ice-cold saline for 5 to 10 min, briefly blotted dry, and then buried in sifted dry ice, in a hollow made to preserve brain shape. When thoroughly frozen, large brains can be blocked to manageable size with a band saw. Blocked or whole brains are secured to cryostat pedestals with embedding matrix (Lipshaw M-1 has the same consistency as frozen brain), and stored in air-tight bags at $-15°C$ or colder. Brains can be preserved at this temperature for a year or longer, though it is likely that some receptors may perish over long periods.

### 2. *Cutting of Sections*

The following procedure has been determined to produce reliable adherence of sections to slides and to preserve tissue morphology. A frozen brain is equilibrated to cryostat temperature, set according to desired section thickness. Sections 25 μm thick are most easily cut at −14 to −16°C; thinner sections can be cut at lower temperatures (Rogers, 1979), although tissue damage might occur at temperatures below −20°C. A small camel hair brush can be used to gently move sections from the microtome blade to cold, gelatin-coated slides; in this manner, several sections can be placed on a single slide and positioned at leisure. Alternatively, a cold slide can be pressed firmly onto a section on the microtome blade. In either case, sections are secured to slides by melting; a finger or warm metal bar moved slowly across the back of a slide produces a controlled wave of heat, preventing the entrapment of air bubbles between tissue and slide. Since refreezing must be avoided at this stage, slides are transferred to a slide box maintained at approximately 0°C. The box is covered to keep sections from gathering condensation.

When a box has been filled with slides, it is transferred to a cold (0 to −10°C), humidity-free container, in which sections dry without freezing. Either a sealed desiccator jar kept in an ice-salt mixture or freezer or a drying box lined with desiccant and kept in a closed cryostat may be used for this purpose. Drying time is about 2 hr and may be accelerated by reduced pressure created with a vacuum pump. In any case sections must be kept out of air currents to prevent freezing.

Sections that have dried properly, at or below 0°C and without freezing, have a glassy, transparent appearance and should adhere to slides even through incubation at physiological temperatures. Sections that have frozen while drying are frosty white. A translucent appearance indicates that sections were too warm while drying. In either instance the sections might fall off the slides during subsequent procedures. After drying, slide boxes are covered and sealed in bags containing desiccant and can be stored indefinitely in a freezer at −15°C or colder. Prior to incubation, slides are transferred from boxes to stainless steel racks (Lipshaw) in the cryostat to prevent water condensation. The racks may be placed in bags, removed from the cryostat to warm up, and then placed in the first solution.

## C. Incubation and Validation of Specific Binding

### 1. *Incubation*

Since nonspecific binding increases linearly as a function of radiolabeled ligand concentration, while specific binding increases asymptotically as recep-

tors are saturated, a fairly low (below $K_d$) tritiated ligand concentration usually yields the highest possible ratio of total-to-nonspecific binding. However, binding at low concentrations of [$^3$H]ligand requires a long exposure time for autoradiographic visualization, even if the specific activity of the ligand is high. When $^{125}$I-labeled ligands are used, binding at very low ligand concentrations can be successful and may be necessary for subsequent visualization. Low ligand concentrations are useful for the selective labeling of the higher-affinity site of two receptor subtypes that cannot be differentiated by other means.

Binding can be profoundly influenced by the presence of various allosteric effectors in the incubating solution. For example, chloride ions have been demonstrated to enhance [$^3$H]diazepam binding (e.g., Costa *et al.*, 1979), and sodium ions to promote [$^3$H]naloxone binding (Pert *et al.*, 1973). GTP and divalent cations were used by Bowen *et al.* (1981) to alter the binding selectivity of D-[$^3$H]-Ala-DLeu-enkephalin.

High concentrations of unlabeled ligand can be used to assess the ratio of total to nonspecific binding. Competitive ligand is added to the incubating solution in the smallest concentration that will inhibit a maximal amount of radiolabeled ligand binding (this amount is experimentally determined, using scintillation spectophotometry as a measure of displacement). Enantiomers with drastically different pharmacological potencies (e.g., levorphanol/dextrorphan or levallorphenol/dextrallorphan) may be used in an elegant demonstration of the stereospecificity of binding (Goldstein *et al.*, 1971). In our experience at least 50% specific binding is necessary for adequate receptor visualization.

Incubation temperature is a critical variable. Many of the ions which can be used as allosteric effectors in the incubating solution function as such only at certain temperatures. The chloride and sodium effects mentioned above, for example, occur only at low temperatures; the binding of opiate receptor alkaloid agonists, in contrast, is optimal at physiological temperatures (Creese and Snyder, 1975). Incubation and subsequent washing should take place in neutral buffered solutions. Isotonicity helps to maintain tissue quality and can be achieved with the addition of sucrose if NaCl cannot be used. Bovine serum albumin is added to keep hydrophobic ligands in solution. Protease inhibitors may be used to preserve the structures of peptides subject to degradation by tissue proteases.

The beneficial effects of preincubation in buffer, which can double the amount of specific binding in subsequent incubation, are often attributed to the removal of competing endogenous ligands from the tissue in brain homogenates (Pasternak *et al.*, 1975). Furthermore, preincubation in various ionic media can couple or uncouple receptors and other membrane components and thus stabilize ligand selectivity patterns in the form most desirable for the study of the binding of a particular tritiated ligand to a receptor subtype (Bowen *et al.*, 1981). Optimal preincubation conditions should be determined empirically by varying tem-

perature and ionic content of solution, as well as length of preincubation, in a systematic manner.

*2. Washing at the Termination of Incubation*

High-affinity specific receptor binding generally dissociates at a temperature-dependent rate. By contrast, nonspecific binding is not temperature-dependent and is most effectively eliminated by the largest possible number of successive immersions into ice-cold (nonradioactive) buffered washes in the smallest amount of time. Generally, we have found that three to six 400-ml washes are sufficient to minimize nonspecific binding. Sodium is included in the washing medium if it does not promote the dissociation of the receptor–ligand complex. The duration of washing must be empirically determined to optimize the amount of specific binding retained. Total wash time may vary from 1 to 60 min, depending on receptor affinity and dissociation rate of the ligand used.

*3. Drying at the Termination of Washing*

Incubated and washed sections should be dried as quickly as possible to prevent the diffusion of reversibly bound ligand. A hand-held hair dryer or air hose can be used to blow room temperature air over slides at a strength sufficient to remove most water within a few seconds and complete the drying process within a few minutes. Presumably, evaporation keeps the tissue cold.

*4. Scintillation Counting*

The amount of radiolabeled ligand remaining in tissue sections after incubation, washing, and drying can be determined with a scintillation counter. Slides are scored and broken; section-bearing fragments are placed in scintillation vials and, after the addition of Protosol and/or 10 ml of detergent fluor and vigorous agitation, counted by scintillation spectrophotometry. The scintillation counter is also used to establish relative potencies among unlabeled displacers. Though kinetic studies are useful in validating receptor binding (e.g., Meibach *et al.*, 1980; Palacios *et al.*, 1981a; Penney *et al.*, 1981; Quirion *et al.*, 1981; Wamsley *et al.*, 1980, 1981a; Young and Kuhar, 1979a, 1980b), examination of relative displacement potencies among a series of ligand analogs provides more compelling evidence for receptor binding specificity (Pert and Herkenham, 1981). There is often a high degree of correlation between the ability of a series of analogs to inhibit the binding of the radiolabeled ligand and their ability to produce particular behavioral or physiological effects (Table II). The binding of a ligand to nonbiological substances such as talc can mimic receptor binding if kinetics alone are considered (Hollenberg and Cuatrecacas, 1976); it does not appear, however, that such substances can mimic receptors in tests involving correlation

TABLE II

Comparison of Ligand Selectivity Patterns for Opiate Receptors from Different Sources[a]

| Opiate | $ID_{50}$ of [$^3$H]naloxone displacement from slide-mounted brain slices[b] (n*M*) | $ED_{50}$ of suppression of muscular contraction in guinea pig ileum[d] (n*M*) | $ID_{50}$ of [$^3$H]naloxone displacement from brain membrane[e] (n*M*) |
|---|---|---|---|
| Etorphine | 0.2 | 0.25 | 0.6 |
| Levallorphan | 3.0 | 2.9 | 2 |
| Levorphanol | 10 | 30 | 15 |
| Methadone | 100 | 80 | 200 |
| D($Ala_2$)-Met-enkephalin | 500 | 600[c] | 400[g] |
| Morphine | 100 | 200 | 100 |
| Propoxyphene (Darvon) | 1,000 | 2,000 | 12,000 |
| Codeine | 10,000 | 15,000 | 10,000 |
| Dextrallorphan | 10,000 | 2,800 | 7,000[h] |
| Dextrorphanol | 10,000 | 8,500 | 8,000[h] |

[a]For the ten analogs of morphine tested for binding potency on brain slices prepared as in Methods, values are directly proportional and nearly perfectly correlated[c] with both pharmacological activity in the guinea pig ileum[d] and binding potencies in brain homogenates[e]. It has previously been shown that the correlation between pharmacological activity and binding in the guinea pig intestine is excellent ($p < .001$)[d] and that the ligand selectivity pattern of the intestinal receptor is indistinguishable ($p < .005$) from displacement of [$^3$H]naloxone from brain membranes and ability to produce "analgesia" in the mouse hot plate test (Wilson *et al.*, 1975).

[b]Herkenham and Pert (1980).

[c]The data were logged to achieve a more normal distribution; "y on x" and "x on y" regression analyses were performed comparing binding on slides with intestinal pharmacological potency and binding on slides with binding to homogenates. None of the values were significantly different from 1 ($p < .01$); the structural regression slopes (Munson and Rodbard, 1981) were 1.05 from slices versus intestinal potency and .98 for slices versus homogenates, and $r = .985$ for the intestinal comparison and .97 for the homogenate comparison.

[d]Creese and Snyder (1975).

[e]Pert and Garland (1978).

[f]Lord *et al.* (1977).

[g]Pert *et al.* (1976b).

[h]Pert and Snyder (1973).

between the relative potencies of ligands and their behavioral and physiological effects.

## D. Autoradiography

### *1. Tritium-Sensitive Film Autoradiography*

Incubated slides are arranged in standard X-ray cassettes (we use Wolf) and covered in total darkness with sheets of tritium-sensitive film ($^3$H-Ultrofilm,

LKB, Gaithersburg, Maryland). Cassettes are kept at room temperature for 2 to 20 weeks when using $^{3}$H-ligands, and for 1 to 20 days when using $^{125}$I-labeled ligands. Film can be developed under very dim safelight conditions with Kodak D-19 Developer (5 min at 20°C). The apposition of film does not alter tissue in any way; after film has been developed, sections may be used for subsequent film exposures, further processed, or dipped in liquid emulsion for wet autoradiography.

### 2. *Liquid Emulsion Autoradiography*

*a. Fixation.* Slides to be coated with liquid emulsion must undergo a fixation procedure to keep the ligand–receptor complex intact. Liquid vapors or immersion in liquid fixatives can be effective (Chan-Palay, 1978; Duka *et al.*, 1981; Hamel and Beaudet, 1982) but may cause chemography (MH, unpublished). Vapors from paraformaldehyde powder heated to 80°C are an extremely effective fixative and cause no chemography. When incubated sections are thoroughly dry, slide racks are placed in a desiccator jar over a Petri dish containing 10–30 g of paraformaldehyde powder that has equilibrated to room humidity (40–60%) for at least 2 days. A vacuum pump reduces pressure to maintain a seal between jar and lid, and the jar is heated in an oven at 80°C for 2 hr. Under these conditions, formaldehyde vapors fix sections without condensing on them or altering their appearance. The jar should be opened under a hood, and racks must be removed immediately to prevent the cooling vapor from condensing on slides. The powder can be reused indefinitely. The sections are left in a drafted hood overnight to rid them of residual vapors. Though fixation is not necessary for autoradiography with tritium-sensitive film, it helps to maintain tissue quality for subsequent emulsion analysis and/or staining. Formaldehyde vapors produce no chemographic artifact on either film or emulsion.

Not all ligands are trapped to an equal degree by the fixation process. The chemistry of vapor fixation is mysterious, but it is possible that covalent bonds between ligands of suitable chemistry and reactive groups on receptor-vicinity proteins contribute to the fixation process (Pearse, 1980). The primary amino groups on peptides seem to be ideal sites for such bonds, suggesting the universal applicability of this method to studies of peptide receptor localization. Ligands with moieties subject to electrophilic or nucleophilic attack, such as secondary amines or ketone groups, may be fixed to varying degrees.

For ligands with less reactive moieties, vapor fixation may trap only some proportion of the bound material (Kuhar and Unnerstall, 1982). We have found almost no loss of bound [$^{3}$H]naloxone after fixing and extensive washing in aqueous media (Herkenham and Pert, 1982) but significant loss of bound [$^{3}$H]dihydromorphine (MH, unpublished). In both instances quantitative analysis of films by computer-assisted densitometry revealed no selective loss or re-

distribution of label in the autoradiographs. Since the patterns of labeling were qualitatively identical in films from sections exposed before and after aqueous washes, we conclude that washing removes specifically and nonspecifically bound ligand in equal proportions. Defatting removes from the tissue the lipids that absorb (quench) the weak tritium emissions (Alexander *et al.*, 1981) and therefore, corrects for this artifact in the autoradiograph (Herkenham and Sokoloff, 1983).

If loss of ligand is unacceptable for the purposes of a particular study, the dry autoradiographic techniques are still available. The film technique has been described above. High-resolution dry autoradiography can be achieved by apposing predried emulsion-coated coverslips to the incubated tissue sections according to the method of Young and Kuhar (1979a).

*b. Defatting.* Delipidation ensures good register between emulsion and sections, reduces quenching of β-particles from tritium emissions, improves staining characteristics, and removes any unfixed, diffusible radiolabeled ligand. We routinely defat our sections by passing slide racks through a succession of aqueous alcohol solutions increasing in strength from 70 to 100%, followed by several baths of xylene and a second succession of alcohol solutions, diminishing in strength, with two final washes in distilled water (Hendrickson and Edwards, 1978). The slides are dried in a 37°C oven overnight.

*c. Emulsion Coating.* The dipping procedure is identical to that previously described for autoradiographic tracing of axonal connections in the central nervous system (Cowan *et al.*, 1972). The dark room is brought to at least 80% humidity. Under safelight conditions, Kodak NTB-2 emulsion is melted in a water bath maintained at 38°C, then diluted 1:1 in a filtered solution of 0.1% Dreft detergent in distilled water, and stirred carefully but thoroughly. Slides are individually dipped and set vertically to dry slowly in the damp air. After 2 hr, they are placed in light-tight boxes containing desiccant capsules. Boxes are sealed and stored in a freezer at −15°C for periods of weeks to months before developing in Kodak D-19 (2 min at 17°C). Developed slides are fixed, washed, and stained with thionin to mark cell bodies.

## III. VISUALIZATION OF RECEPTOR LOCATIONS

### A. Film Autoradiographs (Figure 1)

Sections of rhesus monkey brain were prepared as described here and in Wise and Herkenham (1982) and Herkenham *et al.* (1982). Adjacent sections were incubated for 60 min at 0°C in 2.5 n*M* [$^3$H]naloxone in 50 m*M* Tris–HCl buffer,

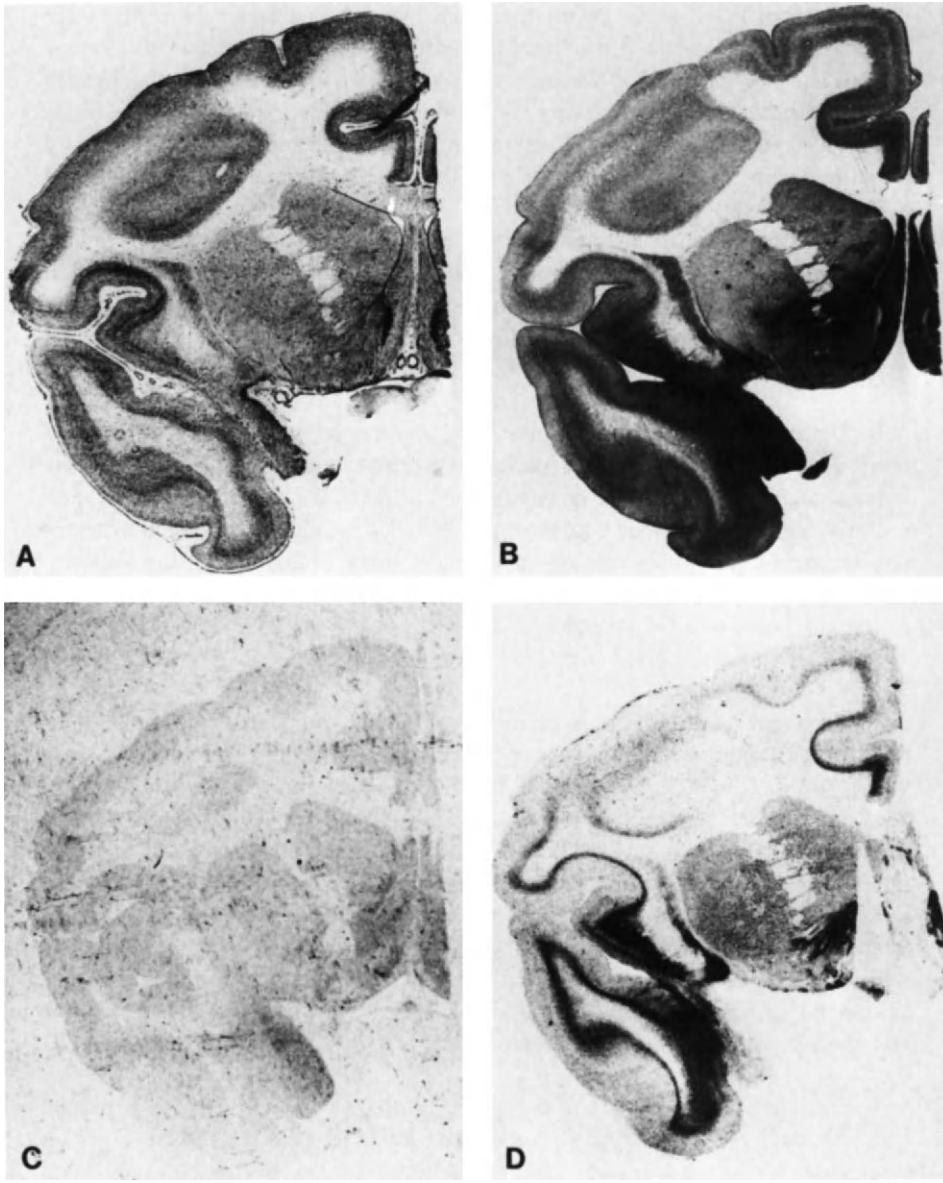


**Fig. 1.** Tritium-sensitive film autoradiography. Nissl stain (A) and film image (B) of rhesus monkey section incubated in [$^3$H]naloxone. A blank was generated (C) by incubating a section in the same [$^3$H]naloxone solution to which was added 1 $\mu M$ etorphine as a competitive inhibitor. Photograph was overexposed to make the image visible. Adjacent section (D) incubated in [$^3$H]neurotensin.

pH 7.4, plus 100 m*M* NaCl to label the opiate receptor, or in 4 n*M* [$^3$H]neurotensin in the same Tris buffer plus 1% bovine serum albumin and 0.5 mg/ml bacitracin to label the neurotensin receptor. Blanks were generated by incubating sections in the same solutions containing 1 $\mu M$ etorphine or neurotensin, respectively. The film autoradiographs can be compared to show in overview many common areas of receptor localization in the forebrain. Both opiate and neurotensin receptors show dense binding in deep cortical layers, portions of the claustral complex, and the ventromedial striatum. In contrast to widespread [$^3$H]naloxone labeling, dense [$^3$H]neurotensin binding is discretely localized to the cingulate, insular, and temporal polar cortices, the endopiriform nucleus, and the lateral edge of the nucleus accumbens. The neurotensin receptor, therefore, appears to be a more selective "limbic" marker (though the septum, a core limbic structure, is unlabeled). Opiate receptors marked by [$^3$H]naloxone are found in varying densities in all portions of monkey forebrain. Principles of laminar and regional variations in cortex have been elaborated (Wise and Herkenham, 1982). The striatal labeling shows distinct patterns, which can be correlated both with the appearance of cell clusters (Goldman-Rakic, 1982), seen in the Nissl-stained section, and with patterns of enkephalin immunoreactivity (Haber and Elde, 1982).

## B. Emulsion Autoradiographs (Figure 2)

Rat brain sections cut in the sagittal plane at the level of the striatum (Figs. 2A and B) or in the coronal plane at the level of the locus coeruleus (Figs. 2C–E) were incubated in 1 n*M* [$^3$H]naloxone (Figs. 2A, C–E) or in 1 n*M* D-[$^3$H]Ala-DLeu-enkephalin (Fig. 2B) under conditions described by Herkenham and Pert (1982) to selectively label the $\mu$ and $\delta$ opiate receptor subtypes, respectively. These sections were fixed, defatted, and dipped in emulsion. At striatal levels the choice of opiate ligand, allosteric effectors, and temperature of incubation have profound effects on patterns of receptor distribution.

The binding of [$^3$H]naloxone at brainstem levels (Figs. 2C–E) shows the degree of resolution possible with emulsion autoradiography. Figures 2C and 2D are brightfield and darkfield microphotographs of the same section, showing dense [$^3$H]naloxone binding throughout the locus coeruleus and in a thin strip of the ventrolateral portion of the parabrachial nuclei. The huge cells of the sensory nucleus of the trigeminus are unlabeled and provide a very thin zone of separation between the two labeled nuclei. Figure 2E, a high-magnification photomicrograph of this area, shows three cells of the sensory nucleus at left and the lateralmost cells of the locus coeruleus at right. The silver grains in the overlying emulsion mark the locations of bound [$^3$H]naloxone in the neuropil at right. The selective localization

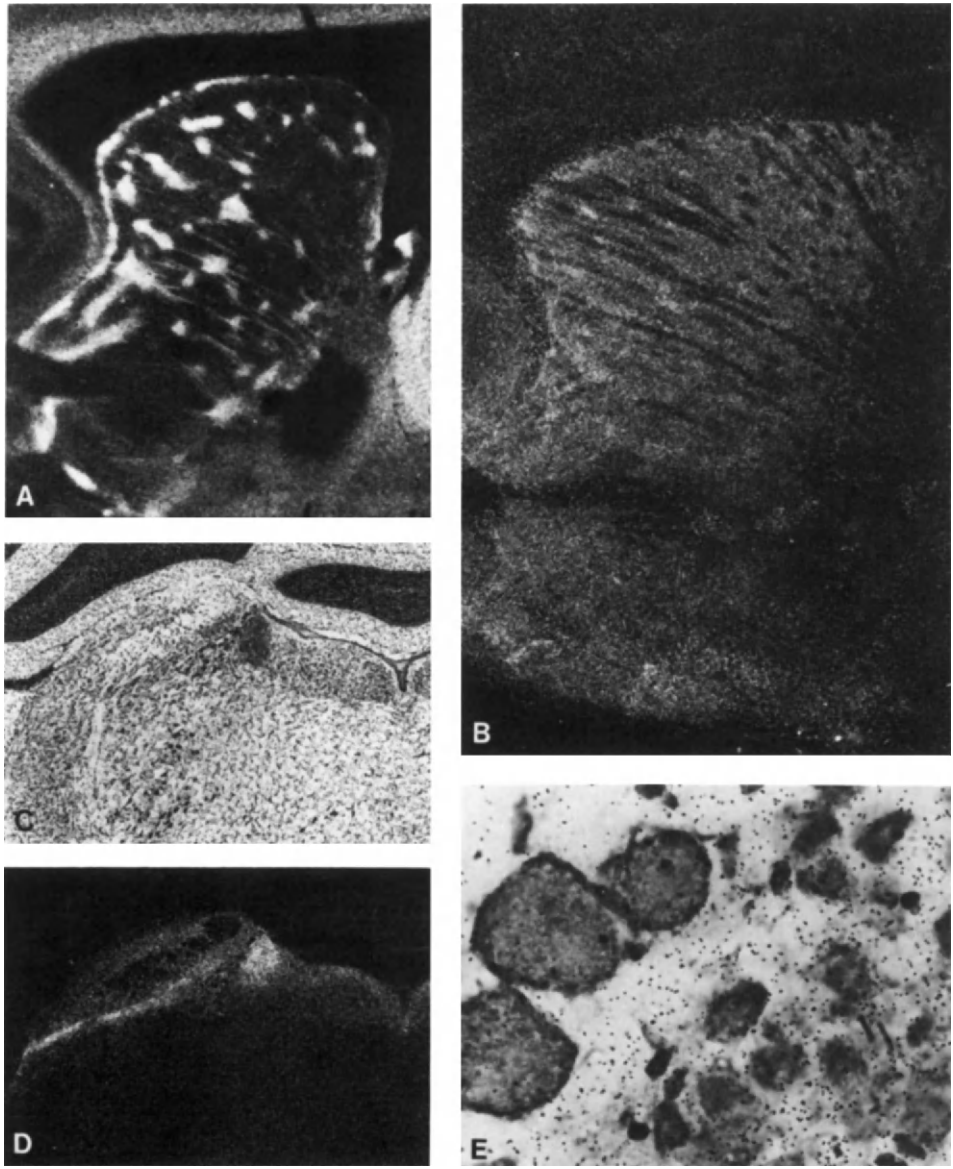


**Fig. 2.** Wet emulsion autoradiography of [$^3$H]naloxone (A,C–E) and D-[$^3$H]Ala-DLeu-enkephalin binding (B) in rat brain.

of opiate receptors to dendrites of the locus coeruleus neurons can be surmised from this labeling patten.

## IV. VARIATIONS IN AUTORADIOGRAPHIC STRATEGY

The decisions to use *in vivo* or *in vitro* receptor binding, fixed or unfixed tissue, and dry emulsion, wet emulsion, or film autoradiography to localize receptors in tissue sections will depend on a number of factors. The nature of the problem under investigation will largely determine (i) the selection and preparation of tissue, (ii) the choice of ligand, and (iii) the requirements for resolution and quantification of autoradiographic image. The advantages of *in vitro* binding have already been discussed. Following *in vitro* binding, either of two major methods of autoradiography may be employed to localize receptors. Dry and wet techniques each have certain advantages and disadvantages and are each particularly well suited to serve certain purposes.

### A. High-Resolution Autoradiography

Certain studies require localization at the limits of light microscopic resolution. For these, some form of emulsion coating is required. Perfect apposition and register and high light microscopic resolution can be achieved by dipping slides in liquid emulsion. Receptors in small tissues (e.g., Kent *et al.*, 1982) or small regions of tissue can be analyzed and compared with other markers for morphology in the same or adjacent sections (Herkenham and Pert, 1981; Herkenham *et al.*, 1983). The localization of receptors to specific parts of a cell may be achieved by high-power light microscopy. To date, receptors on cells grown in culture have been localized by techniques that involve either dipping in wet emulsion (Chan-Palay *et al.*, 1978) or apposing dry emulsion by the loop (Hösli *et al.*, 1980, 1983), flat substrate (Lane *et al.*, 1977; James and Klein, 1982), or coverslip methods (Terracio *et al.*, 1982). Similarly, localization of binding to specific cell types within a tissue (Hartzell, 1980; Barnes *et al.*, 1982; Manning *et al.*, 1982) is made possible by the emulsion technique. Emulsion applied by the loop and flat substrate techniques forms a monolayer, permitting accurate receptor quantification (e.g., Lane *et al.*, 1977) and its use at the electron microscopic level (Hendrickson, 1975).

### B. Fixation

Depending again on the problem at hand, tissues for receptor autoradiography may be processed unfixed, or they may be fixed by intravenous perfusion and/or

by fixation on the slide. In the case of *in vivo* binding, perfusion fixation can fix the tissue as well as the ligand–receptor complex if the ligand has suitable chemistry for forming covalent links with the receptor–membrane environment. This is likely to be true for proteins and peptides such as insulin (van Houten and Posner, 1979), angiotensin (van Houten *et al.*, 1980), and synthetic enkephalin (Beaudet *et al.*, 1979). This procedure lends itself to electron microscopic autoradiography, for which covalent links and excellent tissue preservation are essential.

Surprisingly, perfusion fixation does not necessarily destroy the biological activity of the receptor; thus, it permits postfixation incubations *in vitro*. Receptor localization in tissues from animals perfused with relatively high aldehyde concentrations has been demonstrated from α-bungarotoxin binding to the nicotinic cholinergic receptor (Arimatsu *et al.*, 1978, 1981) and for naloxone binding to the μ opiate receptor (Lewis *et al.*, 1982). The drawback to this approach is that not all receptors survive the fixation, and those that do must be shown not to be artifactual binding sites by comparison of the localization patterns and structure–activity relationships in fixed and unfixed tissue.

Often, perfusion with very low concentrations of aldehyde fixative is used to preserve tissue integrity during incubation (Young and Kuhar, 1979a; Wilkin *et al.*, 1981). However, if unfixed tissues are prepared as described in this chapter, tissues adhere quite well to the slide; the necessity for validating binding to fixed tissues is obviated, and subtle demonstrations of receptor maleability (Bowen *et al.*, 1981) are more likely to be successful.

Tissues may be fixed after the incubation, washing, and drying steps. This is necessary if wet autoradiography is to be used and, regardless of autoradiographic techniques employed, is recommended to preserve quality during the later stages of development and staining. Postincubation fixation will trap ligands of suitable chemistry at the receptor (Herkenham and Pert, 1982), while those that do not contain reactive groups will be washed out during aqueous processing (Kuhar and Unnerstall, 1982).

The defatting step is necessary to guarantee adherence of emulsion to sections. It also results in excellent cell staining and minimizes the differential absorption of $^3H$ emissions from gray, white, and mixed gray/white matter structures, permitting more accurate quantitative measures between structures of different lipid contents (Herkenham and Sokoloff, 1983). Thus, even when there is partial loss of ligand in washing and defatting, this procedure is recommended for quantitative studies and for achieving the highest quality autoradiographs at the light microscopic level (Herkenham and Pert, 1982). It is essential for the processing of tissues to be used at the electron microscopic level (Hamel and Beaudet, 1982).

When several different radiolabeled ligands are available for the study of a given receptor, the selection of a particular ligand for autoradiography might be

based on its degree of fixation by aldehydes. Other options include many ligands which bind irreversibly (toxins and, e.g., Rice *et al.*, 1983) and photolabile ligands such as flunitrazepam, which can be "fixed" by irradiation with UV light (Hösli *et al.*, 1980).

Dry autoradiography does not require successful fixation of the ligand to the receptor; it can be executed with predried emulsion films, emulsion-coated coverslips, or tritium-sensitive sheet film. The irregular relief of tissue sections and differential pressure applied to the emulsion layer over the section render dry media subject to problems of apposition, which may result in poor resolution or pressure artifacts, especially if tritium is used as the radiolabel (Rogers, 1979). Relatively large silver grains and high background in tritium-sensitive film compound the problem of poor resolution. The use of film entails loss of register between image and tissue; dried emulsion techniques, in contrast, guarantee good register.

## C. Quantitative Autoradiography

Of all autoradiographic media, film is perhaps neatest and easiest to use (Larsson and Ullberg, 1977). Film images can be examined by direct viewing and are particularly suitable for large tissues. Autoradiographic density bears a direct relationship to receptor density in the underlying tissue, and film images are easily quantified by microdensitometry, which can be computer-assisted (Palacios *et al.*, 1981e; Penney *et al.*, 1981; Quirion *et al.*, 1981; Rainbow *et al.*, 1982b; Unnerstall *et al.*, 1982). Quantitative autoradiography is useful as an adjunct to the assessment by liquid scintillation spectrophotometry of binding in whole sections and the evaluation of drug (Hammer *et al.*, 1982) and lesion effects (Palacios and Kuhar, 1981a; Penney *et al.*, 1981; Pan *et al.*, 1983).

The number of developed silver grains in an exposed emulsion or film is proportional within a certain range to the amount of underlying radioactivity and, hence, bound ligand (Penney *et al.*, 1981; Unnerstall *et al.*, 1982). Standards of known radioactivity and protein content can be autoradiographed with the labeled tissue to permit regional determination of fmoles ligand bound per mg protein. This is an important emerging application of autoradiography, and a few notes of caution are in order.

Commercially made plastic standards for tritium are now available in the radioactivity range useful for receptor labeling. They can also be made in the laboratory (Alexander *et al.*, 1981; Geary and Wooten, 1983). A problem with these is that radioactive decay must be taken into acount. Some researchers (Pan *et al.*, 1983) claim that $^{14}C$ standards may be substituted for $^{3}H$, but others (Lysz *et al.*, 1982) argue that the two isotopes do not covary at different exposure times. Tissue standards made from cryostat-cut pastes or homogenates may be

slightly inaccurate if they are permeated with air pockets and if protein content was determined from partially dehydrated frozen sections. Furthermore, the protein content determined for a tissue homogenate will not precisely reflect the differential protein contents of different brain structures. Standards made from whole brain homogenates do not permit discrimination between absorption of tritium emissions by gray matter and white matter (Alexander *et al.*, 1981). The normal variability of background density across a large sheet of tritium-sensitive film and among several films can pose problems in comparative quantification of images at different locations on the films. Finally, the image that is produced represents total binding; specific binding would have to be calculated by subtracting the value of the blank in each region.

### D. Other Isotopes

Though tritium is perhaps the isotope most frequently used in autoradiography, other isotopes can be used (Table I) and are better suited to certain studies. Higher-energy isotopes such as $^{14}C$ (high-energy $\beta$ emitter) and $^{125}I$ (weak-energy $\gamma$ emitter) are not differentially absorbed in different tissues. However, unlike $^{3}H$, they produce images of density proportional to section thickness; pilot studies in which section thickness was altered reveal that $^{125}I$ emissions pass through more than 50 $\mu$m of tissue (MH, unpublished). Variable image density makes standardization difficult.

Many peptides and hormones can be iodinated directly; others must be coupled to the isotope with Bolton-Hunter reagent. The principal advantage in using $^{125}I$ is the speed with which autoradiographs can be produced (in a matter of days, rather than months). An added advantage is the high specific activity of iodinated ligands (~2000 Ci/mmol), which permits selective binding to a high-affinity receptor subtype with ligand concentrations of 0.05 to 0.10 n*M* in the incubation medium. Rapid exposure times are especially valuable to pharmacologists who seek information about binding conditions before attempting a series of experiments. Neuroanatomists might wish to use iodinated ligands in double-label experiments.

Problems of light microscopic resolution in $^{125}I$ autoradiography can be minimized by using thin sections (4–15 $\mu$m thick). If ultrathin sections are cut for electron microscopy, $^{125}I$ resolution can rival that of tritium (van Houten and Posner, 1979; van Houten *et al.*, 1980).

One disadvantage in using $^{125}I$ for autoradiography is its short half-life (60 days). Another drawback is that biological characteristics of the ligand can be altered by the addition of the iodine molecule; appropriate tests of kinetics must be performed to ensure the biological similarity of labeled and unlabeled ligand. The problem of variable image density and the difficulty of preparing standards for quantitative autoradiography have been discussed above.

## V. APPLICATIONS

Receptor localization by autoradiography represents the union of traditional neuroanatomy with more recent developments in neuropharmacology. The information provided by this technique advances both fields of neuroscience—neuroanatomists can see new forms of organization in the brain, and neuropharmacologists can visualize the consequences of pharmacological manipulations and analyze binding kinetics in separate regions.

The neuroanatomist can examine "chemoarchitecture," together with cyto- and myeloarchitecture, to identify functional subdivisions of the nervous system. Cortical subdivisions, for example, are difficult to visualize in cell and fiber stains and are often best located on the basis of electrophysiology. Transitions in opiate receptor lamination and density patterns occur at recognized architectural and physiological borders (Wise and Herkenham, 1982). Furthermore, μ-opiate receptor density progressively increases along successive stations of sensory processing (Lewis *et al.*, 1981). Together these findings indicate a promising future for correlative studies of structure and function in the cerebral cortex and elsewhere.

Receptor autoradiography affords neuropharmacologists new insights into structure–function relationships. Their enhanced understanding will aid in the identification of clinically beneficial drugs that have specific targets of action in the nervous system. Ligands with selective affinities have revealed distributions of receptor subtypes (Goodman *et al.*, 1980; Young and Kuhar, 1980b; Wamsley *et al.*, 1981a; Lewis *et al.*, 1983). Pharmacology of binding can be analyzed region by region (Penney *et al.*, 1981; Geary and Wooten, 1983; Pan *et al.*, 1983).

The value of receptor autoradiographic studies depends on the quality of the anatomy and histology and the accurate pharmacological validation of a "meaningful" binding site. Perhaps because the field is still relatively new, a large number of published papers are sorely lacking in one or both respects. Poor tissue preservation, misidentification of anatomical structures, and failure to validate binding conditions properly necessitate replication of much of the original work. Future work will improve substantially as individuals or groups trained in both anatomy and histology and pharmacology perform receptor autoradiography.

Receptor autoradiography can be used in conjunction with other techniques to benefit several major areas of neuroscience, including immunology (Lewis *et al.*, 1982), physiology (Lee *et al.*, 1983), cerebral metabolism (Hammer *et al.*, 1982), behavior, and clinical neuropathology (Larsen *et al.*, 1982); examples follow. Correlative demonstrations of neurotransmitter and receptor distribution by immunohistochemistry and autoradiography, respectively, are possible in the same (Keefer, 1980) or adjacent (Lewis *et al.*, 1982) tissue sections. The ade-

nosine-evoked depression of neuronal activity in hippocampal slices can be correlated with autoradiographs of adenosine receptors in the same preparation (Lee *et al.*, 1983). Ketamine-altered metabolic activity in rat hippocampus, demonstrated by 2-deoxyglucose autoradiography, corresponds to phencyclidine and opiate receptor distributions in adjacent sections from the same animal (Hammer *et al.*, 1982). Functional interactions can also be demonstrated by alterations in receptor densities after lesions (Penney *et al.*, 1981; Pan *et al.*, 1983) or chronic drug treatment (Moon Edley, 1983). Finally, diseases of the human nervous system that have correlates in receptor distributions or densities can be identified postmortem by autoradiography (Larsen *et al.*, 1982; Palacios, 1982; Penney and Young, 1982; Lang and Henke, 1983) or in living subjects by positron emission tomography.

## VI. CONCLUSIONS

Localization of receptor distributions in the brain or other tissues can be achieved by a number of autoradiographic techniques. The choice of a particular technique depends on several factors, including the method of initial binding (*in vivo* or *in vitro*), chemistry of the ligand (suitable for perfusion fixation, postincubation vapor fixation, or not fixable), its binding affinity (reversible or irreversible binding), and the need for autoradiographic resolution weighed against simplicity and/or demands for density quantification (emulsion or film autoradiography). The tissue preparation procedure described here will ensure optimal preservation of unfixed tissues in aqueous media, regardless of choice of autoradiographic technique.

## ACKNOWLEDGMENTS

I thank Jennifer Stuart for assistance in the preparation of this chapter, Dr. Steven P. Wise for preparation of primate material and interpretation of the data, Drs. Wayne Bowen and Remi Quirion for determining opiate and neurotensin binding conditions, and Dr. Candace B. Pert for close involvement in all phases of this work.

## REFERENCES

Alexander, G. M., Schwartzman, R. J., Bell, R. D., Yu, J., and Renthal, A. (1981). *Brain Res.* **223,** 59–67.

Arimatsu, Y., Seto, A., and Amano, T. (1978). *Brain Res.* **147,** 165–169.

Arimatsu, Y., Seto, A., and Amano, T. (1981). *J. Comp. Neurol.* **198,** 603–631.

Atweh, S. F., and Kuhar, M. J. (1977a). *Brain Res.* **124,** 53–67.
Atweh, S. F., and Kuhar, M. J. (1977b). *Brain Res.* **129,** 1–12.
Atweh, S. F., and Kuhar, M. J. (1977c). *Brain Res.* **134,** 393–405.
Atweh, S. F., Murrin, L. C., and Kuhar, M. J. (1978). *Neuropharmacology* **17,** 65–71.
Barnes, P. J., Basbaum, C. B., Nadel, J. A., and Roberts, J. M. (1982). *Nature (London)* **299,** 444–447.
Barnes, P. J., Nadel, J. A., Roberts, J. M., and Basbaum, C. B. (1983). *Eur. J. Pharmacol.* **86,** 103–106.
Baskin, D. G., Petracca, F., and Dorsa, D. M. (1983). *Eur. J. Pharmacol.* **90,** 155–157.
Beaudet, A., Tremeau, O., Menez, A., and Droz, B. (1979). *C.R. Hebd. Seances Acad. Sci.* **289,** 591–594.
Biegon, A., and Rainbow, T. C. (1982). *Eur. J. Pharmacol.* **82,** 245–246.
Biegon, A., and Rainbow, T. C. (1983a). *Neurosci. Lett.* **37,** 209–214.
Biegon, A., and Rainbow, T. C. (1983b). *J. Neurosci.* **3,** 1069–1076.
Biegon, A., Rainbow, T. C., Mann, J. J., and McEwen, B. S. (1982). *Brain Res.* **247,** 379–382.
Bowen, W. D., Gentleman, S., Herkenham, M., and Pert, C. B. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 4818–4822.
Buckley, N., and Burnstock, G. (1983). *Brain Res.* **279,** 374–377.
Caro, L. G., and Van Tuburgen, R. P. (1962). *J. Cell Biol.* **15,** 173–188.
Chan-Palay, V. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 2516–2520.
Chan-Palay, V., Yonezawa, T., Yoshida, S., and Palay, S. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 6281–6284.
Costa, R., Rodbard, D., and Pert, C. B. (1979). *Nature (London)* **277,** 315–316.
Cowan, W. M., Gottlieb, D. I., Hendrickson, A. E., Price, J. L., and Woolsey, T. A. (1972). *Brain Res.* **37,** 21–51.
Creese, I., and Snyder, S. H. (1975). *J. Pharmacol. Exp. Ther.* **194,** 205–219.
Dashwood, M. R. (1983). *Eur. J. Pharmacol.* **86,** 51–58.
Duka, T., Wüster, M., Schubert, P., Stoiber, R., and Herz, A. (1981). *Brain Res.* **205,** 181–186.
Fuxe, K., Calza, L., Benfenati, F., Zini, I., and Agnati, L. F. (1983). *Proc. Natl. Acad. Sci. U.S.A.* **80,** 3836–3840.
Geary, W. A., II, and Wooten, G. F. (1983). *J. Pharmacol. Exp. Ther.* **225,** 234–240.
Goldman-Rakic, P. S. (1982). *J. Comp. Neurol.* **205,** 398–413.
Goldstein, A., Lowney, L. J., and Pal, B. K. (1971). *Proc. Natl. Acad. Sci. U.S.A.* **68,** 1742–1747.
Goodman, R. R., and Snyder, S. H. (1982a). *J. Neurosci.* **2,** 1230–1241.
Goodman, R. R., and Snyder, S. H. (1982b). *Proc. Natl. Acad. Sci. U.S.A.* **18,** 5703–5707.
Goodman, R. R., Snyder, S. H., Kuhar, M. J., and Young, W. S., III (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 6239–6243.
Goodman, R. R., Kuhar, M. J., Hester, L., and Snyder, S. H. (1983). Science **220,** 967–969.
Grabowsky, K. I., McCabe, R. T., and Wamsley, J. K. (1983). *Life Sci.* **32,** 2355–2361.
Greenamyre, J. T., Young, A. B., and Penney, J. B. (1983). *Neurosci. Lett.* **37,** 155–160.
Haber, S., and Elde, R. (1982). *Neuroscience* **7,** 1049–1095.
Halpain, S., Parson, B., and Rainbow, T. C. (1983). *Eur. J. Pharmacol.* **86,** 313–314.
Hamel, E., and Beaudet, A. (1982). *Soc. Neurosci. Abstr.* **8,** 221.
Hammer, R. P., Jr., Herkenham, M., Pert, C. B., and Quirion, R. (1982). *Proc. Natl. Acad. Sci. U.S.A.* **79,** 3067–3070.
Hartzell, H. C. (1980). *J. Cell Biol.* **86,** 6–20.
Hendrickson, A. (1975). *Brain Res.* **85,** 241–247.
Hendrickson, A., and Edwards, S. B. (1978). *In* "Neuroanatomical Research Techniques" (R. T. Robertson, ed.), pp. 241–290. Academic Press, New York.
Herkenham, M., and Pert, C. B. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 5532–5536.

Herkenham, M., and Pert, C. B. (1981). *Nature (London)* **291,** 415–418.
Herkenham, M., and Pert, C. B. (1982). *J. Neurosci.* **2,** 1129–1149.
Herkenham, M., and Sokoloff, L. (1983). *Soc. Neurosci. Abstr.* **9,** 329.
Herkenham, M., Wise, S. P., Quirion, R., and Pert, C. B. (1982). *Soc. Neurosci. Abstr.* **8,** 647.
Herkenham, M., Moon Edley, S., and Stuart, J. (1984). Neuroscience **11,** 561–593.
Hollenberg, M. D., and Cuatrecasas, P. (1976). *Methods Mol. Biol.* **9,** Part II, 429–477.
Hösli, E., Möhler, H., Richards, J. G., and Hösli, L. (1980). *Neuroscience* **5,** 1657–1665.
Hösli, E., Krugsgaard-Larsen, P., and Hösli, L. (1983). *Brain Res.* **268,** 177–180.
Hunt, S. P., and Schmidt, J. (1978). *Brain Res.* **142,** 152–159.
James, W. L., and Klein, W. L. (1982). *Neurosci Lett.* **32,** 5–10.
Keefer, D. A. (1980). *Cell Tissue Res.* **209,** 167–175.
Kent, J. L., Pert, C. B., and Herkenham, M. (1982). *Dev. Brain Res.* **2,** 487–504.
Klemm, N., Murrin, L. C., and Kuhar, M. J. (1979). *Brain Res.* **169,** 1–9.
Kuhar, M. J., and Unnerstall, J. R. (1982). *Brain Res.* **244,** 178–181.
Kuhar, M. J., and Yamamura, H. I. (1975). *Nature (London)* **253,** 560–561.
Kuhar, M. J., Murrin, L. C., and Malouf, A. T. (1978). *Life Sci.* **22,** 203–210.
Kuhar, M. J., Taylor, N., Wamsley, J. K., Hulme, E. C., and Birdsall, N. J. M. (1981). *Brain Res.* **216,** 1–10.
Lane, M., Sastre, A., Law, M. and Salpeter, M. M. (1977). *Dev. Biol.* **57,** 254–269.
Lang, W., and Henke, H. (1983). *Brain Res.* **267,** 271–280.
Larsen, A., Calne, D., Quirion, R., Herkenham, M., and Pert, C. B. (1982). *Soc. Neurosci. Abstr.* **8,** 563.
Larsson, B., and Ullberg, S. (1977). *Acta Pharmacol. Toxicol.* **41,** Suppl. 1, 48–49.
Lee, K. S., Schubert, P., Reddington, M., and Kreutzberg, G. W. (1983). *Neurosci. Lett.* **37,** 81–85.
Lewis, M. E., Patel, J., Moon Edley, S., and Marangos, P. J. (1981). *Eur. J. Pharmacol.* **73,** 109–110.
Lewis, M. E., Khachaturian, H., and Watson, S. J. (1982). *Life Sci.* **31,** 1347–1350.
Lewis, M. E., Pert, A., Pert, C. B., and Herkenham, M. (1983). *J. Comp Neurol.* **216,** 339–358.
Lord, J. A. H., Waterfield, A. A., Hughes, J., and Kosterlitz, H. W. (1977). *Nature (London)* **267,** 495–499.
Lysz, T. W., Toga, A. W., and Geary, W. A., III (1982). *Neurosci. Abstr.* **8,** 645.
Manning, D. C., Snyder, S. H., Kachur, J. F., Miller, R. J., and Fields, M. (1982). *Nature (London)* **299,** 256–259.
Meibach, R. C., and Maayani, S. (1980). *Eur. J. Pharmacol.* **68,** 175–179.
Meibach, R. C., Maayani, S., and Green, J. P. (1980). *Eur. J. Pharmacol.* **67,** 371–382.
Minkovic, M., Hunt, S. P., and Kelley, J. S. (1981). *Brain Res.* **230,** 111–119.
Monaghan, D. T., and Cotman, C. W. (1982). *Brain Res.* **252,** 91–100.
Moon Edley, S. (1983). *Anat. Rec.* **205,** 133A.
Moon Edley, S., Hall, L., Herkenham, M., and Pert, C. B. (1982). *Brain Res.* **249,** 184–188.
Munson, P. J., and Rodbard, D. (1981). *In* "Quality Control in Clinical Endocrinology" (D. W. Webster, S. J. Gaskell, and K. W. Kemp, eds.), pp. 89–98. Alpha Omega, Cardiff.
Murphy, K. M. M., Gould, R. J., and Snyder, S. H. (1982). *Eur. J. Pharmacol.* **81,** 517–519.
Murrin, L. C., and Kuhar, M. J. (1979). *Brain Res.* **177,** 279–285.
Murrin, L. C., Gale, K., and Kuhar, M. J. (1979). *Eur. J. Pharmacol.* **60,** 229–235.
Olgiati, V. R., Guidobono, F., Netti, C., and Pecile, A. (1983). *Brain Res.* **265,** 209–215.
Palacios, J. M. (1982). *Brain Res.* **243,** 173–175.
Palacios, J. M., and Kuhar, M. J. (1980a). *Science* **208,** 1378–1380.
Palacios, J. M., and Kuhar, M. J. (1980b). *Brain Res. Bull.* **5,** Suppl. 2, 145–148.
Palacios, J. M., and Kuhar, M. J. (1981a). *Nature (London)* **294,** 587–589.

Palacios, J. M., and Kuhar, M. J. (1981b). *Dev. Brain Res.* **2,** 531–540.
Palacios, J. M., Young, W. S., III, and Kuhar, M. J. (1979). *Eur. J. Pharmacol.* **58,** 295–304.
Palacios, J. M., Young, W. S., III, and Kuhar, M. J. (1980). Localization of gamma amino butyric-acid receptors in the rat cerebellum. *Proc. Natl. Acad. Sci. U.S.A.* **77,** 670–674.
Palacios, J. M., Niehoff, D. L., and Kuhar, M. J. (1981a). *Brain Res.* **213,** 277–290.
Palacios, J. M., Wamsley, J. K., and Kuhar, M. J. (1981b). *Brain Res.* **214,** 155–162.
Palacios, J. M., Wamsley, J. K., and Kuhar, M. J. (1981c). *Brain Res.* **222,** 285–307.
Palacios, J. M., Wamsley, J. K., and Kuhar, M. J. (1981d). *Neuroscience* **6,** 15–38.
Palacios, J. M., Niehoff, D. L., and Kuhar, M. J. (1981e). *Neursci. Lett.* **25,** 101–105.
Pan, H. S., Frey, K. A., Young, A. B., and Penney, J. B., Jr. (1983). *J. Neurosci.* **3,** 1189–1198.
Parsons, B., and Rainbow, T. C. (1983). *Neurosci. Lett.* **36,** 9–12.
Pasternak, G. W., Wilson, R. H., and Snyder, S. H. (1975). *Mol. Pharmacol.* **11,** 340–351.
Pearse, A. G. E. (1980). "Histochemistry: Theoretical and Applied," 4th ed., Vol. I. Churchill-Livingstone, Edinburgh and London.
Pearson, J., Brandeis, L., Simon, Ed. and Hillar, J. M. (1980). *Life Sci.* **26,** 1047–1052.
Penney, J. B., Jr., and Young, A. B. (1982). *Neurology* **32,** 1391–1395.
Penney, J. B., Jr., Pan, H. S., Young, A. B., Frey, K. A., and Dauth, G. W. (1981). *Science* **214,** 1036–1038.
Pert, C. B., and Garland, B. (1978). *Recept. Horm. Act.* **3,** 535–551.
Pert, C. B., and Herkenham, M. (1981). *In* "Biochemistry of Taste and Olfaction" (R. H. Cagan and M. R. Kare, eds.), pp. 511–522. Academic Press, New York.
Pert, C. B., and Snyder, S. H. (1973). *Science* **179,** 1011–1014.
Pert, C. B., Pasternak, G., and Snyder, S. H. (1973). *Science* **182,** 1359–1361.
Pert, C. B., Kuhar, M. J., and Snyder, S. H. (1975). *Life Sci.* **16,** 1849–1854.
Pert, C. B., Pert, A., Chang, J. K., and Fong, B. T. W. (1976a). *Science* **194,** 330–332.
Pert, C. B., Kuhar, M. J., and Snyder, S. H. (1976b). *Proc. Natl. Acad. Sci. U.S.A.* **73,** 3729–3733.
Pfaff, D. W. (1968). *Science* **161,** 1355–1356.
Polz-Tejera, G., Schmidt, J., and Karten, H. J. (1975). *Nature (London)* **258,** 349–351.
Quirion, R. (1983). *Neurosci. Lett.* **36,** 267–271.
Quirion, R., Hammer, R. P., Jr., Herkenham, M., and Pert, C. B. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 5881–5885.
Quirion, R., Gaudreau, P., St.-Pierre, S., Rioux, F., and Pert, C. B. (1982a). *Peptides* **3,** 757–763.
Quirion, R., Gaudreau, P., St.-Pierre, S., and Rioux, F. (1982b). *Peptides* **3,** 765–769.
Quirion, R., Bowen, W., Herkenham, M., and Pert, C. B. (1983a). *Cell. Mol. Neurobiol.* **2,** 333–346.
Quirion, R., Shulfs, C. W., Moody, T. W., Pert, C. B., Chase, T. N., and O'Donohue, T. L. (1983b). *Nature (London)* **303,** 714–716.
Rainbow, T. C., and Biegon, A. (1983). *Brain Res.* **262,** 319–322.
Rainbow, T. C., Biegon, A., and McEwen, B. S. (1982a). *Eur. J. Pharmacol.* **77,** 363–364.
Rainbow, T. C., Bleisch, W. V., Biegon, A., and McEwen, B. S. (1982b). *J. Neurosci. Methods* **5,** 127–138.
Rice, K. C., Jacobson, A. E., Burke, T. R., Bajwa, B. S., Streaty, R. A., and Klee, W. A. (1983). *Science* **220,** 314–316.
Rogers, A. W. (1979). "Techniques of Autoradiography." Elsevier/North-Holland, New York.
Rotter, A., Birdsall, N. J. M., Burgen, A. S. V., Field, P. M., Hulme, E. C., and Raisman, G. (1979a). *Brain Res. Rev.* **1,** 141–165.
Rotter, A., Birdsall, N. J. M., Field, P. M., and Raisman, G. (1979b). *Brain Res. Rev.* **1,** 167–184.
Salpeter, M. M., and McHenry, F. A. (1973). *In* "Advances of Techniques in Biological Electron Microscopy" (J. K. Koehler, ed.), pp. 113–152. Springer-Verlag, Berlin and New York.

Schlumpf, M., Richards, J. G., Lichtensteiger, W., and Möhler, H. (1983). *J. Neurosci.* **3,** 1478–1487.
Simon, E. J., Hiller, J. M., and Edelman, I. (1973). *Proc. Natl. Acad. Sci. U.S.A.* **70,** 1947–1949.
Slater, P., Crossman, A. R., and Sambrook, M. A. (1982). *Neurosci. Lett.* **31,** 259–263.
Snyder, S. H. (1978). *In* "Neurotransmitter Receptor Binding" (H. I. Yamamura, S. J. Enna, and M. J. Kuhar, eds.), pp. 1–11. Raven Press, New York.
Stumpf, W. E. (1968). *Science* **162,** 1001–1003.
Stumpf, W. E. (1976). *Methods Cell Biol.* **13,** 171–193.
Terenius, L. (1973). *Acta Pharmacol. Toxicol.* **33,** 377–384.
Terracio, L., Beitz, A. J., Wells, W. E., and Buggy, J. (1982). *Neurosci. Abstr.* **8,** 20.
Unnerstall, J. R., and Wamsley, J. K. (1983). *Eur. J. Pharmacol.* **86,** 361–371.
Unnerstall, J. R., Kuhar, M. J., Niehoff, D. L., and Palacios, J. M. (1981). *J. Pharmacol. Exp. Ther.* **218,** 797–804.
Unnerstall, J. R., Niehoff, D. L., Kuhar, M. J., and Palacios, J. M. (1982). *J. Neurosci. Methods* **6,** 59–73.
Unnerstall, J. R., Molliver, M. E., Kuhar, M. J., and Palacios, J. M. (1983). *Dev. Brain Res.* **7,** 157–169.
van Houten, M., and Posner, B. I. (1979). *Nature (London)* **282,** 623–625.
van Houten, M., Schiffrin, E. L., Mann, J. F. E., Posner, B. I., and Boucher, R. (1980). *Brain Res.* **186,** 480–485.
Wamsley, J. K., Zarbin, M., Birdsall, N., and Kuhar, M. J. (1980). *Brain Res.* **200,** 1–12.
Wamsley, J. K., Lewis, M. S., Young, W. S., III, and Kuhar, M. J. (1981a). *J. Neurosci.* **1,** 176–191.
Wamsley, J. K., Palacios, J. M., and Kuhar, M. J. (1981b). *Neurosci. Lett.* **27,** 19–24.
Wamsley, J. K., Zarbin, M. A., Young, W. S., III, and Kuhar, M. J. (1982). *Neuroscience* **7,** 595–613.
Wilkin, G. P., Hudson, A. L., Hill, D. R., and Bowery, N. G. (1981). *Nature (London)* **294,** 584–587.
Wilson, R. S., Rogers, M. E., Pert, C. B., and Snyder, S. H. (1975). *J. Med. Chem.* **18,** 240–242.
Wise, S. P., and Herkenham, M. (1982). *Science* **218,** 387–389.
Yamamura, H. I., Kuhar, M. J., and Snyder, S. H. (1974). *Brain Res.* **80,** 170–176.
Young, W. S., III, and Kuhar, M. J. (1979a). *Brain Res.* **179,** 255–270.
Young, W. S., III, and Kuhar, M. J. (1979b). *Nature (London)* **280,** 393–394.
Young, W. S., III, and Kuhar, M. J. (1980a). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 1696–1700.
Young, W. S., III, and Kuhar, M. J. (1980b). *J. Pharmacol. Exp. Ther.* **212,** 337–346.
Young, W. S., III, and Kuhar, M. J. (1980c). *Eur. J. Pharmacol.* **62,** 237–239.
Young, W. S., III, and Kuhar, M. J. (1981). *Brain Res.* **206,** 273–285.
Young, W. S., III, Wamsley, J. K., Zarbin, M. A., and Kuhar, M. J. (1980). *Science* **210,** 76–78.
Young, W. S., III, Niehoff, D., Kuhar, M. J., Beer, B., and Lippa, A. S. (1981). *J. Pharmacol. Exp. Ther.* **216,** 425–430.
Zarbin, M. A., Innis, R. B., Wamsley, J. K., Snyder, S. H., and Kuhar, M. J. (1983). *J. Neurosci.* **3,** 877–906.

# *Chapter 9*

# *AFFINITY AND PHOTOAFFINITY LABELING OF RECEPTORS*

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## I. INTRODUCTION

The method of affinity labeling is based on the fact that the key step in biological reactions is the specific recognition and binding of a ligand to the receptor macromolecule, whether we consider enzymes, immune proteins, transport proteins, and receptor proteins. A small and well-defined part of the protein surface is presumed to be involved in this selective, high-affinity interaction with the specific ligand, and this part is referred to as the binding site. The definition of affinity labeling thus must contain two distinct steps: selective binding and covalent bond formation. This is achieved by the incorporation of a chemically labile group into a ligand with the retention of most of the structural features that are required for recognition by the receptor macromolecule. The modified ligand can be detected by virtue of a tailored marker that is radioactive, fluorescent, or spin-labeled. The ligand can then become an affinity label, provided that appro-




ISBN 0-12-470350-X

priate chemical reactivity is present at the ligand binding site. The proximity effect of placing the functional group of the analog in direct juxtaposition to its reaction partner is very significant here. It means that the effective reagent concentration is much higher near the binding site residue than near any of the other residues of the same kind in the protein. Even for sluggish reagents and for relatively low-affinity sites, the reaction at the binding site residue will consequently be favored.

The term *receptor* in the context used currently in drug and hormonal studies and in this review is defined operationally as those molecules that specifically recognize and bind the neurotransmitter, hormone, or drugs and, as a consequence of this recognition, can lead to other changes, which ultimately result in biological response. Most drug and hormone receptors that are membrane-bound are in the category of integral membrane proteins; that is, they are not readily solubilized from the membrane and require detergents or other hydrophobic bond–breaking agents to release them. After release of the receptor from the membrane, the physiological functions by which the receptor activity is recognized are usually lost, and even the capacity of the receptor to bind its specific drug or hormone may be destroyed or radically modified. Positive identification of receptors in preparations of subcellular fractions is feasible only if the receptor protein has been labeled before the disruption of the membrane. The labeling techniques commonly employed for the identification of receptor proteins are inadequate, however, since the latter constitute only a minor component of the total cell proteins. In principle, affinity labeling provides the high specificity needed for this purpose. It is of interest to note that affinity labeling of the norepinephrine α-receptor was reported as early as 1949 (Nickerson and Gump, 1949). The elegant demonstrations of the feasibility and utility of the general approach of affinity labeling or active-site–directed reagents (Baker *et al.*, 1961; Wofsy *et al.*, 1962; Schoellman and Shaw, 1962; Baker, 1967) certainly gave a tremendous impact to the study of protein structure–function relationships. This approach has significantly contributed and will continue to contribute both to our fundamental understanding of how proteins carry out their biological functions and to the applied areas of drug design and chemotherapy.

In choosing a reagent suitable for affinity labeling of a receptor, two main factors have to be considered: (*a*) the affinity constant of the reagent toward the target receptor, and (*b*) the nature of the chemically reactive moiety. The armamentarium of the protein chemist contains many group-selective reagents from which one capable of modifying some residues located at the desired site can often be chosen. Some examples of reactive groups that have been employed for affinity labeling are as follows: Haloacetyl (i.e., $BrCH_2CO$), sulfonyl halides ($SO_2F$), haloketones, isothiocyanates (RNCS), epoxides, aziridines, and diazo derivatives. It is beyond the scope of this chapter to review the many types of functional groups that have been used or could be used as affinity labeling reagents and how they react with the different amino acid sidechains in proteins.

However, it may be worth emphasizing that the old prejudices that some residues (Val, Ile, Leu) are inert in modification reactions can be discarded with the introduction of reagents with such functional groups as carbenes and nitrenes, which react almost indiscriminately with many types of side chains. This chapter covers literature published up to February 1982.

## II. CRITERIA FOR AFFINITY LABELING

The complementarity between the ligand-like moiety of the affinity label and the binding site of the protein promotes, but does not necessarily determine, the binding of the compound at or near the site normally occupied by the substrate. The amino acid residue attacked may be one directly involved in binding. It may also be a residue in the neighborhood of the site, so that when the covalent link with the affinity label is formed, the substrate-like moiety either rests in the substrate binding site or is sufficiently close so that the substrate is blocked from access to its binding site and the receptor is thus modified. Successful application of the affinity labeling method depends on the degree to which the receptor molecule has been exclusively labeled at the specificity site. A number of criteria should be applied in the design and evaluation of such experiments:

1. The compound should be either a ligand for the receptor or closely resemble a ligand. This is a necessary but not a sufficient condition for concluding that the label is at the ligand binding site.

2. Specific protection against inactivation. If a specific binding is inactivated by reaction with an affinity label, the inactivation should be slower when the reaction is carried out in the presence of a specific protector (reversible inhibitor, agonist, or antagonist) of the site in question. The slower rate of inactivation, which is determined in the presence of excess labeling agent, is usually pseudo-first order. However, inactivation may result also from reaction conditions or from nonspecific modification of the receptor protein; the effect on the receptor of a nonspecific analog that possesses the same reactive group as the affinity label should therefore be examined in control experiments. This criterion is not altogether adequate, since a particular receptor will probably account for less than 1% of the total cellular protein, and specific labeling of a single protein when this protein is a minor component in a mixture has to be demonstrated. Therefore, the following criteria must also be met.

3. Exclusivity (Singer, 1970). The estimation of nonspecific labeling of proteins other than the receptor by the affinity label may be achieved by the differential labeling technique. A preparation to be labeled is first treated with a nonradioactive form of the affinity labeling reagent in the presence of a reversible protective agent (inhibitor, agonist, or antagonist). The protective agent is then removed, for example, by dialysis or centrifugation, and the sample is exposed

to the radioactive affinity labeling reagent. Comparison of the results of this experiment with those of direct labeling of another portion of the sample with a radioactive agent allows an estimate of the exclusivity of the procedure. Furthermore, the exclusivity measurements should be performed with suitable controls, since exposure of new nonspecific sites might occur during labeling of the receptor with the nonradioactive form of the reagent. Thus, the sample that was modified in the presence of the protector is divided into two portions, to one of which the protector is added back. Both portions are then treated with the radioactive form of the reagent.

4. Saturation kinetics of the inactivation reaction. Affinity labeling takes place in two steps: The first is the reversible formation of a Michaelis–Menten complex, followed by the formation of an irreversible covalent bond to the protein. Therefore, a plot of the rate constant for inactivation against affinity label concentration will reach a plateau. However, in contrast to affinity labeling of soluble enzymes, saturability and stoichometric inactivation are not always applicable to receptors because of the high degree of nonspecific labeling that usually occurs.

A theoretical kinetic description of the affinity labeling process was carried out by Metzger *et al.* (1963). This includes the formation of a reversible complex ($L\cdots R$) that, while in the complexed form, undergoes an accelerated covalent bond-forming reaction with one or more appropriately located residues ($L—R$)

$$L + R \underset{k_2}{\overset{k_1}{\rightleftarrows}} L\cdots R \xrightarrow{k_3} L—R \tag{1}$$

where $L$ = affinity label reagent and $R$ = receptor.

The affinity labeling reagent may react simultaneously, although usually at a slower rate, in a bimolecular process with amino acid residues outside the active site. Reaction with sites of lower specificity would yield a series of undesired products ($L—A_i$).

$$L + A_i \xrightarrow{k_{4i}} L—A_i \tag{2}$$

The ratio between the specific $L—R$ and the sum of the nonspecific products, $L—A_i$, obtained after a reaction time $t$, will markedly influence the choice of a labeling reagent. Let us assume for simplicity that all nonspecific sites are equally reactive, that is, that the individual rate constants can be approximated by a mean rate constant, $\bar{k}_4$ ($k_{4i} = \bar{k}_4$). Assume also that the total sum of nonspecific sites available for modification, $A$, is equal to $\sum A_i$, and that the total amount of nonspecifically bound reagent, $L—A$, is equal to $\sum L—A$. Then the parameters that measure the specificity, $X_{(t)}$, of the reaction can be reduced to

$$X_{(t)} = \frac{(LR)}{(L—A)} = \frac{k_3 \cdot (k_1/k_2)\,(R)}{k_4\,(A)} \tag{3}$$

Equation (3) shows that specificity is directly proportional to the equilibrium constant, $K$, for the formation of the complex $R{\cdots}L$ ($K = k_1/k_2$) [Eq. (1)]. Hence, the greater the association constant of the reagent with the receptor, the greater the specificity of labeling. Specificity also depends on the ratio $k_3/\bar{k}_4$, that is, on the relative reactivity of the labeling groups in and out of the binding site. This implies that the absolute reactivity of the reactive group of the reagent is not very important. However, its selectivity may be crucial, since a group of low selectivity will have more sites to react with. An increase in $A$ ($\sum A_i$) would thus lead to a corresponding decrease in specificity. Nevertheless, reactivity considerations are important because the reactivity of the labeling group and the reaction conditions can determine which residues are labeled. Thus, bromoacetyl groups have little selectivity, since they will react with amino, phenolic, sulfhydryl, and carboxyl residues. However, performing the reaction at a pH lower than 7 might increase selectivity. It is also obvious that, since specificity [$X_{(t)}$] [Eq. (3)] is proportional to the free receptor concentration ($R$), it will be markedly reduced when the receptor comprises only a minor fraction of the total groups exposed.

## III. PHOTOAFFINITY LABELING

The majority of chemical affinity labels are susceptible to hydrolysis. Therefore, the labeling depends upon the successful competition between a nucleophile at the binding site and the 55 $M$ water in which the labeling reaction is carried out. It should be noted that in some cases this is not always a limitation; for example, oxonium salts, which have a half-life of the order of seconds in water, have been used successfully in affinity labeling of the nicotinic receptor (Chao *et al.*, 1975). Furthermore, it is becoming clear that some biological problems require a reagent whose reactivity remains masked until the experimenter chooses to activate it. For example, how could one label the inside components of a cell or a vesicle without modifying the outside? With classical chemical reagents, it is obvious that reactive groups on the outside would be extensively labeled with reagent prior to the incorporation of any radioactive reagent into the cytoplasm. In principle, the use of photoactive labels (Knowles, 1972; Chowdhry and Westheimer, 1979) has a great potential in circumventing these limitations. In this method (photoaffinity labeling) a reagent is used that can combine specifically and reversibly with the binding site and contains a photolyzable group, $P$. $P$ is unreactive in the dark but when photolyzed, it is converted to an extremely reactive intermediate, $P^*$, if certain conditions are satisfied. $P^*$ may then react to form a covalent bond with residue(s) in the binding site of the receptor before the reagent dissociates from the site [Eq. (4)].

$$\begin{array}{l} L + R_p \underset{k_2}{\overset{k_1}{\rightleftarrows}} L\cdots R_p \xrightarrow{h\nu} L\cdots R_p^* \xrightarrow{k_3^*} L{-}R \\ \qquad\qquad\qquad\qquad\quad k_1^* \downarrow\uparrow k_2^* \\ \qquad\qquad\qquad\qquad\quad L \;+\; R_p^* \end{array} \tag{4}$$

Under such circumstances, $k_3^* > k_2^*$, and the formation of a covalent $L{-}R$ bond will occur before the affinity label can dissociate from the receptor. As noted by Kiefer *et al.* (1970) these conditions could lead to a very high degree of specificity of labeling at binding sites; any reagent molecules that underwent photolysis in free solution would be expected to react with the solvent or with an added scavenger, and nonspecific reactions with the membrane proteins might be greatly reduced. The photolyzable groups commonly employed are diazoacyl reagents and arylazides, which upon irradiation are converted to carbene and nitrene radicals, respectively. However, these reagents exhibit a high degree of nonspecificity either because $k_3^*$ [Eq. (4)] is not sufficiently high or because of the low selectivity of the carbene or nitrene moieties formed in the photolytic process, which react by a variety of insertions at any chemical bonds, including C—H bonds, in their vicinity (for review see Bayley and Knowles, 1977). The reactivity of nitrenes is considerably lower than that of carbenes. Nitrenes are less indiscriminate than carbenes in their reactions with primary, secondary, and tertiary carbon–hydrogen bonds, for instance. Nitrenes are also somewhat electrophilic, preferring an O—H over a C—H bond.

One great advantage of photolabile reagents over chemical affinity reagents should always be exploited: Binding or activity assays can be carried out with the modified ligand before photoactivation, and such tests will often confirm the site specificity of the ligand analog.

## IV. UV-INDUCED LABELING OF RECEPTORS

As stated in the preceding section, photoaffinity labeling requires synthesis of a ligand with a photolabile group. In recent years it was noted that with some ligands such as benzodiazepines (Möhler *et al.*, 1980; Sieghart and Karobath, 1980), phencyclidine, and chloropromazine (Oswald and Changeux, 1981) simple UV irradiation in the presence of the receptor appears sufficient to link the ligand covalently into the protein. This approach has been sparked by the photo-cross-linking of protein–nucleic acid complexes through the action of UV light (for reviews see Schimmel and Budzik, 1977; Antonoff *et al.*, 1977); for example, there are indications that 10 of the amino acid side chains can be joined to uracil (Smith, 1969). This photochemical approach has advantages, for example, of simplicity, since no extraneous labels or chemicals are required to achieve cross-linking. Benzodiazepine receptors were visualized recently in regions of

synaptic contact by EM autoradiography using [$^3$H]flunitrazepine as the label in rat brain tissue (Möhler *et al.*, 1981). Moreover, UV-induced labeling of the δ-chain of the membrane-bound nicotinic acetylcholine receptor from *Torpedo marmorata* using various reversible noncompetitive blockers of this receptor has been achieved (Oswald and Changeux, 1981). A disadvantage of the method is that the efficiency of labeling is lower than that found with standard photoaffinity ligands. Such low yields preclude, for instance, direct estimation of site stoichiometries. Even though the chemistry involved is still largely unelucidated and the irradiation conditions are often harsh, this technique might be helpful in delineating the regions of contact between the ligand and the receptor.

## V. TECHNICAL CONSIDERATIONS

1. The tighter the binding of the affinity and/or photolabile ligand to the receptor, the more successful the labeling will be.

2. Radiolabeled molecules should be synthesized so that the labeled atom is located as close to the affinity labile group as possible and should not be separated from it by any linkages that might be cleaved during subsequent manipulations. It should be kept in mind that the modification of a natural ligand to give an analog that retains good binding and functional properties may involve a part of the ligand that is unimportant in recognition and binding. This means that the modified part of the molecule may be exposed to the bulk solvent, to the lipid phase of a membrane, or to a neighboring macromolecule and that little labeling of the target macromolecule will occur on reaction. In practice, then, it is advisable to synthesize a range of nonradioactive reagents to evaluate inactivation before investing in a radioactive synthesis.

3. It is essential to have a reagent of high purity, both chemical and radiochemical, since small quantities of impurities with structures similar to that of the reagent under study may have tight binding constants to the target locus or unsuspected binding properties to other loci and thereby prevent efficient labeling or give misleading binding patterns.

4. Often the reagent must of necessity be added in organic solvent. It is essential that the quantity of solvent be minute, since subtle changes can be induced in biological preparations by organic solvents.

5. The labeling site should be both saturable and show saturation kinetics for photochemical labeling. The half-maximally effective ligand concentration should be close to the binding or inhibition constant of the ligand in the binding assay.

6. The labeling site should be protected by the natural ligand. In photoaffinity labeling protection by nonphotolabile ligand analog should be observed.

7. In experiments involving photolysis the following precautions should be observed:

a. Prolonged photolysis can result in undesirable effects such as nonspecific labeling by photolysis products and destruction or alteration of the binding site. It should be established that the preparation does not react with the ligand in the dark during the time required for the photolysis.
b. Repeated treatment: A single period of irradiation of a ligand–receptor complex may not yield complete labeling. Repeated treatments can be effective, but since the noncovalently bound reaction products can block the receptor site, products should be removed before further treatment.
c. The receptor preparation should be photolyzed alone, and the ability of the receptor to continue to bind its ligands should be assessed.
d. Prephotolysis of the reagent is important in order to establish whether the ligand photoproducts react with the receptor or protect the receptor from further modification by the reagent.
e. Certain thiols, which are commonly added to protect proteins against inactivation, can cause reduction of the diazo group (a carbene precursor) to the corresponding hydrazone (Takagaki *et al.*, 1980).

## VI. INFORMATION AND USE OF AFFINITY LABELING

The most significant contributions of affinity labeling techniques to date have been in enzyme chemistry, in the elucidation of active site structures and in unraveling mechanistic details in the catalytic processes. The incorporation of the affinity label provides the necessary handle to pull out the proper peptide sequences from the labeled proteins and, with due care, to draw conclusions about the amino acid sequences in or around the active sites. By the use of differently designed reagents with the reactive group in various geometrical relationships to the affinity group, it has been possible to involve a variety of residues in the covalent modification. In this way one can build up an inventory of the residues in different peptide sequences that presumably constitute the active site proper. Thus, a strategy for obtaining an active, detergent-soluble receptor preparation based on affinity labeling was recently described (Niedel, 1981). Because the method focuses directly on those parts of the molecule that are of most immediate interest, it has been possible to accumulate relevant sequence data on families of proteins and do comparative studies on active site peptides in cases in which complete sequences would take years to produce. The reader is also referred to the methodology of Simons *et al.* (1979) for an approach defined as chemoaffinity labeling. This approach appears to combine

some of the desirable features associated with either affinity or photoaffinity labeling, for example, (*a*) specificity for $NH_2$ and SH groups; (*b*) fast reaction; and (*c*) the product of the reaction is intensely fluorescent.

The application of affinity labeling and photoaffinity labeling to receptor sites may have one of the following several objectives.

## A. Identification and Quantitation

Specific labeling that may be achieved by affinity labels is often required in order to follow changes in the quantity or distribution of certain receptors. For example, in developing or differentiating systems, the correlation between the appearance of a functional activity and a particular receptor population or its ultrastructural distribution may be significant. As mentioned above, general labeling of membranes by nonspecific nonpermanent reagents is not satisfactory, since the receptor proteins in question often make up only a very small fraction of the total protein of the membrane preparation. Table I compiles only recent published examples (1980–1982) regarding the application of affinity and photoaffinity labeling to the identification and quantitation of receptors. For previously published data see, for example, reviews by Bayley and Knowles (1977), Zisapel and Sokolovsky (1977), and Chowdhry and Westheimer (1979).

## B. Purification

After the membrane is disrupted or the receptor protein extracted, the physiological functions by which its activity is recognized are usually lost. For the detection of receptor proteins during isolation, labels that are firmly attached and easy to recognize should be used. Disaggregation or denaturation of the receptor in the course of such manipulations will not then affect the experimenter's ability to detect the receptor constituents. Moreover, in such cases it should be demonstrated that the labeled receptors have preserved their biochemical characteristics. This is of major importance in order to ensure that the isolated protein represents not only one conformational type of the receptor, but also the average population of receptor molecules. Furthermore, if the receptor is an aggregated protein complex, only the chains that contain the labeled binding site would be detectable during purification, and, since the state of aggregation of the receptor may depend on the binding of the drug, an isolation procedure based on the monitoring of an affinity label may result in the loss of some structural components of receptor constituents. It must be emphasized that attempts to isolate receptor material by using the affinity labeling method have generally not yet resulted in success.

TABLE I

Identification of Receptors by Reagents for Affinity and Photoaffinity Labeling

| Reagent | Receptor | Tissue | References |
|---|---|---|---|
| Azidobenzoyl-LHRH | LHRH | Rat pituitary | Hazum, 1981 |
| 2-Nitro-5-azidobenzoyl-angiotensin A II | Angiotensin | Rabbit aorta | Galardy and Lavorgna 1981 |
| | | Dog adrenal cortex and uterus | Capponi and Catt, 1980 |
| Azidoatrazine | Herbicide | Chloroplast membranes | Pfister *et al.*, 1981 |
| 21-, 16α- and 11α-Bromo-acetoxy progesterone | Progesterone | Human uterus | Holmes *et al.*, 1981 |
| 17α-21-Dimethyl-19-norpregin-4,9-diene-3,20 dione | Progesterone | Chick oviduct and *Xenopus* oocytes | Dure *et al.*, 1980<br>Sadler and Maller 1982 |
| Arylazide enkephaline | Enkephalin | NG-108 | Lee *et al.*, 1979 |
| Bromoacetyl and 4-azido-2-nitrophenyl derivatives of formyl peptides | Chemotactic | Human neutropil membranes | Niedel, 1981 |
| [*p*-Azidophenylalanine$^{13}$]α-MSH | α-Melanotropin | *Xenopus laevis* oocytes | DeGraan *et al.*, 1981 |
| Hydroxysuccinimidyl-*p*-azidobenzoate | Glucagon | Rat liver | Johnson *et al.*, 1981 |
| Dexamethasone-21-mesylate | Glucocorticoid | Rat hepatoma cells | Simons and Thompson, 1981 |
| 6-(4′-Azido-2′-nitrophenylamino)-hexanayl-somatomedin (basic) | Somatomedin | Human placenta | Bhaumick *et al.*, 1981 |
| Bromoacetyl derivative of L-throxine and L-thyrone | Thyroid | Rat liver | Nikodem *et al.*, 1980 |
| (5-Azido-*w*-nitrobenzoyl) scorpion toxin | Sodium channel | Neuroblastoma cells | Beneski and Catterall, 1980 |
| ($N^{\varepsilon}$-$B^{29}$-Azidobenzoyl) insulin | Insulin | Rat brain and liver | Yip *et al.*, 1980 |
| N-Methyl-4-piperidyl-*p*-azidobenzilate | Muscarinic | Rat cortex | Amitai *et al.*, 1982 |
| Azidobenzylcarazolol | β-Adrenergic | | Lavin *et al.*, 1981 |
| Iodoazidobenzyl pidolol | β-Adrenergic | Duck erythrocyte | Rashidbaigi and Ruoho, 1981 |

## C. Biochemical Structure–Function Studies

Many receptors exhibit multiple modes of binding with agonists and antagonists. Often, cooperativity (either negative or positive) is observed. The effects of covalent binding of a drug or drug analog to its receptor on the kinetic parameters of reversible interactions with other drugs can be used to study the characteristics of binding processes, as well as such other phenomena as desensitization of receptors and the existence of low- and high-affinity binding sites. These techniques might prove very helpful in the biochemical characterization of processes such as isomerization and induced interconversion of high- and low-affinity binding states of receptors, e.g., by GTP, thermal exposure, (Gurwitz and Sokolovsky, 1980), metal ions, which were observed, for example, in the

TABLE II

Structure–Function Studies

| Reagent | Receptor | Tissue | References |
|---|---|---|---|
| Bromoacetylcholine | Nicotinic | Mammalian skeletal muscle | Shorr *et al.*, 1981 |
| Bromoacetylcholine | Nicotinic | *Torpedo marmorata* electric organ | Barrantes, 1980 |
| 5-Iodonaphthyl-1-azide | IgE | Normal and tumor mast cells | Holowka *et al.*, 1981 |
| 8-Azido-cAMP | cAMP-binding protein | Mouse, rat, cow, and sheep brains | Panter *et al.*, 1981 |
| (4-Azido-2-nitrophenyl)-acetylinsulin | Insulin | Rat liver | Baron *et al.*, 1981 |
| ($N^{\alpha B1}$-*p*-Azidobenzoyl)-insulin | Insulin | Rat liver | Yeung *et al.*, 1980 |
| [(2-Nitro-5-azidophenylsulfenyl)-$Trp^{9}$]-ACTH | Corticotropin | Rat adrenocortical cells | Ramachandran *et al.*, 1980 |
| 5-Azido-trimethisoquine | Nicotinic | *Torpedo marmorata* electric organ | Wennogle *et al.*, 1981 |
| [*p*-Azidophenylalanine$^{13}$]α-MSH | α-MSH | *Xenopus laevis* oocytes | DeGraan *et al.*, 1981 |
| 4-Substituted deoxy hexestrol derivatives (haloketone, epoxy, sulfonyl fluoride, and azido) | Estrogen | Lamb uterus | Katzenellenbogen *et al.*, 1981 |
| Arylazidoaminopropionyl-ATP | Purine | Guinea pig vas deferens | Fedan *et al.*, 1981 |
| Azido-acebutolol | β-Adrenergic | Rat reticulocyte membranes | Wrenn and Homcy, 1980 |

muscarinic system (for review see Sokolovsky and Bartfai, 1981). Such investigations carried out with intact cells, membrane fragments, or purified receptors can supply valuable information on the basis of which the characterization of differences in type and site of binding could be attained. (See Table II for recently published examples.)

The use of antagonists' and agonists' analogs can be utilized to establish the identity or nonidentity of these sites. Furthermore, reagents of systematically increasing size (length and flexibility) can be used as a molecular ruler for measuring distances at the binding site. This approach can be used also in a multicomponent system to locate components that are at different distances from the ligand binding site. Moreover, in multichain proteins, in which more than one chain forms the binding site(s), it is possible to design bifunctional reagents that will cross-link the two chains participating in the site. Affinity labeling and cross-linking proved very helpful in providing direct evidence for the presence of a heterogenous population of three major insulin receptor structures in membranes from rat and human tissues (Massague *et al.*, 1980). Furthermore, the technique of photoaffinity cross-linking with agents such as hydroxysuccinimidyl-*p*-azidobenzoate provides a rapid, simple method of covalently attaching ligands to their putative receptors (Johnson *et al.*, 1981). Photoaffinity cross-linking does not require chemical modification of the labeled ligand and has a less stringent requirement for specific reactive groups than the commonly used bifunctional cross-linking reagents.

Clearly, affinity labeling can be used to study the primary structure of the receptor binding site and to identify the amino acid residues in its close vicinity. It can also serve as a model for the "turned on" receptor to be compared structurally with a "turned off," ligand-free receptor, "open" and "closed" ion channels, etc. It can resolve heterogeneity problems, as was recently shown for the benzodiazepine receptors (Sieghart and Karobath, 1980). A special variant of affinity labeling will be the result of attempts to obtain "superactivated" receptors by chemical modification. This approach was demonstrated with enzymes (see Blumberg and Vallee, 1975, and references therein).

It should be emphasized that the absence of a covalent reaction does not necessarily mean that a particular component is not located at that binding site. The lack of reaction may mean that the reactive residues of the component in question are inaccessible to the probe, or that the rate of reaction with a different nearby component is much higher than with the component of interest.

The reactive moieties themselves may influence the reaction products obtained for reasons other than the reaction selectivity just discussed. For example, a hydrophobic probe will tend to bind to any hydrophobic region that is available to it. Thus, it will most likely react while in this region, and the reaction products will be biased. This is of particular concern in the photoaffinity experiments that use substituted aromatic compounds as reactive groups.

### D. Pharmacological Studies

Covalent binding of a drug to its receptor either *in vivo* or *in vitro* produces long-lasting inactivation or stimulation of the receptor system. This type of perturbation can be utilized to follow biochemical, physiological, and behavioral molecular events that follow receptor stimulation. Affinity labeling can also be used to evaluate stoichiometric relationships between the state of occupancy of receptors and the response. The spare receptor theory, which states that maximum response does not require total occupation of the receptors by an agonist, and theories related to activity of partial agonists may thereby be examined.

## VII. EPILOGUE

Although affinity and photoaffinity labeling have important contributions to make in solving the complex problems of receptor structure–function relationship, the technique must be used with caution since the perturbation caused by the presence of a covalently bound ligand is capable of altering the system under study in ways that are not yet fully understood.

## REFERENCES

Amitai, G., Avissar, S., Balderman, D., and Sokolovsky, M. (1982). *Proc. Natl. Acad. Sci. U.S.A.* **79,** 243–247.

Antonoff, R. S., Obrig, T., and Ferguson, J. J. (1977). *In* "Methods in Enzymology" (W. B. Jakoby and M. Wilchek, eds.), vol. 46, pp. 335–339. Academic Press, New York.

Baker, B. R. (1967). "Design of Active Site Directed Irreversible Enzyme Inhibitors." Wiley, New York.

Baker, B. R., Lee, W. W., Tong, E., and Ross, L. O. (1961). *J. Am. Chem. Soc.* **83,** 3713–3714.

Baron, M. D., Wisher, M. H., Thamm, P. M., Saunders, D. J., Brandenburg, D., and Sonksen, P. H. (1981). *Biochemistry* 20, 4156–4161.

Barrantes, F. J. (1980). *Eur. J. Pharmacol.* **65,** 49–53.

Bayley, H., and Knowles, J. R. (1977). *In* "Methods in Enzymology" (W. B. Jakoby and M. Wilchek, eds.), vol. 46, pp. 69–91. Academic Press, New York.

Beneski, D. A., and Catterall, W. A. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 639–643.

Bhaumick, B., Bala, R. M., and Hollenberg, M. D. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 4279–4283.

Blumberg, S., and Vallee, B. L. (1975). *Biochemistry* **14,** 2410–2419.

Capponi, A. M., and Catt, K. J. (1980). *J. Biol. Chem.* **255,** 12081–12086.

Chao, Y., Vandlen, R. L., and Raftery, M. A. (1975). *Biochem. Biophys. Res. Commun.* **63,** 300–307.

Chowdhry, V., and Westheimer, F. H. (1979). *Annu. Rev. Biochem.* **48,** 293–325.

DeGraan, P. N. E., Eberle, A. N., and Van de Veerdonk, F. C. G. (1981). *FEBS Lett.* **129,** 113–116.

Dure, L. S., IV, Schrader, W. T., and O'Malley, B. W. (1980). *Nature (London)* **283,** 784–786.

Fedan, J. S., Hogaboom, G. K., O'Donnell, J. P., Colby, J., and Westfall, D. P. (1981). *Eur. J. Pharmacol.* **69,** 41–54.

Galardy, R. E., and Lavorgna, K. A. (1981). *J. Med. Chem.* **24,** 362–366.

Gurwitz, D., and Sokolovsky, M. (1980). *Biochem. Biophys. Res. Commun.* **94,** 493–500.

Hazum, E. (1981). *Endocrinology* **109,** 1281–1283.

Holmes, S. D., Van, N. T., Stevens, S., and Smith, R. G. (1981). *Endocrinology* **109,** 670–672.

Holowka, D., Gitler, C., Bercovici, T., and Metzger, H. (1981). *Nature (London)* **289,** 806–808.

Johnson, G. L., Macandrew, V. I., Jr., and Pilch, P. F. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 875–878.

Katzenellenbogen, J. A., McGorrin, R. J., Tatee, T., Kempton, R. J., Carlson, K. E., and Kinder, D. H. (1981). *J. Med. Chem.* **24,** 435–450.

Kiefer, H., Lindström, J. Q., Lennox, E. S., and Singer, S. J. (1970). *Proc. Natl. Acad. Sci. U.S.A.* **67,** 1688–1694.

Knowles, J. R. (1972). *Acc. Chem. Res.* **5,** 155–160.

Lavin, T. N., Heald, S. L., Jeffs, P. W., Storr, R. G. L., Lefkowitz, R. J., and Caron, M. G. (1981). *J. Biol. Chem.* **256,** 11944–11950.

Lee, T. T., Williams, R. E., and Fox, C. F. (1979). *J. Biol. Chem.* **254,** 11787–11790.

Massague, J., Pildi, P. F., and Czech, M. P. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 7137–7141.

Metzger, H., Wofsy, L., and Singer, S. J. (1963). *Biochemistry* **2,** 979–988.

Möhler, H., Battersby, M., and Richards, J. G. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 1666–1670.

Möhler, H., Richards, J. G., and Wu, J. Y. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 1935–1938.

Nickerson, M., and Gump, W. S. (1949). *J. Pharmacol. Exp. Ther.* **97,** 25–47.

Niedel, J. (1981). *J. Biol. Chem.* **256,** 9295–9299.

Nikodem. V. M., Cheng, S.-Y., and Rall, J. E. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 7064–7068.

Oswald, R., and Changeux, J. P. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 3925–3929.

Panter, S. S., Butley, M. S., and Malkinson, A. M. (1981). *J. Neurochem.* **36,** 2080–2085.

Pfister, K., Steinback, K. E., Gardner, G., and Arntzen, C. J. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 981–985.

Ramachandran, J., Muramoto, K., Kenez-Keri, M., Keri, G., and Buckley, D. I. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 3967–3970.

Rashidbaigi, A., and Ruoho, A. E. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 1609–1613.

Sadler, S. E., and Maller, J. L. (1982). *J. Biol. Chem.* **257,** 355–361.

Schimmel, P. R., and Budzik, G. P. (1977). *In* "Methods in Enzymology" (W. B. Jakoby and M. Wilchek, eds.), Vol. 46, pp. 168–180. Academic Press, New York.

Schoellman, G., and Shaw, E. (1962). *Biochem. Biophys. Res. Commun.* **7,** 36–40.

Shorr, R. G., Lyddiatt, A., Lo, M. M. S., Dolly, J. O., and Barnard, E. A. (1981). *Eur. J. Biochem.* **116,** 143–154.

Sieghart, W., and Karobath, M. (1980). *Nature (London)* **286,** 285–287.

Simons, S. S., Jr., and Thompson, E. B. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 3541–3545.

Simons, S. S., Jr., Thompson, E. B., and Johnson, D. F. (1979). *Biochemistry* **18,** 4915–4922.

Singer, S. J. (1970). *In* "Molecular Properties of Drug Receptors" (R. Porter and M. O'Conner, eds.), p. 229. Churchill, London.

Smith, K. C. (1969). *Biochem. Biophys. Res. Commun.* **34,** 354–357.

Sokolovsky, M., and Bartfai, T. (1981). *Trends Biochem. Sci.* **6,** 303–306.

Takagaki, Y., Gupta, C. M., and Khorana, H. G. (1980). *Biochem. Biophys. Res. Commun.* **95,** 589–595.

Wennogle, L. P., Oswald, R., Saitoh, T., and Changeux, J. P. (1981). *Biochemistry* **20,** 2492–2497.

Wofsy, L., Metzger, H., and Singer, S. J. (1962). *Biochemistry* **1,** 1031–1039.

Wrenn, S. M., and Homcy, C. J. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 4449–4453.

Yeung, C. W. T., Moule, M. L., and Yip, C. C. (1980). *Biochemistry* **19,** 2196–2203.

Yip, C. C., Moule, M. L., and Yeung, C. W. T. (1980). *Biochem. Biophys. Res. Commun.* **96,** 1671–1678.

Zisapel, N., and Sokolovsky, M. (1977). *In* "Methods in Enzymology" (W. B. Jakoby and M. Wilchek, eds.), Vol. 46, pp. 572–582. Academic Press, New York.

# *Chapter 10*

# *CYCLIC NUCLEOTIDES AND ADENYLATE CYCLASE IN BRAIN: ELECTROPHYSIOLOGICAL STUDIES*

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## I. INTRODUCTION

The discovery of cyclic 3′,5′-adenosine monophosphate (cAMP) as a possible intracellular mediator of the glycogenolysis produced by adrenaline in the liver (Sutherland and Robison, 1966) initiated the search in a multitude of laboratories for an analogous function in other systems. These studies have ranged from bacteria (Moses, 1976) to man (Cramer, 1977) and from the control of gene transcription (Cramer and Schultz, 1977) to the interactions between brain cells (Daly, 1977). As a putative intracellular mediator of hormonal actions on a variety of cells, cAMP was dubbed a "second messenger" (Robison *et al.*, 1971). The possible role of cyclic 3′,5′-guanosine monophosphate (cGMP) was not appreciated until some years after the discovery of cAMP.




ISBN 0-12-470350-X

This chapter is concerned with the examination of cyclic nucleotide function in the mammalian brain using electrophysiological methods. The rationale underlying such studies is that the application of exogenous cyclic nucleotides should mimic the cellular changes thought to be mediated by the intracellular compounds.

## II. THE ELECTROPHYSIOLOGICAL ANALYSIS OF CYCLIC NUCLEOTIDE ACTION

Electrophysiological studies of cyclic nucleotide responses have been performed on anesthetized animals *in vivo* and on slices of brain *in vitro*. In addition, the techniques used fall into two groups: those in which compounds are superfused over an area of brain (normally *in vitro*) and those in which compounds are applied into the vicinity of individual cells in the brain by microiontophoresis (Curtis, 1964).

Microiontophoresis (also known as microionophoresis and microelectrophoresis) involves the passage of nanoampere currents through drug solutions contained in glass capillary electrodes having a tip size of about 1 μm. The passage of a positive (outward or cationic) current through the solution to ground or an indifferent electrode elsewhere in the animal will cause a positively charged ion to pass out of the barrel into the brain. Conversely, a negative (inward or anionic) current will effect the ejection of an anion.

Microiontophoresis was originally used at the neuromuscular junction (Nastuk, 1952; Del Castillo and Katz, 1955) and was later applied to the CNS by Curtis and Eccles (1958). As usually practiced the technique involves a multibarreled micropipette (five or seven barrels) to permit a comparison of responses to several agents.

With that brief introductory background, these techniques will now be considered in more detail.

### A. Studies *in Vivo*

#### *1. Animal Preparation*

Rats or cats are usually used for electrophysiological experiments, full surgical anesthesia being induced by urethane (Sigma Chemicals), 1.5 g/g, given intraperitoneally (ip) as a 25% w/v solution in water; pentobarbitone, 40 mg/kg (ip); or halothane (Fluothane ICI) (0.8 to 1% in air or oxygen) by continuous inhalation. In rats urethane will produce anesthesia lasting at least 12 hr, whereas pentobarbitone requires additional injections to maintain anesthesia. Some work-

ers insert a tracheal cannula at this stage to facilitate the clearing of mucus in long experiments. Users of halothane may induce anesthesia with ether, insert a tracheal cannula, and then administer halothane directly into the cannula. If it is intended to record intracellularly from neurons, then additional measures, such as the production of bilateral pneumothorax (with a consequent need for artificial respiration by a small animal pump), may be required to stabilize the preparation.

When anesthetized, the animal is mounted in a frame designed to prevent head movements. (Suitable stereotaxic frames are made by Kopf Instruments and by Trent-Wells.) In ambient temperatures of about 20°C the animal's core temperature must be maintained by either an infrared lamp or a heating blanket beneath the body. An inexpensive unit for maintaining body temperature by a blanket regulated automatically from a rectal probe is available from Bioscience.

The area of CNS of interest may now be exposed and the dura mater cut and folded back. It is particularly important from this point to ensure that the exposed brain surface is not allowed to become dry. It may be covered with cotton wool soaked with artificial cerebrospinal fluid (CSF) or with soft petroleum jelly or agar. Alternatively, a barrier can be constructed around the skull either by stitching the scalp to a ring held above the head or by building a wall of molten paraffin wax in which a pool of artificial CSF can be maintained. It should be noted that some authors use a pool of saline, not CSF, but over the course of many hours (often 12–24 hr) over which these experiments are conducted, the use of saline may cause some local alteration of extracellular calcium and magnesium levels.

Stability of the brain is rarely a problem for extracellular recordings of unit activity, particularly if the cells of interest are several millimeters deep. For intracellular work and studies of superficial neurons, however, some measures may be needed to reduce cardiovascular and respiratory pulsations. The use of bilateral pneumothorax and artificial respiration has been mentioned above. The exposed surface of brain may also be covered with a firm (4 or 5%) solution of agar, or a small rigid plate with a hole through which the electrode can pass may be held in place on the brain surface. It is essential, of course, that the plate pressure does not compromise the patency of the superficial blood vessels.

### 2. *Electrodes*

Electrodes are the most critical components of an electrophysiological experiment. For recording the activity extracellularly of single neurons, microelectrodes may be constructed of glass or metal. However, glass electrodes are much simpler and more rapid in construction and are cheaper. They are preferred for acute experiments, especially when recording is to be combined with microiontophoresis. Metal electrodes will not, therefore, be discussed further here, but

their manufacture has been described by other authors (Hubel, 1957; Merrill and Ainsworth, 1972).

In constructing a single recording microelectrode, a suitable piece of glass tubing is mounted so that heat can be applied, usually by a metal coil around the center of the tube. Tubing is normally of borosilicate glass and can have any diameter desired, though 1 mm internal diameter would be typical. If plain glass capillaries are used, a special filling procedure will be required later. It is infinitely preferable to use "omega-dot" tubing, which has a glass fiber fused along the length of the tube on its internal wall. This glass filament allows pulled electrodes to be filled simply and quickly by capillary action immediately before use, any solution introduced into a barrel being drawn the length of the pipette into the extreme tip. This tubing can be purchased from WPI Instruments (United States) or Clark Electromedical (United Kingdom).

The tubing is "pulled" to give two microelectrodes by heating the middle of the tube and pulling apart the two halves. Although this process was performed by hand in the early days of electrophysiology, it is not to be recommended if any degree of reproducibility is to be attained. A variety of pullers are available commercially, some of which are organized vertically [Kopf Instruments (United States); Bioscience and Campden Instruments (United Kingdom); Narashige (Japan)] and some horizontally [Clark Electromedical (United Kingdom); Narashige (Japan)]. Choice of puller is largely personal, though horizontal machines are often preferred for the finer, intracellular electrodes, while the vertical Narashige may be needed to accommodate large-diameter tubing and large multibarrels.

Electrode characteristics, such as the length of shaft, angle of taper, size of pulled tip, and ease of breaking, can be altered to a large degree by changing the degree of heating, the amount of pull, and the timing of the pull of the electrode. The best combination for any particular situation is best found by trial and error. For extracellular recording the majority of pulled electrodes will have too small a tip and too high a resistance and require breaking back. This is readily achieved by positioning the electrode on a microscope stage and with a magnification of about 50× bringing it up to just touch a solid object held in the field of view. (The author uses a small glass sphere, which can be moved by a micromanipulator in the field of view.)

The object of breaking back is to produce a tip resistance of less than 10 MΩ when containing electrolyte. Depending on the other electrode characteristics, this should involve a tip opening of ~1–2 μm. Once pulled and broken, therefore, each pipette requires filling and its resistance must be measured.

For filling, filament-containing electrodes merely require solution to be introduced into the barrel by a fine plastic or metal cannula as mentioned earlier. Electrodes without filaments may be filled by immersing in methanol under vacuum and subsequently replacing the methanol with electrolyte solution. Time

must then be allowed for diffusional exchange of methanol and electrolyte. Alternatively, solution may be placed into the body of the electrode, and this then centrifuged at low speed so as to force fluid into the tips. All solutions used in micropipettes should be as free as possible of solid matter (such as undissolved drug and dust particles) as these will tend to block the pipette orifice and thus increase electrode resistance.

Although most of the above comments refer to single microelectrodes, exactly similar considerations apply to multibarrel assemblies. Some workers use very large assemblies such as those from Wesley Coe (Cambridge, United Kingdom), in which each barrel has a diameter of 4 mm at its outer end. The outer 2 or 3 cm of each barrel are bent outward to reduce the possibility of interbarrel contamination, which can occur with nonsplayed varieties. The splayed arrangement also facilitates the introduction of the current-carrying lead. These larger assemblies, which are partially pulled during manufacture, require a large, heavy puller such as the vertical Narashige. Smaller-scale versions of these splayed blanks, compatible with iontophoretic and pressure ejection, are available from Medical Systems Corporation (United States).

Most popular are the fused assemblies of five or seven filament-containing tubes available from WPI or Clark Electromedical. These can be pulled perfectly well on a smaller puller, broken back to 4 to 8 μm overall tip diameter, and filled by capillary action after introducing solution into the barrel. Great care must be taken, however, to prevent spillover between barrels. The cut-open end of the electrode must be kept perfectly dry at all times.

A majority of workers using microiontophoresis record cellular activity through one barrel of the multibarrel assembly, often the central one as this tends to have a slightly larger tip and thus lower resistance. However, if a separate single electrode is used for recording and attached to the multibarrel (Stone, 1973), then far superior recordings can be obtained and smaller cells can be samples. In addition, this kind of "piggy-back" arrangement allows a variable separation between the recording and drug ejection sites, which means that drugs could be applied, for example, to cortical pyramidal cell or cerebellar Purkinje cell dendrites while recording from the cell bodies several tens of μm distant. This type of electrode arrangement would also be necessary for recording intracellular responses to extracellularly applied substances.

Although a variety of specialized manipulators have been described for aligning microelectrodes, it is perfectly feasible to construct an arrangement of this type in less than 10 min with no special apparatus. The two electrodes can be approximately aligned using Plasticine, small adjustments to the desired position being made under microscopic control. A suitable resin such as Rapid-Araldite (Ciba), which hardens in about 5 min, is then carefully applied between the electrodes, giving time for final adjustment microscopically. Alignment is greatly assisted by bending the single electrode at the shank.

### 3. *Solutions*

In theory the concentration of drug in solution should not affect its ejection by microiontophoresis. This is because, assuming the drug is dissolved by distilled or preferably deionized water, any current passed should be carried by drug ion, since

$$I = it/Fn$$

where $I$ = ionic flux in gram ions $sec^{-1}$, $i$ = current flow, $n$ = valency, $F$ = Faraday (96,500 coulombs), $t$ = transport number (fractional current carried by ion).

In practice, however, there are several complicating factors. First, because of the physical properties of glass, there is an electrical double layer inside the tip such that the solution itself carried a positive charge. The passage of outward (positive) current will therefore tend to eject bulk solution. Second, it is almost impossible to prevent some diffusion of drug out of the pipette tip, even when a small holding (*braking, backing,* or *retaining*) current is applied. For these two reasons it is desirable to reduce drug concentrations.

On the other hand, a 10 or 20 m*M* solution may result in the influx of water by osmosis, with resulting local distortion of the tissue. Most authors, therefore, use solutions which are approximately isoosmolar with body fluids or, if very potent compounds are involved, solutions which are brought to isoosmolarity with NaCl.

Some attention also has to be paid to the p*K* of atoms or groupings in the drug molecule, with adjustment of pH being made where necessary to ensure that a substantial fraction of the compound is in one or other ionic form. It is clearly unsatisfactory to use a compound which exists largely as a zwitterion or as a mixture of cationic and anionic form, as the use of a "retaining" current to prevent spontaneous diffusional leakage would be impracticable. If pH adjustment is made, it is always advisable to perform control ejections of $H^+$ or $OH^-$ from saline or CSF at that pH. It seems to be the case, however, that the ejection of these ions is only problematic from solutions of pH 3 or less or pH 10 and above, unless very large amounts of charge are being passed (say 100 nA for 10 min) or large molecules are being studied whose ionic mobility is much less than $H^+$ or $OH^-$.

### 4. *Penetration*

Whether one barrel of the multibarrel or a separate single electrode are used for recording, it should be filled with a strong electrolyte such as 3 *M* NaCl, KCl, or potassium acetate, the latter being used routinely in the author's laboratory.

The electrode assembly is now mounted in a suitable micromanipulator. Satis-

factory extracellular recordings can be achieved using a direct manually driven manipulator, but hydraulic systems which can be operated remotely either manually (Clark Electromedical, United Kingdom) or electronically (Trent-Wells, Kopf, Haer, Narashige, Burleigh) are preferable.

### 5. *Extracellular Recording*

In order to minimize the loss of recordable signal, an amplifier is required which has reasonably high input impedance at the input stage or probe (usually around $10^{10}$ to $10^{12}$ $\Omega$) and which is designed for AC recording. There are literally dozens of excellent, suitable amplifiers currently available, and the choice can thus be made by consultation, depending on specific usage and compatibility with existing equipment. The various modular systems available present maximum flexibility with moderate price.

For extracellular recording an amplification of at least 100 should be used in order to boost the recorded signals (around 100–1000 $\mu$V) to levels observable on an oscilloscope. Amplifiers should also be low noise so as to facilitate separation of signal from baseline. The recording lead into the electrode solution should be of platinum or chlorided silver to reduce noise and polarization artifacts.

Careful screening and grounding of the animal and surrounding equipment should eliminate much of the interference picked up from fluorescent lights and AC cables when recording low-level signals, but some additional filtering will usually be required. Unless DC lighting is available, as in the author's laboratory, it may be preferable to experiment without lights.

In hospital environments radiofrequency interference may be a nuisance from call systems, but these can be eliminated by the use of suitable screening and a radiofrequency filter.

After the amplification stage action potentials may be displayed on oscilloscopes and passed through an audio amplifier. The latter greatly improves the detection of cell activity and is an invaluable aid at monitoring continuously the state of a cell or the action of a drug while the experimenter is performing some other task.

Permanent records of the raw signal may be stored on magnetic tape, perhaps at a sufficiently high speed to allow later replay within the frequency capability of a pen recorder or on photographic film. The Grass and Nihon Koden oscilloscope cameras are ideal for this purpose.

Alternatively, data may be processed before recording. In many microiontophoresis laboratories this is achieved by discriminating action potentials from background noise (physical and/or biological) using a window discriminator and then counting the discriminated output pulses on a ratemeter. The ratemeter output can then be fed into a chart recorder to give a hand copy of firing rate in spikes per second.

### 6. *Microiontophoresis*

The last 5 years have seen the introduction of several types of microiontophoresis equipment from commercial suppliers whereas previously, equipment was homemade. Those available are from WPI, Dagan, and Medical Systems Corporation. The latter (Neurophore) has the best specifications of the three systems and offers the very advantageous flexibility of interchanging modules for microiontophoretic ejection with modules for pressure ejection. This last facility is invaluable when using nonionized or high molecular weight molecules, which are difficult to apply by microiontophoresis.

Using these systems alone or in combination with an external clock, sequences of compounds can be applied in carefully regulated time cycles. This is an important consideration because the passage of current during an ejection period alters the ionic composition of fluid at the barrel tip. During the "pause" period the solution will tend to return toward equilibrium. Only by having a constant cycle and eject period, therefore, will the ejection conditions be exactly reproducible from one cycle to the next. This problem is made greater if a retaining current, which tends to remove ions from the barrel tip, is applied during the pause phase. These ideas have been developed by Bradshaw *et al.* (1973).

For similar reasons several ejections of a drug may be required before an equilibrium is established within the electrode tip and responses become constant, though it must be confessed that the relative importance of equilibrium within the tip and outside it (diffusion, uptake, metabolism) is unknown.

The possible need for pH controls has been mentioned above. Probably the greatest source of confusion in microiontophoresis, though, is from the use of electrical current very close to cell bodies. The direction of response is normally predictable: an outward (positive) current causing neuronal inhibition and an inward (negative) current excitation, though if the electrode tip is pressing on the cell membrane or has damaged some part of the cell such as a large dendrite, the opposite pattern can be seen. It is therefore always advisable to include NaCl or CSF in one barrel of a multibarrel so that the effect of current alone can be tested on a cell. Current artifacts may also be controlled for to some extent by "current balancing," which is achieved by passing through a NaCl barrel a current equal to the sum of the currents passing through the other barrels but opposite in sign, or by returning the drug barrel currents to ground through the saline barrel. The object of the exercise is to minimize current passage away from the electrode tip through the neurons.

One of the greatest drawbacks of microiontophoresis is that the drug concentration at the cell surface is unknown, and hence, drug dosages are normally expressed as the neuroamperes of current, or the charge in nanocoulombs (nA $\times$ sec) used to eject the compounds. The amount of drug ejected for a given current, however, may be estimated by ejecting the radioactively labeled mate-

rial into saline, agar blocks, or blocks of brain, and this has been attempted for cAMP by Shoemaker *et al.* (1975).

### 7. *Intracellular Recording*

Besides several extra rations of patience, intracellular recording *in vivo* necessitates some modifications of the preceding methods. First, the preparation has to be absolutely stable, and aids to achieving this, including pneumothorax with artifical respiration and a firm covering or pressure plate over the exposed brain, have already been mentioned. In addition, stability may be helped by preventing any increase of CSF pressure within the ventricular system. If the cranio-vertebral junction is exposed, a fine needle can be used to penetrate the dura at the cisterna magna, though it may be necessary to leave the needle or a fine cannula in position so as to ensure a free exit for the CSF.

Second, electrodes must be fine enough to penetrate cell membranes without causing severe damage. The construction of suitable electrodes approaches an art, which can only be learned by trial and error. The use of a horizontal puller and thin (e.g., 1 mm internal diameter) capillary tubing should facilitate the production of good electrodes. Freshly pulled and unbroken electrodes should have resistances greater than about 50 MΩ and are adequate for most intracellular work. A relatively recent innovation has been the beveling of electrode tips. The freshly pulled electrode is held firmly in a micromanipulator and lowered at an angle, tip downwards, toward a flat rotating disc. Commercial apparatus is available (WPI Instruments). The object is to produce a beveled tip at which the angled orifice has a much lower resistance (10–20 MΩ) than originally, but which is at least as fine at the extreme tip so as to ensure clean cell penetration.

A DC amplifier is used for intracellular work and some, such as the WPI M-707 microprobe system, possess many features of great usefulness. Particularly important is a current injection facility so that small (nanoampere) currents can be injected via the microelectrode. The size of the resulting voltage change on the oscilloscope recording is a reflection of cell membrane resistance.

## B. Studies *in Vitro*

Most of the above considerations of electrodes, equipment, etc., apply equally to *in vitro* studies on brain slices. The main difference is in the setting up of a suitable preparation. Slices of various regions of brain have been successfully studied *in vitro*, including cerebral cortex (Li and McIlwain, 1957; Yamomoto and McIlwain, 1966), olfactory cortex (Richards and Sercombe, 1968; Richards, 1981), cerebellum (Yamamoto, 1974), and, most popularly, hippocampus

(Schwartzkroin, 1975, 1981; Richards, 1981). Such slices have the advantages that no anesthetic is present in the slice [animals may be initially anesthetized with urethane, as this anesthetic washes out of brain tissue very rapidly (Bagust and Kerkut, 1981)], small areas of brain can be localized directly by vision, and the local conditions of oxygen tension, temperature, ionic concentrations, etc., can be precisely regulated and monitored. In addition, drugs can be perfused over the preparation at known concentrations and good intracellular recordings are more feasible because of the lack of tissue movement.

In essence, the desired region of brain is dissected out of the brain as rapidly as possible after death and transferred into artificial CSF. Most workers keep this solution at 0 to 5°C to reduce tissue metabolism and improve the firmness of the tissue for chopping. The tissue is next chopped into slices, using either a vibratome or a McIlwain tissue chopper (Mickle Labs, United Kingdom). Slices are usually cut about 200 to 400 μm thick, depending on the size of cells to be studied and how much internal organization of circuitry it is hoped to preserve. It is generally considered that oxygen diffusion will maintain cells up to 300 μm below each surface of the slice (McIlwain, 1975). Individual slices may now be moved into the recording chamber. The temperature of the CSF bathing slices should be around 36°C, with a flow of moistened oxygen over any surface which is left exposed for visualization or access purposes. The solution itself must be thoroughly oxygenated. After allowing 1–2 hr for a slice to equilibrate at the fluid temperature and ionic gradients to reestablish themselves following the trauma of preparation, recordings may be made as for *in vivo* studies. Further discussion of slice techniques and their uses may be found in works by McIlwain (1975) and Kerkut and Wheal (1981).

## III. A REVIEW OF THE LITERATURE

The literature concerning cyclic nucleotides and the nervous system is vast, and in the following sections an attempt will be made to review only those aspects of cyclic nucleotides in which the author has a particular interest, namely, the interactions with amino acids and their electrophysiological actions. Other useful sources of reference are Daly (1977), Cramer and Schultz (1977), Robison *et al.* (1971), Daly (1976), Drummond (1973), Phillis, (1977), Kupferman (1980), Bloom (1975), Nathanson, (1977), and Greengard (1978).

Amines as well as amino acids have been shown to elevate the levels of cAMP in brain (Sattin and Rall, 1970; Sattin *et al.*, 1975; Daly, 1976, 1977), and the question arises whether the cyclic nucleotides mediate the acute effects of these putative neurotransmitters on the postsynaptic neuron, as envisaged by the "second messenger" hypothesis. Such changes would include effects on membrane potential and ionic conductance.

Very little electrophysiological work, however, seems to have researched the possible involvement of nucleotides in amino acid responses. Emphasis has, however, been placed on the amines, and this work will form the bulk of the following summary.

## A. Amines and cAMP

One of the first electrophysiological studies of cAMP responses in the CNS was performed by Hoffer *et al.* (1971). This group demonstrated that the application of cAMP by microiontophoresis to Purkinje cells in the cerebellum of anesthetized rats produced a depression of spontaneous activity exactly similar to that produced by noradrenaline (NA). It was also found that amine responses could be prevented by applying β-adrenoceptor blocking drugs, while the cAMP responses were unchanged. However, the application of prostaglandin E, which suppresses adenylate cyclase activation in brain, prevented the responses to NA but not cAMP. Papaverine and other inhibitors of cyclic nucleotide phosphodiesterase potentiated responses to both. Taken together with the elegant demonstrations from the same laboratory that NA elevated Purkinje cell cAMP levels, as revealed by cytochemical methods (Siggins *et al.*, 1973), these results implied that adenylate cyclase activation may be involved in the depression of neuronal firing by NA (Bloom *et al.*, 1973, 1975). Later, it was shown that the depression of firing induced by electrical stimulation of the noradrenergic neurons of the locus coeruleus could be modulated in similar fashion by agents altering cyclase activity (Hoffer *et al.*, 1973).

Intracellular recordings further revealed that both NA and cAMP produced hyperpolarization of the Purkinje cells but that this effect was mediated, unusually, by an *increase* of membrane resistance (Siggins *et al.*, 1971a) (Fig. 1). The unique nature of this inhibition served strongly to support the view that the same membrane mechanisms were being activated by NA and cAMP.

Since those early experiments, similar work has suggested the involvement of cAMP in amine responses in the hippocampus (Segal and Bloom, 1974), caudate nucleus (Siggins *et al.*, 1974; Bunney and Aghajanian, 1974), and cerebral cortex (Stone and Taylor, 1977; Jones and Roberts, 1979). Both NA and cAMP were also found to depress cell firing in the brainstem (Anderson *et al.*, 1973), although no compelling evidence was produced for a meaningful relationship between these effects.

Some of the observations described so far have been challenged by other groups who have failed to repeat critical observations or have emphasized the pharmacological nonspecificity of some of the drugs used (Lake and Jordan, 1974; Godfraind and Pumain, 1971). These critical reports and the replies to them (Siggins *et al.*, 1971b; Bloom *et al.*, 1974; Bloom, 1975) are most instructive for anyone contemplating microiontophoresis.

One such criticism was that cAMP would not be able to pass across cell

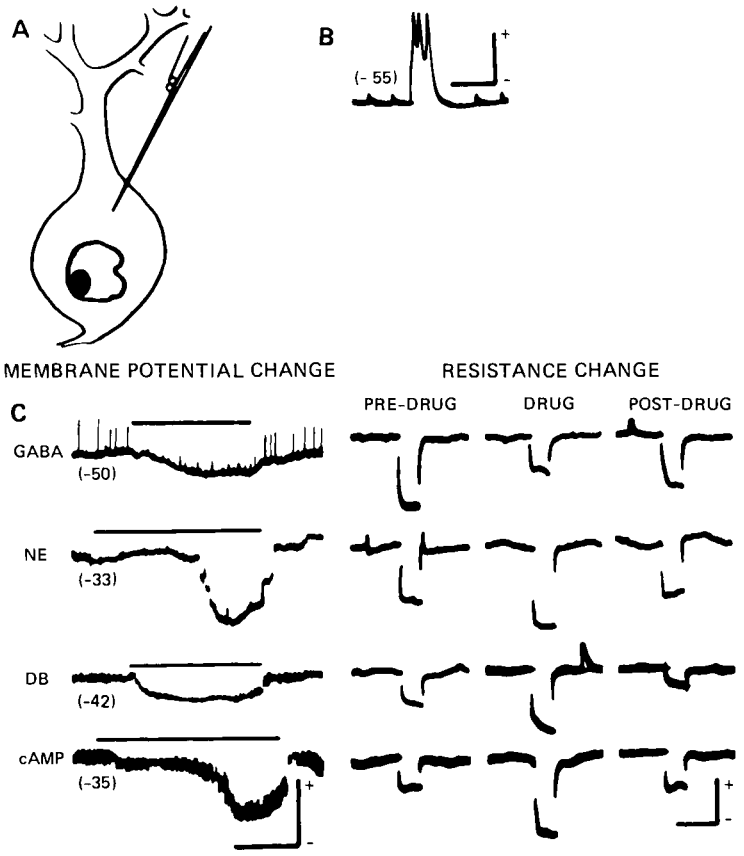


**Fig. 1.** Intracellular recordings from rat cerebellar Purkinje cells. (A) Schematic representation of a three-barreled micropipette with a Purkinje cell. The intracellular electrode protrudes beyond the orifices of the two extracellular microelectrophoretic barrels. (B) Multispiked spontaneous climbing fiber discharge obtained during intracellular recording from a Purkinje cell. Number in parentheses is resting potential in millivolts (mV); calibration bars are 20 msec and 25 mV. (C) Changes in membrane potential and membrane resistance of four Purkinje cells in response to γ-aminobutyrate (GABA), noradrenaline (NE), dibutyryl cAMP (DB), and cAMP. All specimens in each horizontal row of records are from the same cell. Solid bar above each record indicates the extracellular electrophoresis of the indicated drug (100–150 nA). Number in parentheses below each recording is resting potential (mV); calibration bar under membrane potential record is 10 sec and 20 mV for NE, DB, and cAMP, and is 5 sec and 10 mV for GABA. The effective input resistance was judged by the size of pulses resulting from the passage across the membrane of a brief constant current (1 nA) pulse before, during, and after electrophoresis of the respective drugs (1 mV = 1 MΩ). Discontinuities in the fast transients of the pulses result from the loss of high frequencies (>10 kHz) and from the chopped nature of the frequency-modulated magnetic tape recording used. All "pulse" records have been graphically normalized to the same baseline level. Calibration bar on right indicates 80 msec and 15 mV for all pulse records. (From Siggins *et al.*, 1971a, with permission of the American Association for the Advancement of Science. Copyright © 1971 by the American Association for the Advancement of Science.)

membranes, and with the demonstration that adenosine itself could depress cell firing (Phillis *et al.*, 1974), it became important to show that the depressant effects of these two purines could be distinguished. This was reported in 1978 (Taylor and Stone, 1978), using theophylline to block the adenosine receptor.

As it became clear that an important action of cAMP was the activation of protein kinases, an ingenious test of the cAMP hypothesis was carried out by Siggins and Henriksen (1975). This group applied by microiontophoresis to Purkinje cells a series of cAMP analogs with differing abilities to activate cellular protein kinases. The results implied a clear correlation between protein kinase activation and neuronal depression.

As neurochemical research began to suggest that the activation of cholinergic muscarinic receptors induced an increase in the concentration of cGMP, a study was carried out to compare cAMP and cGMP responses. A clear correlation was shown between neuronal depressant responses to NA and cAMP (Stone *et al.*, 1975; Jones and Roberts, 1979), but it was also noted that cGMP applied by microiontophoresis increased the firing of most cells tested (Stone *et al.*, 1975) (Fig. 2). This excitation correlated remarkably well with excitatory responses to acetylcholine. In addition, cells depressed by acetylcholine were depressed by cGMP.

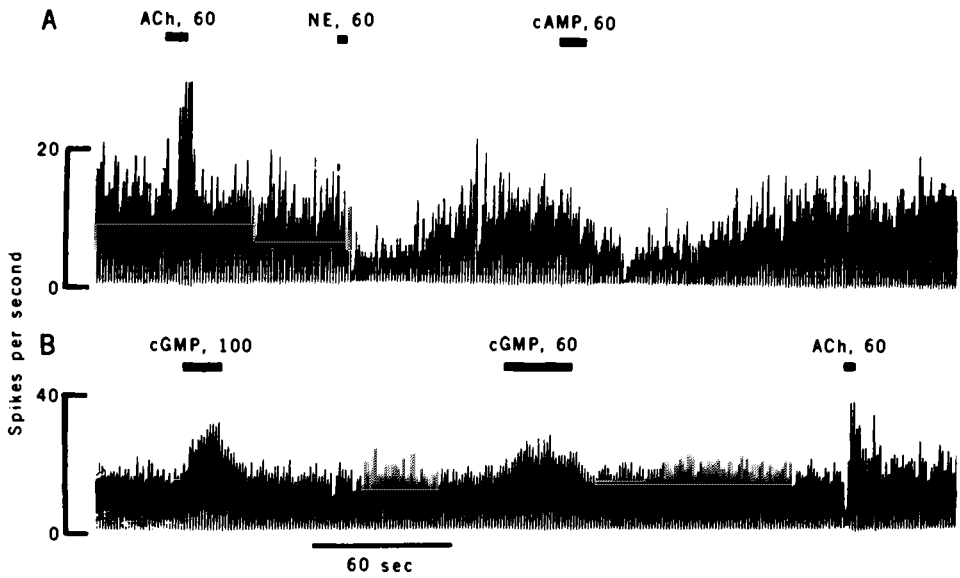


**Fig. 2.** Rate meter records of the firing rates of two different neurons in the cerebral cortex in response to the microiontophoretic application of (A) acetylcholine (ACh), 60 nA; norepinephrine (NE), 60 nA; and dibutyryl cAMP, 60 nA; and (B) cGMP, 100 nA and 60 nA; and ACh, 60 nA. The duration of drug application is indicated by the bars. (From Stone *et al.*, 1975. Copyright © 1975 by the American Association for the Advancement of Science.)

An extracellular study by Phillis *et al.* (1974) observed an excitation by cGMP of more than half the cells tested in cerebral cortex, although the authors were not able to correlate these with sensitivity to acetylcholine. Hoffer *et al.* (1971) had earlier reported a depressant action of cGMP on cerebellar Purkinje neurons, but most of these cells do not normally respond to acetylcholine. Purkinje cells in the mouse, however, are usually excited by acetylcholine, and many of these cells proved to be activated also by cGMP (Siggins *et al.*, 1976). In the hippocampus pyramidal cells tend to be excited by both acetylcholine and cGMP (Hoffer *et al.*, 1977).

Several groups have attempted to repeat and extend the original extracellular work discussed above. Probably the clearest of these studies was by Swartz and Woody, (1979) who applied cGMP intracellularly to neurons in the cerebral cortex and observed a decrease of membrane conductance, which could also be obtained by the extracellular application of acetylcholine.

A pair of papers appeared from Krnjević *et al.* (1976) and Krnjević and Van Meter (1976), reporting studies in which cyclic nucleotides and acetylcholine were applied by microiontophoresis to spinal motoneurons. These papers provide the most detailed insight into the possible role of intracellular nucleotides of any comparable mammalian study. Thus, cGMP injected intracellularly produced an increased membrane conductance, an accelerated time course of action potentials, and an enhancement of postspike hyperpolarization. Changes of membrane potential were variable but usually depolarizing in direction. As acetylcholine also produced depolarization, this study supports qualitatively the similarity of action of acetylcholine and cGMP reported in cerebral cortex (Stone *et al.*, 1975). Indeed, Krnjević *et al.* (1976) also noted that larger amounts of cGMP ejected extracellularly induced a similar spectrum of actions as when applied intracellularly, implying that some of the nucleotide can cross cell membranes. Although this finding seems to have come as a surprise to the observers, it is well known that cyclic nucleotides can pass across cell membranes in an outward direction (Cramer, 1977).

Apart from the similar changes of membrane potential, most of the effects of cGMP and acetylcholine were found to be dissimilar, acetylcholine producing, for example, an increase of membrane resistance, a slowing of spike repolarization and no effect on the postspike hyperpolarization (Krnjević *et al.*, 1976). The authors therefore concluded that cGMP could not be viewed as a second messenger for cholinergic actions. There are, however, two major criticisms of this work which should be emphasized. The first is generally applicable to studies in which compounds are applied intracellularly, and is that the compounds are being injected into an exceedingly complex system in which substances would normally be restricted to specific metabolic compartmentation. Responses to injected materials may therefore never be observed physiologically.

Second, Krnjević *et al.* (1976) took the unorthodox step of applying the

acetylcholine intracellularly also, a site which presumably is never normally reached by acetylcholine. Interesting though their results are, then, it is totally unclear whether these observations can have any relevance to the question of whether external transmitter acetylcholine can produce the same effects as the intracellular cGMP whose formation it engenders.

In a separate study from the same laboratory (Krnjević and Van Meter, 1976), it was noted that intracellularly applied cAMP tended to hyperpolarize cells, a finding consistent with many of the studies described above and supporting the view that cAMP and cGMP may have opposite effects on membrane potential (Stone *et al.*, 1975). In addition, it was reported that the cAMP hyperpolarization was associated with an increased membrane resistance, as noted by Siggins *et al.* (1971a).

## B. Adenosine and cAMP

Adenosine is a potent inhibitor of neurotransmitter release from peripheral and central nerve terminals (see Stone, 1981). Adenosine is also known to increase neuronal cAMP concentrations (Sattin and Rall, 1970; Sattin *et al.*, 1975; Daly, 1976, 1977), and the question arises whether any relationship exists between these actions. In early studies a marked similarity was noted between adenosine's inhibition of synaptic activity in the olfactory cortex slice and its activation of cyclase (Kuroda *et al.*, 1976). Thus, both actions exhibited a similar time course, and a correlation was apparent between the effects of various purine analogs on synaptic potentials and cAMP.

However, in later studies using techniques which permitted nucleotide analysis on a time scale of seconds, it was found that the changes of cAMP levels lagged appreciably behind the electrophysiological changes (Kuroda, 1978). Related observations were made by Reddington and Schubert (1979).

Dunwiddie and Hoffer (1980) and Smellie *et al.* (1979) have demonstrated opposite effects of adenosine and 8-parachlorophenylthio cAMP on hippocampal postsynaptic potentials with no correlation between their electrophysiological actions and activation of adenylate cyclase. Scholfield (1978) has also reported the failure of cAMP and dibutyryl cAMP to mimic the depressant effects of adenosine.

## C. Opiates

In spite of the interest which has developed in the possible involvement of adenylate cyclase in opiate tolerance and dependence (Brandt *et al.*, 1976, 1980; Minneman and Iversen, 1976; Collier, 1980) dating from the *in vivo* demonstration that cAMP could reverse morphine analgesia (Ho *et al.*, 1973)

and that morphine would suppress the activation of cyclase by prostaglandins (see Collier, 1980), relatively few electrophysiological studies have pursued this interaction. Although evidence has been produced for the blockade of opiate effects by methylxanthines (Stone and Perkins,1979; Perkins and Stone, 1980), this has been related more to an adenosine receptor than to cAMP production (Stone, 1982). Studies of myenteric neurons (Karras and North, 1979), spinal cord neurons (Duggan and Griersmith, 1979), and most recently brainstem neurons (Hosford and Haigler, 1981) have failed to modify opiate actions electrophysiologically by substances thought to alter the cyclase pathway.

### D. The Use of Drugs

One of the lessons which has been learned during studies of cyclic nucleotides is that the specificity of any compounds used to modify the system must be as great as possible. For many years methylxanthines such as caffeine and theophylline have been employed as inhibitors of cyclic nucleotide phosphodiesterase. It is now widely recognized that this action is only significant at concentrations an order of magnitude greater than those which block extracellular receptors for adenosine (Sattin and Rall, 1970; Burnstock, 1978; Perkins and Stone, 1980; Daly *et al.*, 1981). It is essential to use only compounds with a relatively specific action such as Ro 20-1724 (Roche) and ICI 63,197 (ICI) as phosphodiesterase inhibitors.

### E. Studies on Peripheral Systems

Although it would be inappropriate to discuss peripheral systems in detail in a chapter devoted to cyclases in brain, a great deal of information on the relationships between acetylcholine, catecholamines, cyclic nucleotides, and membrane potential has been obtained from studies of peripheral ganglia, and these should be consulted for further details (Brown *et al.*, 1979; Busis *et al.*, 1978; Dun *et al.*, 1977; Gallagher *et al.*, 1977; Kobayashi *et al.*, 1978; Hashiguchi *et al.*, 1978; McAfee and Greengard, 1972; Greengard, 1976).

### F. Long-Term cAMP Effects

This chapter has been concerned only with the acute effects of cyclic nucleotides. It has been suggested by McIlwain (1976, 1977, 1978) that intracellular cyclic nucleotides mediate communication between neurons, and between different parts of any one neuron on a time scale of minutes or longer, a role which may have more relevance to neuronal plasticity and the control of

neuronal growth and development than to synaptic transmission as such. It is likely that much more experimental attention will be paid to this area in the future.

## REFERENCES


Anderson, E. G., Haas, H. L., and Hösli, L. (1973). *Brain Res.* **49,** 471–475.

Bagust, J., and Kerkut, G. A. (1981). *Neuropharmacology* **20,** 73–78.

Bloom, F. E. (1975). *Rev. Physiol., Biochem. Pharmacol.* **74,** 1–104.

Bloom, F. E., Chu, N. S. Hoffer, B. J., Nelson, C. N., and Siggins, G. R. (1973). *Neurosci. Res.* **5,** 53–72.

Bloom, F. E., Siggins, G. R., and Hoffer, B. J. (1974). *Science* **185,** 627–629.

Bloom, F. E., Siggins, G. R., Hoffer, B. J., Segal, M., and Oliver, A. P. (1975). *Adv. Cyclic Nucleotide Res.* **5,** 603–618.

Bradshaw, C. M., Roberts, M. H. T., and Szabadi, E. (1973). *Br. J. Pharmacol.* **49,** 667–677.

Brandt, M., Gullis, R. J., Fischer, K., Buchen, C., Hamprecht, B., Moröder, L., and Wunsch, E. (1976). *Nature (London)* **262,** 311–313.

Brandt, M., Buchen, C., and Hamprecht, B. (1980). *J. Neurochem.* **34,** 643–651.

Brown, D. A., Caulfield, M. P., and Kirby, P. (1979). *J. Physiol. (London)* **290,** 441–451.

Bunney, W. E., and Aghajanian, G. K. (1974). *Life Sci.* **15,** 309–318.

Burnstock, G. (1978). *In* "Cell Membrane Receptors for Drugs and Hormones" (L. Bolis and R. W. Straub, eds.), pp. 107–118. Raven Press, New York.

Busis, N. A., Weight, F. F., and Smith, P. A. (1978). *Science* **200,** 1079–1080.

Collier, H. O. J. (1980). *Nature (London)* **283,** 625–629.

Cramer, H. (1977). *J. Neurosci. Res.* **3,** 241–246.

Cramer, H., and Schultz, J. (1977). "Cyclic 3′5′ Nucleotides: Mechanisms of Action." Wiley, New York.

Curtis, D. R. (1964). *In* "Physical Techniques in Biological Research" (W. L. Nastuk, ed.), pp. 144–183. Academic Press, New York.

Curtis, D. R., and Eccles, R. M. (1958). *J. Physiol. (London)* **141,** 435–445.

Daly, J. W. (1976). *Life Sci.* **18,** 1349–1358.

Daly, J. W. (1977). "Cyclic Nucleotides in the Nervous System." Pergamon, Oxford.

Daly, J. W., Bruns, R. F., and Snyder, S. H. (1981). *Life Sci.* **28,** 2083–2097.

Del Castillo, J., and Katz, B. (1955). *J. Physiol. (London)* **128,** 157–181.

Drummond, G. I. (1973). *Prog. Neurobiol.* **2,** 121–176.

Duggan, A. W., and Griersmith, B. T. (1979). *Br. J. Pharmacol.* **67,** 51–58.

Dun, N. J., Kaibara, K., and Karczmar, A. G. (1977). *Neuropharmacology* **16,** 715–717.

Dunwiddie, T. V., and Hoffer, B. J. (1980). *Br. J. Pharmacol.* **69,** 59–68.

Gallagher, J. P., and Shinnick-Gallagher, P. (1977). *Science* **198,** 851–853.

Godfraind, J. M., and Pumain, R. (1971). *Science* **174,** 1257–1258.

Greengard, P. (1976). *Nature (London)* **260,** 101–107.

Greengard, P. (1978). *Science* **199,** 146–152.

Hashiguchi, T., Ushiyama, N. S., Kobayashi, H., and Libet, B. (1978). *Nature (London)* **271,** 267–268.

Ho, I. K., Loh, H. H., and Way, E. L. (1973). *J. Pharmacol. Exp. Ther.* **185,** 336–340.

Hoffer, B. J., Siggins, G. R., Oliver, A. P., and Bloom, F. E. (1971). *Ann. N.Y. Acad. Sci.* **185,** 531–549.

Hoffer, B. J., Siggins, G. R., Oliver, A. P., and Bloom, F. E. (1973). *J. Pharmacol. Exp. Ther.* **184,** 553–569.
Hoffer, B. J., Seiger, A., Freedman, R., Olson, L., and Taylor, D. (1977). *Brain Res.* **119,** 107–132.
Hosford, D. A., and Haighler, H. J. (1981). *J. Pharmacol. Exp. Ther.* **219,** 496–504.
Hubel, D. H. (1957). *Science* **125,** 549–550.
Jones, R. S. G., and Roberts, M. H. T. (1979). *Neuropharmacology* **18,** 701–704.
Karras, P. J., and North, R. A. (1979). *Br. J. Pharmacol.* **65,** 647–652.
Kerkut, G. A., and Wheal, H. V., eds. (1981). "Electrophysiology of Isolated Mammalian CNS Preparations." Academic Press, New York.
Kobayashi, H., Hashiguchi, T., and Ushiyama, N. S. (1978), *Nature* (*London*) **271,** 268–270.
Krnjević, K., and Van Meter, W. G. (1976). *Can. J. Physiol. Pharmacol.* **54,** 416–521.
Krnjević, K., Piul, E., and Werman, R. (1976). *Can. J. Physiol. Pharmacol.* **54,** 172–176.
Kupferman, I. (1980). *Annu. Rev. Physiol.* **42,** 629–641.
Kuroda, Y. (1978). *J. Physiol.* (*Paris*) **74,** 463–470.
Kuroda, Y., Saito, M., and Kobayashi, K. (1976). *Brain Res.* **109,** 196–201.
Lake, N., and Jordan, L. M. (1974). *Science* **183,** 663–664.
Li, C.-L., and McIlwain, H. (1957). *J. Physiol.* (*London*) **139,** 178–190.
McAfee, D. A., and Greengard, P. (1972). *Science* **178,** 310–312.
McIlwain, H. (1975). *In* "Practical Neurochemistry" (H. McIlwain, ed.), pp. 105–132. Churchill-Livingstone, Edinburgh and London.
McIlwain, H. (1976). *Neurochem. Res.* **1,** 351–368.
McIlwain, H. (1977). *Neuroscience* **2,** 357–372.
McIlwain, H. (1978). *Prog. Neurobiol.* **11,** 189–203.
Merrill, E. G., and Ainsworth, A. (1972). *Med. Biol. Eng.* **10,** 662–672.
Minneman, K. P., and Iversen, L. L. (1976). *Nature* (*London*) **262,** 313–314.
Moses, V. (1976). *Sci. Prog.* (*Oxford*) **63,** 503–520.
Nastuk, W. L. (1952). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **10,** 96.
Nathanson, J. A. (1977). *Physiol. Rev.* **57,** 157–256.
Perkins, M. N., and Stone, T. W. (1980). *Br. J. Pharmacol.* **69,** 131–137.
Phillis, J. W. (1977). *Can. J. Neurol. Sci.* **4,** 152–195.
Phillis, J. W., Kostopoulos, G. K., and Limacher, J. J. (1974). *Can. J. Physiol. Pharmacol.* **52,** 1226–1229.
Reddington, M., and Schubert, P. (1979). *Neurosci. Lett.* **14,** 37–42.
Richards, C. D. (1981). *In* "Electrophysiology of Isolated Mammalian CNS Preparations" (G. A. Kerkut and H. V. Wheal, eds.), pp. 107–132. Academic Press, New York.
Richards, C. D., and Sercombe, R. (1968). *J. Physiol.* (*London*) **197,** 667–683.
Robison, G. A., Sutherland, E. W. and Butcher, R. W. (1971). "Cyclic AMP." Academic Press, New York.
Sattin, A., and Rall, T. W. (1970). *Mol. Pharmacol.* **6,** 13–23.
Sattin, A., Rall, T. W., and Zanella, J. (1975). *J. Pharmacol. Exp. Ther.* **192,** 22–32.
Scholfield, C. N. (1978). *Br. J. Pharmacol.* **63,** 239–244.
Schwartzkroin, P. A. (1975). *Brain Res.* **85,** 423–436.
Schwartzkroin, P. A. (1981). *In* "Electrophysiology of Isolated Mammalian CNS Preparations" (G. A. Kerkut and H. V. Wheal, eds.), pp. 15–50. Academic Press, New York.
Segal, M., and Bloom, F. E. (1974). *Brain Res.* **72,** 79–97.
Shoemaker, W. J., Balentine, L. T., Siggins, G. R., Hoffer, B. J., Henriksen, S. J., and Bloom, F. E. (1975). *J. Cyclic Nucleotide Res.* **1,** 97–106.
Siggins, G. R., and Henriksen, S. J. (1975). *Science* **189,** 559–560.
Siggins, G. R., Oliver, A. P., Hoffer, B. J., and Bloom, F. E. (1971a). *Science* **171,** 192–194.

Siggins, G. R., Hoffer, B. J., and Bloom, F. E. (1971b). *Science* **174,** 1258–1259.
Siggins, G. R., Battenberg, E. F., Hoffer, B. J., Bloom, F. E., and Steiner, A. L. (1973). *Science* **179,** 585–588.
Siggins, G. R., Hoffer, B. J., and Ungerstedt, U. (1974). *Life Sci.* **15,** 779–792.
Siggins, G. R., Henriksen, S. J., and Landis, S. C. (1976). *Brain Res.* **114,** 53–69.
Smellie, F. W., Daly, J. W., Dunwiddie, T. V., and Hoffer, B. J. (1979). *Life Sci.* **25,** 1739–1748.
Stone, T. W. (1973). *J. Physiol. (London)* **233,** 211–225.
Stone, T. W. (1981). *Neuroscience* **6,** 523–555.
Stone, T. W. (1982). *Biosci. Rep.* **2,** 77–90.
Stone, T. W., and Perkins, M. N. (1979). *Nature (London)* **281,** 227–228.
Stone, T. W., and Taylor, D. A. (1977). *J. Physiol. (London)* **266,** 523–543.
Stone, T. W., Taylor, D. A., and Bloom, F. E. (1975). *Science* **187,** 845–847.
Sutherland, E. W., and Robison, A. R. (1966). *Pharmacol. Rev.* **18,** 145–161.
Swartz, B. E., and Woody, C. D. (1979). *J. Neurobiol.* **10,** 465–488.
Taylor, D. A., and Stone, T. W. (1978). *Experientia* **34,** 481–482.
Yamamoto, C. (1974). *Exp. Brain Res.* **19,** 271–281.
Yamamoto, C., and McIlwain, H. (1966). *J. Neurochem.* **13,** 1333–1343.

# *Chapter 11*

# *CALMODULIN IN THE NERVOUS SYSTEM*

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## I. INTRODUCTION

Investigations over the past two decades have elucidated an expanding role of the calcium ion in nervous system physiology. Calcium is now recognized as an important mediator of stimulus–secretion coupling in both the nervous system (Katz and Miledi, 1969; Miledi, 1973) and in a number of other secretory tissues (Douglas, 1968). In addition, over the past decade a variety of calcium-dependent processes (Rasmussen, 1981) and other second messenger functions (Rasmussen and Goodman, 1977) have been elucidated in many different cel-




ISBN 0-12-470350-X

lular systems. Recently, investigations from several laboratories have demonstrated that many, but not all (Wise *et al.*, 1982), of these calcium-dependent actions are mediated by a specific calcium-dependent regulator protein, calmodulin (Cheung, 1980; Wang and Waisman, 1979). The ubiquitous and highly conserved nature of this calcium binding protein suggest its fundamental status as a regulator of cellular dynamics in the nervous system and elsewhere.

## A. Structural Aspects of Calmodulin

Calmodulin is a heat-stable calcium binding protein with a molecular weight of ~16,700. As sequenced by Watterson *et al.* (1980), the single polypeptide chain contains no cysteine, tryptophan, or hydroxyproline residues but does contain one trimethyllysine, an unusual amino acid. All calmodulins presently characterized contain one trimethyllysine residue. Thirty percent of the molecule is composed of negatively charged aspartate and glutamate residues, accounting for the protein's low p*I* of 4.3. The absence of cysteine and proline residues confers upon calmodulin a rather flexible tertiary structure (Means and Dedman, 1980). This flexibility may play an important role in the protein's interaction with both calcium and target proteins. Calmodulin contains two types of binding domains: one group of four calcium binding sites and a second enzyme–protein binding site. Calmodulin contains four calcium binding sites with varying affinities according to occupancy (Crouch and Klee, 1980). These binding sites may be able to function as sensitive detectors of calcium concentration (Huang *et al.*, 1981). Calmodulin also contains an enzyme–protein binding site with significant hydrophobic character (Tanaka and Hidaka, 1980). This hydrophobic site is only available when calcium is bound to the calmodulin molecule. Several antipsychotic drugs such as trifluoperazine and chlorpromazine, which have significant hydrophobic properties, bind to the hydrophobic site on calmodulin (Levin and Weiss, 1979; Roufogalis, 1981). These drugs are extremely useful in the purification of calmodulin and as inhibitors of calmodulin-dependent processes.

## B. Calmodulin-Dependent Processes in the Brain

Calmodulin was initially described by Cheung as the heat-stable calcium-dependent activator of cyclic nucleotide phosphodiesterase (Cheung, 1970, 1971). Similar results were independently obtained by Kakiuchi and Yamazaki (Kakiuchi *et al.*, 1970) in rat. Since these initial discoveries, several groups have elucidated calcium–calmodulin-regulated enzymatic systems.

Broström and co-workers (1978) originally reported that the presence of the heat-stable regulator protein altered the activity of adenylate cyclase. Interactions of calmodulin with the regulatory G-unit of the cyclase have been described

(Wescott *et al.*, 1979), as well as interactions with the G-unit and tubulin (Rasenick *et al.*, 1982). A calmodulin-dependent $Ca^{2+}$-ATPase from brain has been isolated using calmodulin affinity chromatography (Papazian *et al.*, 1982). These ATPases demonstrate an absolute requirement for calmodulin to achieve maximal activity. Klee and coworkers (Stewart *et al.*, 1982) have demonstrated recently that the calmodulin binding protein calcineurin is actually a calmodulin-dependent phosphatase. Finally, calcium-dependent Phospholipase A also depends on activation by calmodulin (Moskowitz *et al.*, 1982).

A number of groups have investigated calmodulin-dependent kinases in the brain. Shulman and Greengard (1978a,b) observed that a number of brain substrates were phosphorylated in a calcium–calmodulin-dependent manner. Synaptic vesicle proteins were also shown to be phosphorylated in a calcium–calmodulin-dependent manner (DeLorenzo *et al.*, 1979). Greengard and his collegues have partially purified from brain membrane and cytosol a calmodulin dependent kinase that phosphorylates Protein I (Kennedy and Greengard, 1981). Yamauchi and Fujisawa (1978) and Kuhn and Lovenberg (1982) have isolated a calcium–calmodulin-dependent tryptophan hydroxylase kinase that may regulate the production of the neurotransmitter, 5-hydroxytryptamine. Fukunga *et al.* (1982) have isolated a calmodulin-dependent kinase from rat brain that phosphorylates a number of different brain substrates. Calmodulin-dependent tubulin kinase activity has been demonstrated in synaptosomes (Burke and DeLorenzo, 1981), synaptic vesicles (Burke and DeLorenzo, 1982a), and synaptic cytoplasm (Burke and DeLorenzo, 1982b). We have purified to homogeneity a calmodulin-dependent kinase from brain cytosol that phosphorylates tubulin and microtubule-associated proteins (Goldenring *et al.*, 1982, 1983). All of these investigations suggest that calcium–calmodulin may regulate a large number of dynamic processes in nerve cells (Cheung, 1980; Klee *et al.*, 1980; Means and Dedman, 1980).

## II. PREPARATION OF CALMODULIN

Several properties of calmodulin simplify its purification on a large scale. First, brain tissue, especially the cortex and striatum, are rich sources of calmodulin, providing a readily available source for purification. Second, the heat stability of calmodulin allows rapid purification at room temperature. Third, the highly acidic nature of the protein facilitates its purification on ion exchange chromatography. Utilizing these three fundamental properties, Lin *et al.* (1974) published the first total purification of calmodulin utilizing DEAE chromatography of boiled brain supernatant followed by final purification on gel chromatography.

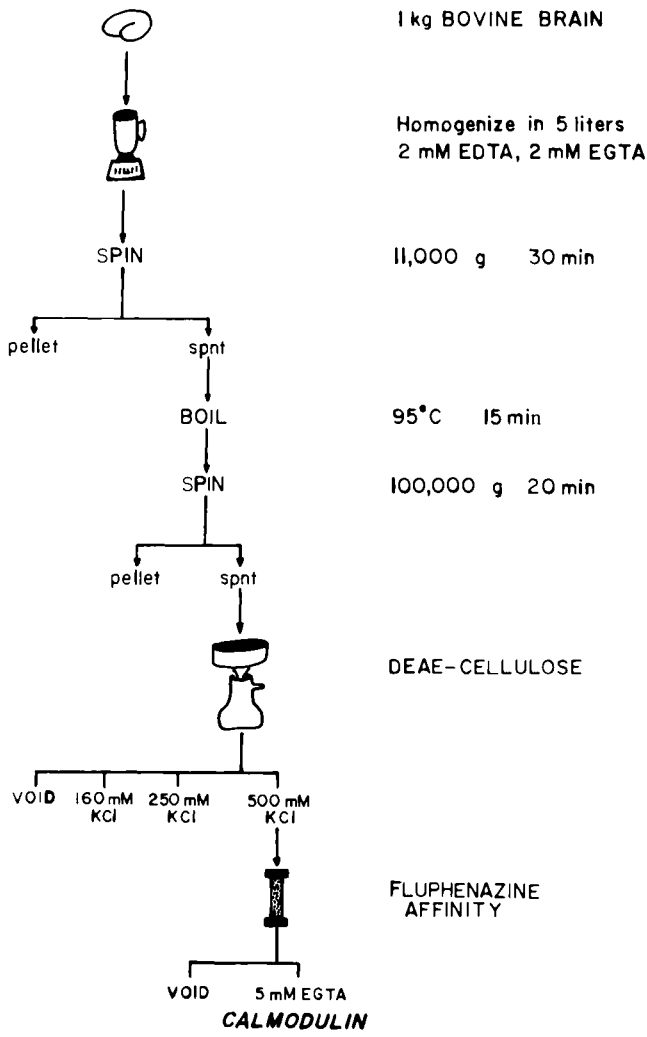


**Fig. 1.** Purification of calmodulin from bovine brain.

More recently, several groups (Charbonneau and Cormier, 1979; Marshak *et al.*, 1981; Kakiuchi *et al.*, 1981), have employed a fourth property of calmodulin, its ability to bind to antipsychotic drugs (Weiss *et al.*, 1980) such as fluphenazine to affinity purify calmodulin. This step as a final purification is much more effective than gel chromatography for large-scale calmodulin preparation. However, although initial reports indicated that calmodulin might be totally purified directly from boiled supernatant by fluphenazine affinity chromatography, it is now clear that the DEAE chromatography step is essential for preparation of homogeneous calmodulin (Moore and Dedman, 1982). The following protocol is

a modification of previously published methods and has been utilized in our laboratory for rapid, efficient purification of large amounts of homogeneous calmodulin (Fig. 1). This preparation technique serves as a rapid and inexpensive method to produce maximum quantities of purified calmodulin.

Bovine brain is one of the most common sources of tissue used to prepare calmodulin because of the amount of tissue obtainable and the relatively low cost; thus, we employ bovine brain as the main source of calmodulin. However, the same protocol described here can be adapted for purification of calmodulin from rat brain or other tissue sources. One kg of bovine brain, with cerebellum and brainstem removed, is homogenized in 5 liters of 2 m*M* EDTA and 2 m*M* EGTA in a Waring blender for 5 min (200 gm of brain in 1 liter of buffer per homogenization). Chelation of brain homogenate with EDTA and EGTA significantly increases the final yield of calmodulin, apparently by releasing the regulator from binding sites associated with membrane. The resulting homogenate is spun for 30 min at 11,000 *g* in a Sorvall GSA rotor. The supernatant is pooled and rechelated to a concentration of 2 m*M* EDTA and 2 m*M* EGTA. This rechelated supernatant is then boiled for 15 min at 95°C on a stirring hot plate. The boiled mixture is then centrifuged for 20 min at 100,000 *g* to yield a clear yellow boiled supernatant.

## A. DEAE Chromatography

We have devised the following batch method to facilitate rapid chromatography of large amounts of supernatant on DEAE cellulose. The procedure is carried out at room temperature. Three hundred ml of packed DEAE cellulose (Whatman, DE-52) equilibrated in 160 m*M* KCl is packed as a cake on Whatman 1 filter paper in a 16-cm-diameter Buchler funnel mounted on a 4 liter sidearm flask. The boiled supernatant (approximately 2 liters) is made 160 m*M* KCl and poured carefully over the DEAE under moderate vacuum filtration so as to maintain a flow rate of approximately 50 ml per minute. Care must be taken to avoid disruption of the DEAE cake, which may create channels in the resin. Thus, the supernatant and washes should be poured onto the resin slowly. Following application of the supernatant, the resin is washed with 600 ml of 160 m*M* KCl followed by 1500 ml of 250 m*M* KCl. The calmodulin-containing fraction is then eluted from the resin with 750 ml of 500 m*M* KCl. The 500 m*M* KCl eluate may sometimes contain some DEAE fines if these are not carefully removed during the equilibration of the DEAE resin. These can be easily removed from the eluate by centrifugation for 20 min at 100,000 *g*.

The resulting preparation contains greater than 50% calmodulin, and this level of purity may be acceptable for some applications. However, in many instances the use of this partially purified preparation may be inappropriate. DeMartino and Blumenthal (1982) have recently shown that this DEAE-purified preparation

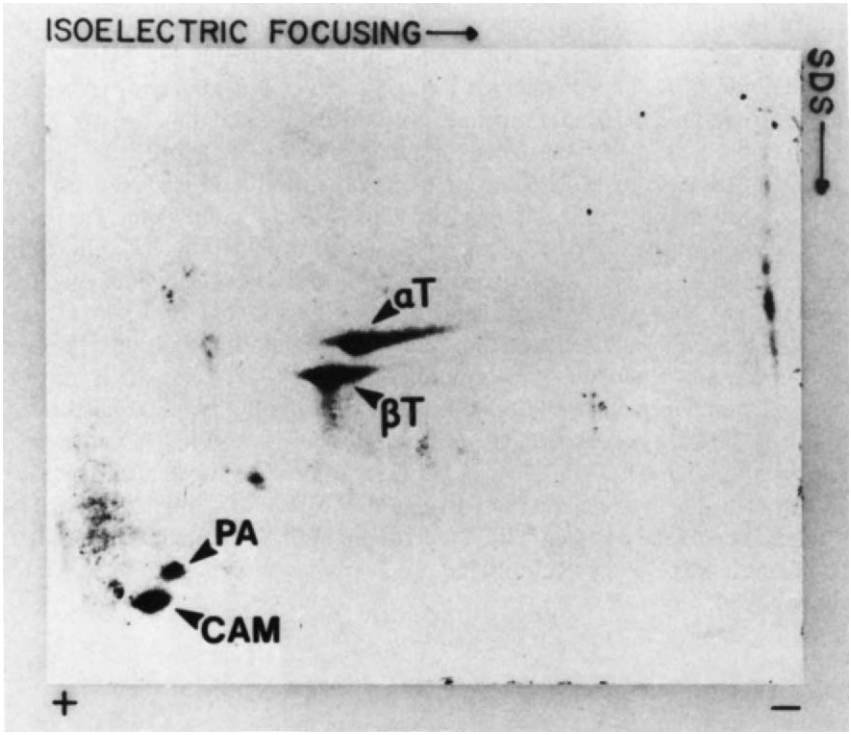


**Fig. 2.** Calcium-dependent protease activator contamination of DEAE-purified calmodulin preparations. A DEAE-purified calmodulin preparation (20 μg) was resolved on two-dimensional gel electrophoresis. Calmodulin (CAM) focused with an approximate pI of 4.3, while the calcium-dependent protease activator (PA) focused at an approximate pI of 4.5 and also displayed a higher molecular weight than calmodulin. α-Tubulin (αT) and β-tubulin (βT) were resolved on the same gel as isoelectric point references.

contains a 20,000-dalton activator of calcium-dependent protease. We have found that this protein can be extremely prominent in preparations from rat and bovine brain. Figure 2 shows that the protease activator displays only a slightly higher molecular weight and only a slightly more basic pH than calmodulin. These differences may be overlooked in normal SDS–polyacrylamide gel electrophoresis (SDS–PAGE). Because this protease-activating protein does not adhere to fluphenazine, fluphenazine affinity–purified calmodulin should be used in any studies in which the identity of calmodulin is critical, such as in the construction of calmodulin affinity columns and in calmodulin binding studies.

### B. Fluphenazine Affinity Purification

We have employed Affigel–Fluphenazine resin manufactured by Biorad; however, Sepharose–fluphenazine and Affigel–fluphenazine resin can be employed by the investigator following previously published procedures (Charbonneau and

Cormier, 1979; Kakiuchi *et al.*, 1981; Marshak *et al.*, 1981). The Biorad Affigel–Fluphenazine resin displays a working capacity of approximately 1 mg per ml of resin. Fluphenazine is light-sensitive, so the resin must be kept in a light-tight column to maintain activity. Since the affinity of calmodulin for fluphenazine is temperature-dependent, chromatography should be performed at room temperature. The Affigel–Fluphenazine resin (40 ml bed vol) is prewashed with two bed vol of 8 *M* urea and then equilibrated in 50 m*M* Tris–HCl, pH 7.5, 200 μ*M* $CaCl_2$, 250 m*M* KCl, 1 m*M* 2-mercapteothanol. The DEAE eluate is diluted to 250 m*M* KCl and made 50 m*M* Tris–HCl, pH 7.5, 200 μ*M* $CaCl_2$, 1 m*M* 2-mercaptoethanol and then applied to the fluphenazine affinity resin at a flow rate of approximately 60 ml/hr. Following application of the sample, the column is washed extensively with the equilibration buffer. The calmodulin is eluted from the resin with 50 m*M* Tris–HCl, pH 7.5, 5 m*M* EGTA.

Because of the absence of tryptophan residues in calmodulin, monitoring the elution of calmodulin at 280 nm is difficult. Nevertheless, monitoring of the elution from fluphenazine affinity resin can be highly advantageous. When appropriate monitoring equipment is available, the absorbance can be easily monitored at 220 nm. Nevertheless, we have found that when a large preparation is conducted the concentrations of calmodulin eluting are so high that the 280 nm absorbance of the tyrosine residues in calmodulin is adequate for monitoring the protein (Fig. 3).

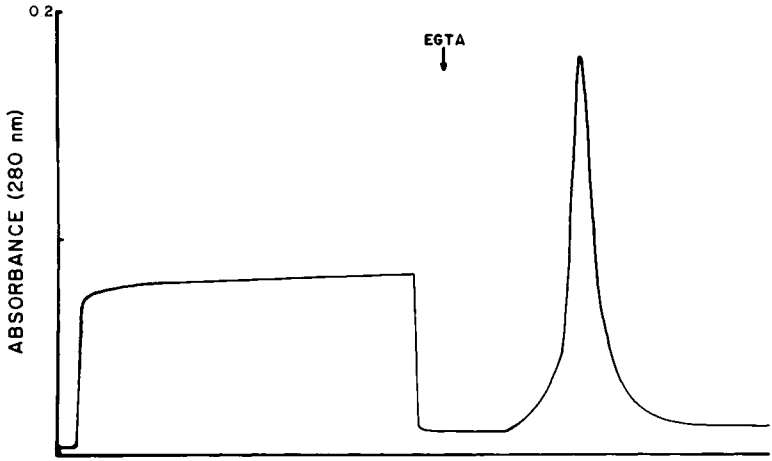


**Fig. 3.** Final purification of calmodulin on Affigel–fluphenazine affinity resin. The 500 m*M* KCl eluate from DEAE cellulose was diluted by half (approximately 1500 ml) and loaded on Affigel–fluphenzaine resin (40 ml bed volume) at a rate of 60 ml per hr. Following complete application of the sample, the column was washed with 250 m*M* KCl, 50 mM Tris–HCl, pH 7.5, 1 m*M* 2-mercaptoethanol, until the absorbance reached baseline. The calmodulin was eluted with 5 m*M* EGTA, 50 m*M* Tris–HCl, pH 7.5 (EGTA). The figure shows the protein trace at 280 nm. Chart speed was accelerated 20-fold during the chelator elution to better visualize the peak. Two-milliliter fractions were collected during the elution.

Using this preparation technique, 30–40 mg of homogeneous calmodulin can be prepared from 1 kg of bovine brain within 4 to 5 days. The calmodulin, as assessed by Coomassie blue staining of protein resolved on SDS–PAGE gels, is more than 98% pure and retains complete activity. Since calmodulin does not contain any cysteine residues, it fails to stain properly with silver procedures and this method should not be used for assessing calmodulin concentration. The calmodulin is stored in aliquots in the elution buffer containing chelator at −20°C. If calmodulin preparations are to be used for procedures in which Tris buffer is undesirable, such as attachment to succinamide ester–modified resins (Affigel 10 or 15) or labeling with Bolton–Hunter reagent, fluphenazine column buffers can be used containing a sulfonic acid buffer such as MOPS (3-[*N*-morpholino]propane sulfonic acid).

## III. CALMODULIN AT THE SYNAPSE

The role of calcium in synaptic function is well established. The molecular mechanism mediating the effects of calcium on synaptic activity is a major question in neuroscience research at the present. Research in this laboratory has been directed at providing a molecular approach to studying the biochemistry of the calcium signal in neurotransmitter release and synaptic modulation. *In vitro* and *in vivo* preparations were developed and employed to study the effects of calcium on neurotransmitter release (DeLorenzo, 1980a; DeLorenzo *et al.*, 1979), synaptic protein phosphorylation (DeLorenzo *et al.*, 1979; DeLorenzo, 1976, 1977, 1980a, 1982), and synaptic vesicle and synaptic membrane interactions (DeLorenzo, 1980a, 1981a, 1982). These studies provided an experimental framework to suggest that calmodulin modulates many of the biochemical effects of calcium on synaptic preparations. From this evidence the calmodulin hypothesis of neuronal transmission was developed (DeLorenzo, 1981a). This hypothesis states that as calcium enters the presynaptic nerve terminal, it binds to calmodulin and activates several calcium–calmodulin-regulated processes that modulate synaptic activity. At this time the evidence from this laboratory and several others indicates that several calcium–calmodulin-dependent processes exist both pre- and postsynaptically (DeLorenzo, 1982). However, the full functional role of calmodulin in synaptic modulation is yet to be fully elucidated. The following material presents some of the background and techniques used in our laboratory and others for approaching this area of neurochemical research.

### A. Pre- and Postsynaptic Calmodulin

As recently reviewed (Cheung, 1980; Klee *et al.*, 1980), it is now becoming accepted that calmodulin may be a ubiquitous receptor, mediating many of the

diverse effects of calcium on cellular functions. Calmodulin is found in high concentrations in brain (Cheung, 1980). However, to implicate this calcium regulator protein in synaptic function, it is necessary to demonstrate that calmodulin is present at the synapse. Calmodulin has been isolated from enriched preparations of synaptic fractions.

A vesicle-bound, heat-stable protein that had the same molecular weight as calmodulin was isolated from highly enriched preparations of synaptic vesicles from rat cortex (DeLorenzo, 1980a). This vesicle-bound protein could be removed from the vesicles in the presence of EGTA and was found to bind calcium at micromolar concentrations (DeLorenzo, 1981a,b). When compared to calmodulin isolated from whole rat brain, the vesicle-bound calcium binding protein

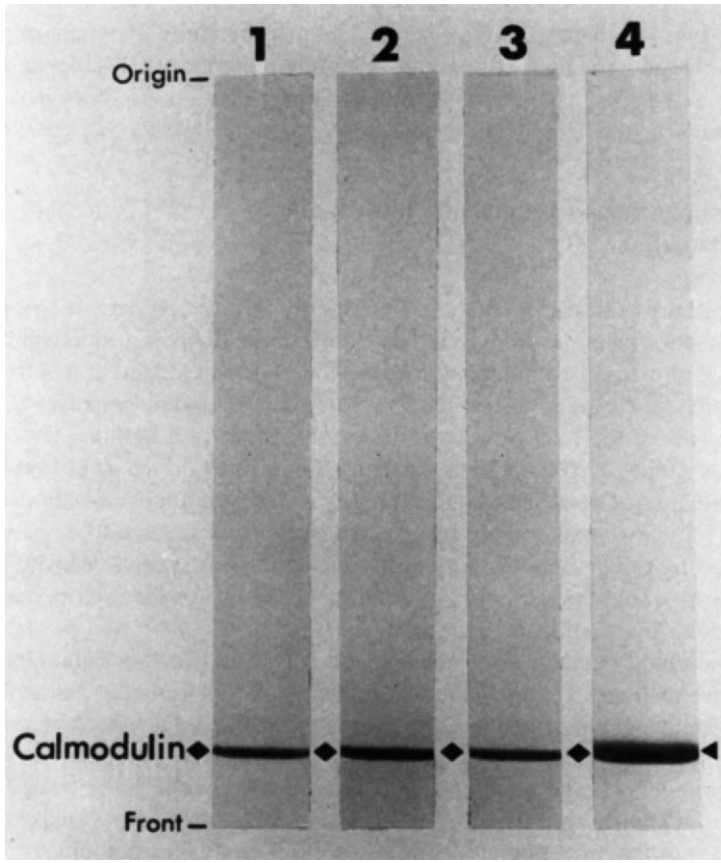


**Fig. 4.** Electrophoretic protein patterns on SDS–polyacrylamide gel of calmodulin isolated from whole rat brain, synaptosome cytoplasm, and synaptic vesicles, and a mixture of all three calmodulin preparations are shown in channels 1–4, respectively. Calmodulin was isolated by the method described in Fig. 1. Protein was stained with Coomassie blue. Reproduced from DeLorenzo (1980a).

was found to be identical to calmodulin in molecular weight, amino acid composition, isoelectric point, and its ability to stimulate vesicle protein kinase, adenylate cyclase, and phosphodiesterase activities. Vesicle calmodulin represented 0.92% of the total protein in synaptic vesicle fractions.

A heat-stable, calcium binding protein was also isolated from nerve terminal synaptoplasm prepared by standard procedures (DeLorenzo, 1980a, 1981b). Approximately 80–90% of the protein in this synaptoplasmic preparation has been shown to originate in the presynaptic terminal (Barondes, 1974). This synaptic protein (Fig. 4) was found to be identical to whole brain calmodulin in molecular weight, isoelectric point, amino acid composition, and ability to stimulate protein kinase and phosphodiesterase activities (DeLorenzo, 1980a). Synaptic calmodulin was 0.71% of the total protein in the synaptoplasm preparation. Because the concentration of calmodulin in whole brain fractions is approximately 1% of the total brain protein, the high percentage of calmodulin in synaptoplasm and synaptic vesicle fractions strongly indicates the presence of this calcium receptor protein in the presynaptic nerve terminal. Calmodulin has also been isolated and characterized from postsynaptic density preparations (Grab *et al.*, 1979, 1980).

## B. Possible Role of Calmodulin in Synaptic Modulation

The ability to isolate calmodulin from highly enriched preparations of pre- and postsynaptic fractions strongly indicates that calmodulin is a transsynaptic protein. Therefore, calmodulin may mediate the effects of calcium on both the pre- and postsynaptic sides of the synapse. Because calmodulin represents approximately 1% of the total protein in these subfraction preparations, it is unlikely that the calmodulin in these isolated fractions was derived from cytoplasmic contamination. Calmodulin concentrations in whole brain cytoplasm do not exceed 1% of the total soluble protein (Cheung, 1980). Therefore, partial contamination of the synaptic cytoplasm, vesicle, and postsynaptic density preparations with whole brain cytoplasm could not account for the high percentage of calmodulin found in these fractions.

These studies demonstrate the methodology for isolating calmodulin from synaptic fractions. The technique described above for isolating calmodlin from whole brain can be applied to different brain regions or other secretory systems. Following the demonstration of calmodulin in synaptic preparations, several calcium-regulated synaptic processes have been shown to be regulated by calmodulin (DeLorenzo, 1981a,b, 1982). To discuss each of these areas is beyond the scope of this presentation. Thus, we will present the methodology for studying calmodulin-dependent protein phosphorylation as a representative example of a synaptic calmodulin system.

## IV. CALCIUM–CALMODULIN-STIMULATED PROTEIN PHOSPHORYLATION IN SYNAPTIC FRACTIONS

### A. Calcium-Stimulated Protein Phosphorylation in Brain

Calcium-stimulated protein phosphorylation in brain was initially described in whole brain homogenates and highly enriched preparations of synaptosomes (DeLorenzo, 1976). These results demonstrated that calcium stimulated the endogenous phosphorylation of many brain proteins, but particularly proteins in the molecular weight ranges 10,000–20,000; 50,000–54,000; 60,000–64,000; and 150,000–300,000. Two proteins with molecular weights of 52,000 to 54,000 and 60,000 to 64,000 (proteins DPH-M and DPH-L, respectively) were of particular interest, for they were most dramatically stimulated by calcium and inhibited by phenytoin, an anticonvulsant that blocks several calcium-dependent processes including neurotransmitter release (DeLorenzo, 1980a). These findings suggested that calcium-dependent protein phosphorylation distinct from cAMP-dependent phosphorylation systems may regulate the effects of calcium on synaptic function and neurotransmitter release (DeLorenzo, 1976, 1980a, 1981b; DeLorenzo *et al.*, 1977).

Depolarization-dependent calcium uptake in intact synaptosomes was shown to stimulate the phosphorylation of an 80,000-dalton protein (Protein I) and possibly other proteins in intact synaptosomes (Krueger *et al.*, 1977). The levels of phosphorylation of proteins with identical molecular weights to proteins DPH-L and DPH-M were shown to be stimulated in intact synaptosomes by depolarizing conditions that stimulated calcium entry and stimultaneously initiated neurotransmitter release from intact synaptosomes (DeLorenzo *et al.*, 1979; DeLorenzo, 1980a). The level of phosphorylation of 50,000- to 60,000-dalton proteins has also been observed to correlate with norepinephrine release in intact adrenal medulla cells (Amy and Kirshner, 1981).

### B. Calmodulin Mediates Some of the Effects of Calcium on Protein Phosphorylation

The calcium-stimulated endogenous phosphorylation pattern described in synaptosomes (DeLorenzo, 1976) was shown to be dependent on calmodulin in crude preparations of brain membrane and in several other tissues (Shulman and Greengard, 1978a,b). Calmodulin was subsequently shown to mediate the effect of calcium on the phosphorylation of specific synaptic vesicle proteins (DeLorenzo *et al.*, 1979). Calmodulin was also shown to modulate the effects of

calcium on the endogenous phosphorylation of highly enriched synaptic membrane (DeLorenzo, 1980a,b), synaptic junctional complex (DeLorenzo, 1980a), and postsynaptic density (DeLorenzo, 1980; Grab, *et al.*, 1979, 1980) preparations. These studies employed calmodulin-depleted membrane preparations by removing calmodulin through chelation with EGTA and then determining if calmodulin and calcium could restore phosphorylation to the fractions. Thus, the preparation of calmodulin-depleted fractions and the conditions of phosphorylation are useful techniques to employ in studying a new system to determine if calmodulin kinase activity is present.

### C. Phosphorylation Conditions: Isolated Fractions

The standard reaction mixture employed in our laboratory for routinely studying the effects of calcium, calmodulin, drugs, or other compounds on endogenous protein phosphorylation (Burke and DeLorenzo, 1982a) contains 50–120 μg of protein, 10 m*M* piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), pH 7.4, 5–10 μ*M* [γ-$^{32}$P]ATP, 2 m*M* EDTA, and 1 m*M* EGTA to chelate divalent cations endogenous to the proteins. $MgCl_2$ is added in quantities sufficient to yield a final concentration of 4 m*M* free magnesium. Reactions are initiated by addition of 10 μl $CaCl_2$ or deionized water in the case of controls. Free calcium in the final reaction mixture is determined by using a calcium–EGTA buffer system, taking the apparent binding constant for calcium–EGTA to be $7.61 \times 10^{-6}$ *M* as described by Portzehl *et al.* (1964) and verified with a calcium-specific electrode (Orion) as 5–50 μ*M*. Tubes are incubated for 1 min at 37°C and final reaction volumes are 0.1 ml. Reactions are terminated and samples prepared for electrophoresis by addition of a stop solution. For one-dimensional SDS–PAGE, 50 μl of a solution containing 360 m*M* Tris–HCl (pH 7.5), 36 m*M* EDTA, 6% SDS, 3 *M* sucrose, and Pyronin Y are added. Samples are heated to 98°C for 3 min and after cooling 20 μl of 2-mercaptoethanol (2-ME) are added. For two-dimensional electrophoresis, reactions are terminated with 100 μl of a urea stop solution containing 10% (v/v) 2-ME, 2% (w/v) SDS, 4% (v/v) Triton X-100, 4% (v/v) ampholytes (3.2% 5–7; 0.8% 3–10), and 9.5 *M* urea. These samples are frozen without boiling to avoid breakdown of the 2-ME and carbamylation of proteins by urea.

### D. Intact Synaptosome Studies

Intact synaptosomes are prepared (DeLorenzo *et al.*, 1979) and characterized for calcium uptake and neurotransmitter release by the methods of Blaustein's group (Blaustein *et al.*, 1972). Briefly, synaptosomes are obtained from whole rat brains, equilibrated (4°C) with calcium-free medium (medium A), containing

TABLE I
Effects of High K+ and Scorpion Venom (SV) in the Presence and Absence of Tectodotoxin (TTX) on Intact Synaptosome $Ca^{2+}$ Uptake, Neurotransmitter Release, and Protein Phosphorylation[a]

| Condition | $Ca^{2+}$ uptake (%) | Norepinephrine release (%) | Protein phosphorylation (cpm) | | |
|---|---|---|---|---|---|
| | | | Whole synaptosome | Synaptic membrane | Synaptic vesicles |
| Control | — | 46 | 372 | 405 | 765 |
| $Ca^{2+}$ | 55 | 53 | 391 | 418 | 776 |
| $Ca^{2+}$, K+ | 100[b] | 100[b] | 674[b] | 762[b] | 1978[b] |
| $Ca^{2+}$, K+, TTX | 97[b] | 95[b] | 675[b] | 751[b] | 1892[b] |
| $Ca^{2+}$, SV | 89[b] | 83[b] | 588[b] | 692[b] | 1807[b] |
| $Ca^{2+}$, SV, TTX | 51 | 55 | 430 | 459 | 873 |

[a]Intact synaptosomes were incubated under various conditions after preincubation with $^{32}P$ as described previously (DeLorenzo *et al.*, 1979). Following the reactions, synaptic membrane and synaptic vesicles were isolated from each reaction mixture and quantitated for incorporation of $^{32}P$-phosphate into protein DPH-M. Data give the mean values of six determinations and are representative of two separate experiments. $^{32}P$-Phosphate incorporation into protein DPH-M is expressed as cpm per 500 μg protein and was representative of several other synaptosomal phosphoproteins (DeLorenzo *et al.*, 1979). $Ca^{2+}$ uptake and norepinephrine release were determined by established procedures (DeLorenzo, 1980; DeLorenzo *et al.*, 1979) and are expressed as a percentage of the maximal condition.

[b]$p < .001$ in comparison to control or $Ca^{2+}$ alone conditions.

132 m*M* NaCl/5 m*M* KCl/1.3 m*M* $MgCl_2$/1.3 m*M* $MgCl_2$/10 m*M* glucose/20 m*M* Tris maleate buffer (pH 7.45), and then preincubated with medium A for 12 min at 30°C. After preincubation addition aliquots of test solutions are added containing calcium (for calcium uptake studies) or various test agents (potassium, veratridine, scorpion venom, or tetrodotoxin) plus calcium, and further incubation is carried out (Table I). Each reaction is terminated and quantitated for calcium uptake and norepinephrine release as described (Blaustein *et al.*, 1972). It is important to be sure in each laboratory that conditions of depolarization employed to alter protein phosphorylation are indeed altering calcium uptake and neurotransmitter release.

Krueger *et al.* (1977) provided the first description of the effects of depolarization and calcium on protein phosphorylation in intact synaptosomes, employing the techniques developed by Blaustein *et al.* (1972) to depolarize synaptosome fractions. These techniques have also been employed in this laboratory to study the effects of depolarization on protein phosphorylation in intact nerve terminal preparations (DeLorenzo *et al.*, 1979; DeLorenzo, 1980a,b).

Phosphorylation studies are carried out identically to calcium uptake and neurotransmitter release experiments, except that $^{32}P$ is added to the initial equilibration medium (0.1–0.2 mCi/ml). After equilibration for 10 min at 4°C, 4 ml

aliquots of synaptosome suspension (10 mg of protein per ml) are preincubated for 12 min at 30°C and test aliquots (1 ml) are then added to each tube and incubated for various time intervals (Table I). For quantitation of the effects of different conditions on total synaptosome protein phosphorylation, each reaction is terminated by addition of 2.5 ml of SDS stop solution and assayed as described above for the synaptic vesicles. $^{32}$P-Phosphate incorporation into synaptic vesicle proteins isolated from intact synaptosomes that had been incubated with $^{32}P_i$ can be measured by terminating each reaction by osmotically shocking the incubated synaptosome preparation at 4°C and rapidly isolating the vesicles from each reaction mixture by differential centrifugation (DeLorenzo *et al.*, 1979). Vesicles are isolated in less than 3 hr, and the resultant vesicle pellets are immediately soubilized with SDS stop solution. In some experiments reactions with intact synaptosomes are terminated by osmotic shock and the mixtures maintained at 4°C until synaptic vesicles are isolated from other reaction mixtures. Protein phosphorylation is then measured with the vesicle fractions to determine the effect of osmotic shock and incubation at 4°C on synaptosomal protein phosphorylation.

## E. Pharmacological Manipulation of Calmodulin Kinase Activity

### *1. Calmodulin Inhibitors*

In some preparations it is difficult to remove calmodulin from the preparation. Under these circumstances it is useful to employ the phenothiazines to inactivate calmodulin in the preparation and then add back excess calmodulin to overcome this effect. Trifluoperazine is the classical calmodulin inhibitor (Weiss *et al.*, 1980). Weiss's group has provided extensive evidence, supported by other laboratories, that trifluoperazine and numerous other phenothiazines bind to and inactivate calmodulin. However, it is clear that this is not the only effect of these compounds on nervous tissue. Thus, trifluoperazine can be employed to inactivate calmodulin, but it may also inactivate several other enzyme systems in a preparation.

When employed properly, trifluoperazine can be a useful pharmacological tool to inactivate calmodulin, especially in studying calmodulin kinase activity. Each preparation should be characterized for its sensitivity to calmodulin. Employing the dose–response curve for calmodulin, the concentration of calmodulin that just produces a maximal kinase activation is selected. Then at this calmodulin concentration, a dose response curve for trifluoperazine is obtained, usually ranging from $10^{-7}$ to $10^{-3}M$. The lowest concentration of trifluoperazine that produces approximately 50–70% inhibition of the kinase is selected. Employing

this trifluoperazine concentration, increasing concentrations of calmodulin are then added back to the reaction mixture. If calmodulin can overcome the trifluoperazine inhibition of the kinase, this is strong evidence that calmodulin is specifically activating this kinase system.

The trifluoperazine technique described above is particularly helpful in studying endogenous calmodulin kinase activity in crude preparations that are in very limited quantity. Invertebrate ganglia, pooled cells from a ganglion, isolated anatomical regions, and cultured cells are common examples of preparations that initially may yield small quantities of tissue. Thus, it is often difficult to prepare calmodulin-depleted membranes from these sources. A standard approach for studying this type of system is to produce a crude homogenate and quantitate the endogenous phosphorylation of the mixture under standard conditions in the presence of magnesium, magnesium plus calcium, and magnesium plus calcium plus calmodulin.

Following phosphorylation, the samples are subjected to SDS–PAGE and quantitation. If the tissue is saturated with calmodulin, calcium will produce a stimulation in the phosphorylation of several specific peptides. However, addition of calmodulin may have no effect since it is already present in saturating conditions in the reaction tubes. The calmodulin in the reaction tubes can be "pharmacologically depleted" by adding trifluoperazine to the raction mixture. This "calmodulin-depleted" fraction can now be studied to determine whether added calmodulin restores the ability of calcium to stimulate protein phosphorylation. This technique was employed to demonstrate the existence of calmodulin tubulin kinase activity in whole brain cytoplasm (Burke and DeLorenzo, 1980a).

### 2. *Calmodulin Kinase Inhibitors*

In addition to inhibiting calmodulin, it has been demonstrated that the benzodiazipines (DeLorenzo *et al.*, 1981) and phenytoin (DeLorenzo, 1980b) inhibit calmodulin kinase activity without inactivating calmodulin. These drugs inhibit the calmodulin target enzyme system. The major difference between these compounds and the phenothiazines is that their inhibitory effects cannot be significantly overcome by adding back excess calmodulin. These compounds are useful as an adjunct to trifluoperazine in evaluating calmodulin kinase systems in intact preparations, as shown in the synaptosome preparation (DeLorenzo, 1982).

## ACKNOWLEDGMENTS

This research was supported by Research Career Development Award NS1-EA-1-KO5-NS245 and Grants NS 1352 and AFOSR F49620-83-K-0035 to R. J. DeLorenzo. James Goldenring is a recipient of an MSTP Fellowship.

## REFERENCES


Amy, C. M., and Kirschner, N. (1981). *J. Neurochem.* **3,** 847–854.

Barondes, S. H. (1974). *Annu. Rev. Biochem.* **43,** 147–194.

Blaustein, M. P., Johnson, E. M., and Needleman, P. (1972). *Proc. Natl. Acad. Sci. U.S.A.* **69,** 2237–2240.

Broström, M. A., Broström, C. O., Breckenridge, B. M., and Wolff, D. J. (1978). *Adv. Cyclic Nucleotide Res.* **9,** 85–99.

Burke, B. E., and DeLorenzo, R. J. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 991–995.

Burke, B. E., and DeLorenzo, R. J. (1982a). *Brain Res.* **236,** 393–415.

Burke, B. E., and DeLorenzo, R. J. (1982b). *Neurochemistry* **38,** 1205–1218.

Charbonneau, H., and Cormier, M. J. (1979). *Biochem. Biophys. Res. Commun.* **90,** 1039–1047.

Cheung, W. Y. (1970). *Biochem. Biophys. Res. Commun.* **38,** 533–558.

Cheung, W. Y. (1971). *J. Biol. Chem.* **246,** 3859–2869.

Cheung, W. Y. (1980). *Science* **207,** 19–27.

Crouch, T. H., and Klee, C. B. (1980). *Biochemistry* **19,** 3692–3698.

DeLorenzo, R. J. (1976). *Biochem. Biophys. Res. Commun.* **71,** 590–597.

DeLorenzo, R. J. (1977). *Brain Res.* **134,** 125–138.

DeLorenzo, R. J. (1980a). *Ann. N.Y. Acad. Sci.* **356,** 92–109.

DeLorenzo, R. J. (1980b). *In* "Antiepileptic Drugs: Mechanisms of Action" (G. H. Glaser, J. K. Penry, and D. M. Woodbury, eds.), pp. 399–414. Raven Press, New York.

DeLorenzo, R. J. (1981a). *Cell Calcium* **2,** 365–385.

DeLorenzo, R. J. (1981b). *In* "Regulatory Mechanisms of Synaptic Transmission" (R. Tapia and C. W. Cotman, eds.), pp. 205–240. Plenum, New York.

DeLorenzo, R. J. (1982). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **41,** 2265–2272.

DeLorenzo, R. J., and Fredman, S. D. (1977). *Biochem. Biophys. Res. Commun.* **77,** 1036–1043.

DeLorenzo, R. J., and Freedman, S. D. (1978). *Biochem. Biophys. Res. Commun.* **80,** 183–192.

DeLorenzo, R. J., Emple, G. P., and Glaser, G. H. (1977). *J. Neurochem.* **28,** 21–30.

DeLorenzo, R. J., Freedman, S. D., Yohe, W. B., and Maurer, S. C. (1979). *Proc. Natl. Acad. Sci. U.S.A.* **76,** 1838–1842.

DeLorenzo, R. J., Burdette, S., and Holderness, J. (1981). *Science* **212,** 1157–1159.

DeMartino, G. N., and Blumenthal, D. K. (1982). *Biochemistry* **21,** 4297–4303.

Douglas, W. W. (1968). *Br. J. Pharmacol.* **34,** 451–474.

Fukunga, K., Yamamoto, H., Matsue, K., Higushi, K., and Myamoto, E. (1982). *J. Neurochem.* **39,** 1607–1617.

Goldenring, J. R., Gonzalez, B., and DeLorenzo, R. J. (1982). *Biochem. Biophys. Res. Commun.* **108,** 421–428.

Goldenring, J. R., Gonzalez, B., McGuire, J. S., Jr., and DeLorenzo, R. J. (1983). *J.Biol. Chem.* (in press).

Grab, D. J., Berzins, K., Cohen, R. S., and Siekevitz, P. (1979). *J. Biol. Chem.* **254,** 8690–8696.

Grab, D. J., Carlin, R. K., and Siekevitz, P. (1980). *Ann. N.Y. Acad. Sci.* **356,** 55–72.

Greengard, P. G. (1978). *Science* **199,** 146–152.

Huang, C. Y., Chau, V., Chock, P. B., Wang, J. H., and Sharma, R. U. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 871–874.

Kakiuchi, S., Yamazaki, R., and Nauajima, H. (1970). *Proc. Jpn. Acad.* **46,** 587–592.

Kakiuchi, S., Sobue, U., Yamazaki, R., Kambayashi, J., Sakon, M., and Kosaqi, G. (1981). *FEBS Lett.* **126,** 203–207.

Katz, B., and Miledi, R. (1969). *J. Physiol. (London)* **203,** 689–706.

Kennedy, M. B., and Greengard, P. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 1293–1297.

Klee, C. B., Crouch, T. H., and Richman, P. G. (1980). *Annu. Rev. Biochem.* **49,** 489–515.
Krueger, B., Forn, J., and Greengard, P. (1977). *J. Biol. Chem.* **252,** 2764–2773.
Kuhn, M., and Lovenberg, W. M. (1982). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **41,** 2258–2264.
Levin, R. M., and Weiss, B. (1979). *J. Pharmacol. Exp. Ther.* **208,** 454–459.
Lin, Y. M., Lin, Y. P., and Cheung, W. T. (1974). *J. Biol. Chem.* **249,** 4943–4954.
Marshak, D. R., Watterson, D. M., and Van Eldik, L. J. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **28,** 6793–6797.
Means, A. R., and Dedman, J. R. (1980). *Nature (London)* **285,** 73–77.
Niledi, R. (1973). *Proc. R. Soc. London* **183,** 421–425.
Moore, P. B., and Dedman, J. R. (1982). *J. Biol. Chem.* **95,** 5a.
Moskownitz, N., Schook, W., and Puszkin, S. (1982). *Science* **216,** 305–307.
Papazlian, D. M., Rahamimoff, H., and Goldin, S. M. (1982). *Neurosci. Abstr.* **8,** 692.
Portzehl, H., Caldwell, P. C., and Ruegg, J. C. (1964). *Biochim. Biophys. Acta* **79,** 581–591.
Rasenick, M. M., Kosack, C. M., and DeLorenzo, R. J. (1982). *Neurosci. Abstr.* **8,** 344.
Rasmussen, H. (1981). "Calcium and cAMP as Synarchic Messengers." Wiley, New York.
Rasmussen, H.,and Goodman, D. B. P. (1977). *Phys. Rev.* **57,** 421–509.
Roufogalis, B. D. (1981). *Can. J. Biol.* **30,** 607–613.
Shulman, H., and Greengard, P. (1978a). *Nature (London)* **271,** 478–479.
Shulman, H., and Greengard, P. (1978b). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 5432–5436.
Stewart, A. A., Ingebriten, T. S., Manalan, A., Klee, C. B., and Cohen, P. (1982). *FEBS Lett.* **137,** 80–84.
Tanaka, T., and Hidaka, H. (1980). *J. Biol. Chem.* **255,** 11078–11080.
Wang, J. H., and Waisman, D. M. (1979). *Curr. Top. Cell Regul.* **15,** 47–107.
Watterson, D. W., Sharief, F., and Vannaman, T. C. (1980). *J. Biol. Chem.* **255,** 962–975.
Weiss, B., Proxialeck, W., Cimino, M., Barnette, M. S., and Wallace, T. L. (1980). *Ann. N.Y. Acad. Sci.* **356,** 319–345.
Wescott, K. R., Laporte, D. C., and Storm, D. R. (1979). *Proc. Natl. Acad. Sci. U.S.A.* **76,** 204–208.
Wise, B. C., Raynor, R. L., and Kuo, J. F. (1982). *J. Biol. Chem.* **257,** 8481–8488.
Yamauchi, T., and Fujisawa, H. (1978). *Biochem. Biophys. Res. Commun.* **90,** 28–35.

# Chapter 12

# PREPARATION OF THE CATALYTIC SUBUNIT OF cAMP-DEPENDENT PROTEIN KINASE[1]

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## I. INTRODUCTION

In recent years, it has been possible to gain insight into cellular events controlled by cAMP through purification and study of the cAMP-dependent protein kinase. [For reviews of the properties and substrates of this class of enzymes, see Beavo and Krebs (1979), Flockhart and Corbin (1982), and Cohen (1978).] This enzyme appears to be the principal cellular receptor for cAMP in eukaryotic cells. Thus, it is responsible for the effects of many hormones, neurotransmit-

[1]This work was supported by Research Grants AM 15988 and GM 30953 from the National Institutes of Health.




ISBN 0-12-470350-X

ters, and other agents on biological processes. It has two types of subunit, a regulatory subunit (R subunit), which inhibits the enzymatic activity in the absence of cAMP, and a catalytic subunit (C subunit), which catalyzes the phosphorylation of a wide variety of protein substrates when cAMP is elevated. When cAMP is bound to the R subunit, the inhibitory activity is prevented (Corbin *et al.*, 1978). There appear to be a number of different types of R subunit which are responsible for the different isozyme types of cAMP-dependent protein kinase found in tissues. Several different types of R subunit have been identified up to this time. Two main classes (types I and II) are present in many tissues in varying amounts and are named on the basis of their elution from DEAE–cellulose with salt (Corbin *et al.*, 1975; Hofmann *et al.*, 1975).

There is one isozyme which has DEAE elution characteristics similar to those of type II but which possesses different immunological properties and is present in bovine neural tissues (Erlichman *et al.*, 1980). It has been found in bovine cerebral cortex, cerebellum, medulla, brainstem, midbrain, anterior pituitary, adrenal medulla, and pineal gland but not in bovine kidney, heart, skeletal muscle, liver, or adrenal cortex. Erlichman *et al.* (1980) have pointed out the great potential value of this subclass of isozyme in developmental, anatomical, and pathological studies of the brain. Since types I and II are known to be both tissue- and species-specific (Flockhart and Corbin, 1982), the form believed to be a neural subclass may also be, and searches for it should be extended to include other tissues and species. Very few significant differences have been demonstrated between samples of C subunit isolated from a wide variety of tissues (for a review see Flockhart and Corbin, 1982). The proteins isolated from either the type I isozyme or the type II isozyme or obtained from any of these tissues have similar chemical (Peters *et al.*, 1977), physical (Bechtel *et al.*, 1977), catalytic (Glass and Krebs, 1980), and immunological (Schwoch *et al.*, 1980) properties, as well as similar $K_m$ for ATP (Yamamura *et al.*, 1973), protein substrate specificity (Beavo and Krebs, 1979; Cohen, 1978; Hofmann *et al.*, 1977), and the ability to interact with either the type I or type II R subunit (Yamamura *et al.*, 1973; Bohnert *et al.*, 1982). These similarities make it possible to use the enzyme isolated from one source in the study of other tissues or animals.

The purified C subunit is useful for a number of specific types of experiments. It can be used to determine precise substrate kinetics *in vitro* in order to demonstrate that a protein which can be phosphorylated has the kinetic characteristics required of an *in vivo* substrate. These characteristics have been defined by Krebs and Beavo (1979). It is possible to remove phosphate from phosphorylated protein substrates by the use of C subunit in the presence of high concentrations of Mg.ADP (Flockhart, 1983; Shizuta *et al.*, 1975; Rosen and Erlichman, 1975). The purified enzyme has also been used in the reconstitution of complicated biochemical systems such as the glycogen particle system (Cohen, 1978) and skinned muscle systems (Ruegg and Paul, 1982), in which direct effects of the

added enzyme can be demonstrated in the presence of appropriate concentrations of magnesium and ATP. Finally, the availability of pure enzyme preparations has made possible the microinjection of C subunit into whole cells to examine whether responses to cellular effectors can be mimicked by the protein kinase (Osterreider *et al.*, 1982).

A number of different purification schemes have been published and reviewed by Carlson *et al.* (1979). The protocol described here represents an improved version of the original procedure described for bovine liver by Sugden *et al.* (1976) and since modified by Reimann and Beham (1983). This method has been optimized for bovine heart since this tissue contains a relatively high specific activity of the enzyme (0.007–0.16 units per gram of tissue) in a form which can be easily isolated.

## II. PRINCIPLE

The protocol involves the use of the large difference in isoelectric points between the C subunit and either the R subunit or the holoenzyme complex. In general, the holoenzymes and R subunits focus on isoelectric focusing gels at pH 5–6, whereas the C subunit is found at pH 6.9–9.1 (Sugden *et al.*, 1976; Rangel-Aldao *et al.*, 1979). As a result, the free C subunit undergoes ion exclusion during DEAE chromatography at pH 6.8, whereas the holoenzyme complex and R subunits are bound to the resin. This procedure therefore involves chromatography of tissue extract at pH 6.8 in the absence of cAMP, followed by extensive washing of the resin with buffer and then elution by addition of cAMP to the chromatography buffer. The eluted protein is then further purified and concentrated by hydroxylapatite chromatography.

## III. MATERIALS

DEAE–cellulose (DE-11 or DE-22) is obtained from Whatman. Hydroxylapatite is obtained from BDH Biochemicals (Spheroidal) or BioRad (Bio-gel HTP). The sodium salt of cAMP or 8-(6-aminohexylamino)cAMP (8-AHAcAMP), histone (type IIA), Triton X-100, and EDTA are obtained from Sigma. [$\gamma$-$^{32}$P]ATP is prepared by the method of Walseth and Johnson (1979).

## IV. REAGENTS

The following solutions are made up on the day before tissue is used and chilled overnight in a cold room at 0 to 4°C. The volumes given are appropriate

for a preparation from four beef hearts. All potassium phosphate (KP) solutions contain equal parts of monobasic potassium phosphate and dibasic potassium phosphate, which are made up from 1 *M* stocks of these solutions. All EDTA solutions are made up from a stock of 200 m*M* EDTA, pH 6.8.

24 liters of 10 m*M* KP, 1 m*M* EDTA, 0.1% Triton X-100, 0.1 m*M* DTT, pH 6.8 (Buffer I)
50 liters of 55 m*M* KP, 1 m*M* EDTA, 0.1 m*M* DTT, pH 6.8
6 liters of 45 m*M* KP, 0.1 m*M* DTT, 0.1 m*M* cAMP, pH 6.8
3 liters of 40 m*M* KP, 0.1 m*M* DTT, pH 6.8 (Buffer II)
500 ml of 350 m*M* KP, 0.1 m*M* DTT, pH 6.8
500 ml of 5 m*M* KP, 0.1 m*M* DTT, pH 6.8

About 2 liters of DE-11 or DE-22 are equilibrated in Buffer I. The conductivity of the buffer after equilibrium should be ~1.1 mΩ. Two hundred ml of hydroxylapatite are equilibrated in Buffer II.

## V. PROTEIN KINASE ASSAY

The enzyme is assayed according to the filter paper method of Corbin and Reimann (1974), the only modification being that 10 m*M* sodium pyrophosphate is included in the 10% trichloracetic acid used to precipitate protein onto the filter papers. The specific activity of the ATP is 20–50 cpm/pmol. One unit of enzyme activity = 1 μmol $^{32}P$ incorporated into histone in 1 min.

## VI. PROCEDURE

Four fresh bovine hearts are obtained from a local slaughterhouse. The hearts are put on ice immediately after removal from the animal and used within 2 hr. All steps are performed at 4°C. The hearts are trimmed of fat tissue and then cut into small pieces with a sharp knife, minced with a heavy-duty meat grinder, and then homogenized in a large Waring Blendor, using a 1:3 homogenate in Buffer I. The homogenate is then centrifuged at 10,000 rpm ($r_{av}$ = 9.5 cm) for 30 min in a Beckman JA-10 Rotor and the supernatant (15–20 liters) saved. The pellets are resuspended with 100 ml each of Buffer I, recentrifuged, and the supernatant combined with the original. This latter step can be omitted, although it significantly increases the yield of enzyme. The combined supernatants are then filtered through glass wool and added with stirring to 2 liters of settled acid- and base-washed DEAE–cellulose. The resin is then left to stand overnight, or for 2 hr, with agitation every 15 min in the latter case. Shorter times will result in a fall in the amount of C subunit recovered.

The resin is poured into a column (9.5 × 28 cm) and washed at 30 to 40 ml/min with 55 m*M* KP, 1 m*M* EDTA, 0.1 m*M* DTT until the absorption at 280 nm of the eluting buffer is less than 0.1. The initial value should be ~0.9 and the wash should take about 24 hr and 40–50 liters of buffer. During this wash, the free C subunit present will be eluted, as will most of the type I holoenzyme. However, bovine heart contains mainly the type II isozyme and this holoenzyme remains bound to the resin under these conditions. An alternative and quicker wash procedure is to use a batchwise method for several washes before pouring the DEAE–cellulose into a large column for further washing.

The bound C subunit is then eluted with 45 m*M* KP, 0.1 m*M* DTT, 0.1 m*M* cAMP and fractions (~500 ml) are collected and assayed (Fig. 1). The elution is followed by monitoring the absorption at 254 nm. When this value appears to have reached a plateau (A ≅ 1.2), the elution is terminated, and fractions are assayed as quickly as possible, since the protein is very dilute at this stage. Lower concentrations of the cyclic nucleotide can be used, but this makes the spectrophotometric monitoring of the elution more difficult. A modified cyclic nucleotide such as 8-AHA-cAMP can be used instead of cAMP because its more basic properties mean that it is retarded less on the resin, resulting in more concentrated protein in the fractions and shortening the elution time. Once the procedure has been performed a number of times, it is possible to use the absorption of nucleotide as a monitor for the elution of activity rather than waiting to do the assay.

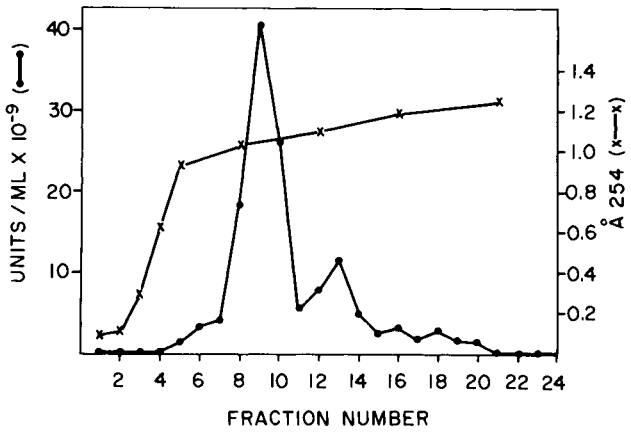


**Fig. 1.** Elution of C subunit from DEAE–cellulose. Two liters of DEAE were equilibrated as described in the text, and 16 liters of 10,000 *g* supernatant were batch adsorbed. The resin was poured into a column (9.5 × 28 cm) and washed extensively with 55 m*M* KP, 1 m*M* EDTA, 0.1 m*M* DTT, pH 6.8, before elution with 45 m*M* KP, 0.1 m*M* DTT, 0.1 m*M* cAMP, pH 6.8. Fractions (~500 ml) were collected and assayed for C subunit and absorbance at 254 nm as described.

TABLE I

Summary of C Subunit Purification from Four Beef Hearts

| Step | Volume (ml) | Protein (mg/ml) | Activity (U/ml) | Specific activity (U/mg) | Total activity (units) | Yield (%) | Purification (fold) |
|---|---|---|---|---|---|---|---|
| Whole homogenate | 17,500 | 21 | 0.00596 | 0.000284 | 104 | 100 | 1 |
| 10,000 × *g* Supernatant | 16,000 | 13 | 0.00713 | 0.000549 | 114 | 109.3 | 2 |
| DEAE-cellulose cAMP eluate | 5,500 | 0.087 | 0.0118 | 0.136 | 63.2 | 60.5 | 479 |
| Hydroxylapatite column I | 120 | 0.192 | 0.504 | 2.56 | 60.6 | 58.0 | 9,021 |
| column II | 11 | 1.47 | 4.65 | 3.17 | 51.3 | 49.1 | 11,144 |

The eluted enzyme is then further purified and concentrated by applying the pooled, active fractions to a 200 ml column of hydroxylapatite equilibrated in 40 m*M* KP, 0.1 m*M* DTT at 5 ml/min. The protein is eluted with a 250 ml linear gradient using 40 m*M* KP, 0.1 m*M* DTT in one chamber and 350 m*M* KP, 0.1 m*M* DTT in the other. The activity elutes as a single peak at 170 to 220 m*M* KP.

The last step is repeated by diluting the eluant three-fold with $H_2O$ and applying it to a smaller column (~2.6 × 2 cm) in order to concentrate the enzyme further. In this case it is preferable to do a batchwise elution with 350 m*M* KP, 0.1 m*M* DTT. The enzyme ($M_r$ = 40,000) is more than 99% pure as judged by SDS gel electrophoresis. The purified protein thus obtained is dialyzed overnight against a 1:1 (v/v) solution of glycerol–350 m*M* KP, 0.1 m*M* DTT, pH 6.8, and stored at −20°C. Under these conditions and with protein concentrations greater than ~0.5 mg/ml, the enzyme is stable for several months. It should have a specific activity of about 3 units/mg. The enzyme can also be stored frozen at −70°C in the absence of dialysis versus glycerol. In this case it is stable for several years with loss of about half the original activity by the freezing–thawing process. The specific activities of cAMP-dependent protein kinase and the yields at each stage of the purification are summarized in Table I.

## REFERENCES

Beavo, J. A., and Krebs, E. G. (1979). *Annu. Rev. Biochem.* **48**, 923.

Bechtel, P. J., Beavo, J. A., and Krebs, E. G. (1977). *J. Biol. Chem.* **252**, 2691.

Bohnert, J. L., Malencik, D. A., Anderson, S. R., Teller, D., and Fischer, E. H. (1982). *Biochemistry* **21**, 5563.

Carlson, G. M., Bechtel, P. J., and Graves, D. J. (1979). *Adv. Enzymol.* **50,** 41.
Cohen, P. (1978). *Curr. Top. Cell. Regul.* **14,** 118.
Corbin, J. D., and Reimann, E. M. (1974). *In* "Methods in Enzymology" (J. G. Hardman and B. W. O'Malley, eds.), Vol. 38, p. 287. Academic Press, New York.
Corbin, J. D., Keely, S. L., and Park, C. R. (1975). *J. Biol. Chem.* **250,** 218.
Corbin, J. D., Sugden, P. H., West, L., Flockhart, D. A., Lincoln, T. M., and McCarthy, D. (1978). *J. Biol. Chem.* **253,** 3997.
Ehrlichman, J., Sarkar, D., Fleischer, N., and Rubin, C. S. (1980). *J. Biol. Chem.* **225,** 8179.
Flockhart, D. A. (1983). *In* "Methods in Enzymology" (J. D. Corbin and J. G. Hardman, eds.) Vol. 99, p. 14, Academic Press, New York.
Flockhart, D. A., and Corbin, J. D. (1982). *CRC Crit. Rev. Biochem.* **12,** 133.
Glass, D. B., and Krebs, E. G. (1980). *Annu. Rev. Pharmacol. Toxicol.* **20,** 363.
Hofmann, F., Beavo, J. A., Bechtel, P. J., and Krebs, E. G. (1975). *J. Biol. Chem.* **250,** 7795.
Hofmann, F., Bechtel, P. J., and Krebs, E. G. (1977). *J. Biol. Chem.* **252,** 1441.
Osterreider, W., Brum, G., Hescheler, J., Trautwein, W., Flockerzi, V., and Hofmann, F. (1982). *Nature (London)* **298,** 576.
Peters, K. A., Demaille, J. G., and Fischer, E. H. (1977). *Biochemistry* **16,** 5691.
Rangel-Aldao, R., Kupiec, J. W., and Rosen, O. M. (1979). *J. Biol. Chem.* **254,** 2499.
Reimann, E., and Beham, R. A. (1983). *In* "Methods in Enzymology" (J. D. Corbin and J. G. Hardman, eds.) Vol. 99, p. 51, Academic Press, New York.
Rosen, O. M., and Ehrlichman, J. (1975). *J. Biol. Chem.* **250,** 7788.
Ruegg, J. C., and Paul, R. J. (1982). *Circ. Res.* **50,** 394.
Schwoch, G., Hamaan, A., and Hilz, H. (1980). *Biochem. J.* **192,** 223.
Shizuta, Y., Beavo, J. A., Bechtel, P. J., Hofmann, F., and Krebs, E. G. (1975). *J. Biol. Chem.* **250,** 6891.
Sugden, P. H., Holladay, L. A., Reimann, E. M., and Corbin, J. D. (1976). *Biochem. J.* **159,** 409.
Walseth, T. F., and Johnson, R. A. (1979). *Biochim. Biophys. Acta* **562,** 11.
Yamamura, H., Nishiyama, K., Shimomura, R., and Nishizuka, Y. (1973). *Biochemistry* **12,** 856.

# Chapter 13

# PHOSPHOLIPID METHYLATION IN BRAIN AND OTHER TISSUES

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## I. INTRODUCTION

Since the pioneering studies of Hirata and Axelrod demonstrated that norepinephrine increased the methylation of phospholipids in rat reticulocytes, numerous studies have demonstrated that phospholipid methylation is involved in receptor-mediated signal transduction. The phospholipid methyltransferase (EC 2.1.1.17) enzymes (PMTs) *N*-methylate phosphatidylethanolamine (PE) three consecutive times to form phosphatidylcholine (PC), or lecithin. At least two enzymes appear to be involved in these three methylations. Phospholipid methyltransferase activity has been found in almost all tissues investigated. The liver is unique in that it has very high phospholipid methyltransferase activity and actually secretes phosphatidylcholine formed through the methylation pathway into circulation (Bjørnstad and Bremer, 1966). The brain has less activity, similar to that found in most other tissues. Phospholipid methylation has been shown to be activated in different tissues by such receptor agonists as catecholamines (Hirata *et al.*, 1979), benzodiazepines (Strittmatter *et al.*, 1979), lectins (Hirata *et al.*, 1979), antigen–immunoglobin stimulation (Crews *et al.*, 1981), glucagon (Castano *et al.*, 1980), vasopressin (Prasad and Edwards, 1981), and angiotensin (Alemany *et al.*, 1981). The activation is usually very rapid and transient. For




ISBN 0-12-470350-X

example, during stimulation of mast cells with Anti-IgE, the methylation of phospholipids peaks around 15 sec and returns to control values after 30 sec (Ishizaka *et al.*, 1980). This rapid turnover is consistent with the hypothesis that PMTs are involved in receptor-mediated membrane signal transduction.

The mechanism by which PMTs effect membrane signal transduction is thought to be through alterations in membrane fluidity which occur during the synthesis and translocation of the methylated phospholipids across the lipid bilayer membrane (see Hirata and Axelrod, 1980, for review). Using varying concentrations of *S*-adenosyl-L-methionine (SAM), it has been shown in rat reticulocytes that the membrane viscosity, as measured by the fluorescent polarization of diphenylhexatriene, decreased in parallel with the synthesis of phosphatidyl-*N*-monomethylethanolamine (PNE) (Hirata and Axelrod, 1978b). Other studies on chicken erythrocytes have found that concanavalin A will increase phospholipid methylation and simultaneously decrease membrane viscosity (Nakajima *et al.*, 1981). This study used the electron spin resonance spectrum of a steric acid spin label to estimate membrane viscosity and found that methylation inhibitors could block the concanavalin A–induced change in fluidity. These studies are consistent with the hypothesis that activation of phospholipid methylation alters the viscosity of a discrete localized membrane domain which then allows or enhances the receptor mediated signal.

## II. LIPID METHYLATION IN NERVOUS TISSUE

### A. Phospholipid Methyltransferases

Studies in bovine adrenal gland (Hirata *et al.*, 1978), rat brain (Mozzi and Porcellati, 1979; Crews *et al.*, 1980a,b), and bovine brain (Blusztajn *et al.*, 1979) have suggested that at least two enzymes are involved in the methylation of phosphatidylethanolamine to form phosphatidylcholine. The first enzyme, phospholipid methyltransferase I (PMT I), methylates phosphatidylethanolamine once to form phosphatidyl-*N*-monomethylethanolamine. PMT I has an optimum pH of 7.5, is activated by $Mg^{2+}$, and has a low apparent $K_m$ for *S*-adenosyl-L-methionine, the methyl donor. A second enzyme, phospholipid methyltransferase II (PMT II), catalyzes two successive methylations of PNE to form phosphatidyl-*N*,*N*′-dimethylethanolamine (PNNE) and then phosphatidylcholine (PC). PMT II has an optimum pH of 10.5, a high apparent $K_m$ for *S*-adenosyl-L-methionine, and can be differentially solubilized by sonication. The separate pH optima and affinity for SAM suggest the presence of two enzymes. However, these enzymes have proved difficult to separate during purification. It is possible that there may be more than two enzymes involved.

Several lines of evidence suggest that PMT I is the rate-limiting enzyme in the

methylation of phospholipids. The first is that the maximal activity measured in membranes for PMT II is much greater than that for PMT I. A second line of evidence is that in intact cells incubated with [*methyl*-$^3$H]methionine almost all of the methylated phospholipid is PC. The third line of evidence comes from the addition of exogenous phospholipids. Since there is a large amount of PE present in membranes, it is not surprising that the addition of exogenous PE to synaptosomes does not significantly change the methylation of phospholipids (Table I). The addition of PNE greatly increases the methylation of phospholipids, particularly the formation of PNNE. These data suggest that the formation of PNE is the rate-limiting step in the methylation of phospholipids.

Studies of membranes from a variety of tissues have indicated that phospholipids are asymmetrically distributed across the phospholipid bilayer with phosphatidylcholine and sphingomyelin localized on the outside and phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine localized primarily on the inside layer of the bilayer (see Crews, 1982, for more details). Similar to other membranes, synaptosomal plasma membranes appear to have an asymmetric distribution of phospholipids (Fontaine *et al.*, 1980). This asymmetry could be related to the asymmetric distribution of phospholipid methyltransferases.

Evidence for the asymmetric localization of phospholipid methyltransferases in synaptosome membranes has been obtained by selective proteolytic digestion using trypsin in intact and lysed synaptosomes (Crews *et al.*, 1980b). Trypsin treatment caused a small reduction, about 13%, in PMT I activity in intact synaptosomes, while in lysed synaptosomes it destroyed 83% of the activity.

TABLE I

Effects of Exogenous Phospholipids on [$^3$H]Methyl Incorporation into Lipids[a]

| Exogenous substrate | PNE | PNNE (pmol/mg protein/45 min) | PC |
|---|---|---|---|
| O | 7.18 ± 0.87 | 7.73 ± 0.62 | 7.73 ± 0.22 |
| PE | 6.40 ± 0.39 | 8.26 ± 0.38 | 6.65 ± 0.46 |
| PNE | 7.38 ± 1.26 | 68.50 ± 2.12[b] | 9.19 ± 0.18[c] |
| PNNE | 7.16 ± 0.09 | 7.65 ± 0.28 | 91.50 ± 3.57[b] |

[a]Values represent the mean ± SE of three determinations. The synaptosomal fraction from rat brain was incubated with 10 μg of exogenous substrate. Samples were incubated for 45 min with 200 μ*M* SAM. Exogenous substrates were dried under $N_2$ and then suspended in 25 m*M* Tris (pH = 8) by sonication for 30 sec. The products were separated by thin-layer chromatography. PE, Phosphatidylethanolamine; PNE, phosphatidyl-*N*-monomethylethanolamine; PNNE, phosphatidyl-*N*, *N*-dimethylethanolamine; PC, phosphatidylcholine.

[b]$p < .001$ as compared with 0 exogenous substrate.

[c]$p < .01$ as compared with 0 exogenous substrate.

This suggests that PMT I mainly faces the cytoplasmic side of the plasma membrane where trypsin cannot penetrate. Trypsin treatment reduced PMT II activity by 57% in intact synaptosomes and by 95% in lysed synaptosomes. The loss of PMT II in intact synaptosomes after trypsin treatment represents the fraction of the enzyme localized on the exterior side of the plasma membrane and indicates that PMT II is present on the outer surface of the membrane. The additional loss of PMT II activity in lysed preparations probably represents PMT II present on vesicles, mitochondria, and other intrasynaptosomal particles. These results suggest the asymmetric distribution of the two methyltransferases in the synaptosomal plasma membrane; PMT I, which methylates PE to PNE, is mainly exposed on the cytoplasmic side, and PMT II, which catalyzes the two successive methylations of PNE to form PC, faces the exterior sides of the membrane (Fig. 1). Similar experiments using phospholipase C suggested that in intact synaptosomes PNE is buried within the membrane or located on the cytoplasmic side, whereas PNNE and PC are exposed on the outer surface of the membrane (Crews *et al.*, 1980b). The phospholipid methyltransferase enzymes have also been found to be asymmetrically distributed in liver microsomal membranes (Higgins, 1981) and in rat erythrocyte ghosts (Hirata and Axelrod, 1978a). These data suggest the stepwise methylation and translocation of a

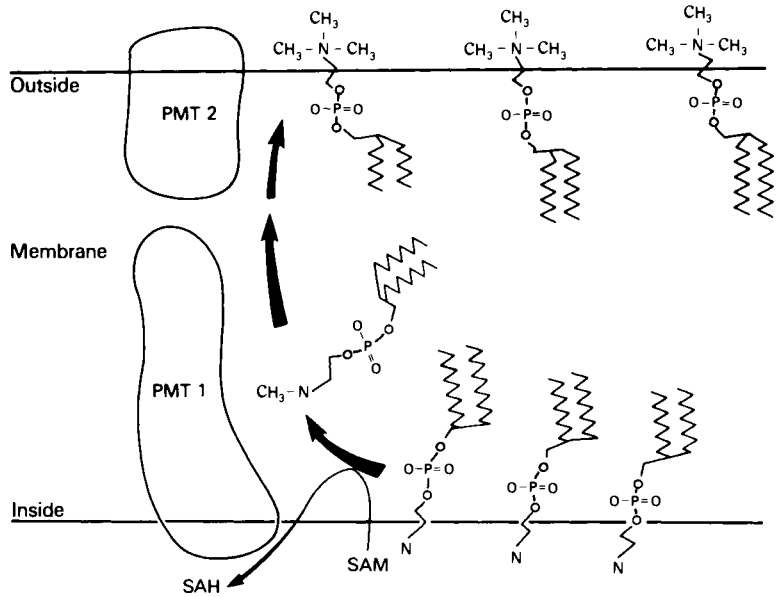


**Fig. 1.** Schematic diagram of methylation and translocation of phospholipids in synaptosomal membranes. SAM, *S*-Adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine; PMT I, phospholipid methyltransferase I; PMT II, phospholipid methyltransferase II.

fraction of the methylated phospholipids from the cytoplasmic side to the outside of the membrane.

## B. Methodology

### *1. Incubation*

Phospholipid methylation can be measured in homogenates and subcellular fractions by incubation with *S*-adenosyl-L-[*methyl*-$^3$H]methionine. In a typical experiment we use 6 ml polypropylene capped tubes (e.g., 12 × 75 mm Falcon tubes). A reaction mixture is prepared containing *S*-adenosyl-L-[*methyl*-$^3$H]methionine (4 μCi), 2 m*M* $MgCl_2$, 25 m*M* Tris–glycylglycine buffer, pH 8.0, and tissue extract (0.2–0.4 mg of protein) in a total volume of 100 ul. The reaction mixture is prepared at 4°C. *S*-Adenosyl-L-[*methyl*-$^3$H]methionine ([$^3$H]SAM) requires special care. It is not very stable chemically, particularly in alkaline solutions. Commercial [$^3$H]SAM should be separated into aliquots to avoid decomposition during freezing and thawing. We have always used high specific activity [$^3$H]SAM, that is, 55–90 Ci/mmol. Care must be used in adding [$^3$H]SAM to the incubation mixture since commercial sources provide [$^3$H]SAM in a sulfuric acid–ethanol (9:1) mixture. The ethanol can be removed by blowing under $N_2$. The pH of the incubation mixture should be checked to be sure the sulfuric acid has not overcome the buffer. PMT I is best assayed with low concentrations of [$^3$H]SAM, that is, less than 1 μ*M*. This is easily prepared using high specific activity SAM. With [$^3$H]SAM of 60 Ci/mmol add 4 μCi/100 μl of incubation buffer for a final SAM concentration of 0.67 μ*M*. To determine PMT II activity, cold SAM should be added for a final SAM concentration of about 100 to 200 μ*M*. In addition, exogenous PNE or PNNE may be added. Once the reaction mixture is prepared on ice, that is, 4°C, the reaction may be started by placing the mixture in a 37°C water bath.

We have used several methods to stop the reaction. Each method has some problems. The easiest method stops the reaction by adding 3 ml of chloroform–methanol–hydrochloric acid (2/1/0.02, v/v/v) and immediately starts the extraction. High blank values can be a problem with this method. We have obtained lower blanks by stopping the reaction with 10% TCA and 10% $Na_2TCA$, pH 7, to precipitate protein. The protein is pelleted by centrifugation, the TCA aspirated, and the pellet extracted. The problem with 10% TCA is that plasmalogen phospholipids are hydrolysed by acid treatment and in brain tissue they represent a significant fraction of the methylated phospholipids (Mozzi *et al.*, 1981). Although $Na_2TCA$ is neutral, we have had considerable problems with obtaining consistent precipitation of proteins. Although there are problems with each of these, we have successfully used them all.

### 2. *Phospholipid Methylation in Intact Cells*

The methylation of phospholipids is easily measured in cell culture. In fact, studies on receptor stimulation of phospholipid methylation have been done predominantly in intact cells. Homogenization of certain tissues may disrupt the coupling of receptors to the phospholipid methyltransferases. Phospholipid methylation can be measured using L-[*methyl*-$^3$H]methionine. The cell will synthesize *S*-adenosyl-L-[*methyl*-$^3$H]methionine from this precursor. Since most cell culture media preparations contain 100 μ*M* methionine, media for a methylation experiment can be easily prepared by placing a trace amount of [*methyl*-$^3$H]methionine directly into the media. High specific activity [*methyl*-$^3$H]methionine, that is, 50–70 Ci/mmol, is commercially available and can be used to make a final [*methyl*-$^3$H]methionine specific activity in the medium of between 0.25 and 1.5 Ci/mmol. In our studies, we usually incubate $1–5 \times 10^6$ cells/ml in media of this composition. In general, rapidly dividing cells have a much higher rate of phospholipid methylation. For experiments on receptor stimulation, this background rate can be reduced in confluent cultures of cells which exhibit contact inhibition. The primary methylated phospholipid formed under these conditions is usually phosphatidylcholine. We have used several methods to stop the methylation reaction. In detached cell experiments, rapid cooling with a large volume of cold buffer followed by centrifugation works well. The media is easily removed by aspiration, and the phospholipids can be extracted with chloroform–methanol. With attached cells in wells, we have stopped the reaction by aspirating the media, washing the well with ice-cold buffer, and adding 10% TCA. The cells can then be scraped with a rubber policeman, pelleted, and the phospholipids extracted.

### 3. *Lipid Extraction*

A simple method for extracting pellets is as follows: Three ml of chloroform–methanol (2:1 v/v) containing 50 μg/ml of the antioxidant butylated toulene are added to the tubes. The pellets are dispersed and the phospholipids extracted by vigorous shaking for 10 min. After the addition of 2 ml or 0.1 *M* KCl–50% methanol (1:1, v/v), the tubes are again shaken for 10 min and centrifuged at 2000 *g* for 10 min. The aqueous (upper) phase is aspirated, the chloroform phase again washed with 2 ml of 0.1 *M* KCl–50% methanol, and the upper phase aspirated. For total incorporation of [$^3$H]methyl groups into phospholipids, an aliquot of the chloroform phase is transferred to a vial, the solvent evaporated to dryness at 80°C in an oven, and 10 ml of scintillation fluid is added, the residue solubilized, and the radioactivity measured. Evaporation is important to remove any [$^3$H]methanol formed from [$^3$H]SAM. The remaining chloroform fraction can be stored at −20°C for later identification of individual phospholipids. Approximately 100 mg of $Na_2SO_4$ can be added to remove ex-

cess $H_2O$ and reduce oxidation during storage. We have found this extraction protocol to be relatively easy and to extract approximately 80–90% of the total phospholipids as determined by phospholipid phosphate analysis. Bligh and Dyer (1959) have described a slightly more involved chloroform–methanol extraction procedure which, we have found, recovers more than 90% of the total phospholipid. In our experiments on phospholipid metabolism which use radiolabeled precursors, we have found no major differences in any of these extraction procedures.

*4. Separation of Reaction Products*

To an aliquot of the remaining chloroform phase, 2 μg each of phosphatidylethanolamine, phosphatidylmonomethylethanolamine, phosphatidyldimethylethanolmine, and phosphatidylcholine should be added as carriers. To avoid oxidation of phospholipids, the chloroform aliquot can be dried under a stream of nitrogen gas. We have recently successfully used a Savant Speed Vacuum centrifuge concentrator to evaporate our samples. The residue is dissolved in 50 to 150 μl of chloroform–methanol (1:1 v/v). The samples are applied on a silica gel plate (Uniplate, Analtech, Inc., or LK5 Whatman Silica Gel Plates) under a stream of nitrogen. For identification of products by one-dimensional thin layer chromatography (TLC), the following solvent systems can be used: (*a*) chloroform–propionic acid–*n*-propyl alcohol–water (2/2/3/1 by vol), (*b*) chloroform–methanol–2 propranol–0.25% KCl–triethylamine (30/9/25/6/18 by vol), (*c*) chloroform–methanol–7 *M* ammonia (60/35/5 by vol), (*d*) chloroform–methanol–water (65/25/4 by vol), and (*e*) *n*-butyl alcohol–acetic acid–water (6/2/2 by vol). Products can also be separated using two-dimensional TLC, with the first solvent system of chloroform–methanol–28% ammonia (65/35/3 by vol) followed by chloroform–acetone–methanol–acetic acid–water (5/2/1/1/0.5 by vol). Solvent systems *a* and *b* are best for the separation of PC, PNE, and PNNE, the three compounds produced by phospholipid methyltransferases (Table II). Although some phospholipids migrate close to the methylated phospholipids, only the methylated phospholipids are labeled by [$^3$H]methionine and [$^3$H]SAM.

In addition to the methylation of phospholipids, there are neutral lipids which are formed in certain tissues during incubation with [$^3$H]methionine and [$^3$H]SAM. These nonpolar lipids include methylated fatty acids, ubiquinone, and 2-(methylthio)benzothiazole (Kloog *et al.*, 1982). In the solvent systems described above, they all migrate as a radioactive peak at the solvent front. Methylated fatty acids can be formed in significant amounts in homogenates incubated with [$^3$H]SAM (Kloog *et al.*, 1982). Homogenization of brain, lung, and other tissues releases fatty acids which can then be methylated (Zatz *et al.*, 1981). Much smaller amounts of nonpolar lipids are formed during incubations with purified subcellular fractions or in intact cells. The formation of these nonpolar methylated

TABLE II

Mobility of Phospholipids in Solvent Systems *a* and *b*[a]

| Phospholipid | $R_f$ Solvent *a* | $R_f$ Solvent *b* |
|---|---|---|
| PC | 0.48 | 0.19 |
| PNE | 0.74 | 0.41 |
| PNNE | 0.62 | 0.54 |
| PE | 0.78 | 0.40 |
| PS | 0.55 | 0.30 |
| PI | 0.58 | 0.33 |
| SPM | 0.54 | — |
| Chol | 0.98 | 0.69 |
| PA | 0.74 | 0.37 |

[a]Solvent system *a*: Chloroform–proprionic acid–1-propanol–$H_2O$ (2/2/3/1). Solvent system *b*: Chloroform–methanol–2-propanol–0.25% KCl–triethylamine (30/9/25/6/18). PC, Phosphatidyl choline; PNE, phosphatidyl-N-monomethylethanolamine; PNNE, phosphatidyl-*N*,*N*-dimethyl-ethanolamine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; SPM, sphingomyelin; Chol, cholesterol; PA, phosphatidic acid.

lipids makes product separation and identification an essential feature of experiments studying receptor-mediated stimulation of phospholipid methylation.

### 5. *Visualization of Phospholipids*

We have used several methods to visualize phospholipids separated by TLC. One simple method is to place the plate in a spare chromatography tank which contains iodine. Iodine will stain all the compounds on the TLC plate a light brown color in 5 to 10 min. At least 10 μg of phospholipid standards should be used for iodine identification. A second method uses diphenylhexatriene (DPH). DPH is dissolved in petroleum ether (10 mg/100 ml) and sprayed onto the thin layer plate. Lipids can then be visualized with an ultraviolet light.

Once the lipids are visualized, the spots can be scraped off the TLC plate into scintillation vials. The phospholipids are eluted with 1 ml of methanol, scintillation fluid added, and the radioactivity determined.

## III. INTERACTIONS OF PHOSPHOLIPID METHYLATION WITH RECEPTORS IN NERVOUS TISSUES

Phospholipid methylation has been shown to alter receptor-mediated signal transduction as well as the density of receptor binding sites in a large variety of tissues (see Hirata and Axelrod, 1980; Crews, 1982). In general, PMTs appear to

enhance the coupling of receptors to second messenger responses, for example, changes in membrane permeability or activation of adenylate cyclase. This review will briefly cover receptor–phospholipid methylation interactions in nervous tissue. Strittmatter *et al.* (1979), using a particular cell line, C6-astrocytoma cells, demonstrated that both β-adrenergic agonists and benzodiazepines stimulate PMTs in a dose-dependent manner. When β-adrenergic and benzodiazepine agonists were added together, PMT activity was increased in an additive manner. This suggests that different receptors are located in separate areas of the membrane and are associated with their own component of methyltransferase enzymes. Thus, PMTs may act as coupling factors to the receptors' primary signals, that is, the β receptor is coupled to adenylate cyclase and the benzodiazepine receptor to the chloride channel–GABA receptor complex. More recent studies have shown that PMT activity is stimulated by norepinephrine and dopamine in rat brain synaptosomes (Leprohon *et al.*, 1983). In other studies, the binding of [$^3$H]diazepam as well as β-[$^3$H]carboline-3-carboxylic acid ethyl ester ([$^3$H]βCCE) to synaptosomal membranes can be increased almost twofold by incubating membranes with SAM (DePerri *et al.*, 1983). The increase in the density of benzodiazepine receptors (i.e., [$^3$H]diazepam) and GABA receptors (i.e., [$^3$H]βCCE) is correlated to and dependent upon the increases in methylated phospholipids. In addition to these neurotransmitter–receptor interactions with PMTs, nerve growth factor has been shown to stimulate PMTs in cultured rat superior cervical ganglia (Pfenninger and Johnson, 1981). Thus, several types of receptors in nervous tissue appear to interact with PMTs.

In addition to receptor–PMT interactions in the nervous system, PMTs may play a unique and important role in nervous tissue as a source of choline. Choline availability is thought to be an important factor in acetylcholine synthesis. Phospholipid methylation is the only known metabolic route by which *de novo* choline is formed. Studies in rat brain synaptosomes have shown that phospholipid methylation can rapidly generate free choline (Blusztajn and Wurtman, 1981). Thus, PMT activity may provide a portion of the choline for acetylcholine synthesis.

The activity of PMTs in rat brain have been shown to change during development and aging. During development, the activity of PMT I is highest neonatally, whereas PMT II activity is highest approximately 2 weeks after birth (Blusztajn *et al.*, 1982; Hitzemann, 1982). The activity of PMT I and II remains relatively constant in rats 1–5 months old. In contrast to many enzymes, as rats age the activity of PMT I increases. The activity of PMT I increases from 0.49 ± 0.02 pmol methyl-$^3$H incorporation/mg protein/30 min in one-month-old rats to 0.64 ± 0.03 pmol methyl-$^3$H incorporation/mg protein/30 min in 21-month-old rats (Crews *et al.*, 1981). The activity of PMT II does not change during aging. The significance of these changes in PMT activity during development and aging is not clear.

In summary, PMTs may modulate receptor-mediated signal transduction as

well as the number of receptor binding sites in nervous tissue. In addition, they may play a role in regulating membrane fluidity during development and/or aging as well as providing a source of choline for acetylcholine synthesis. Although the exact role of PMTs in neurotransmission is still uncertain, there are clearly strong indications for several significant sites at which PMTs may be involved in brain function.

## REFERENCES


Alemany, S., Varela, I., and Mato, J. M. (1981). *FEBS Lett.* **135,** 111–114.

Bjørnstad, P., and Bremer, J. (1966). *J. Lipid Res.* **7,** 38–45.

Bligh, E. G., and Dyer, W. J. (1959). *Can. J. Biochem. Physiol.* **37,** 911–924.

Blusztajn, J. K., and Wurtman, R. J. (1981). *Nature (London)* **290,** 417–418.

Blusztajn, J. K., Zeisel, S. H., and Wurtman R. J. (1979). *Brain Res.* **179,** 319–327.

Blusztajn, J. K., Zeisel, S. H., and Wurtman, R. J. (1982). *In* "Biochemistry of S-Adenosylmethionine and Related Compounds" (E. Usdin, R. I. Borchardt, and C. Creveling, eds.), pp. 155–162. Macmillan, New York.

Bremer, J., and Greenberg, D. M. (1961). *Biochim. Biophys. Acta* **46,** 205–218.

Castano, J. G., Alemany, S., Nicto, A., and Mato, J. M. (1980). *J. Biol. Chem.* **255,** 9041–9043.

Crews, F. T. (1982). *Int. Rev. Neurobiol.* **23,** 141–163.

Crews, F. T., Hirata, F., and Axelrod, J. (1980a). *J. Neurochem.* **34,** 1491–1498.

Crews, F. T., Hirata, F., and Axelrod, J. (1980b). *Neurochem. Res.* **5,** 983–991.

Crews, F. T., Calderini, G., Battistella, A., and Toffano, G. (1981). *Brain Res.* **229,** 256–259.

DePerri, B., Calderini, G., Battistella, A., and Toffano, G. (1983). *J. Neurochem.* **41,** 302–308.

Fontaine, R. N., Harris, R. A., and Schroeder, F. (1980). *J. Neurochem.* **34,** 269–277.

Higgins, J. A. (1981). *Biochim. Biophys. Acta* **640,** 1–5.

Hirata, F., and Axelrod, J. (1978a). *Proc. Natl. Acad. U.S.A.* **75,** 2348–2352.

Hirata, F., and Axelrod, J. (1978b). *Nature (London)* **275,** 219–220.

Hirata, F., and Axelrod, J. (1980). *Science* **209,** 1082–1090.

Hirata, F., Viveros, O. H., Diliberto, E. J., Jr., and Axelrod, J. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 1718–1721.

Hirata, F., Axelrod, J., and Crews, F. T. (1979). *Proc. Natl. Acad. Sci. U.S.A.* **76,** 4813–4816.

Hitzemann, R. (1982). *Life Sci.* **30,** 1297–1301.

Ishizaka, T., Hirata, F., Ishizaka, K., and Axelrod, J. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 1903–1906.

Kloog, Y., Zatz, M., Rivnay, B., Dudley, P., and Markey, S. (1982). *Biochem. Pharmacol.* **31,** 753–759.

Leprohon, C. E., Blusztajn, J. K., and Wurtman, R. J. (1983). *Proc. Natl. Acad. Sci. U.S.A.* **80,** 2063–2066.

Mozzi, R., and Porcellati, G. (1979). *FEBS Lett.* **100,** 363–366.

Mozzi, R., Siepe, O., Andredi, V., and Porcellati, G. (1981). *FEBS Lett.* **131,** 115–119.

Nakajima, M., Tamura, E., Irimura, T., Toyoskima, S., Hirano, H., and Osawa, T. (1981). *J. Biochem. (Tokyo)* **89,** 665–675.

Pfenninger, K. H., and Johnson, M. P. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 7797–7800.

Prasad, C., and Edwards, R. M. (1981). *Biochem. Biophys. Res. Commun.* **103,** 559–564.

Strittmatter, W. J., Hirata, F., Axelrod, J., Mallorga, P., Tallman, J. F., and Henneberry, R. C. (1979). *Nature (London)* **282,** 857–859.

Zatz, M., Dudley, P. A., Kloog, Y., and Markey, S. P. (1981). *J. Biol. Chem.* **256,** 10028–10031.

# *Chapter 14*

# *β-ADRENERGIC RECEPTORS*

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ISBN 0-12-470350-X

# I. INTRODUCTION AND HISTORICAL PERSPECTIVE

Although extensive pharmacological studies of adrenergic receptors have been performed for several decades, their identification by radioligand binding studies lagged behind those of certain polypeptide hormone receptors and the nicotinic cholinergic receptors. Pharmacological reviews such as those of Ariens (1967) documented in detail the structure–activity relationship responsible for eliciting adrenergic responses and discussed the evidence for the existence of at least two distinct types of adrenergic receptors, those of the α and β type (Ahlquist, 1948). By the late 1960s the limitations of classic pharmacological methods for the understanding of adrenergic responses were becoming apparent. A new technique, similar to that already applied to radioimmunoassay, allowed the direct identification of receptors by radioligand binding methods.

One of the earliest attempts at using a radioactively labeled compound to study β-adrenergic receptors was that of Potter (1967). He tried to bind the radioactive antagonist (±)-[$^3$H]propranolol to fragments of atrial muscle. However, under the conditions utilized, the binding appeared to be nonsaturable and did not have the specific characterization expected of a β-adrenergic receptor. Subsequently, during the period from 1967 to 1974, several investigators attempted to use different radioligands, especially [$^3$H]catecholamines, in a number of tissues. Unfortunately, the binding sites in these studies also did not have the specific binding characteristics of β-adrenergic receptors. Thus, by 1974 there had still been no reports of successful identification of β-adrenergic receptors using radioactively labeled ligands. In that year, three groups independently developed methods for the direct identification of β-adrenergic receptors. Levitzki *et al.* (1974) and Atlas *et al.* (1974) used (±)-[$^3$H]propranolol, Lefkowitz *et al.* (1974) used (−)-[$^3$H]dihydroalprenolol, and Aurbach *et al.* (1974) developed the iodinated radioligand (±)-[$^{125}$I]iodohydroxybenzylpindolol.

Since then, much methodological progress has been achieved and a number of other suitable radioligands have become commercially available. By 1977, β-adrenergic receptor sites could be labeled by both agonists and antagonists (Lefkowitz and Williams, 1977; Williams and Lefkowitz, 1977, 1978) and could be identified in plasma membrane preparations from a variety of tissues and also in a solubilized form. In 1980 computer programs became available for analysis of β-adrenergic receptor and other ligand binding data (Kent *et al.*, 1980; Munson and Rodbard, 1980). In the past few years interpretation of even complex β-adrenergic receptor interactions has been made possible through computer methodology.

## II. RADIOLIGANDS

A list of β-adrenergic radioligands is given in Table I. Most of these are commercially available and their appropriateness for studying β-adrenergic receptors is well documented. A short description of these radioligands is given below.

### A. Antagonist Radioligands

During the past few years the most widely used radioligands for the characterization and quantification of β-adrenergic receptors have been (−)-[$^3$H]DHA [(−)-[$^3$H]dihydroalprenolol] and (±)-[$^{125}$I]HYP [(±)-[$^{125}$I]iodohydroxybenzylpindolol]. For review see also Maguire *et al.* (1977) and Williams and Lefkowitz (1978). The iodinated HYP has the inherent advantage of possessing a very high affinity ($K_D$ approximately 50 p*M*) and a specific radioactivity of 2200 Ci/mMol. This radioligand therefore allows the quantification of femtomolar amounts of β-adrenergic receptor binding. However, (±)-[$^{125}$I]HYP is not yet available as the pure (−) stereoisomer, and this may complicate data analysis. Problems arising from the use of racemic radioligands are discussed in Section IV,C.

If receptor concentration is not a limiting factor, a pure stereoisomer such as (−)-[$^3$H]DHA, which can be stored for several months without significant chemical or radiochemical degradation, is preferable. Because of the relatively short half-life of $^{125}$I, (±)-[$^{125}$I]HYP has to be prepared or purchased more frequently. Historically, an antagonist radioligand that was used for β-adrenergic receptor binding studies was (±)-[$^3$H]propranolol (Levitzki *et al.*, 1974; Nahorski, 1976). Although the (−) stereoisomer is commercially available, its specific radioactivity and affinity is low, and its nonspecific binding is therefore quite high. The pure stereoisomer (−)-[$^3$H]carazolol has an extremely high affinity ($K_D$ approximately 10–20 p*M*; Bürgisser *et al.*, 1981a) and because of its very slow dissociation kinetics ($t_{1/2}$ = 4–5 hr) (Bürgisser *et al.*, 1981b), such a high-affinity radioligand may be especially useful in nonequilibrium experiments in which slow dissociation is important. However, for equilibrium experiments, extremely high-affinity radioligands require longer incubation times (>1 hr), and this could lead to significant degradation of receptors and radioligand.

A new analog of pindolol is (±)-[$^{125}$I]iodocyanopindolol ([$^{125}$I]CYP) (Engel *et al.*, 1981; Hoyer *et al.*, 1982), which combines very high affinity ($K_D \sim 50$ p*M* for the racemic mixture) and high specific radioactivity; it has been shown that [$^{125}$I]CYP interacts much less with other non-β-adrenergic receptor sites

TABLE I

Radioligands for the β-Adrenergic Receptor

| Radioligand | | Max specific radioactivity (Ci/mmol) | $K_D$ (approx.) | Specificity | Commercial code[a] | Comments |
|---|---|---|---|---|---|---|
| Short name | Compound | | | | | |
| Antagonists | | | | | | |
| (−)-[$^3$H]DHA | (−)-[$^3$H]Dihydroalprenolol | 120 | 0.5–2 n*M* | $\beta_1 = \beta_2$ | N-507/N-720<br>A-551/A-649 | Most frequently used radioligand |
| (±)-[$^3$H]PRO | (±)-[$^3$H]Propranolol | 30 | 1–10 n*M*[b] | $\beta_1 = \beta_2$ | A-495 | |
| (−)-[$^3$H]PRO | (−)-[$^3$H]Propranolol | 30 | 1–10 n*M* | $\beta_1 = \beta_2$ | N-515 | |
| (±)-[$^3$H]CAR | (±)-[$^3$H]Carazolol | 40 | 30–200 p*M*[b] | $\beta_1 = \beta_2$ | N-604 | Innis *et al.* (1979) |
| (−)-[$^3$H]CAR | (−)-[$^3$H]Carazolol | 24 | 10 p*M* | $\beta_1 = \beta_2$ | — | Very high affinity tritiated radioligand |
| (−)-[$^3$H]BUP | (−)-[$^3$H]Bupranolol | 18 | 650 p*M* | $\beta_1 = \beta_2$ | — | Kaumann (1981); Morris *et al.* (1981) |
| (±)-[$^{125}$I]GCP | (±)-[$^3$H]CPG-12177 | | 370 p*M*[b] | | — | Very hydrophilic |
| (±)-[$^{125}$I]HYP | (±)-[$^{125}$I]Iodohydroxybenzylpindolol | 2200 | 10–1000 p*M*[b] | $\beta_1 = \beta_2$ | N-125<br>A-111 | Most frequently used iodinated radioligand |

| | | | | | | |
|---|---|---|---|---|---|---|
| (±)-[$^{125}$I]CYP | (±)-[$^{125}$I]Iodocyanopindolol | 2200 | 40–100 p*M*[b] | $\beta_1 = \beta_2$ | N-174<br>A-115 | Very high affinity iodinated radioligand |
| (−)-[$^{125}$I]CYP | (−)-[$^{125}$I]Iodocyanopindolol | 2200 | 10–20 p*M* | $\beta_1 = \beta_2$ | A-142/N-189 | The only iodinated radioligands available as pure (−) steroisomers |
| (±)-[$^{125}$I]PIN | (±)-[$^{125}$I]Iodopindolol | 2200 | 130 p*M*[b] | | | |
| (−)-[$^{125}$I]PIN | (−)-[$^{125}$I]Iodopindolol | 2200 | 90 p*M* | | | |
| Agonists | | | | | | |
| (±)-[$^{3}$H]HBI | (±)-[$^{3}$H]Hydroxybenzylisoproterenol | 20 | 1–10 n*M*[b,c] | $\beta_2 >> \beta_1$ | N-587 | The most frequently used agonist-radioligand |
| (−)-[$^{3}$H]EPI | (−)-[$^{3}$H]Epinephrine | 145 | 80 n*M*[c] | $\beta_2 >> \beta_1$ | N-696<br>A-598 | Also α-adrenergic |
| (−)-[$^{3}$H]NEP | (−)-[$^{3}$H]Norepinephrine | 60 | | $\beta_1 >> \beta_2$ | N-678<br>A-584 | Also α-adrenergic |
| (±)-[$^{3}$H]ISO | (±)-[$^{3}$H]Isoproterenol | 15 | 150 n*M*[b,c] | $\beta_1 = \beta_2$ | N-142<br>A-295 | |

[a]N denotes material available from New England Nuclear, A from Amersham.
[b]Value for racemic radioligand.
[c]Value from high-affinity site.

(e.g., α-adrenergic and 5-HT receptors) compared to (±)-[$^{125}$I]HYP (Wolfe and Harden, 1981). Recently, the pure (−) stereoisomer of [$^{125}$I]CYP became commercially available. This compound has the potential for being one of the most suitable β-adrenergic radioligands. Both the racemic mixture (Ezrailson *et al.*, 1981) and the (−) stereoisomer (Barovsky and Brooker, 1980; Wolfe and Harden, 1981) of [$^{125}$I]iodopindolol ([$^{125}$I]PIN) have been synthesized and their binding properties investigated. High affinity ($K_D$ = 130 p*M*) was found for the racemic radioligand in membranes of rat skeletal muscle with much lower nonspecific binding than (±)-[$^{125}$I]HYP. Although there is little information in the literature concerning the (−) stereoisomer of [$^{125}$I]iodopindolol similar to (−)-[$^{125}$I]CYP, this new radioligand combines the advantages of high affinity ($K_D \sim 50$ p*M*) and high specific radioactivity without the disadvantages of a racemic mixture (Bürgisser *et al.*, 1981a,b). However, further studies are required to determine whether or not [$^{125}$I]PIN also binds to non-β-adrenergic receptor sites and whether it possesses partial agonistic activity.

Most of these antagonist radioligands are rather hydrophobic and may therefore be unsuitable for studying intact cells, although (−)-[$^{125}$I]PIN seems useful for this purpose. A highly hydrophilic radioligand (±)-[$^{3}$H]CGP-12177 has been synthesized, and its binding characteristics in membrane preparations and in intact cells have been investigated (Staehelin and Simons, 1982; Porzig, 1982; Porzig *et al.*, 1982). These studies indicate that this ligand may bind exclusively to receptors on the outside surface of the plasma membrane.

## B. Agonist Radioligands

At the present time there are four adrenergic agonist radioligands commercially available, these being (±)-[$^{3}$H]hydroxybenzylisoproterenol ([$^{3}$H]-HBI), (−)-[$^{3}$H]isoproterenol, (−)-[$^{3}$H]epinephrine, and (−)-[$^{3}$H]norepinephrine. The latter two radioligands are also α-adrenergic agonists, whereas (±)-[$^{3}$H]HBI and (−)-[$^{3}$H]isoproterenol selectively bind to β-adrenergic receptors. (−)-Isoproterenol does not distinguish between $\beta_1$ and $\beta_2$ subtypes but (±)-HBI is, in frog heart membranes, 29-fold selective for $\beta_2$ versus $\beta_1$ receptors (Hancock *et al.*, 1979).

All the agonist radioligands for β-adrenergic receptors possess significantly lower affinities than do their antagonist counterparts. Thus, much higher radioligand concentrations are required to occupy a given number of receptors. This, in consequence, increases nonspecific binding and scatter of data points. When using agonist radioligands, a very important fact should be kept in mind: β-Adrenergic agonists generally bind to two interconvertible and GTP-dependent affinity states of the receptor (Stadel *et al.*, 1982). In frog erythrocyte membranes, the $K_D$ values for (−)-epinephrine binding to these receptor states were

found to be 80 and 10,000 n*M* as measured by competition with [$^3$H]DHA (Kent *et al.*, 1980). Somewhat higher affinities were found for (−)-isoproterenol ($K_H$ = 32, $K_L$ = 2500 n*M*) and for (±)-[$^3$H]HBI ($K_H$ = 6, $K_L$ = 800 n*M*) (Kent *et al.*, 1980). As a consequence of this receptor site heterogeneity and relative low affinity even for the high-affinity agonist state, adrenergic agonist radioligands are generally not used for routine quantification and characterization of β-adrenergic receptors. However, for studies of the mechanism of agonist actions they have been quite useful. For example, (±)-[$^3$H]HBI has been used to label the β-adrenergic receptor guanine nucleotide regulatory site complex prior to detergent solubilization (Limbird and Lefkowitz, 1978; Limbird *et al.*, 1979).

## C. Irreversible Radioligands

The radioligands listed in Table II bind covalently to the β-adrenergic receptor. Because of the formation of an irreversible ligand receptor complex, such interactions do not follow the Law of Mass Action, and this situation needs to be distinguished from the equilibrium binding experiments described above. Tritiated and iodinated photoaffinity labels for targeting β-adrenergic receptors have recently been developed (Rashidbaigi and Ruoho, 1981; Lavin *et al.*, 1981, 1982; Burgermeister *et al.*, 1982; Caron *et al.*, 1982; Shorr *et al.*, 1982a,b). Since photoaffinity labels bind reversibly before exposure to light, it is essential that the affinity for the receptors be very high and the nonspecific binding low. At equilibrium, the unbound radioligand is washed off, and the remaining bound photosensitive ligand is exposed to light to allow the formation of a covalent complex. The availability of high-affinity radioactively labeled photoactive β-adrenergic antagonists has proven to be a uniquely useful means for monitoring solubilized and purified β-adrenergic receptors. At the present time there are several reports dealing with synthesis and characterization of photoaffinity labels which seem to fulfil the requirements for probing the β-adrenergic receptor. [$^{125}$I]Azidobenzylpindolol ([$^{125}$I]ABP) labels two polypeptides in duck erythrocyte membranes (Rashidbaigi and Ruoho, 1981). Its affinity is 220 p*M*, and the specific binding better than 70% in that system. A series of high-affinity β-adrenergic antagonists based on the structure of carazolol have been developed as photoaffinity probes for the β-adrenergic receptor by Lavin *et al.*, (1981, 1982), Caron *et al.* (1982), and Shorr *et al.* (1982a,b). Two of these compounds, [$^{125}$I]*p*AMIBC (15-[3′-iodo-4′-aminobenzyl]carazolol) and [$^{125}$I]*p*AMBIC (15-[4′-aminobenzyl]-(1-iodo)-carazolol), though not photoactive themselves, can be covalently incorporated into the β-adrenergic binding site by using a bifunctional photoactive cross linker, *N*-succinimidyl 6-(4′-azido-2′-nitrophenylamino)hexanoate (SANAH). These compounds show extremely high affinity [$K_D$ value for [$^{125}$I]*p*AMIBC is 5–20 p*M*) and specific binding greater than

TABLE II

Potential Irreversible Radioligands for β-Adrenergic Receptors

| Radioligand | | Max specific radioactivity (Ci/mmol) | $K_D$ (approx.) | Commercial | Comments |
|---|---|---|---|---|---|
| Short name | Compound | | | | |
| (±)-[$^{125}$I]ABP | (±)-[$^{125}$I]Iodoazidobenzylpindolol | 1300 | 220 p$M$[a] | | Photoactive |
| (±)-[$^{125}$I]CPA-1 | (±)-[$^{125}$I]Iodocyanopindolol-azide-1 | 2200 | 40 p$M$[a] | | Photoactive |
| (±)-[$^{125}$I]CPA-2 | (±)-[$^{125}$I]Iodocyanopindolol-azide-2 | 2200 | 40 p$M$[a] | | Photoactive |
| (±)-[$^{125}$I]*p*AMIBC | (±)-[$^{125}$I]*para*-Aminoiodobenzylcarazolol | 2200 | 5–20 p$M$[a] | N-177[c] | Not photoactive but cross-linkable to photoactive compound |
| (±)-[$^{125}$I]*p*AMBIC | (±)-[$^{125}$I]*para*-Aminobenzyliodocarazolol | 2200 | 5–20 p$M$[a] | [b] | Not photoactive but cross-linkable to photoactive group |
| (±)-[$^{125}$I]*p*AIBC | (±)-[$^{125}$I]*para*-Azidoiodobenzylcarazolol | 2200 | 10–30 p$M$[a] | NEX-180 | Photoactive |
| (±)-[$^{125}$I]*p*ABIC | (±)-[$^{125}$I]*para*-Azidobenzyliodocarazolol | 2200 | 10–30 p$M$[a] | [b] | Photoactive |
| (−)-[$^{3}$H]*p*ABC | (−)-[$^{3}$H]*para*-Azidobenzylcarazolol | 60 | 50 p$M$ | NET-604 | Photoactive |

[a] $K_D$ value for racemic radioligand.

[b] Available at NEN upon request.

[c] N denotes material from New England Nuclear.

80% (Shorr *et al.*, 1982a)]. The photoactive labels [$^{125}$I]*p*AIBC (15-[4′-azido(3′-iodo)benzyl]carazolol) and [$^{125}$I]*p*ABIC (15-[4′-azidobenzyl]-(1-iodo)-carazolol) may be even more useful since after washing off the unbound ligand they can immediately be covalently bound to β-adrenergic sites by exposure to light. These ligands display the specific β-adrenergic characteristics of stereoselectively for agonists and antagonists with an appropriate potency series. In the absence of light, both photosensitive affinity ligands bind to $\beta_1$ and $\beta_2$ adrenergic receptors in a saturable fashion with very high affinity ($K_D$ values = 20–30 p*M*). Depending on the system used, their specific binding ranges from 50 to 80%. Nonspecifically bound photoaffinity label seems not to be covalently incorporated into nonreceptor proteins as demonstrated by SDS–polyacrylamide gel electrophoresis (Lavin *et al.*, 1982). The tritiated version of *para*-azidobenzylcarazolol ([$^3$H]*p*ABC) shows binding characteristics similar to its iodinated form (Lavin *et al.*, 1981) and may be useful as an autoradiographic receptor probe for cells or whole tissue preparations. These various carazolol derivatives are now commercially available (Caron *et al.*, 1982). Since it has been shown that the carazolol-derived photoaffinity labels inhibit isoproterenol-stimulated adenylate cyclase, it can be concluded that these compounds label the β-adrenergic receptor in a highly specific manner.

Another photoaffinity compound based on derivatives of iodocyanopindolol with very high affinity ($K_D$ = 40–45 p*M*) is reported to covalently bind into the β-adrenergic receptor of turkey erythrocyte membranes (Burgermeister *et al.*, 1982). These [$^{125}$I]CYP-azides-1 and -2 show very low nonspecific binding, probably due to their hydrophilic structure.

To date there has been no direct comparative study involving these three different groups of photoaffinity labels. However, each of them seem to satisfy the criteria for irreversibly labeling β-adrenergic receptors. Earlier reports of covalently attached β-adrenergic ligands (Atlas and Levitzki, 1978; Wrenn and Homcy, 1980) showed $K_D$ values about 100 n*M* and have therefore not been proven to be useful means for probing the β-adrenergic receptor.

## III. METHODS OF ASSAYING β-ADRENERGIC RECEPTOR RADIOLIGAND BINDING

### A. Characterization of a Radioligand/Receptor Binding System

#### *1. Types of Binding Experiments*

There are basically two types of radioligand binding experiments which can be performed: equilibrium (steady state) and kinetic (time course) experiments. An equilibrium experiment can take the form of either a saturation or a competition

binding experiment, whereas time course experiments consist of association (on-rate kinetics) or dissociation (off-rate kinetics) curves. In the literature, competition binding experiments are often erroneously referred to as "displacement" experiments. Indeed, only in a dissociation kinetics experiment is the radioligand displaced by a large excess of an unlabeled ligand. In a competition experiment the binding reaction is begun in the presence of both the radioligand and nonradioactive competitor.

### 2. *Evaluation of Experimental Conditions from One-Point Studies*

In the course of searching for the optimal experimental conditions for a radioligand binding experiment, it is often convenient to first perform a series of binding experiments at a constant radioligand and receptor assay concentration. Buffer, pH, ionic strength, different nonradioactive competitors, incubation time, temperature, and potential modulators such as guanine nucleotide analogs can then be tested in one assay. A radioligand concentration in the neighborhood of the $K_D$ value is recommended.

### 3. *Determination of Time Course of Association and Reversibility by Dissociation Kinetics*

To optimize the incubation time at a given incubation temperature, the time course of association has to be explored. Kinetic experiments are easier to perform and more accurate if incubation takes place in one large tube and aliquots are removed for filtration at indicated time points. It is necessary to perform such experiments with more than a single radioligand concentration, and ideally, they should be performed using a low, medium, and high radioligand concentration. The time required for the lowest radioligand concentration to approach a steady state determines the incubation time for equilibrium experiments. For β-adrenergic radioligands, 30–90 min of incubation is generally sufficient. After any association time, the dissociation of radioligand/receptor complex can be initiated by adding approximately a 1000-fold excess of an equipotent nonradioactive ligand to the incubation mixture. This type of experiment demonstrates the reversibility of the binding. If a biphasic dissociation curve is observed, it suggests a complex binding model, for example, racemic radioligands (see also Section IV,D). Initiation of dissociation only by dilution of the reaction mixture is not recommended because of the possibility of rebinding.

### 4. *Competition Binding Experiments with Agonists and Antagonists*

In a competition binding experiment, the radioligand concentration is kept constant and the nonradioactive competitor concentration is varied on a log-

arithmic scale over a range of several orders of magnitude. Any β-adrenergic system which has been characterized by radioligand binding typically shows stereospecificity for agonists and antagonists. In all cases the (−) stereoisomer is more potent than its (+) counterpart. For antagonists, the $K_D$ ratio of the stereoisomers generally ranges from 30 to 50, whereas for agonists it may be as low as 10-fold for norepinephrine, 20- to 30-fold for epinephrine, and as high as 300-fold for isoproterenol. Another characteristic of β-adrenergic receptors and a criterion for distinguishing the $\beta_1$ from the $\beta_2$ subtype of receptor is the potency order of the agonists. For the $\beta_1$ subtype, (−)-norepinephrine is equipotent with or a little more potent than (−)-epinephrine, whereas $\beta_2$-adrenergic receptors exhibit much higher potency for (−)-epinephrine as compared to (−)-norepinephrine. For both subtypes (−)-isoproterenol is more potent than (−)-epinephrine. However, since agonist competition curves are less steep than antagonist competition curves $EC_{50}$ values cannot easily be converted into dissociation constants. Further, computer modeling has revealed that there are two interconvertible and guanine nucleotide-dependent agonist binding sites (Kent *et al.*, 1980) (see also Section IV,A).

## B. Separation of Free and Bound Radioligand

### *1. Choice of Separation Method*

All radioligand binding techniques require a physical separation of free and bound radioligand. The choice of the method for this separation depends on whether one is dealing with membranes, whole cells, or solubilized receptors. Several separation methods have been used for nonsolubilized receptor preparations, including equilibrium dialysis, centrifugation, and vacuum filtration. In the past few years rapid vacuum filtration has become very popular for assaying β-adrenergic receptors because of its reliability and simplicity. This technique will be discussed in detail in the following section. Equilibrium dialysis is laborious and seems to have few advantages over filtration assay, whereas the centrifugation method lacks flexibility and is not suitable for kinetic experiments.

For assaying solubilized β-adrenergic receptors, the previously described methods are not applicable because of particle size. Charcoal separation techniques similar to those used for radioimmunoassays can therefore be used for solubilized receptor preparations. Ammonium sulfate precipitation methods have been shown not to be applicable for digitonin-solubilized β-adrenergic receptors. The best experience in the past has been with gel filtration separation on Sephadex G-50 (fine) (Caron and Lefkowitz, 1976a). A rack with 100 small columns (0.6 × 12 cm) is set up in such a manner that the radioactivity eluted in the void volume (bound radioligand) can be collected directly into scintillation vials. Typically 0.5

ml of solubilized receptor is put onto the columns, which are washed with 0.6–0.7 ml of buffer (50 m*M* Tris–HCl, 10 m*M* $MgCl_2$, pH 7.4) and subsequently eluted with 1 ml buffer. The columns then are recycled by washing with 15–20 ml of buffer. This procedure is performed in the cold (4°C).

*2. Rapid Vacuum Filtration Assay*

In the past several years the rapid vacuum filtration technique for the separation of bound and free radioligand has become the method of choice not only in the field of β-adrenergic receptor research but also for many other receptor systems. Equilibrium as well as kinetic experiments can be easily performed by one person. However, in order to take full advantage of this method, several requirements should be fulfilled: (1) A multifiltration device with 60–120 holes should be available; (2) all parts which come in contact with the incubation mixture before the filter should be made of material with low absorption properties, such as stainless steel or Teflon; and (3) the suction unit should be devised in such a manner that backsplashing of the filtrate is impossible. Although there are several vacuum filtration setups commercially available, any research workshop will be able to construct such an instrument.

Of particular importance is the choice of the filter. Based on several years of experience with vacuum filtration assays for β-adrenergic receptors and many other receptor systems, it has been found that glass fiber filters such as Whatman GF/C are the most useful. In any case, the adsorption of a radioligand to the filter must be very low, for example, for (−)-[$^3$H]DHA it is generally found to be less than 0.1% of total radioligand.

## C. Incubation Conditions

The incubation of an equilibrium binding reaction should be started by adding the receptor material to test tubes which contain all other drugs and chemicals. The incubation should take place in a constant temperature environment (±0.5°C) such as a shaking water bath. The incubation time for an equilibrium binding experiment should be determined by association kinetic experiments, as discussed in Section III,A,3. Other conditions, such as buffer, pH, and ionic strength, must be evaluated for each particular system. In order to prevent lysis of intact cells, such studies must be performed in an isotonic medium. For binding experiments with β-adrenergic radioligands and membrane preparations, the following incubation buffer is very often used: 50 m*M* Tris buffer, 10 m*M* $MgCl_2$, 1 m*M* EDTA, pH 7.4.

The final assay volume for a filtration experiment may be varied from 0.2 to

10 ml/tube. As a standard assay, 1 ml assay volume is usually sufficiently accurate and convenient to pipet.

## D. Pitfalls, Limits, and Trouble Shooting

### *1. Pipetting and Adsorption Problems*

Some radioligands as well as nonradioactive chemicals tend to adsorb to the surface of test tubes and pipette tips. As a consequence, their final concentrations may deviate from the expected values. Such an artifact can be detected by plotting the measured radioactivity of a serial dilution versus the calculated expected radioactivity. A systematic deviation from linearity clearly indicates either imprecise pipettes or adsorption problems, whereas a scatter around the straight line is usually caused by inadequate pipetting technique. We have found that most of the adrenergic radioligands adsorb much less to plastic tubes and tips if the serial dilution is done in 1 m*M* HCl (pH 3). The addition of 100–200 μl of 1 m*M* HCl in a final 1 ml volume of 50 m*M* Tris buffer will not affect pH. Furthermore, nonspecific adsorption is often very temperature dependent. Holding the bottom of an ice-cold test tube by hand for a few minutes may change the radioactivity in an aliquot from the first through the last tubes significantly. Vigorous mixing, such as vortexing of the test tubes, is not necessary. On the contrary, the enlarged surface increases nonspecific adsorption significantly. Finally, for pipetting radioligands, drugs, or any chemicals from stock solutions into incubation vials, it is best to equilibrate the pipette tip by several strokes before transferring the aliquot to a vial or test tube.

### *2. Assay Volume and Sequence of Pipetting*

As mentioned previously, the final assay volume for a radioligand/β-adrenergic receptor binding assay can be varied from ~0.2 to 10 ml. An assay volume of 1 ml in a 15 ml polypropylene tube is generally convenient. We have had the best results by pipetting the radioligand and any drugs or effectors whose concentration has to be very accurate in 100 μl aliquots, whereas all chemicals which are in saturating concentrations (e.g., drugs for nonspecific binding) have been added in 10 μl aliquots without correcting for this neglectible small change in volume.

As a general rule, one should first pipette those additives which cannot affect the samples which follow. Serial dilutions should be pipetted from low to high concentrations. If the incubation of the binding reaction is started by adding a larger volume (700–900 μl) of membranes or cells, one has to be sure that the stock solution of receptor preparation is not contaminated by potent drugs. If, for

example, two competition binding experiments are performed consecutively, and it happens that at the highest competitor concentration of the first experiment (e.g., $10^{-2}$ *M*) a drop of only 10 μl is transferred to the receptor stock solution of 100 ml, the original $10^{-2}$ *M* concentration is diluted to $10^{-7}$ *M*. This is above the $EC_{50}$ for many β-adrenergic drugs. Therefore, the stock solution of receptor material should be aliquoted.

### 3. *Filtration Assay*

The rapid vacuum filtration assay method, as described in Section III,B,2, is very reliable and relatively problem free. Glass fiber filters such as Whatman GF/C should be wet, otherwise the vacuum will drop and the flow rate will decrease. If too much membrane or cells are filtered, glass fiber filters tend to become clogged. This can be circumvented by using filters with larger pore size, by assaying a sample over more than one filter, or by attempting to further purify the receptor material.

Any new radioligand must be checked for its filter blank. A standard washing buffer is 50 m*M* Tris base containing 10 m*M* $MgCl_2$, pH 7.4 at 0°C. After washing with ice-cold buffer the filter blank should be minimal. For β-adrenergic radioligands 10–20 ml has proven to be sufficient. If the filter blank is high, either the washing buffer can be changed or the filters may be presoaked in bovine serum albumin (BSA) (0.1 mg/ml). In some cases the addition of 100 m*M* NaCl may help to reduce the filter blank.

### 4. *Scintillation Counting*

Since the counting statistics are proportional to the square root of the total counts, in order to double the precision a sample has to be counted four times longer. Usually 2–5 min counting time is sufficient if the signal/noise ratio is acceptable. One should pay particular attention to counter background and avoid introduction of contamination with the samples. We have found that after filtration, the filter need not be completely dry when placed into scintillation fluid. However, samples should be refrigerated in their vials with scintillation fluid for about 1 hr before liquid scintillation counting. If the counter does not automatically correct for quenching, this parameter has to be taken into account for the calculation of molar concentrations. In some cases the choice of scintillation fluid is essential. For measuring the radioactivity on the filters, the addition of a detergent (e.g., Triton X-100) to the scintillation cocktail is necessary. Surprising results are obtained if the radioligand is very hydrophilic, such as [$^3$H]CGP-12177, and a common scintillation fluid is used. In such a case only a fraction of the radioactivity in the vial is dissolved and counted and therefore the result is unusable.

## IV. DATA ANALYSIS OF β-ADRENERGIC RADIOLIGAND BINDING STUDIES

### A. Resolution of Receptor Subtypes by Competition Experiments

One of the most interesting questions in the β-adrenergic receptor field is the distribution of $\beta_1$ and $\beta_2$ receptor subtypes. Since the commonly used antagonist radioligands such as [$^3$H]DHA, [$^{125}$I]HYP, or [$^{125}$I]CYP are nonselective with regard to $\beta_1/\beta_2$ subtypes, competition with selective agonists or antagonists is necessary to determine the proportion of receptor subtypes. Such competition curves have slope factors significantly less steep than unity or in extreme cases appear biphasic even to the naked eye. In attempting to analyze such complex competition binding curves, several procedures have been proposed. Unfortunately, the frequently applied modified Scatchard transformation has proven unsuitable for this type of analysis. Another method for transformed data was introduced by Minneman *et al.* (1979). Whereas this procedure is applicable under limited circumstances, the best results have been achieved with the nonlinear least-squares regression method of untransformed data according to the model described by Hancock *et al.* (1979). They have performed extensive studies on $\beta_1/\beta_2$ receptor subtype analysis and have confirmed the applicability, reliability, and limitations of this method by Monte Carlo simulation techniques (De Lean *et al.*, 1982). In this study, predetermined mixtures of pure turkey ($\beta_1$) and frog erythrocytes ($\beta_2$) were assayed for subtype selectivity using various agonists and antagonists. Finally, the selectivity for $\beta_1$ and $\beta_2$ subtypes was confirmed by computer-assisted data analysis. In Table III these results are summarized (for further details, see De Lean *et al.*, 1982). In addition to real experimental data, Monte Carlo simulations of receptor subtype analysis have been performed. The "phase diagram" in Fig. 1 (De Lean *et al.*, 1982) depicts the conditions under which two affinity states can be resolved using selective competitors. As one would intuitively expect, the chance of obtaining a significant improvement in the goodness-of-fit is large if the affinity ratio of the two competitors is large and the proportion of sites is approximately 1:1. Interestingly, below an affinity ratio of about 7, no resolution of these sites can be expected. The experimental design for these Monte Carlo simulations mimics high-quality experiments. For less accurate binding curves the "phase diagram" in Fig. 1 would shift upwards. This study has convincingly demonstrated that the method of selective competition versus nonselective radioligands is, in combination with appropriate computer modeling techniques, a very useful tool for receptor subtype analysis.

TABLE III

Computer Estimates of Affinity Constants ($M^{-1}$) from Competition Curves[a]

| Ligand and affinity constant | Receptor | | | |
|---|---|---|---|---|
| | Pure turkey ($\beta_1$) or pure frog ($\beta_2$) | 10% Turkey and 90% frog | 50% Turkey and 50% frog | 90% Turkey and 10% frog[c] |
| MJ9184 (Zinterol) | | | | |
| $K_{\beta_1}$ | 1.1 (± 0.03) × $10^6$ (3) | 1.5 (± 0.5) × $10^7$ (2) | 1.6 (± 0.03) × $10^6$ (3) | 1.9 (± 0.2) × $10^6$ (3) |
| $K_{\beta_2}$ | 2.2 (± 0.1) × $10^8$ (3)[b] | 2.1 (± 0.1) × $10^8$ (2) | 1.1 (± 0.2) × $10^8$ (3) | 1.3 (± 0.6) × $10^8$ (3) |
| (−)-Norepinephrine | | | | |
| $K_{\beta_1}$ | 3.1 (± 0.1) × $10^5$ (3) | 6.9 (± 2.0) × $10^5$ (2) | 3.1 (± 0.5) × $10^5$ (3) | 3.5 (± 0.2) × $10^5$ (3) |
| $K_{\beta_2}$ | 4.6 (± 0.3) × $10^3$ (3)[b] | 4.9 (± 0.2) × $10^3$ (2) | 4.8 (± 0.4) × $10^3$ (3) | 1.1 (± 0.2) × $10^4$ (3) |
| (−)-Epinephrine | | | | |
| $K_{\beta_1}$ | 2.6 (± 0.3) × $10^5$ (3) | ND | 4.3 (± 0.9) × $10^5$ (2) | ND |
| $K_{\beta_2}$ | 4.6 (± 0.5) × $10^4$ (3)[b] | ND | 8.3 (± 0.09) × $10^4$ (2) | ND |
| (−)-Isoproterenol | | | | |
| $K_{\beta_1}$ | 2.3 (± 0.2) × $10^6$ (4) | ND | 6.2 (± 1.3) × $10^6$ (3) | ND |
| $K_{\beta_2}$ | 2.8 (± 0.2) × $10^5$ (3)[b] | ND | 7.1 (± 0.3) × $10^5$ (3) | ND |
| Butoxamine | | | | |
| $K_{\beta_1}$ | 1.7 (± 0.1) × $10^5$ (3) | ND | 2.6 × $10^5$ (1) | ND |
| $K_{\beta_2}$ | 3.4 (± 0.1) × $10^5$ (3) | ND | 2.6 × $10^5$ (1)[d] | ND |
| (±)-Propranolol | | | | |
| $K_{\beta_1}$ | 1.8 (± 0.2) × $10^8$ (3) | ND | 2.6 (± 0.04) × $10^8$ (2) | ND |
| $K_{\beta_2}$ | 2.0 (± 0.3) × $10^8$ (3) | ND | 2.6 (± 0.04) × $10^8$ (2)[c] | ND |

[a]Geometric means (± SEM when appropriate) are shown. Numbers in parentheses indicate the number of experiments performed.

[b]$K_{\beta_1}$ is significantly different from $_{\beta_2}$.

[c]ND, Not determined.

[d]Two components could not be resolved.

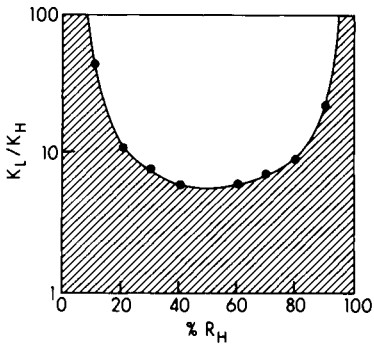



**Fig. 1.** Phase diagram of the resolution limit of the computer modeling technique as determined by Monte Carlo simulations. The points represent the selectivity ratio $K_L/K_H$ of a selective ligand able significantly to resolve $R_H$ and $R_L$ in 50% of the simulated experiments for a given proportion of the mixture (% $R_H$). The cross-hatched area indicates conditions under which a resolution of two sites cannot be expected.

## B. Resolution of Receptor Subtypes by Saturation Experiments

As is documented by the large number of publications presenting curvilinear Scatchard plots, the quantification of receptor subtypes is an increasingly important issue. In Fig. 2 a two-site saturation curve is shown. The same data, as generated by computer simulation according to the two-site model, are depicted in the untransformed direct plot and in the Scatchard representation (for details, see Bürgisser, 1983). Whereas for weighted nonlinear least-squares regression analysis is highly significant two-site fit ($p < .001$) was obtained, no significant improvement in the goodness-of-fit was achieved for the transformed data in the Scatchard analysis. This exemplifies the disadvantage of analyzing transformed

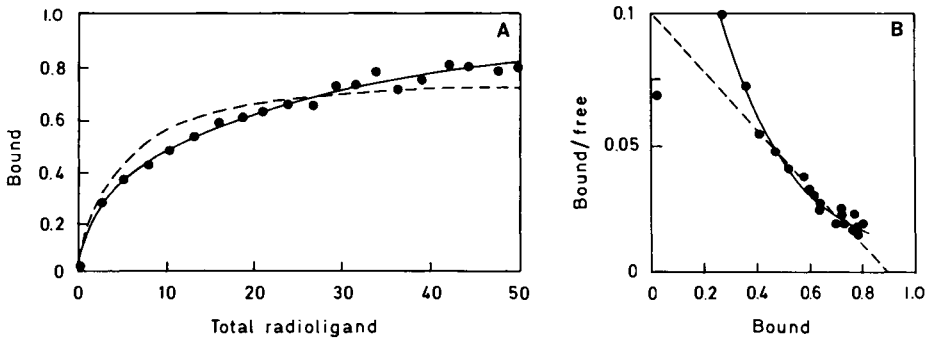



**Fig. 2.** Two-site saturation binding curve (A). A two-site saturation curve was constructed and analyzed by Monte Carlo simulation techniques. Under the conditions chosen for this example, all 30 simulated experiments yielded a significantly better two-site fit as analyzed by nonlinear regression. $K_H = 1$; $K_L = 20$; $R_H = 25\%$. Scatchard representation of the same data is depicted in B. The fit curves of the Scatchard plots are taken from nonlinear regression.

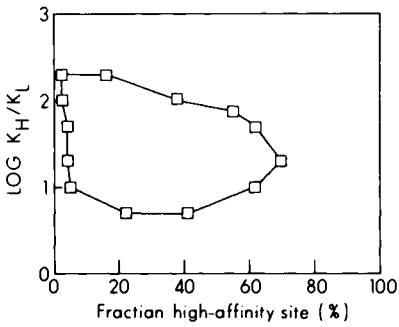


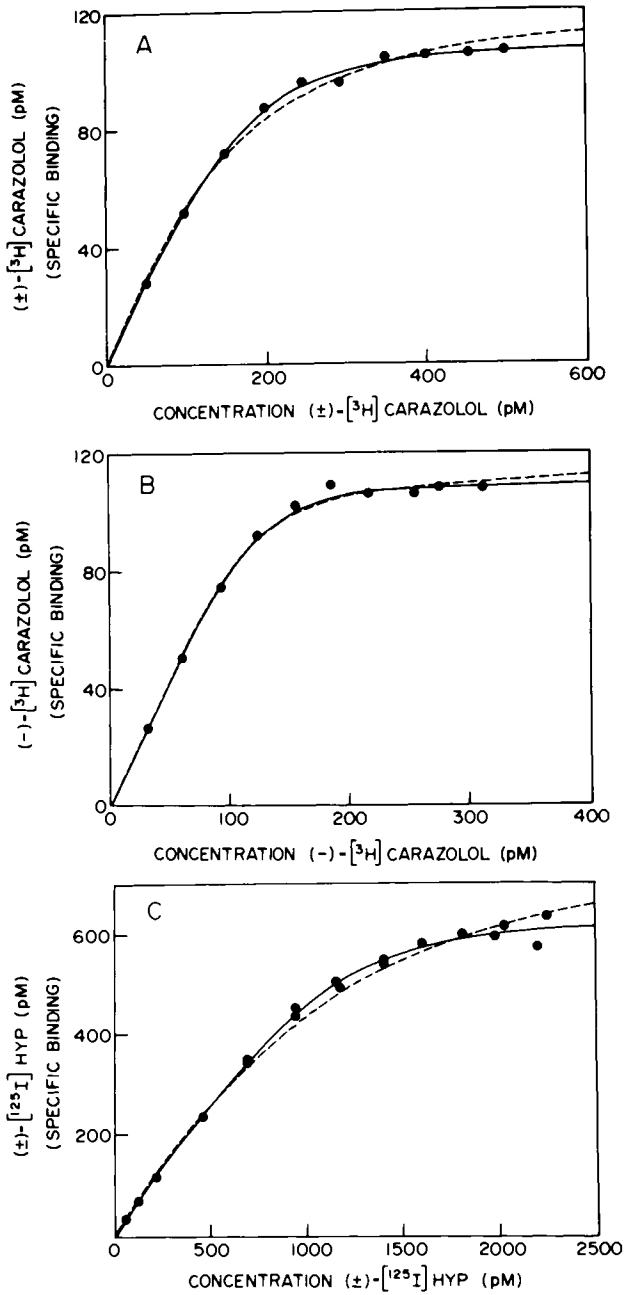

**Fig. 3.** Conditions for a significant resolution of two sites from a saturation experiment. By analogy with the phase diagram in Fig. 1, Monte Carlo simulation techniques were applied to investigate the conditions under which two sites can be resolved from a saturation binding experiment. The curve encloses the area in which a significantly better two-site fit ($p = .05$) was obtained in 95% of 30 simulated low-scatter experiments.

data. In an extensive Monte Carlo study (Bürgisser, 1983), we have investigated the conditions under which a two-site fit can be successfully obtained. It was found that: (1) the random scatter of the data points is the most limiting parameter and (2) the number and distribution of data points are of importance. The probability of significant resolution of two sites is shown in Fig. 3. This relationship depends on the ratio of the $K_D$ values and the proportion of high- and low-affinity sites. This result is representative of high-quality binding experiments. From these studies we conclude that under optimal experimental conditions for saturation binding experiments the resolution of two affinity forms is repeatedly highly significant only when analyzed with weighted nonlinear least-squares regression methods. More dubious, however, are two-site models stemming from lower quality experiments with 10 or fewer data points, especially if analyzed by the Scatchard method.

## C. Racemic Radioligands

Many of the β-adrenergic radioligands are racemic mixtures (see Table I). Since the affinity of the (+) isomer generally is 10- to 200-fold lower than the affinity of the (−) isomer, only a small fraction of the (+) isomer is bound. Under certain circumstances, systematic artifacts may be observed. As we have shown in detailed studies (Bürgisser *et al.*, 1981a), a saturation binding curve with the racemic radioligand (±)-[$^3$H]carazolol and (±)-[$^{125}$I]HYP does not fit ideally to the simple Mass Action law model. This is shown in Fig. 4. As verified

**Fig. 4.** Computer curve fitting according to a one- and two-ligand model for saturation binding experiments with racemic and nonracemic radioligands. Frog erythrocyte membranes were incubated with increasing concentrations of (±)-[$^3$H]carazolol (A), (−)-[$^3$H]carazolol (B), and (±)-[$^{125}$I]HYP (C). A significant improvement of the computer fit ($p < .05$) for Model II (———, two-ligand model) over Model I (- - - -, one-ligand model) was achieved in the case of the racemic radioligand (A and C). No significant improvement for Model II over Model I could be observed for (−)-[$^3$H]carazolol (B). In A, $K_{Dav} = 61 \pm 6$ p*M*; $K_{D(-)} = 6.8 \pm 2$ p*M*; $K_{D(+)} = 279 \pm 50$ p*M*; $p = .002$. In B, $K_{D(-)} = 9.1 \pm 1.5$ p*M*; $p = .06$. In C, $K_{Dav} = 540 \pm 43$ p*M*; $K_{D(-)} = 32 \pm 7$ p*M*; $K_{D(+)} = 2900 \pm 400$ p*M*; $p < .001$.


A
(±)-[3H] CARAZOLOL (pM)
(SPECIFIC BINDING)
CONCENTRATION (±)-[3H] CARAZOLOL (pM)
B
(-)-[3H] CARAZOLOL (pM)
(SPECIFIC BINDING)
CONCENTRATION (-)-[3H] CARAZOLOL (pM)
C
(±)-[125I] HYP (pM)
(SPECIFIC BINDING)
CONCENTRATION (±)-[125I] HYP (pM)

by Monte Carlo simulation studies, the degree of the underestimation of affinity of the (−) isomer varies from $\sim 2K_{D(-)}$ to $\frac{1}{2}\ K_{D(+)}$, depending on the assay receptor concentration. It may be argued that because the receptor assay concentration is usually far below the $K_D$ of radioligand, the contribution of the (+) isomer is negligible, and the $K_D$ of the (−) isomer can be obtained by dividing the total radioligand concentration by a factor of two. However, since very high-affinity radioligands such as (±)-[$^{125}$I]HYP, (±)-[$^{125}$I]CYP, and (±)-[$^{3}$H]carazolol have become available, a receptor assay concentration in the range of 10–100 p*M* is comparable to, or higher than, the $K_D$ values of these (−) isomers. Although with computer modeling techniques, the proper analysis of saturation binding curves involving racemic radioligands is feasible, it should be emphasized that, whenever possible, a pure stereoisomer radioligand is preferred. In addition to the previously described anomalous binding curves obtained with racemic radioligands, they may also obscure the existence of two affinity states. We have shown that to be the case for (±)-[$^{3}$H]quinuclidinyl benzylate (QNB) binding to the muscarinic receptor (Bürgisser *et al.*, 1982). As will be discussed in the following section, racemic radioligands may also show artifically complex binding kinetics.

## D. Kinetics

The mathematical treatment of two-ligand or two-site on-rate kinetics is rather complex, and the analysis of such models often exceeds the limitations of the information content of the data. However, if the off-rate constants $k_{-1}$ and $k_{-2}$ are determined separately from dissociation experiments, the on-rate constants may be estimated with sufficient accuracy. Nevertheless, a systematic deviation from a simple bimolecular reaction model points toward a more complex model. In such a case experiments other than on-rate kinetics have to be designed in order to elucidate the nature of the reaction mechanism. In the case of racemic radioligands, we have successfully applied nonlinear regression analysis in order to determine the association and dissociation rate constants for both stereoisomers (Bürgisser *et al.*, 1981b). In Fig. 5 the dissociation kinetics of (±)- and (−)-[$^{3}$H]carazolol are shown. Both curves were analyzed simultaneously by computer modeling and by sharing the off-rate constant for the (−) isomer. The rate constants were found to be $2.5 \times 10^{-3}$/min and $8.5 \times 10^{-2}$/min for the (−) and the (+) stereoisomer.

In closing this discussion of data analysis, it should be emphasized that a good fit of experimental data to a given receptor model does not, of course, prove its validity. Yet, such techniques can provide valuable tools for exploring the likelihood of different receptor/ligand interaction models.

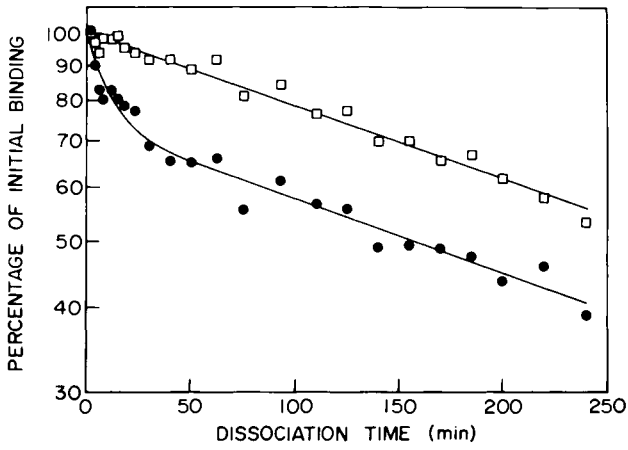



**Fig. 5.** Off-rate kinetics of a racemic and a pure stereoisomer radioligand. (−)- and (±)-[$^3$H]Carazolol (represented in the figure by □—□ and ●—●, respectively) are displaced from a single class of sites in frog heart membranes. The fast component in the curve of the racemic radioligand is due to the less tightly bound (+) stereoisomer.

## V. MEASUREMENT OF COUPLING BETWEEN β-ADRENERGIC RECEPTORS AND ADENYLATE CYCLASE

### A. Resolution of High- and Low-Affinity Agonist Binding States

Radioligand binding studies may lead not only to quantitative measurement of ligand/receptor affinity and binding capacity but also can provide certain insights into subsequent molecular events, such as coupling to guanine nucleotide regulatory proteins (N) (for review, see Limbird, 1981; Stadel *et al.*, 1982). It was obsered that when β-adrenergic agonists were allowed to compete with radioactively labeled antagonists, the competition curves were less steep than those of unlabeled antagonists. Computer-assisted analysis revealed that agonists bind to two affinity states of the receptor, whereas β-adrenergic antagonists showed monophasic competition curves in accordance with a single affinity state. Furthermore, it was shown that the ratio of the dissociation constants $K_L/K_H$ for an agonist correlated with the intrinsic activity (adenylate cyclase activity) of that agonist (Kent *et al.*, 1980). Biochemical studies of solubilized β-adrenergic receptors such as those described by Limbird *et al.* (1979) confirmed and extended the results obtained by radioligand binding experiments. By employing agonist (±)-[$^3$H]HBI, it was demonstrated that the apparent molecular weight of the β-adrenergic receptors of frog erythrocytes and rat reticulocytes was larger if

the membranes were first exposed to agonists prior to solubilization. The larger complex has been shown to consist of a receptor–nucleotide regulatory protein complex stabilized by agonists but not antagonists. Other lines of evidence, such as the studies by Ross and Gilman (1977), Cassel and Pfeuffer (1978), and Northup *et al.* (1980), also support the concept of a guanine nucleotide regulatory protein which serves as a shuttle between the β-adrenergic receptor and adenylate cyclase (for review, see Stadel *et al.*, 1982).

### B. The Effect of Guanine Nucleotides and Ions on High- and Low-Affinity Agonist Binding States

Guanine nucleotides, such as GTP and its nonhydrolizable analog Gpp(NH)p, were found to modulate agonist binding to the β-adrenergic receptor, whereas antagonist interactions were not affected (Maguire *et al.*, 1976; Lefkowitz *et al.*, 1976). The order of potencies of the nucleotides in changing the proportion of high- and low-affinity agonist states in different tissues is Gpp(NH)p > GTP >> ATP ≅ GMP. The maximum guanine nucleotide effect can be achieved at a concentration of ~10 μ*M* for Gpp(NH)p and ~100 μ*M* for GTP. Interesting, in many membrane preparations guanine nucleotides do not cause a complete shift to only low-affinity agonist states. The reason for this is at present not clear. It is possible that a fraction of the membrane preparation consists of vesicles, in which guanine nucleotides have no access to the inner surface of the membrane-localized guanine nucleotide regulatory protein. Another explanation for this phenomenon might be incomplete uncoupling of β-adrenergic receptor from the guanine nucleotide regulatory protein.

In many β-adrenergic systems, such as frog and turkey erythrocytes and different cell lines, it has been observed that magnesium is necessary to promote formation of the high-affinity agonist state of the receptors. Therefore, in standard assays 10 m*M* $Mg^{2+}$ in excess of 1 m*M* EDTA is used. Another divalent cation, $Mn^{2+}$, was shown to lead to progressive loss of hormone-stimulated adenylate cyclase activity in the concentration range from 5 to 20 m*M*. However, in contrast to $Mg^{2+}$ it seems to have no significant effect on agonist binding (Limbird *et al.*, 1979).

## VI. ASSAYING SOLUBILIZED AND PURIFIED β-ADRENERGIC RECEPTORS

### A. Solubilization and Purification of β-Adrenergic Receptors

A major breakthrough in attempts to purify and characterize the β-adrenergic receptor of frog erythrocytes was the discovery that among a wide variety of detergents tested, only digitonin could solubilize the receptors in an active form

(Caron and Lefkowitz, 1976a,b). A currently used method for preparation of solubilized β-adrenergic receptors (Shorr *et al.*, 1982b) involves the use of 2% digitonin in 100 m*M* NaCl, 10 m*M* Tris–HCl at pH 7.2, $3 \times 10^{-5}$*M* phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml soybean trypsin inhibitor. A protocol for preparation of turkey erythrocyte membranes and solubilization of β-adrenergic receptor is given here as an example. After removing white cells and serum by centrifugation the erythrocytes are washed in buffered saline (140 m*M* NaCl in 10 m*M* Tris–HCl pH 7.4, 10 m *M* $MgCl_2$). Then the red cells are incubated for 90 min (25°C) in a nitrogen bomb (Parr Inst. Co1, Moline, Illinois) under 800 psi pressure and lysed by quick release of the pressure. Remaining unlysed cells and unruptured nuclei are removed by centrifugation (4°C, 800 × *g*, 20 min). Membranes are then obtained by centrifugation of the supernatant (40,000 × *g*, 15 min) and washing three times with 75 m*M* Tris–HCl, 25 m*M* $MgCl_2$ at pH 7.4. Membranes can be stored by the addition of protease inhibitors at −80°C. The thawed or fresh membranes are pelleted and resuspended in the solubilization buffer as described previously. After Dounce homogenization the membranes are stirred on ice for 45 min. The unsolubilized material is then removed by centrifugation (240,000 × *g*, 45 min). Soluble β-adrenergic receptor can be assayed by radioligand binding as described in Section III,B,1.

Further purification of β-adrenergic receptor by the use of Sepharose–alprenolol affinity chromatography and gel permeation HPLC, as described by Shorr *et al.* (1982), yields highly purified β-adrenergic receptor within 2 days. This material can be labeled with recently developed photoaffinity probes and subsequently subjected to SDS–polyacrylamide gel electrophoresis.

## B. Photoaffinity Labeling of β-Adrenergic Receptors

The covalent incorporation of a radioactively labeled photoaffinity ligand into β-adrenergic receptors is a very powerful tool for studying solubilized and purified receptors. Recently high-affinity, $^{125}$I-labeled photoaffinity ligands have been developed and successfully applied to the β-adrenergic receptor (see also Section II,B,3). A procedure for labeling various membrane preparations is as follows (Lavin *et al.*, 1982): Membranes are diluted in 25 m*M* Tris–HCl, 2 m*M* $MgCl_2$ for $\beta_2$ and 75 m*M* Tris–HCl, 25 m*M* $MgCl_2$ for $\beta_1$ receptors with pH 7.4 at 25°C to obtain a final receptor assay concentration of 20–30 p*M*. Either photoaffinity label, [$^{125}$I]*p*AIBC or [$^{125}$I]*p*ABIC, (see Table II) is used at equal concentration of 20–30 p*M*. After incubation at 25°C for 60–90 min in the absence of light, the reaction mixture is centrifuged at 40,000 *g* for 15 min. The resulting pellet is resuspended in Tris buffer containing 0.5% BSA and washed three times. BSA is then removed from the pellet by sedimentation in Tris–HCl, $MgCl_2$ buffer, and the membranes are subjected to photolysis for 90 sec, 12 cm from a Hanovia 450W medium pressure mercury lamp filtered with 5 mm of Pyrex glass. After photolysis, the membranes are centrifuged at 40,000

*g* and resuspended in a small volume (1–2 ml) of 50 m*M* Tris–HCl pH 6.8 at room temperature. This material can be suspended in SDS–PAGE sample buffer.

## REFERENCES


Ahlquist, R. P. (1948). *Am. J. Physiol.* **153,** 586–600.

Ariens, E. J. (1967). *Ann. N.Y. Acad. Sci.* **139,** 606–631.

Atlas, D., and Levitzki, A. (1978). *Nature (London)* **272,** 370–371.

Atlas, D., Steer, M. L., and Levitzki, A. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71,** 4246–4248.

Aurbach, G. D., Fedak, S. A., Woodward, C. J., Palmer, J. S., Hauser, D., and Troxler, T. (1974). *Science* **186,** 1223–1224.

Barovsky, K., and Brooker, G. (1980). *J. Cyclic Nucleotide Res.* **6,** 297–307.

Burgermeister, W., Hekman, M., and Helmreich, E. J. M. (1982). *J. Biol. Chem.* **257,** 5306–5311.

Bürgisser, E. (1983). *J. Recept. Res.* **3,** 261–281.

Bürgisser, E., Hancock, A., Lefkowitz, R. J., and De Lean, A. (1981a). *Mol. Pharmacol.* **19,** 205–216.

Bürgisser, E., Lefkowitz, R. J., and De Lean, A. (1981b). *Mol. Pharmacol.* **19,** 509–512.

Bürgisser, E., De Lean, A., and Lefkowitz, R. J. (1982). *Proc. Natl. Acad. Sci. U.S.A.* **79,** 1732–1736.

Caron, M. G., and Lefkowitz, R. J. (1976a). *J. Biol. Chem.* **251,** 2374–2384.

Caron, M. G., and Lefkowitz, R. J. (1976b). *Biochem. Biophys. Res. Commun.* **68,** 315–322.

Caron, M. G., Lavin, T. N., Heald, S. L., Nambi, P., Jeffs, P. W., and Lefkowitz, R. J. (1982). *New Prod. News,* N. Eng. Nucl. **1,** No. 7.

Cassel, D., and Pfeuffer, T. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 2669–2673.

De Lean, A., Hancock, A. A., and Lefkowitz, R. J. (1982). *Mol. Pharmacol.* **21,** 5–16.

Engel, G., Hoyer, D., Berthold, R., and Wagner, H. (1981). *Naunyn-Schmiedeberg's Arch. Pharmacol.* **317,** 277–285.

Ezrailson, E. G., Garber, A. J., Munson, P. J., Swartz, T. L., Birnbaumer, L., and Entman, M. L. (1981). *J. Cyclic Nucleotide Res.* **7,** 13–26.

Hancock, A. A., De Lean, A., and Lefkowitz, R. J. (1979). *Mol. Pharmacol.* **16,** 1–9.

Hoyer, D., Engel, G., and Berthold, R. (1982). *Naunyn-Schmiedeberg's Arch. Pharmacol.* **318,** 319–329.

Kaumann, A. J. (1981). *Naunyn-Schmiedeberg's Arch. Pharmacol.* **317,** 13–18.

Kent, R. S., De Lean, A., and Lefkowitz, R. J. (1980). *Mol. Pharmacol.* **17,** 14–23.

Lavin, T. N., Heald, S. L., Jeffs, P. W., Shorr, R. G. L., Lefkowitz, R. J., and Caron, M. G. (1981). *J. Biol. Chem.* **256,** 11944–11950.

Lavin, T. N., Nambi, P., Heald, S. L., Jeffs, P. W., Lefkowitz, R. J., and Caron, M. G. (1982). *J. Biol. Chem.* **257,** 12332–12340.

Lefkowitz, R. J., and Williams, L. T. (1977). *Proc. Natl. Acad. Sci. U.S.A.* **74,** 515–519.

Lefkowitz, R. J., Mukherjee, C., Coverstone, M., and Caron, M. G. (1974). *Biochem. Biophys. Res. Commun.* **69,** 703–710.

Lefkowitz, R. J., Mullikin, D., and Caron, M. G. (1976). *J. Biol. Chem.* **251,** 4686–4692.

Levitzki, A., Atlas, D., and Steer, M. L. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71,** 2773–2776.

Limbird, L. E. (1981). *Biochem. J.* **195,** 1–13.

Limbird, L. E., and Lefkowitz, R. J. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 228–232.

Limbird, L. E., Hickey, A. R., and Lefkowitz, R. J. (1979). *J. Biol. Chem.* **254,** 2677–2683.

Maguire, M. E., Van Arsdale, P. M., and Gilman, A. G. (1976). *Mol. Pharmacol.* **12,** 335–339.
Maguire, M. E., Ross, E. M., and Gilman, A. G. (1977). *Adv. Cyclic Nucleotide Res.* **8,** 1–83.
Minneman, K, P., Hegstrand, L. R., and Molinoff, P. B. (1979). *Mol. Pharmacol.* **16,** 21–33.
Morris, T. H., Sandrock, K., and Kaumann, A. J. (1981). *Naunyn's-Schmiedeberg's Arch. Pharmacol.* **317,** 19–25.
Munson, P. J., and Rodbard, D. (1980). *Anal. Biochem.* **107,** 220–239.
Nahorski, S. R. (1976). *Nature (London)* **259,** 488–489.
Northup, J. K., Sternweis, P. C., Smigel, M. D., Schleifer, L. S., Ross, E. M., and Gilman, A. G. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 6516–6520.
Porzig, H. (1982). *Trends Pharmacol. Sci.* **3,** 75–78.
Porzig, H., Becker, C., and Reuter, H. (1982). *Naunyn-Schmiedeberg's Arch. Pharmacol.* **321,** 89–99.
Potter, L. T. (1967). *J. Pharmacol. Exp. Ther.* **155,** 91–100.
Rashidbaigi, A., and Ruoho, A. E. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 1609–1613.
Ross, E. M., and Gilman, A. G. (1977). *Proc. Natl. Acad. Sci. U.S.A.* **74,** 3715–3719.
Shorr, R. G. L., Heald, S. L., Jeffs, P. W., Lavin, T. N., Strohsacker, M. W., Lefkowitz, R. J., and Caron, M. G. (1982a). *Proc. Natl. Acad. Sci. U.S.A.* **79,** 2778–2782.
Shorr, R. G. L., Strohsacker, M. W., Lavin, T. N., Lefkowitz, R. J., and Caron, M. G. (1982b). *J. Biol. Chem.* **257,** 12341–12350.
Stadel, J. M., De Leon, A., and Lefkowitz, R. J. (1982). *Adv. Enzymol.* **53,** 1–43.
Staehelin, M., and Hertel, C. (1982). *J. Recept. Res.* **3,** 35–43.
Staehelin, M., and Simons, P. (1982). *EMBO J.* **1,** 187–190.
Williams, L. T., and Lefkowitz, R. J. (1977). *J. Biol. Chem.* **252,** 7207–7213.
Williams, L. T., and Lefkowitz, R. J. (1978). "Receptor Binding Studies in Adrenergic Pharmacology." Raven Press, New York.
Wolfe, B. B., and Harden, T. K. (1981). *J. Cyclic Nucleotide Res.* **7,** 303–312.
Wrenn, S. M., Jr., and Homcy, C. J. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 4449–4453.

# Chapter 15

# α-ADRENERGIC RECEPTORS IN NEURAL TISSUES: METHODS AND APPLICATIONS OF RADIOLIGAND BINDING ASSAYS

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ISBN 0-12-470350-X

# I. INTRODUCTION

## A. α-Adrenergic Receptors

In the early 1970s Starke and co-workers (1975) discovered that two α-adrenergic responses, the contraction of smooth muscle (presumably a postsynaptic response), and inhibition of norepinephrine overflow (presumably a presynaptic response), demonstrated different rank orders of potency for the agonist compounds naphazoline, oxymetazoline, and phenylephrine. At roughly the same time, Langer and co-workers reported that phenoxybenzamine, an α-selective antagonist, was 30-fold more potent at increasing norepinephrine overflow than it was at inhibiting the contraction of smooth muscle. Both groups inferred the existence of two types of α-adrenergic receptor, and Langer (1974) coined the now standard classification of $\alpha_1$- and $\alpha_2$-receptors. Originally, $\alpha_1$- and $\alpha_2$-adrenergic receptors were anatomically defined as postsynaptic ($\alpha_1$) and presynaptic autoreceptors ($\alpha_2$). Multiple demonstrations of postsynaptic $\alpha_2$-receptors in both peripheral (Timmermans and van Zwieten, 1980; Kobinger and Pichler, 1981; Bylund and Martinez, 1981; McPherson and Summers, 1981) and central (U'Prichard *et al.*, 1979, 1980) tissues as well as preliminary reports of potential presynaptic $\alpha_1$-receptors (Kobinger and Pichler, 1982) have made the anatomical definition of α-adrenergic receptor types unsatisfactory. The critical role of peripheral and central α-adrenergic receptors in a myriad of physiological functions (e.g., regulation of blood pressure, responses to "stress," possible loci of action for antipsychotic, antidepressant, and anti-hypertensive medications, possible primary role in etiology of essential hypertension and depressive psychoses, and secondary roles in other disease processes) has made the study of α-adrenergic receptors an area of intensive research for many disciplines, ranging from molecular pharmacology to physiology to clinical medicine.

A major methodology for the differentiation, localization, quantitation, and characterization of α-adrenergic receptors is direct radioligand labeling of the receptors. This technique involves the binding of a compound with high specific radioactivity to the α-receptor recognition site and often other associated proteins. Because of the simplicity of the methodology, it has been widely used by investigators from all disciplines. Unfortunately, while subtle complexities of radioligand interactions with the α-receptor complex have been demonstrated, a lag in communication across disciplines has led, in some instances, to the less than optimal use and/or interpretation of radioligand binding assays. In many studies nonoptimal preparation of tissue, improper selection of radioligand, blank to define specific binding, assay buffer, and/or other assay constituents (e.g., metal ions and nucleotides), as well as incomplete experimental design, have made published results from adrenergic radioligand binding studies difficult to interpret.

The twofold purpose of this chapter is to outline the current understanding of $\alpha_1$- and $\alpha_2$-adrenergic receptor/effector functioning, and thereby introduce some of the methodological considerations which should dictate optimal design as well as proper methodology for a given experimental goal (e.g., measuring receptor number).

## B. Receptor/Effector Coupling

Physiologically defined adrenergic receptors, like all membrane-bound receptors, consist of a receptor/recognition protein at the active site to which the physiological agonist (and presumably, the radioligand) binds, an intracellularly oriented membrane-bound effector or second messenger system, and in many cases, a protein complex which transduces the extracellular signal (binding of the agonist to the receptor/recognition site) across the membrane to the effector. The separate components are all postulated to exist in an equilibrium which is affected, in a cell lysate preparation, by the presence of agonist, antagonist, nucleotides, ions, pH, temperature, and a variety of other environmental factors. These same factors are believed to influence the components of the receptor system in roughly the same manner in intact cells and *in vivo*. The component(s) to which a given adrenergic radioligand binds are thus dependent upon a variety of ligand, tissue, and assay properties.

### *1. The $\alpha_2$-Adrenergic Receptor/Effector Complex*

The $\alpha_2$-adrenergic receptor/effector complex has been more extensively characterized than the $\alpha_1$-adrenergic receptor complex (Bylund and U'Prichard, 1983; U'Prichard *et al.*, 1983; Hoffman and Lefkowitz, 1980a,b; U'Prichard, 1981; Williams and Lefkowitz, 1978). The proximal biochemical effect of $\alpha_2$-receptor stimulation by agonists in all cells and tissues so far examined, including neural tissue, is a decrease in basal and stimulated membrane adenylate cyclase activity and cellular cyclic AMP production (Jakobs, 1979; Limbird, 1981; Kahn *et al.*, 1982). This negatively coupled $\alpha_2$-receptor/effector complex consists of three major components: $R$, the receptor/recognition site; $N_i$, an inhibitory nucleotide binding protein complex (physically distinct from the $N_s$ for positively coupled receptor cyclase systems such as the β-adrenergic receptor) (Hildebrandt *et al.*, 1983); and $C$, the catalytic moiety of adenylate cyclase. A simple model of the functioning of these components has developed from models of the more completely characterized β-adrenergic receptor and can be illustrated as follows (Fig. 1). The nucleotide-binding complex ($N$) in its native conformation (Fig. 1A) has GDP tightly bound to it; binding of an agonist to $R$ induces a conformational change, allowing the formation of a complex of the $R$ and $N_i$

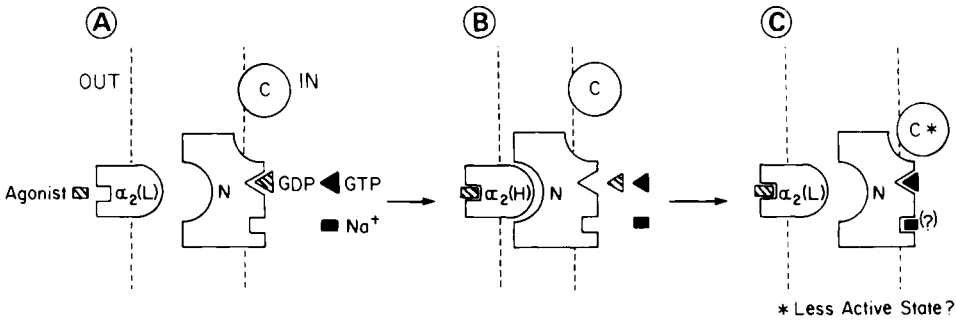


**Fig. 1.** Schematic diagram of the $\alpha_2$-adrenergic receptor/effector complex. The three components—*R* [$\alpha_2$(L)] the receptor/recognition site; *N*, the inhibitory nucleotide binding protein complex; and *C*, the catalytic moiety of adenylate cyclase—interact within the membrane as described in the text (see Section I,B,1).

components (Fig. 1B), which, in turn, favors the dissociation of GDP (and the association of GTP) at the $N_i$ site. Binding of GTP dissociates the $R{\cdot}N_i$ complex, favoring $N_i{\cdot}C$ interactions which "deactivate" adenylate cyclase (Fig. 1C). Hydrolysis of GTP is believed to restore the original resting state (Fig. 1A). In *in vitro* membrane preparations, nucleotides and ions alter the equilibria between these $\alpha_2$-receptor/effector components (Jakobs, 1979; Limbird, 1981; Rodbell, 1980). Guanine nucleotides (e.g., GTP, GMP–PNP) and sodium (probably acting at a physically distinct site) (Michel *et al.*, 1980; Limbird *et al.*, 1982) shift the equilibria in favor of the free *R* state, while divalent cations (e.g., $Mn^{2+}$, $Mg^{2+}$) shift the equilibria in favor of $R{\cdot}N_i$ by stabilizing the complex (U'Prichard and Snyder, 1978; Tsai and Lefkowitz, 1978; Rouot *et al.*, 1980).

For any $\alpha_2$-radioligand binding assay, a critical consideration is to which component or components (under a given experimental condition) the radioligand is preferentially binding at the low concentrations of radioligand typically used in binding assays. Hoffman and Lefkowitz (1980a) and co-workers developed the first working model of $\alpha_2$-adrenergic receptor–radioligand interactions based upon the heterogeneous interactions of agonists, but apparently not antagonists, with the multiple states of the $\alpha_2$-receptor/effector complex. The Hoffman model is based on the postulate that the $\alpha_2$-receptor exists in two affinity states, $\alpha_2$(H) and $\alpha_2$(L), differentiated by high [$\alpha_2$(H)] or low [$\alpha_2$(L)] agonist affinity. Reasonable evidence exists that the $\alpha_2$(H) state is equivalent to the $R{\cdot}N_i$ complex, while the $\alpha_2$(L) state is equivalent to free *R* (Smith and Limbird, 1981). Other investigators have revised the Hoffman model to include more (as many as five) (Glossmann and Hornung, 1980a) affinity states of the $\alpha_2$-receptor/effector complex, including an $\alpha_2$(SH) or super-high affinity state which requires $Mg^{2+}$ (U'Prichard *et al.*, 1982; Mitrius and U'Prichard, 1983). In addition, many $\alpha_2$-antagonists (e.g., yohimbine, rauwolscine) also demon-

strate heterogeneous interactions at the multiple $\alpha_2$-receptor affinity states (Salama *et al.*, 1982; Perry and U'Prichard, 1983a,b,c). Unlike agonists, these antagonists have highest affinity for the $\alpha_2$(L) states (free $R$) and lower affinity for the $\alpha_2$(H) states ($R{\cdot}N_i$ and $R{\cdot}N_i{\cdot}Mg^{2+}$).

Guanine nucleotides and metal ions, because they alter the equilibria between the multiple $\alpha_2$-adrenergic affinity states, will significantly alter the binding properties of [$^3$H]agonist and some [$^3$H]antagonist radioligands (see Section III,A).

### 2. *The $\alpha_1$-Adrenergic Receptor/Effector Complex*

Much less is known about the effector system(s) coupled to $\alpha_1$-adrenergic receptors (Bylund and U'Prichard, 1983). In peripheral tissues, increased phosphoinositide turnover, which in turn has been linked to increased calcium influx and intracellular calcium mobilization, has been associated with $\alpha_1$-receptor stimulation (Burns *et al.*, 1981; Fain and Garcia-Sainz, 1980). In brain tissues $\alpha_1$-receptor stimulation may result in an increase in cyclic nucleotides, possibly through calmodulin-dependent increases in adenylate cyclase activity (Schwabe *et al.*, 1978; Davis *et al.*, 1978). No direct coupling of $\alpha_1$-receptors to adenylate cyclase in plasma membranes has been shown. In many tissues (heart, kidney, brain), nucleotide and metal ion effects on $\alpha_1$-adrenergic radioligand binding characteristics have been inferred to indicate coupling of the $\alpha_1$-receptor to a nucleotide-binding protein (Glossmann and Hornung, 1980b; Yamada *et al.*, 1980; Ernsberger and U'Prichard, 1983). Although complete characterization of $\alpha_1$-receptor/effector complexes has not yet been achieved, binding assays using $\alpha_1$-adrenergic ligands increasingly appear to indicate an ion and nucleotide sensitivity at the recognition site that is similar to, though more variable than, that observed with $\alpha_2$-adrenergic ligands.

## II. DESIGN OF RADIOLIGAND ASSAYS

Although it is not the purpose of this chapter to cover exhaustively radioligand assay designs, the following section will serve as a rough guide to the variety and potential uses of radioligand binding experiments.

### A. Kinetic Studies

A prime consideration for use of any α-adrenergic radioligand is the time at which equilibrium is attained. As the mathematical analyses of the data from saturation, competition, and screening assays all assume that equilibrium has

been attained, time to equilibrium for a given condition, radioligand, and tissue must dictate the incubation time of the assay. A number of excellent, detailed methodological and theoretical reviews of radioligand binding assays cover the specific concerns of these assays (Williams and Lefkowitz, 1978; Yamamura *et al.*, 1978; Weiland and Molinoff, 1981; Molinoff *et al.*, 1981). Briefly, kinetic analysis involves association and dissociation experiments. Association experiments generally employ a single concentration (ideally below the $K_D$) of the radioligand, and samples are incubated for various time periods under conditions identical to those used in other assays with the radioligand (e.g., same temperature, buffer, ionic constituents, and appropriate blank) (see Fig. 2A). Once time to equilibrium has been established, dissociation experiments may be performed. To examine radioligand dissociation, specific binding is allowed to

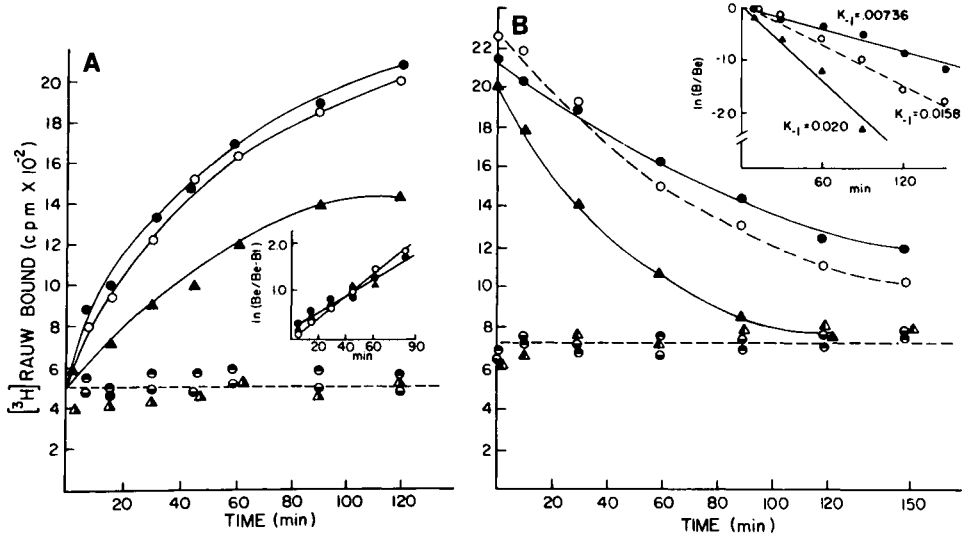


**Fig. 2.** Kinetics at 4°C of [$^3$H]rauwolscine binding to bovine frontal cortex membranes in the absence or presence of 1 and 100 $\mu M$ GTP. (A) Association: 1.0 n$M$ [$^3$H]rauwolscine was incubated with membranes (0.70 mg protein suspended in 50 m$M$ Na–$KPO_4$ buffer) at 4°C for various periods of time, and total binding was determined in the absence (○) or presence of 1.0 (●) or 100 (▲) $\mu M$ GTP. Nonspecific binding was determined by incubations containing 100 $\mu M$ (−)-norepinephrine, in the absence (◓) or presence of 1.0 (◒) or 100 (◭) $\mu M$ GTP. (*Inset*) Pseudo-first-order plot of increase in specific binding with time. (B) Dissociation: 1.0 n$M$ [$^3$H]rauwolscine was incubated to equilibrium (120 min, 4°C) with (half-filled symbols) or without (solid symbols) 100 $\mu M$ (−)-norepinephrine before the addition, at time zero, of 100 $\mu M$ (−)-norepinephrine and either 1.0 (●,◓) or 100 (▲,◭) $\mu M$ GTP, or no GTP (○,◓). Samples were filtered at various times thereafter. (Inset) Semilogarithmic plot of decrease in specific binding with time. Values are from a representative experiment performed in triplicate on the same batch of membranes for association and dissociation experiments.

reach equilibrium (under the particular assay conditions), and then dissociation of the radioligand and binding site is effected by addition of a large excess of an unlabeled agonist or antagonist compound or by rapid, large increase in the assay volume to dilute the radioligand. Although not always necessary, a number of other considerations must be made for thorough kinetic analysis of α-adrenergic ligand assays. Ideally, association and dissociation experiments are carried out using several concentrations of radioligand over a wide range, and dissociation at a single concentration of ligand should be examined with agonist and antagonist blanks and dissociating compounds that are either agonists or antagonists. These studies are particularily important if complex saturation isotherms (see Section II,B,2) are obtained with a given radioligand. In addition, more detailed analysis of kinetics may be made in the presence of nucleotides and/or metal ions to help gain information about the interactions of the given radioligand with the multiple affinity states (e.g., for $\alpha_2$-receptors).

## B. Equilibrium Studies

### *1. Screening Studies*

Once a proper incubation time has been established (note that for almost all currently available α-adrenergic radioligands used in rat, bovine, and human brain, standard assay conditions and incubation times have been established) (see Table I for references), more detailed study of the α-adrenergic binding sites in question can proceed. A commonly used, simple assay involves the use of a single concentration of radioligand in a screening assay. This assay allows one to demonstrate specific binding of the α-adrenergic radioligand in a given situation (e.g., if tissue is limited, if one needs to know if α-adrenergic receptors are present in a given brain region or species, if one wants to compare the effects of experimental treatment as a preliminary to guide further, more detailed radioligand assays). Screening assays, in the absence of characterization of the binding site in the same species, brain region, and experimental condition, do not provide satisfactory information upon which judgements of α-adrenergic receptor number or functioning can be based. Furthermore, unless the binding site has been characterized (as outlined in Sections II,B,2 and 3), one cannot conclude that it is truly an α-adrenergic receptor/recognition site. Single concentration assays do remain, however, useful assays in systems (e.g., [$^3$H]clonidine binding in rat cortex) in which the binding has been characterized, and conclusions about findings are not overinterpreted (e.g., "a decrease in [$^3$H]clonidine binding in rat cortex was observed" and not "a decrease in $\alpha_2$-receptors in rat cortex was observed").

TABLE I

Radioligands Used to Label α-Adrenergic Binding Sites

| Receptor selectivity radioligand | Apparent affinity range[a] ($K_D$ in n*M*) | Reference |
|---|---|---|
| Agonist | | |
| $\alpha_2$ (H) Selective | | |
| [$^3$H]epinephrine | 1.0–10.0 | U'Prichard and Snyder (1977) |
| [$^3$H]norepinephrine | 3.0–10.0 | U'Prichard and Snyder (1977) |
| Partial agonist | | |
| $\alpha_2$ (H) Selective | | |
| [$^3$H]clonidine | 1.5–4.0 | U'Prichard *et al.* (1977a) |
| [$^3$H]*p*-aminoclonidine | 0.5–3.0 | Rouot and Snyder (1979) |
| [$^3$H]guanfacine | 1.5–4.0 | Jarrot *et al.* (1982) |
| Antagonist | | |
| $\alpha_1$ Selective | | |
| [$^3$H]prazosin | 0.10–0.50 | Greengrass and Bremner (1979) |
| [$^{125}$I]BE 2254 | 0.10–0.40 | Engel and Hoyer (1981) |
| $\alpha_2$ (L) Selective | | |
| [$^3$H]rauwolscine | 1.0–4.0 | Perry and U'Prichard (1981) |
| [$^3$H]yohimbine | 1.5–5.0 | Rouot *et al.* (1982) |
| $\alpha_2$ (T) Selective[c] | | |
| [$^3$H]RX 781094 | 2.0–5.0 | Howlett *et al.* (1982) |
| α Selective | | |
| [$^3$H]phentolamine | 6.0–15.0 | Steer *et al.* (1979) |
| [$^3$H]WB 4101 | 0.30–0.80 | U'Prichard *et al.* (1977a) |
| [$^3$H]dihydroazapetine | 4.0–10.0 | Ruffolo *et al.* (1976) |
| α Selective, irreversible | | |
| [$^3$H]phenoxybenzamine | — | Kunos *et al.* (1983) |
| Nonselective | | |
| [$^3$H]dihydroergocryptine | 1.0–6.0 | Greenberg and Snyder (1978) |
| [$^3$H]lisuride | — | Battaglia and Titeler (1980) |
| [$^3$H]mianserin | — | Peroutka and Snyder (1981) |

[a] $K_D$ value ranges shown are for the high-affinity component of binding in brain (if known). Note that "apparent" $K_D$ values are dependent upon assay conditions (see text).

[b] References are for major, primary papers describing binding of the radioligand in brain, or when the radioligand has been used rarely in brain, peripheral tissues.

[c] $\alpha_2$ (T) indicates that [$^3$H]RX 781094 may have roughly equivalent affinities for the $\alpha_2$ (L) and $\alpha_2$ (H) affinity states.

### 2. *Saturation Studies*

Saturation experiments are among the most useful and are certainly the most common procedures in which α-adrenergic radioligands are used. From a saturation experiment, two major results are obtained: a value which is an estimate of the affinity of the radioligand for the binding site (the $K_D$, or equilibrium binding

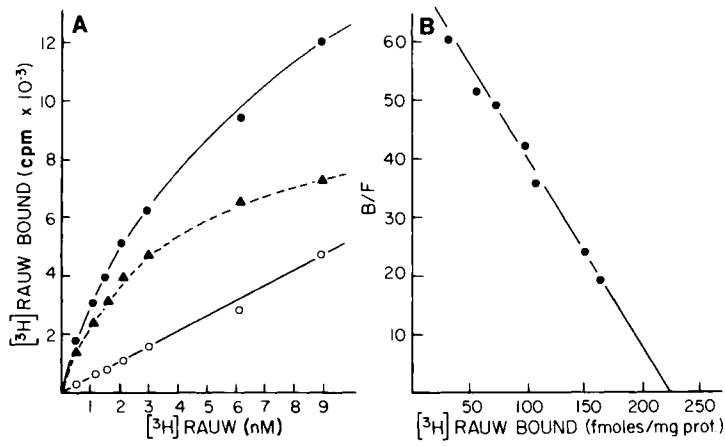


**Fig. 3.** (A) [$^3$H]Rauwolscine binding to bovine frontal cortex membranes as a function of increasing radioligand concentration. Membranes (0.7 mg protein) were incubated at 4°C for 120 min. Nonspecific binding was determined in parallel incubations which included 100 μ*M* (−)-norepinephrine. ●, Total; ▲, specific; ○, nonspecific. (B) Scatchard plot of specific binding from the experiment in A. $K_D$ = 3.20 n*M*.

constant) and a measure of the maximum number of binding sites ($B_{max}$). Both of these values are of importance in the characterization of a radioligand binding site, and particularly with $\alpha_2$-adrenergic radioligands, both are subject to major changes when assay constituents (e.g., nucleotides, metal ions) are varied (see Section III,A). Saturation studies involve incubation of the tissue preparation with increasing concentrations of the radioligand (0.1–10 times the estimated $K_D$ value). For a radioligand with a single site of interaction, such as the $\alpha_1$-adrenergic antagonist radioligands, a linear Scatchard or Eadie-Hofstee transformation is obtained (Fig. 3), and derivation of the $K_D$ and $B_{max}$ values is straightforward. Almost all $\alpha_2$-radioligands (agonist, partial agonist, and antagonist) will yield curvilinear Scatchard or Eadie-Hofstee transformations of the saturation isotherms (see Section III,A and Fig. 4). Derivation of $K_D$ and $B_{max}$ values is much more complex and may require computer-assisted curve fitting methods (see Section IV). Furthermore,nucleotides, metal ions, and other assay properties will determine the radioligand's apparent affinities for, as well as relative amounts of, the high- and low-affinity components of binding. The heterogeneous interactions of agonist and antagonist $\alpha_2$-radioligands at the multiple affinity states of the $\alpha_2$-receptor/effector complex complicate any use of saturation studies using limiting concentrations of the ligands to assess $\alpha_2$-adrenergic receptor number and functional status. Using limiting concentrations of $\alpha_2$-radioligand, changes in $B_{max}$ are often misinterpreted as changes in actual $\alpha_2$-receptor number. In addition, changes in apparent $K_D$ may reflect alterations in

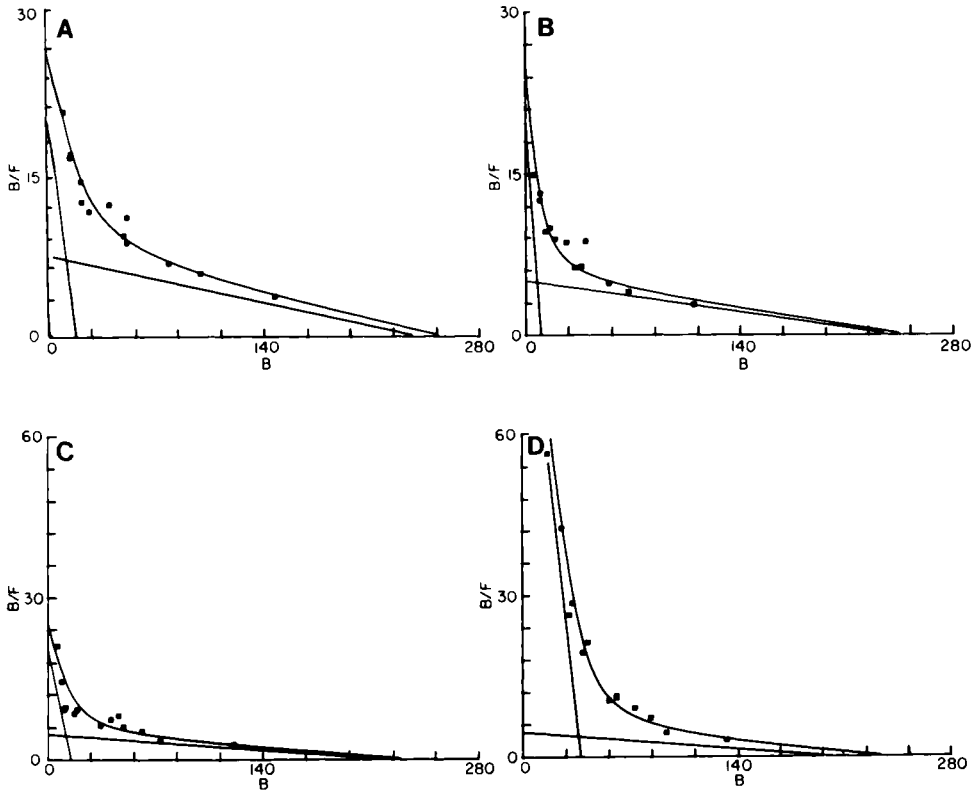


**Fig. 4.** Scatchard plots of [$^3$H]Rauwolscine and [$^3$H]*p*-aminoclonidine binding to $\alpha_2$-receptor sites in bovine frontal cortex membranes. (A) [$^3$H]Rauwolscine in Na–$KPO_4$ buffer. (B) [$^3$H]Rauwolscine in Tris–HCl buffer. (C) [$^3$H]*p*-Aminoclonidine in Na–$KPO_4$ buffer. (D) [$^3$H]*p*-Aminoclonidine in Tris–HCl buffer. Membranes from the same batch (0.5–0.7 mg protein) were incubated at 4°C for 120 min for [$^3$H]Rauwolscine and at 25°C for 30 min for [$^3$H]*p*-aminoclonidine in the absence or presence of (−)-norepinephrine (100 $\mu M$ for [$^3$H]Rauwolscine; 10 $\mu M$ for [$^3$H]*p*-aminoclonidine) to define specific binding. All data were analyzed with the BIPHAS program and fit a two-site model significantly better than a one-site model. The straight lines represent the two components resolved from each binding isotherm. Computer-derived $K_D$ and $B_{max}$ values from these experiments are summarized in Table II.

the ratio of high- and low-affinity components of binding rather than true changes in radioligand affinity. The use of saturation experiments to measure total $\alpha_2$-receptor number may require analysis over a wide range of radioligand concentrations to ensure accurate measurement of both the high- and low-affinity components of binding. Alternatively, in a system such as the $\alpha_2$-receptor, in which, at very low ligand concentrations, [$^3$H]agonist high-affinity binding will estimate the number of $\alpha_2$(H) states, and [$^3$H]antagonist high affinity binding

TABLE II

[$^3$H]Rauwolscine and [$^3$H]*p*-Aminoclonidine Binding Sites in Bovine Cortex[a]

| | Site 1 | | Site 2 | | |
|---|---|---|---|---|---|
| | $K_D$ (n*M*) | $B_{max}$ (fmol/mg) | $K_D$ (n*M*) | $B_{max}$ (fmol/mg) | Total sites (fmol/mg) |
| [$^3$H]Rauwolscine | | | | | |
| Na–$KPO_4$ | 0.54 | 24.2 | 46.0 | 239.2 | 263.4 |
| Tris–HCl | 0.72 | 8.1 | 48.0 | 222.6 | 231.0 |
| [$^3$H]*p*-Aminoclonidine | | | | | |
| Na–$KPO_4$ | 0.74 | 15.2 | 47.0 | 220.0 | 235.2 |
| Tris–HCl | 0.46 | 38.0 | 44.2 | 203.0 | 241.0 |

[a]The same batch of bovine cortex membranes (0.5 mg protein/ml) was incubated in triplicate with 14 concentrations of [$^3$H]*p*-aminoclonidine (0.07–45.5 n*M*) or [$^3$H]rauwolscine (0.1–50.0 n*M*). $K_D$ and $B_{max}$ estimates were computer-derived by obtaining the best fit assuming a two-site model.

will estimate the number of $\alpha_2$(L) states, assays using both agonist and antagonist ligands over a low, restricted concentration range may be a more practical and suitable method of estimating total $\alpha_2$-receptor number (addition of the number of [$^3$H]agonist sites [$\alpha_2$(H)] and [$^3$H]antagonist sites [$\alpha_2$(L)] obtained from essentially linear Scatchard or Eadie-Hofstee plots).

Saturation experiments may also be performed to gain an understanding of the molecular pharmacology of α-receptor/effector functioning. Measurements of the effects of nucleotides and metal ions on radioligand affinites at the multiple states or of the ratio of the high- and low-affinity components of binding can be used to explore further the regulation of α-receptor/effector complexes under various conditions. An example of this kind of saturation analysis is shown in Table II, which is discussed in more detail below (Section III,A,3).

### *3. Competition Studies*

*a. Pharmacological Characterization.* Of prime importance is the demonstration that the radioligand binds specifically to the α-adrenergic receptor subtype of interest. An assessment of the characteristics of competition by a variety of known pharmacological agents at the radioligand binding site will allow such characterization. In competition experiments, a single concentration of radioligand is incubated with the receptor in the presence of increasing concentrations of unlabeled competitor. The concentration of competitor which will inhibit 50% of the specific binding of the radioligand is the $IC_{50}$, which [using the Cheng-Prusoff relation (Cheng and Prusoff, 1973)] can be converted to the apparent $K_i$ value, an estimate of the affinity of the unlabeled competitor for the

TABLE III

Inhibition of [$^3$H]Rauwolscine Specific Binding to $\alpha_2$-Receptor Sites in Rat Cortex Membranes[a]

| Drug | No GTP | | 100 μ*M* GTP | |
|---|---|---|---|---|
| | $K_i$ (n*M*) | $n_H$ | $K_i$ (n*M*) | $n_H$ |
| Catecholamines | | | | |
| (−)-Epinephrine | 220 | 0.57 | 1,430 | 0.91 |
| (−)-α-Me-Norepinephrine | 400 | 0.62 | 1,180 | 0.85 |
| (−)-Norepinephrine | 1,550 | 0.64 | 5,300 | 0.95 |
| (−)-Isoproterenol | 40,000 | — | 50,000 | — |
| Antagonists | | | | |
| Rauwolscine | 6.5 | 1.01 | 6.2 | 0.94 |
| Yohimbine | 6.4 | 0.98 | 7.3 | 0.98 |
| Phentolamine | 15 | 0.94 | 18 | 0.96 |
| Prazosin | 900 | 0.92 | 950 | 0.95 |

[a][$^3$H]Rauwolscine (0.8–1.1 n*M*) was incubated with membranes (0.65 mg protein) at 4°C for 120 min. The $K_D$ of [$^3$H]rauwolscine used to determine $K_i$ values was 2.0 n*M*. Values are means from two to five experiments, each performed in triplicate.

radiolabeled binding sites. With a series of apparent $K_i$ values for a number of competitors, a rank order of potencies is established (see Table III), which can be compared to the rank order of potencies of competitors at other well-characterized α-adrenergic response systems, both *in vivo* and *in vitro*. Assuming a proper selection of competitors, which would include agonist and antagonist compounds selective for the α-adrenergic system as well as a number of compounds with known selectivity for other closely related receptor subtypes, a match of rank order of potency at the radiolabeled binding site with other well characterized systems can ensure that the site corresponds to the α-adrenergic receptor of interest.

Competition studies are useful for other purposes. Of particular interest is the use of a well-characterized binding system (e.g., [$^3$H]rauwolscine binding in bovine frontal cortex) to screen the α-receptor potency and specificity of newer, experimental drugs. Competition studies in a variety of well-characterized brain receptor binding systems can rapidly and inexpensively screen the pharamcological specificity (and thereby the potential therapeutic and adverse effects) of a compound.

For the majority of competition assays, eight or more concentrations of unlabeled competitor should be employed and the concentration of radioligand should be at or below the $K_D$ value; the heterogeneous interactions of $\alpha_2$-adrenergic radioligands make routine competition assays more complex and require the use of more competitor concentrations. One concern with these complex ligand binding systems is to establish the rank order of potency of various

compounds at the low-affinity component of binding. This can be accomplished by repeating the series of competition experiments at a much higher concentration of radioligand. If, in fact, the low-affinity component of binding is one of the α-adrenergic receptor/effector affinity states, the rank order of potency at this site should remain characteristic of the α-adrenergic receptor one is labeling.

*b. Multiple-Site Analysis.* Competition studies are another way in which heterogeneous interactions of various compounds can be investigated. Transformation of standard competition data into pseudo-Hill plots can give a rough estimate of whether the unlabeled compound is probably interacting with a single site (pseudo-Hill slope values within the range of 0.85 to 1.1; see Table III) or multiple binding sites (pseudo-Hill slope values <0.8). For example, agonist compounds competing at [$^3$H]rauwolscine-labeled sites representing $\alpha_2$-receptors in rat cerebral cortex have shallow pseudo-Hill slopes (Fig. 5 and Table III), reflecting interactions at both the $\alpha_2$(H) and $\alpha_2$(L) states of the receptor which [$^3$H]rauwolscine (at a 1.0 n*M* concentration) labels. Promotion of the formation of the $\alpha_2$(L) affinity state by addition of 100 μ*M* GTP is reflected in the competi-

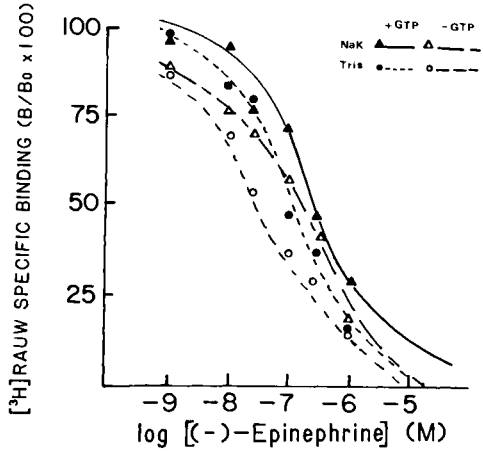


**Fig. 5.** Inhibition of [$^3$H]rauwolscine specific binding by (−)-epinephrine: sodium and GTP effects. 1.0 n*M* [$^3$H]rauwolscine was incubated with bovine frontal cortex membranes [0.65 mg protein suspended in either 50 m*M* Tris–HCl (●,○) or 50 m*M* Na–$KPO_4$ (▲,△) buffer] for 120 min at 4°C in the presence of varying concentrations of unlabeled drug, and in the presence (+GTP, solid symbols) or absence (−GTP, open symbols) of 100 μ*M* GTP. Specific binding was defined by incubations containing 100 μ*M* (−)-norepinephrine. In this experiment, the apparent $K_i$ and pseudo-Hill ($N_H$) values, respectively, for (−)-epinephrine inhibition in different conditions were 32 n*M*, 0.64 (−GTP, Tris–HCl); 157 n*M*, 0.96 (+GTP, Tris–HCl); 115 n*M*, 0.71 (−GTP, Na–$KPO_4$); 365 n*M*, 0.94 (+GTP, Na–$KPO_4$). The heterogeneous interactions of (−)-epinephrine at the [$^3$H]rauwolscine-labeled sites were investigated with a number of similar, more extensive competition experiments; the computer-derived apparent $K_i$ values and fraction of total sites for each component of binding for (−)-epinephrine under the various conditions are summarized in Table IV.

tion assay by a decrease in apparent agonist affinity (a right shift of the curve; Fig. 5) and a steepening of the curve, as shown by the increased pseudo-Hill value (Table III).

Using a few (7–10) concentrations of unlabeled competitor (bracketing the $K_i$ value) can be useful for the crude examination of heterogeneous interactions and nucleotide and metal ion effects upon the multiple affinity states. More sophisticated competition experiments employ a wider range and more (ideally 18 or more) concentrations of unlabeled competitor. From the resulting curve, computer-assisted curve-fitting programs can be used to derive the equilibrium binding constants of the unlabeled competitor for the multiple sites, as well as the relative fraction of total binding sites each component represents (Fig. 6; Tables IV, V). Changes in the ratio of affinity states and changes in affinity of the unlabeled competitor at each state can be assessed following various treatments without the expense and effort of multiple, extended saturation experiments.

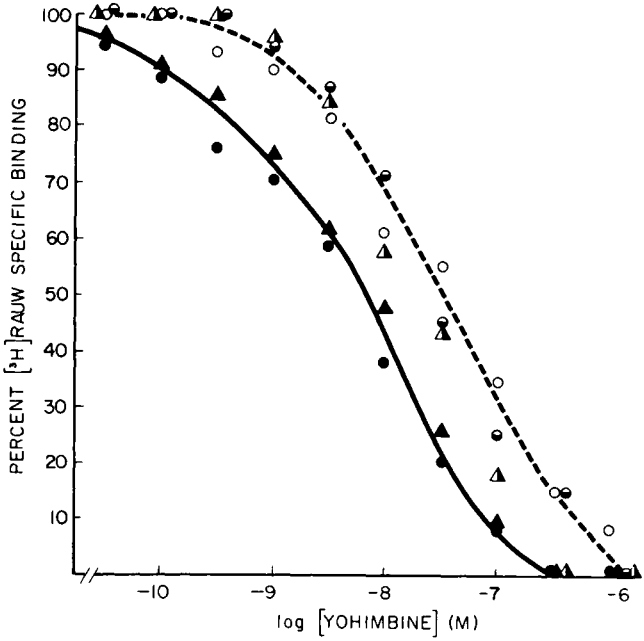


**Fig. 6.** Inhibition of [$^3$H]rauwolscine specific binding by yohimbine: guanine nucleotide effects. 0.8 n*M* [$^3$H]rauwolscine was incubated with bovine frontal cortex membranes (0.6 mg protein suspended in 50 m*M* Na--$KPO_4$ buffer) for 120 min at 4°C in the presence of varying concentrations of unlabeled yohimbine and in the absence (○) or presence of 1 μ*M* GMP–PNP (●), 100 μ*M* GMP–PNP (◒), 1 μ*M* GTP (▲), or 100 μ*M* GTP (Δ). Specific binding was defined by 100 μ*M* (−)-norepinephrine. Values represent means from two to three similar experiments. Computer-derived apparent $K_D$ values and relative fraction of total sites for each component of binding are summarized in Table V.

TABLE IV

(−)-Epinephrine Competition at [$^3$H]Rauwolscine Binding Sites in Bovine Frontal Cortex: Sodium and GTP Effects[a]

| | Site 1 | | Site 2 | |
|---|---|---|---|---|
| | $K_D$ | % Total sites | $K_D$ | % Total sites |
| (−)-Epinephrine | | | | |
| Tris–HCl | 2.1 ± 0.21 | 27.2 ± 5.1 | 64.4 ± 12.0 | 70.3 ± 7.5 |
| NA–$KPO_4$ | 1.8 ± 0.24 | 17.8 ± 2.3 | 80.7 ± 6.8 | 75.9 ± 4.7 |
| Tris–HCl + GTP | — | — | 91.0 ± 15.0 | 100.3 ± 1.0 |
| Na–$KPO_4$ + GTP | — | — | 78.0 ± 17.5 | 92.0 ± 3.6 |

[a][$^3$H]Rauwolscine (0.8–1.0 n*M*) was incubated with membranes at 4°C for 120 min with 8–12 concentrations of (−)-epinephrine. Specific binding was defined with 100 μ*M* (−)-norepinephrine. $K_D$ and $B_{max}$ estimates were computer-derived by obtaining the best fit assuming a two-site model, with % inhibition = bound and the concentration of (−)-epinephrine = free. In the presence of 100 u*M* GTP, the data best fit a one-site model and was analyzed by weighted regression of Eadie-Hofstee plots (Zivin and Waud, 1982). Values are mean ± SEM from three to eight experiments, each performed in triplicate.

### 4. *Metal Ion and Nucleotide Studies*

Because many monovalent and divalent metal ions and guanine nucleotides alter the equilibrium between multiple affinity states of the $\alpha_2$- (and possibly $\alpha_1$-) receptor/effector complex, metal ions and nucleotides will affect α-radioligand binding. Metal ion and nucleotide effects can be examined in kinetic, single-point screening, saturation, and competition assays, each providing a somewhat

TABLE V

Antagonist Competition at $\alpha_2$-Adrenergic Receptor Sites ([$^3$H]Antagonist and [$^3$H] Agonist) in Bovine Frontal Cortex Membranes: Effects of GTP[a]

| | Site 1 | | Site 2 | |
|---|---|---|---|---|
| | $K_D$ (n*M*) | % Total sites | $K_D$ (n*M*) | % Total sites |
| Yohimbine vs [$^3$H]Rauwolscine (Na–$KPO_4$) | | | | |
| Control | 1.8 ± 0.2 | 29.0 ± 3.0 | 52.3 ± 0.8 | 66.8 ± 1.8 |
| 1.0 μ*M* GTP | 1.9 ± 0.3 | 60.7 ± 7.4 | 58.1 ± 20.7 | 39.3 ± 9.2 |
| 1.0 μ*M* GMP–PNP | 2.0 ± 0.2 | 56.0 ± 5.0 | 44.3 ± 2.7 | 51.5 ± 4.5 |
| Rauwolscine vs [$^3$H]*p*-Aminoclonidine (Tris–HCl) | | | | |
| Control | 1.4 ± 0.9 | 32.0 ± 4.0 | 42.0 ± 10.0 | 59.0 ± 4.0 |

[a]Assay was performed and data were analyzed as described in Table IV legend. Values are mean ± SEM from two to five experiments, each performed in triplicate.

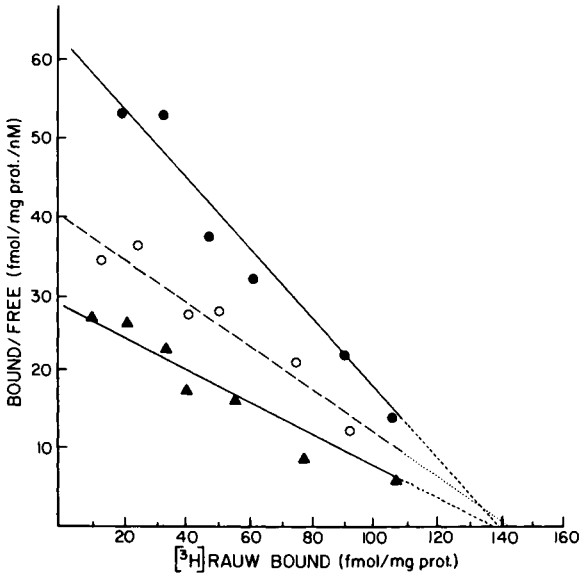


**Fig. 7.** Scatchard plots of equilibrium saturation isotherms of [$^3$H]rauwolscine specific binding to bovine frontal cortex membranes in the absence or presence of GTP. Bovine frontal cortex membranes from the same preparation (0.58 mg protein suspended in 50 m*M* Na–$KPO_4$ buffer) were incubated for 120 min at 4°C with various concentrations of [$^3$H]rauwolscine (0.2–8.0 n*M*), in the presence of 1.0 (●) or 100 (▲) μ*M* GTP, or in the absence of GTP (○). Values represent one of two experiments, each with similar results and each performed in triplicate. For ●, $K_D$ = 2.3 n*M*; ○, $K_D$ = 3.7 n*M*; ▲, 5.0 n*M*.

different view of the radioligand–receptor/effector complex interactions. For example, guanine nucleotides increase the apparent affinity of the $\alpha_2$-antagonist radioligand [$^3$H]rauwolscine when limited saturation studies are employed (Fig. 7). Kinetic analysis (Fig. 2) in this binding system reveals that guanine nucleotides apparently cause this increase in affinity by retarding dissociation of the radioligand. Detailed competition studies in the same system with the antagonist yohimbine reveal that the increase in apparent affinity is caused by an alteration in the ratio of $\alpha_2$(H) to $\alpha_2$(L) (Fig. 6, Table V), rather than an actual change in radioligand affinity. The [$^3$H]rauwolscine saturation experiments in Figs. 3 and 7 used narrow ranges of radioligand, which were insufficient to reveal the low-affinity component of binding (revealed in an extended saturation such as in Fig. 4) and the resulting shift from $\alpha_2$(H) to $\alpha_2$(L), for which [$^3$H]rauwolscine has higher affinity (Perry and U'Prichard, 1983a,b,c). This example reveals the potential problems with interpretation if one is faced with incomplete experimental design of radioligand analysis of a putative α-adrenergic receptor site.

## III. α-ADRENERGIC RADIOLIGAND ASSAY METHODS

### A. Selection of Suitable α-Adrenergic Radioligands

α-Adrenergic binding sites have been radiolabeled in peripheral and central tissues by agonists, partial agonists, and antagonists (Table I). The wide variety and potential experimental uses of these radioligands makes proper selection of a suitable compound essential.

#### *1. Agonist Radioligands*

*a. $\alpha_1$-Agonist Radioligands.* High affinity, $\alpha_1$-specific binding of an agonist radioligand has yet to be demonstrated in brain tissue. In the periphery, only a few laboratories have reported $\alpha_1$-specific binding of [$^3$H]epinephrine and [$^3$H]norepinephrine (Geynet *et al.*, 1981). At present, radioagonist labeling of $\alpha_1$-adrenergic receptor binding sites in brain is not feasible.

*b. $\alpha_2$-Agonist Radioligands.* [$^3$H]Norepinephrine and [$^3$H]epinephrine binding in bovine brain have been extensively characterized (U'Prichard and Snyder, 1977). In brain, these physiological agonists appear to label the same population of $\alpha_2$-receptor binding sites such that at low concentrations (0.1–4.0 n*M*) they bind predominantly to the $\alpha_2$(H) states (*R·N* complex) while at higher concentrations, they label, with lower affinity, $\alpha_2$(L) (free *R*). Extended binding curves, therefore, give rise to nonlinear Scatchard plots, although over working ranges (0.5–10.0 n*M*) [$^3$H]catecholamine Scatchard plots are linear. [$^3$H]Epinephrine has two to threefold higher affinity for the $\alpha_2$(H) states than does [$^3$H]norepinephrine. These [$^3$H]catecholamine ligands are technically the most difficult to use and specific binding is only readily detectable after the inclusion of pyrocatechol (1.0 m*M*) to prevent nonspecific catechol interactions, the use of antioxidants [such as $Na_2EDTA$ (0.1 m*M*), dithiothreitol (0.01 m*M*), and ascorbic acid (0.01%)], and an MAO inhibitor (e.g., 10 μ*M* pargyline) to prevent radioligand degradation. Additionally, with [$^3$H]epinephrine, care must be taken to prevent high-affinity $\beta_2$-adrenergic receptor interactions. Because of technical considerations and the fact that apparently identical labeling can be attained with [$^3$H]imidazoline radioligands, the use of [$^3$H]catecholamines (particularly [$^3$H]norepinephrine) in brain tissue is limited.

#### *2. Partial Agonist Radioligands*

*a. $\alpha_1$-Adrenergic Partial Agonist Radioligands.* Again, high-affinity, $\alpha_1$-specific binding of an $\alpha_1$-partial agonist radioligand has yet to be demonstrated.

*b. $\alpha_2$-Adrenergic Partial Agonist Radioligands.* The imidazoline [$^3$H]clonidine, a substituted clonidine derivative, [$^3$H]*p*-aminoclonidine, and the substituted guanidine compound [$^3$H]guanfacine are all $\alpha_2$-partial agonists and apparently label binding sites which are pharmacologically similar to those labeled by [$^3$H]epinephrine, that is, $\alpha_2$(H) states. These ligands are all stable, with high specific activity and high $\alpha_2$-selectivity, making their use in brain tissue technically much easier than the [$^3$H]catecholamines. Therefore, these ligands have been used widely to label brain $\alpha_2$-receptors. In tissues where direct comparisons have been made, [$^3$H]*p*-aminoclonidine demonstrates higher affinity than either [$^3$H]clonidine or [$^3$H]guanfacine for the $\alpha_2$(H) states and may be more suitable for certain assays in which tissue is limited or receptor density is low.

Although all of the [$^3$H]catecholamines and [$^3$H]imidazolines (at lower concentrations) label the $\alpha_2$(H) states of the $\alpha_2$-receptor/effector complex, at higher concentrations they label the $\alpha_2$(L) states. Biphasic saturation and dissociation with all of the [$^3$H]agonist and [$^3$H]partial agonist radioligands can be expected; furthermore (as would be predicted from the discussion in Section I,B,1), assay constituents will significantly alter the portion or portions of the $\alpha_2$-receptor/effector complex labeled by these radioligands at a given concentration (see Section III,D).

### *3. Antagonist Radioligands*

*a. $\alpha_1$-Antagonist Radioligands.* The development and use of radiolabeled antagonists to label $\alpha_1$-receptor binding sites in brain has been quite successful (Table I). The $\alpha_1$-selective antagonist [$^3$H]prazosin and the less $\alpha_1$-selective [$^3$H]WB 4101 (see Table I for references) label a single class of $\alpha_1$-adrenergic binding sites with high affinity ($K_D$ = 0.10–0.40 n*M*). The sites labeled by [$^3$H]prazosin and [$^3$H]WB 4101 are presumed to be the same class of sites. However, the $\alpha_1$-selectivity of [$^3$H]WB 4101 is questionable (Hoffman and Lefkowitz, 1980c; Perry and U'Prichard, 1983a) and with the ready availablity and easy use of [$^3$H]prazosin, a highly $\alpha_1$-selective radioligand, [$^3$H]WB 4101 should be used only to label $\alpha_1$-receptors in a well-characterized system, if at all. [$^3$H]Dihydroergocryptine will label brain $\alpha_1$-adrenergic receptors; the high-affinity interactions of this radioligand with many other receptor types (5-HT, $\alpha_1$, $\alpha_2$, histamine, etc.) make its use in brain tissue specifically to label $\alpha_1$- or $\alpha_2$-adrenergic receptors quite unsatisfactory.

An iodinated $\alpha_1$-selective radioligand, [$^{125}$I]BE 2254 (HEAT), has been used to label $\alpha_1$-receptors in brain (see Table I). This radioligand has very high specific activity and high affinity ($K_D$ = 0.1 n*M*) for a single class of $\alpha_1$-adrenergic binding sites. Use of this radioligand is recommended when tissue quantity or $\alpha_1$-receptor density is limited.

Many investigators have reported the lack of metal ion and nucleotide effects

upon $\alpha_1$-antagonist radioligand binding (see Section I,B,2). However, several laboratories have observed that $Na^+$, $Mg^{2+}$, or guanine nucleotides will directly influence [$^3$H]prazosin or [$^{125}$I]BE 2254 binding (Glossmann *et al.*, 1981; Ernsberger and U'Prichard, 1983) and the apparent affinities of competing agonist compounds at [$^3$H]prazosin-labeled sites (Glossmann and Hornung, 1980b; Yamada *et al.*, 1980; Ernsberger and U'Prichard, 1983). These results imply that the $\alpha_1$-adrenergic receptor, at least in some tissues, may interact with nucleotide-binding proteins and that nucleotides and metal ions may alter the binding characteristics of [$^3$H]prazosin and [$^{125}$I]BE 2254 in brain.

*b. $\alpha_2$-Antagonist Radioligands.* The first demonstration of α-specific binding was with [$^3$H]dihydroergocryptine (Williams and Lefkowitz, 1976) in rabbit uterus. Shortly thereafter, specific binding of [$^3$H]dihydroergocryptine was demonstrated in brain (Greenberg and Snyder, 1978). The nonselectivity of [$^3$H]dihydroergocryptine has, however, made it an unsuitable radioligand for the specific labeling of brain $\alpha_2$-adrenergic receptors.

The alkaloid yohimbine and its diastereoisomer rauwolscine are $\alpha_2$-selective antagonists in a number of pharmacological systems (Hedler *et al.*, 1981; Tanaka and Starke, 1980), with rauwolscine roughly 20 times more $\alpha_2$-selective. Both have been radiolabeled (Table I) and are very useful $\alpha_2$-receptor ligands. [$^3$H]Rauwolscine and [$^3$H]yohimbine both demonstrate heterogeneous interactions with the multiple affinity states of the $\alpha_2$-receptor (Figs. 4 and 6, Tables II and V). At low concentrations, these antagonists apparently label the $\alpha_2$(L) states. Nucleotides and metal ions influence the binding of these $\alpha_2$-antagonist radioligands (see all figures and tables) in a manner reciprocal to the effects on $\alpha_2$-agonist radioligands (Perry and U'Prichard, 1981, 1983a,b,c). For example, in a direct comparison (Fig. 4, Table II), $Na^+$ (in the Na–$KPO_4$ buffer) increases the number of [$^3$H]rauwolscine high-affinity binding sites [$\alpha_2$(L)] and decreases, to a similar extent, the number of [$^3$H]*p*-aminoclonidine high-affinity sites, all with no changes in apparent affinity of either ligand for the multiple affinity components. A reciprocal guanine nucleotide effect upon agonist and antagonist interactions at $\alpha_2$-affinity states can be seen by comparing Tables IV and V. (−)-Epinephrine interacts with the [$^3$H]rauwolscine-labeled sites in a heterogeneous manner, with apparent affinity constants ($K_D$ values in Table IV) similar to those derived from the biphasic [$^3$H]epinephrine saturation studies. Sodium decreases the percentage of (−)-epinephrine high-affinity sites [$\alpha_2$(H)] as do, to a greater degree, guanine nucleotides. Yohimbine also interacts with [$^3$H]rauwolscine labeled sites in a heterogeneous manner (Fig. 6, Table V) and with computer-derived apparent affinity constants close to those derived from biphasic [$^3$H]yohimbine saturation studies. Addition of guanine nucleotides (low concentrations, i.e., 1.0–10.0 μ*M*) increases the number of yohimbine high-affinity sites [$\alpha_2$(L)], an effect reciprocal to the guanine nucleotide-induced

decrease in (−)-epinephrine high-affinity sites ($\alpha_2$(H)]. Higher nucleotide concentrations decrease antagonist specific binding in a nucleotide-unspecific, little-understood manner.

The fact that limiting concentrations (0.2–8.0 n*M*) of these two antagonist radioligands label only a portion of the total $\alpha_2$-receptor population poses certain methodological problems for investigators wishing to measure $\alpha_2$-receptor number. Apparently not all $\alpha_2$-antagonists, however, demonstrate heterogeneous interactions at the $\alpha_2$-receptor affinity states (Salama *et al.*, 1982; Perry and U'Prichard, 1983b). Preliminary results indicate that the imidazoline RX 781094, a highly $\alpha_2$-selective antagonist, may label both the $\alpha_2$(H) and $\alpha_2$(L) states with equal high affinity (Salama *et al.*, 1983). This compound has been tritium-labeled recently and appears to be an effective radioligand (Howlett *et al.*, 1982). Phentolamine also appears to demonstrate roughly equivalent affinities for the $\alpha_2$(H) and $\alpha_2$(L) states (Salama *et al.*, 1982; Perry and U'Prichard, 1983b), though [$^3$H]phentolamine in brain suffers from relative nonspecific interactions at other receptor subtypes. If preliminary indications are confirmed, [$^3$H]RX 781094 and [$^3$H]phentolamine (when employed with a suitable blank) may be superior radioligands for the purpose of measuring $\alpha_2$-receptor number.

A number of other antagonist radioligands ([$^3$H]mianserin, [$^3$H]lisuride, [$^3$H]dihydroazapetine, [$^3$H]phenoxybenzamine) may label $\alpha_2$-adrenergic binding sites in brain. The nonspecificity (or irreversibility in the case of [$^3$H]phenoxybenzamine) of these agents and the availability of the other more selective $\alpha_2$-antagonists makes routine use of these radioligands specifically to label $\alpha_2$-receptors inadvisable.

## B. Selection of Blank for Nonspecific Binding Determinations

When a radioligand binds to the membrane preparation a number of nonreceptor sites are always labeled (nonspecific binding). In order to define specific binding to the α-receptor of interest, parallel incubations with an excess of unlabeled drug (blank) are performed. Selection of the proper drug (and concentration) is of utmost importance. Using a large excess of the unlabeled form of the radioactive compound to define specific binding is generally not acceptable, as this drug will compete with the same (specific and nonspecific) classes of binding site. It is preferable to select a suitable concentration of the neurotransmitter (norepinephrine or epinephrine) which interacts at the receptor of interest. In brain, for example, for [$^3$H]rauwolscine, [$^3$H]yohimbine, and [$^3$H]prazosin, α-receptor specific binding is suitably defined by 100 μ*M* (−)-norepinephrine; for [$^3$H]clonidine and [$^3$H]*p*-aminoclonidine, 10 μ*M* (−)-nor-

epinephrine is optimal. Ascorbic acid (0.01 %) is generally included to help prevent degradation of the catecholamine blank. (For α-adrenergic ligands, this low concentration of ascorbic acid does not appear to alter binding characteristics.) For the [$^3$H]catecholamines, imidazoline blanks are most appropriate.

In many cases, it is necessary to characterize the specific binding of an α-radioligand; for this purpose multiple blanks should be compared, choosing both unlabeled α-selective agonist and antagonist compounds to demonstrate equivalence of α-specific binding. Lack of additivity of inhibition with high concentrations of two of the proposed blanks in the same assay should also be demonstrated. Characterization should proceed to determine the optimal concentration of the blank, such that the blank defines only receptor-specific and not non-receptor-specific (e.g., norepinephrine re-uptake sites) binding.

## C. Tissue Preparation

### *1. Dissection*

Any study of brain α-adrenergic receptors, particularly $\alpha_2$-adrenergic receptors, must be performed with a due appreciation of the brain regional variation in α-adrenergic receptor number and, possibly, functional status. For example, both the total number of $\alpha_2$-receptors and the ratio of $\alpha_2$(H) to $\alpha_2$(L) states of the $\alpha_2$-receptor/effector complex vary widely from region to region in bovine, rat, and human brain, possibly reflecting brain regional differences in both $\alpha_2$-receptor density and $\alpha_2$-receptor/effector coupling (Perry and U'Prichard, 1983c). Furthermore, species differences (and strain differences within species, Perry *et al.*, 1983) in these differences exist. Dissections which are incomplete (e.g., "whole brain" or "brainstem") can dilute potentially useful findings which may pertain only to discrete brain regions. In addition, if an experimental design investigating a specific, neurally mediated function employs radioligand assays in brain, it is wise to select brain regions known to be relevant to the function of interest (e.g., in investigating α-receptors and blood pressure, assay receptors in medulla/pons and hypothalamus). A different approach is that of *in vitro* autoradiography of α-receptors in brain tissue sections (e.g., Young and Kuhar, 1980). Recent developments in computer-enhanced imaging should allow for a high degree of receptor quantitation in specific brain nuclei.

In general, the method of sacrifice for rodents is cervical dislocation; bovine, sheep, and pig brains may be obtained at local slaughterhouses. While human brain tissue is generally not obtained with the same rapid time course (3–4 hr versus minutes) as other mammalian tissues, no major ill effects upon α-adrenergic binding characteristics have been noted. Furthermore, tissues may be flash-frozen (in dry ice–acetone), stored at −70°C, and prepared for assay at a

later date (up to 1 year) with no apparent ill effects on α-radioligand binding. Membranes prepared immediately after sacrifice and then frozen have a longer storage life.

*2. Washing*

The general methods for the preparation of brain membranes for α-adrenergic radioligand assays are quite consistent for all of the ligands. For example, initial homogenization with a Brinkmann Polytron for 30 sec at setting 7 in 100 volumes of 50 m*M* Tris-HCl, pH 7.7, followed by centrifugation at 50,000 × *g*, resuspension in fresh Tris buffer, and recentrifugation (as below). The final pellet is suspended in the buffer of the assay (see Section III,D)

*3. Special Considerations*

A major consideration is eliminating endogenous agonist (which will significantly alter binding properties, particularly [$^3$H]agonist) from the membranes. This can be effected by preincubation of the final membrane preparation at 37°C for 20 min in the absence of MAO inhibitors and antioxidants to degrade endogenous catecholamines effectively.

Stripping membranes of endogenous divalent cations and nucleotides is often necessary for optimal binding, or desirable if nucleotide and metal ion effects on radioligand binding are to be examined. This can be effected by including 5 m*M* $Na_2EDTA$ in the homogenization and washing buffers and expanding the washing procedure to include an additional, final wash in fresh EDTA-free buffer to wash the EDTA from the membranes.

## D. Selection of Assay Buffer and Ionic Medium

As outlined earlier, metal ions and nucleotides alter $\alpha_1$- and $\alpha_2$,radioligand (both agonist and antagonist) binding. Selection of assay buffer and ionic medium will influence results. The majority of α-radioligand assays have employed Tris–HCl buffers (25–50 m*M*) with pH ranges from 6.8–8.0. Potentially, other buffers, such as HEPES, would be equally serviceable. Each α-radioligand has an optimal assay pH range, which will dictate the buffer pH (e.g., [$^3$H]rauwolscine specific binding decreases outside as physiological pH). In addition, $Na^+$-containing buffers (e.g., 50 m*M* Na–$KPO_4$ or physiological buffers) will increase the high-affinity binding of [$^3$H]rauwolscine and [$^3$H]yohimbine, while it will decrease high-affinity [$^3$H]catecholamine and [$^3$H]imidazoline binding (Figs. 4 and 7; Tables II, IV, and V). Glycylglycine buffer also increases the

apparent affinities ($K_D$ values from limited concentration saturation studies) of [$^3$H]yohimbine and [$^3$H]rauwolscine in most tissues.

The addition of metal ions to the assay medium is, on occasion, useful. Optimizing antagonist radioligand binding can be achieved by addition of $Na^+$ ($EC_{50}$ = 25 m*M*), while optimizing agonist or partial agonist radioligand binding may be achieved by addition of $Mg^{2+}$ or $Mn^{2+}$ ($EC_{50}$ = 1–5 m*M*) to the assay medium. A number of laboratories routinely include 10–15 m*M* $Mg^{2+}$ in the assay medium regardless of the α-radioligand employed. This would appear to be a questionable and unnecessary practice.

## E. Incubation Conditions

For all of the major α-adrenergic radioligands, standard incubation conditions have been established. Incubation time should be long enough to reach equilibrium at sub-$K_D$ ligand concentrations (e.g., 120 min for [$^3$H]rauwolscine at 4°C, 30 min for [$^3$H]*p*-aminoclonidine at 25°C) but should not be long enough to allow degradation or oxidation of the radioligand or blank. Incubation time is influenced by radioligand affinity (high-affinity ligands such as [$^{125}$I]BE 2254 take longer) and by temperature, as well as by metal ions and nucleotides. Temperature also affects (possibly differentially) agonist and antagonist interactions with the α-receptor/effector complexes. For example, [$^3$H]$\alpha_2$-agonist apparent affinities increase with decreasing incubation temperatures; in addition, apparent affinities of agonist compounds increase (lower $K_i$ values) while antagonist apparent affinities decrease when competing at [$^3$H]$\alpha_2$-agonist labeled sites as a function of decreasing incubation temperature.

## F. Termination of Assay

The high affinities of the α-adrenergic radiligands allows vacuum filtration separation of free from bound radioligand. In most cases, the termination of binding assays is effected by rapid filtration over glass fiber microfilters (e.g., Whatman GF/B) under reduced pressure. For certain special cases separation may be effected by centrifugation (e.g., determination of the degradation of radioligand). A number of commercially available filtration devices are optimal for binding assays and include the manual 12- or 45-well filtration apparatus (Millipore, Inc.; Earl Sandbeck and Sons, Inc.) and newer, automatic 24-well "tissue-harvesting" vacuum devices (e.g., Brandel, Inc.). The radioactivity trapped by the glass microfilters (*bound*) is quantitated by adding the glass filters to scintillation vials, adding scintillation cocktail, and counting with liquid scintillation spectrometry.

## IV. DATA ANALYSIS AND INTERPRETATION

A number of excellent reviews cover in detail the specific mathematical analysis of radioligand binding data (Yamamura *et al.*, 1978; Weiland and Molinoff, 1981; Molinoff *et al.*, 1981). In general, bound radioligand (from either screening or saturation studies) should be expressed as fmol or pmol/mg of protein. It is far less satisfactory to express data as fmol or pmol/ mg of wet weight tissue. Analysis of radioligand saturation data can proceed a number of ways. For a single class of binding sites (e.g., [$^3$H]prazosin or [$^3$H]rauwolscine over limiting concentrations) (see Figs. 3 and 7) analysis is optimal using weighted linear regression of Eadie-Hofstee plots (Zivin and Waud, 1982). Data suspected to be biphasic should be analyzed, if at all possible, with one of the computer-assisted curve-fitting programs such as LIGAND (Munson and Rodbard, 1980) or SCTFIT (De Lean *et al.*, 1982). BIPHAS (Perry and U'Prichard, 1983b) is a less sophisticated program using nonlinear, least squares curve-fitting methodology based upon the mass-action interactions of ligand and receptor, assuming a two-site model and using general equations described by Feldman (1972). This program will obtain best estimates of $K_D$ and $B_{max}$ values within a preselected range for each component of binding. Heterogeneous competition curves can be analyzed by these computer programs as well by transforming competition data such that bound = % inhibition and free = the concentration of the unlabeled competitor (see Tables IV and V). If computer analysis of biphasic radioligand interactions is not possible, manual and graphical methods are available (Klotz and Hunston, 1971) but are much less ideal. It is unacceptable to draw "best" lines through data points representing the high- and low-affinity components of a curvilinear Scatchard plot to derive $K_D$ and $B_{max}$ values.

Interpretation of radioligand binding data can be difficult unless proper design and methods have been carried out. Demonstration of true changes in α-receptor number following an experimental manipulation (e.g., chronic drug treatment) requires considerable effort (see Section V,A). Additionally, great caution must be exercised when inferring affinity changes following experimental treatments, as these changes can be readily effected by very small percentage changes in the ratio of multiple affinity states, and, as described previously, in almost all cases (even with computer-assisted data analysis) radioligand assays measure apparent affinities which may be mixtures of true radioligand affinities at multiple affinity states. Assuming a change in radioligand $K_D$ value (derived from a limited saturation study) has been demonstrated following an experimental condition, further (not necessarily time-consuming or expensive) analysis using extended agonist and antagonist competition studies, possibly including metal ion and nucleotide effects, could help determine whether or not a change in affinity did, in fact, take place. Many conditions (e.g., chronic agonist exposure) (U'Prichard

*et al.*, 1982; Perry *et al.*, 1983) apparently alter the ratio of $\alpha_2$(H) and $\alpha_2$(L) (which may be physiologically as important as changes in total receptor number) and thereby result in apparent (although not true) changes in α-radioligand affinities and $B_{max}$ values. Overinterpretation and incomplete design remain major problems with, and have resulted in an undesirable measure of disregard for, radioligand binding methodologies.

## V. OTHER CONSIDERATIONS

### A. Measuring α-Adrenergic Receptor Number

A goal of many investigations which employ α-radioligand assays is quantitation of $\alpha_1$- or $\alpha_2$-receptor number. The preceding discussions of the α-receptor/effector complex–radioligand interactions demonstrate how difficult this can be. It must be stressed that the α-radioligand binds to a portion or portions of the physiological, functionally defined α-receptor. It is an assumption, though not an unreasonable one, that the radioligand, optimally employed, will measure total α-receptor number.

Because of the apparent single-site interactions of the $\alpha_1$-antagonist radioligands with the $\alpha_1$-adrenergic receptor, measuring total $\alpha_1$-receptor number is somewhat easier than for $\alpha_2$-receptors. Optimizing [$^3$H]prazosin or [$^{125}$I]BE 2254 binding in a tissue and obtaining a $B_{max}$ value from a reasonable saturation experiment (7–10 concentrations with a wide ligand concentration range, 0.1–10 times the $K_D$ value) is as good an estimate of $\alpha_1$-adrenergic receptor "number" (actually, $\alpha_1$-adrenergic binding sites) as can be obtained with direct radiolabeling techniques. For $\alpha_2$-adrenergic receptors, obtaining this best estimate of $\alpha_2$-receptor number requires more experiments. Extended (0.1–80.0 n*M*) saturation experiments in bovine frontal cortex using [$^3$H]clonidine, [$^3$H]*p*-aminoclonidine, or [$^3$H]rauwolscine all label the same number of sites (Perry and U'Prichard, 1983b). An extended saturation study with either [$^3$H]imidazoline or [$^3$H]rauwolscine or [$^3$H]yohimbine, therefore, may label all of the $\alpha_2$-specific binding sites. These experiments are costly and lengthy, and optimally require computer-assisted analysis. Separate estimates of subpopulations of $\alpha_2$-receptors, that is, $\alpha_2$(H) and $\alpha_2$(L), may be more practical in many cases. These experiments would employ limited saturations (selecting for the high-affinity component of binding) of both an $\alpha_2$-agonist or partial agonist and an $\alpha_2$-antagonist radioligand to give estimates of the $\alpha_2$(H) and $\alpha_2$(L) populations. These experiments also provide an estimate of the $\alpha_2$(H)/$\alpha_2$(L) ratio, which may be of physiological significance.

### B. Radioligand Assays as a Measure of α-Adrenergic Receptor Function

The proteins (receptor/recognition sites) labeled by the radioligand are only a link in the nuerotransmitter–receptor–effector–cellular response chain. In some systems, changes in total α-receptor binding sites can be observed with no effect upon the proximal biochemical effect (inhibition of adenylate cyclase) (U'Prichard *et al.*, 1982), and in others, changes in receptor number (binding sites) and effector functioning are temporally dissociated (Su *et al.*, 1980). In addition, for the α-adrenergic receptors, the multiple links between the proximal biochemical effector and the end physiological result have yet to be closely correlated. Functional changes in operationally defined, physiological α-receptors cannot always be inferred from the demonstration of changes in radioligand binding properties. However, properly designed, carefully executed experiments, particularly those investigating metal ion and nucleotide effects, may yield useful information about agonist and antagonist interactions with, as well as regulation and functioning of, the multiple affinity states of the α-receptor/effector complexes (which are important in the physiological function and functioning of the α-adrenergic receptors).

### C. Physiological Assays

Assays of the effectors (e.g., $Ca^{2+}$ influx, adenylate cyclase) linked to the α-receptors are very useful and, when technically possible, optimal for study of α-receptors. Together with radioligand binding assays, they allow direct examination of the receptor/effector coupling mechanisms and the effects of various experimental conditions (e.g., chronic agonist exposure) on coupling. Unfortunately, technically simple assays of $\alpha_2$-coupled adenylate cyclase activity in brain have yet to be perfected, and the lack of understanding of $\alpha_1$-coupled effector systems in brain has prevented direct studies of α-receptor/effector systems in brain.

## VI. CONCLUSIONS

Direct radioligand binding methods have allowed investigators to localize, characterize, and investigate the regulation of $\alpha_1$- and $\alpha_2$-adrenergic receptors in brain. Understanding of the complexity of the α-receptor/effector–radioligand interactions dictates that caution must be taken to understand truly the receptor subtype (and portions thereof) to which a given radioligand, under a given set of experimental conditions, binds. Metal ions and nucleotides alter the binding

characteristics of all $\alpha_1$- and $\alpha_2$-radioligands by altering the properties of the multiple affinity states of the α-receptor/effector complexes, indicating the necessity of caution when interpreting changes in radioligand $K_D$ and/or $B_{max}$ following an experimental situation. Proper design and use of radioligand methodologies can provide useful information about the molecular functioning of, as well as the physiological and pathophysiological roles played by, α-adrenergic receptors.

## ACKNOWLEDGMENTS

The research illustrated in this chapter was supported in part by USPHS grants NS 15595 and DA 02763. B.D.P. is the recipient of an ADAMHA fellowship award MH 08834. We would like to thank Dr. S. Grill for writing the BIPHAS program and Barbara L. Perry for manuscript preparation.

## REFERENCES

Battaglia, G., and Titeler, M. (1980). *Neurosci. Abstr.* **6,** 852.

Burns, T. W., Langley, P. E., Terry, B. E., Bylund, D. B., Hoffman, B. B., Tharp, M. D. Lefkowitz, R. J., Garcia-Sainz, J. A., and Fain, J. N. (1981). *J. Clin. Invest.* **67,** 467–475.

Bylund, D. B., and Martinez, J. R. (1981). *J. Neurosci.* **1,** 1003–1007.

Bylund, D. B., and U'Prichard, D. C. (1983). *Int. Rev. Neurobiol.* **24,** 343–431.

Cheng, Y., and Prusoff, W. H. (1973). *Biochem. Pharmacol.* **22,** 3099–3108.

Davis, J. N., Arnett, C. D., Hoyler, E., Stalvey, L. P., Daly, J. W., and Skolnick, P. (1978). *Brain Res.* **159,** 125–135.

De Lean, A., Hancock, A. A., and Lefkowitz, R. J. (1982). *Mol. Pharmacol.* **21,** 5–16.

Engel, G., and Hoyer, D. (1981). *Eur. J. Pharmacol.* **73,** 221–224.

Ernsberger, P., and U'Prichard, D. C. (1984). (Submitted for publication.)

Fain, J. N., and Garcia-Sainz, J. A. (1980). *Life Sci.* **26,** 1183–1194.

Feldman, H. A. (1972). *Anal. Biochem.* **48,** 317–338.

Geynet, P., Ferry, N., Borsodi, A., and Hanoune, J. (1981). *Biochem. Pharmacol.* **30,** 1665–1675.

Glossmann, H., and Hornung, R. (1980a). *Naunyn-Schmeidebergs Arch. Pharmacol.* **314,** 101–109.

Glossmann, H., and Hornung, R. (1980b). *Eur. J. Pharmacol.* **61,** 407–408.

Glossmann, H., Lubbecke, F., and Bellemann, P. (1981). *Naunyn-Schmiedebergs Arch. Pharmacol.* **318,** 1–9.

Greenberg, D. A., and Snyder, S. H. (1978). *Mol. Pharmacol.* **14,** 38–49.

Greengrass, P., and Bremner, R. (1979). *Eur. J. Pharmacol.* **55,** 323–326.

Hedler, M., Stamm, G., Weitzell, R., and Starke, K. (1981). *Eur. J. Pharmacol.* **70,** 43–47.

Hildebrandt, J. D., Sekura, R. D., Codina, J., Iyengar, R., Manclark, C. R., and Birnbaumer, L. (1983). *Nature (London)* **302,** 706–709.

Hoffman, B. B., and Lefkowitz, R. J. (1980a). *Annu. Rev. Pharmacol. Toxicol.* **20,** 581–608.

Hoffman, B. B., and Lefkowitz, R. J. (1980b). *N. Engl. J. Med.* **302,** 1390–1396.

Hoffman, B. B., and Lefkowitz, R. J. (1980c). *Biochem. Pharmacol.* **29,** 1537–1541.

Howlett, D. R., Taylor, P., and Walter, D. S. (1982). *Br. J. Pharmacol.* **76,** 294P.

Jakobs, K. H. (1979). *Mol. Cell. Endocrinol.* **16,** 147–156.
Jarrott, B., Louis, W. J., and Summers, R. J. (1982). *Br. J. Pharmacol.* **75,** 401–408.
Kahn, D. J., Mitrius, J. C., and U'Prichard, D. C. (1982). *Mol. Pharmacol.* **21,** 17–26.
Klotz, I. M., and Hunston, D. L. (1971). *Biochemistry* **10,** 3065–3069.
Kobinger, W., and Pichler, L. (1981). *Eur. J. Pharmacol.* **73,** 313–321.
Kobinger, W., and Pichler, L. (1982). *Eur. J. Pharmacol.* **82,** 203–206.
Kunos, G., Kan, W. H., Gregaski, R., and Venter, J. C. (1983). *J. Biol. Chem.* **258,** 326–332.
Langer, S. Z. (1974). *Biochem. Pharmacol.* **23,** 1793–1800.
Limbird, L. E. (1981). *Biochem. J.* **195,** 1–13.
Limbird, L. E., Speck, J. L., and Smith, S. K. (1982). *Mol. Pharmacol.* **21,** 609–617.
McPherson, G. A., and Summers, R. J. (1981). *J. Pharm. Pharmacol.* **33,** 189–191.
Michel, T., Hoffman, B. B., and Lefkowitz, R. J. (1980). *Nature (London)* **288,** 709–711.
Mitrius, J. C., and U'Prichard, D. C. (1984). (Submitted for publication.)
Molinoff, P. B., Wolfe, B. B., and Weiland, G. A. (1981). *Life Sci.* **29,** 427–443.
Munson, P. J., and Rodbard, D. (1980). *Anal. Biochem.* **107,** 220–239.
Peroutka, S. J., and Snyder, S. H. (1981). *J. Pharmacol. Exp. Ther.* **216,** 142–148.
Perry, B. D., and U'Prichard, D. C. (1981). *Eur. J. Pharmacol.* **76,** 462–464.
Perry, B. D., and U'Prichard, D. C. (1984a). *Life Sci.* (submitted for publication).
Perry, B. D., and U'Prichard, D. C. (1984b). *Mol. Pharmacol.* (submitted for publication).
Perry, B. D., and U'Prichard, D. C. (1984c). *Brain Res.* in press.
Perry, B. D., Stolk, J. M., Vantini, G., Guchhait, R. B., and U'Prichard, D. C. (1983). *Science* **221,** 1297–1299.
Rodbell, M. (1980). *Nature (London)* **284,** 17–22.
Rouot, B., and Snyder, S. H. (1979). *Life Sci.* **25,** 769–774.
Rouot, B., U'Prichard, D. C., and Snyder, S. H. (1980). *J. Neurochem.* **34,** 374–384.
Rouot, B., Quennedey, M. C., and Schwartz, J. (1982). *Naunyn-Schmiedebergs Arch. Pharmacol.* **321,** 253–259.
Ruffolo, R. R., Fowble, J. W., Miller, D. D., and Patil, P. N. (1976). *Proc. Natl. Acad. Sci. U.S.A.* **73,** 2730–2734.
Salama, A. I., Lin, L. L., Repp, L. D., and U'Prichard, D. C. (1982). *Life Sci.* **30,** 1305–1311.
Salama, A. I., Perry, B. D., Lin, L., Mitrius, J. C., and U'Prichard, D. C. (1983). *Pharmacologist* **25,** 166.
Schwabe, U., Ohga, Y., and Daly, J. W. (1978). *Naunyn-Schmiedebergs Arch. Pharmacol.* **302,** 141–151.
Smith, S. K., and Limbird, L. E. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 4026–4030.
Starke, K., Endo, T., and Taube, H. D. (1975). *Naunyn-Schmiedebergs Arch. Pharmacol.* **291,** 55–78.
Steer, M. L., Khorana, J., and Galoci, B. (1979). *Mol. Pharmacol.* **16,** 719–728.
Su, Y. F., Harden, T. K., and Perkins, J. P. (1980). *J. Biol. Chem.* **255,** 7410–7419.
Tanaka, T., and Starke, K. (1980). *Eur. J. Pharmacol.* **63,** 191–195.
Timmermans, P. B. M. W. M., and van Zwieten, P. A. (1980). *Eur. J. Pharmacol.* **63,** 199–202.
Tsai, B. S., and Lefkowitz, R. J. (1978). *Mol. Pharmacol.* **14,** 540–548.
U'Prichard, D. C. (1981). *Neurotransm. Recept.* **1,** 131–178.
U'Prichard, D. C., and Snyder, S. H. (1977). *J. Biol. Chem.* **252,** 6450–6453.
U'Prichard, D. C., and Synder, S. H. (1978). *J. Biol. Chem.* **253,** 3444–3452.
U'Prichard, D. C., Greenberg, D. A., and Snyder, S. H. (1977a). *Mol. Pharmacol.* **13,** 454–473.
U'Prichard, D. C., Greenberg, D. A., Sheehan, P., and Snyder, S. H. (1977b). *Brain Res.* **138,** 151–158.
U'Prichard, D. C., Bechtel, W. D., Rouot, B., and Snyder, S. H. (1979). *Mol. Pharmacol.* **16,** 47–60.

U'Prichard, D. C., Reisine, T. D., Mason, S. T., Fibiger, H. T., and Yamamura, H. I. (1980). *Brain Res.* **187,** 143–154.

U'Prichard, D. C., Braas, K. M., and Kahn, D. J. (1982). *Psychopharmacol. Bull.* **18,** 180–183.

U'Prichard, D. C., Perry, B. D., Wang, C. H., Mitrius, J. C., and Kahn, D. J. (1983). Molecular aspects of regulation of $\alpha_2$-adrenergic receptors. *In* "Frontiers in Neuropsychiatric Research" (E. Usdin *et al.*, eds.), pp. 65–82. Macmillan, New York.

Weiland, G. A., and Molinoff, P. B. (1981). *Life Sci.* **29,** 313–330.

Williams, L. T., and Lefkowitz, R. J. (1976). *Science* **192,** 791–793.

Williams, L. T., and Lefkowitz, R. J. (1978). "Receptor Binding Studies in Adrenergic Pharmacology." Raven Press, New York.

Yamada, S., Yamamura, H. I., and Roeske, W. R. (1980). *Eur. J. Pharmacol.* **63,** 239–241.

Yamamura, H. I., Enna, S. J., and Kuhar, M. J., eds. (1978). "Neurotransmitter Receptor Binding." Raven Press, New York.

Young, W. S., and Kuhar, M. J. (1980). *Proc. Natl. Acad. Sci. U.S.A.* **77,** 1696–1700.

Zivin, J. A., and Waud, D. R. (1982). *Life Sci.* **30,** 1407–1422.

# *Chapter 16*

# *DOPAMINE RECEPTORS IN BRAIN*

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## I. CLASSIFICATION OF DOPAMINE RECEPTORS

The rank order of potencies for dopaminergic agonists *in vitro* is

Bromocryptine > Apomorphine = ADTN* > Dopamine > Noradrenaline

*ADTN is 6,7-dihydroxy-2-aminotetralin.




ISBN 0-12-470350-X

TABLE I

Definitions of Dopaminergic Sites and States[a]

| | Central nervous system | | | Peripheral tissues | |
|---|---|---|---|---|---|
| | $D_1$ | $D_2^{Low} \rightleftharpoons D_2^{High}$ | $D_3$ | $DA_1$ | $DA_2 = D_2^{High}$ |
| Dopamine $C_{50}$ | $\mu M$ | $\mu M \rightleftharpoons nM$ | $nM$ | $\mu M$ | $nM$ |
| Spiperone $C_{50}$ | $\mu M$ | $pM$ | $\mu M$ | $nM$ | $nM$ |
| Sulpiride-sensitive? | No | (*S*)-Sulpiride | No | (*R*)-Sulpiride | (*S*)-Sulpiride |

[a]See Fig. 1 for an explanation of symbols.

Hence, an essential criterion for defining a site as dopaminergic is that the site should be generally sensitive to dopaminergic congeners with the above rank order of potencies.

Subclassification of the dopaminergic sites (and/or states) depends on the absolute molarities of agonists and antagonists to which the sites are sensitive.

The nomenclature used in this laboratory is based primarily on the absolute sensitivities of a site to three drugs: dopamine, spiperone, and sulpiride. This is summarized in Table I and Fig. 1.

This recommended nomenclature (in Table I) differs from an earlier classification proposed by Spano and by Kebabian and Calne (1979), wherein the D-1 receptor was defined as that linked to adenylate cyclase and the D-2 receptor as that not linked to this enzyme. A classification based solely on linkage to adenylate cyclase, however, is not convenient since there is sometimes disagreement as to whether certain tissues contain dopamine-stimulated adenylate cyclase (e.g., anterior pituitary) (Schmidt and Hill, 1977; Kebabian, 1978; Ahn *et al.*, 1979).

A comparison of nomenclatures is given in Table II.

TABLE II

Comparison of Nomenclatures for Dopaminergic Sites and/or States

| | Central nervous system | | | | Peripheral tissues | |
|---|---|---|---|---|---|---|
| Creese (1981) | $D_1$ | $D_2^{Low}$ | $D_2^{High}$ | $D_3$ | | $D_2^{High}$ |
| Seeman (1980) | $D_1$ | $D_2$ | $D_4$ | $D_3$ | | $D_4$ |
| Kebabian and Calne (1979) | $D_1$ | $D_2$ | $D_2$ | | | $D_2$ |
| Sokoloff *et al.* (1980) | $D_1$ | $D_4$ | $D_2$ | $D_3$ | | |
| Labrie; Ferland *et al.* (1982) | $D_+$ | $D_0$ | $D_-$ | | | |
| Goldberg and Kohli (1979) | | | | | $DA_1$ | $DA_2$ |

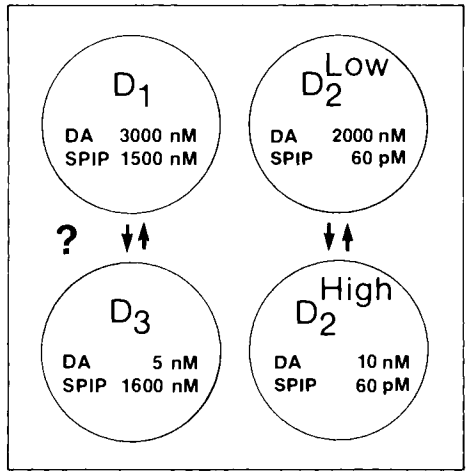


**Fig. 1.** The $D_1$ dopaminergic site is defined as dopamine-stimulated adenylate cyclase. This site is 50% stimulated by micromolar concentrations ($C_{50}$ values) of dopamine and inhibited by micromolar concentrations of neuroleptics such as spiperone; the $D_1$ is not sensitive to sulpiride.

The $D_2$ dopamine receptor is the only dopaminergic site where the drug potencies correlate with their *in vivo* potencies. The $D_2$ receptor has two states (for agonists), both of which have a 60 p*M* dissociation constant ($K_D$) for spiperone. The $D_2^{low}$ state has a low affinity for dopamine, being sensitive to micromolar concentrations of dopamine. The $D_2^{high}$ state has a high affinity for dopamine, since it is sensitive to 10 n*M* dopamine ($C_{50}$ value).

The $D_3$ site has no biological correlate but is a site that is sensitive to nanomolar concentrations of dopamine and micromolar concentrations of spiperone. Since the $D_1$ and $D_3$ sites are both 50% affected by micromolar concentrations of neuroleptics, it is possible that they may be related and even different states of each other. There is no evidence yet supporting this interconversion concept.

The $D_2^{high}$ state can be converted into the $D_2^{low}$ state in the presence of sodium and a guanine nucleotide (George *et al.*, 1983a,b; Grigoriadis and Seeman, 1983; Watanabe *et al.*, 1983). In the past, we had sometimes used the term $D_4$ to indicate the $D_2^{high}$ site, since it was possible that any nonconverting $D_2^{high}$ sites may be biochemically different (Wreggett and Seeman, 1983). Present evidence indicates that all or virtually all the $D_2^{high}$ sites can be converted to $D_2^{low}$ (Grigoriadis and Seeman, 1983; George *et al.*, 1983a,b).

## A. The $D_1$ Site: Dopamine-Stimulated Adenylate Cyclase

$D_1$ signifies dopamine-stimulated adenylate cyclase. It has been generally observed that the $D_1$ site is sensitive to micromolar concentrations of dopamine and to micromolar concentrations of spiperone but is insensitive to the substituted benzamides [(±)-sulpiride or metoclopramide]. These properties thus define the $D_1$ site for any tissue response or for the binding of any [$^3$H]ligand to $D_1$, as summarized in Table III.

TABLE III

Drug Potencies at $D_1$ Sites (Rat Striatum)

| | $C_{50}$ stimulation of adenylate cyclase (n*M*) | $IC_{50}$ (conc. for 50% inhibition of [$^3$H]flupentixol binding) (n*M*) |
|---|---|---|
| Agonists | | |
| Bromocryptine | 500 | 480 |
| Apomorphine | 1,800 | 270 |
| (±)-ADTN | 3,500 | 610 |
| Dopamine | 3,000 | 3,400 |
| Noradrenaline | 40,000 | 62,000 |
| Antagonists | | |
| Spiperone | 1,500 | 1,300 |
| (±)-Sulpiride | 56,000 | 32,000 |
| References | in Seeman (1980) | Hyttel (1980)<br>Cross and Owen (1980) |

There is agreement that (*S*)-sulpiride has no effect on the $D_1$ site (Roufogalis *et al.*, 1976; Rupniak *et al.*, 1981). Nevertheless, the specific binding of [$^3$H]sulpiride is markedly sensitive to guanylate nucleotides (Freedman *et al.*, 1981).

## B. The $DA_1$ Site for Arterial Relaxation

Arteries contain dopamine receptors, termed *$DA_1$ sites* by Goldberg and Kohli (1979), which mediate relaxation of the vessel. As already noted in Table I, these $DA_1$ sites are sensitive to the same drug concentrations as the $D_1$ sites, except that the $DA_1$ sites are sensitive to (*R*)-sulpiride, whereas the $D_1$ sites are not sensitive to either (*S*)- or (*R*)-sulpiride.

In general, it has been found that the concentrations of dopamine and butyrophenone (haloperidol or droperidol) are most effective at micromolar concentrations. Apomorphine is equipotent to dopamine for some arteries (rabbit spleen or ear) but surprisingly less potent than dopamine in other arteries (rabbit renal and mesenteric; dog renal).

## C. The $D_2$ Receptor

The $D_2$ receptor is characterized by its micromolar affinity for dopamine and its nanomolar or picomolar affinity for spiperone (see Table I). This site has been termed as $D_2^{Low}$ by Creese and his colleagues (Creese, 1981; Sibley *et al.*, 1981),

TABLE IV

Drug Potencies (n*M*) at the $D_2$ Receptor
(Rat Striatum)

| | $IC_{50}$ [$^3$H]Spiperone |
|---|---|
| Agonists | |
| Bromocryptine | 38 |
| Apomorphine | 900 |
| (±)-ADTN | 1,500 |
| Dopamine | 19,000 |
| Noradrenaline | 200,000 |
| Antagonists | |
| Spiperone | 0.06 |
| (−)-Sulpiride | 122 |
| (±)-Sulpiride | 250 |
| (+)-Sulpiride | 3,000 |
| References | Seeman (1980)<br>List and Seeman (1981a,b) |

as $D_4$ by Schwartz's group (Sokoloff *et al.*, 1980), and as $D_0$ by Labrie's group (Ferland *et al.*, 1982) (see Table II).

The $D_2$ site is the only dopaminergic site labeled by a [$^3$H]ligand that warrants being called a receptor. This is because the $IC_{50}$ values of agonists and antagonists at this site correlate very well with the doses that elicit various dopaminergic behaviors (rotation, locomotion, anti-Parkinson action, psychotomimetic action, emesis, and stereotypy) (Seeman, 1980). A summary of these $IC_{50}$ values for the main dopaminergic congeners is given in Table IV.

As shown in Table IV, the rank order of dopaminergic agonists is basically the same as for the $D_1$ site (compare Tables III and IV). The $D_2$ receptors, however, are several orders of concentration more sensitive than the $D_1$ sites to the neuroleptic drugs. For example, the $IC_{50}$ value of spiperone on the $D_1$ site is about 1400 n*M*, while that on the $D_2$ receptor is 60 p*M*.

It is known that the $D_2$ dopamine receptors may also interfere with or inhibit the formation of cyclic AMP. This interfering action has been clearly demonstrated in the intermediate lobe of the pituitary (Meunier *et al.*, 1980; Munemura *et al.*, 1980; Ferland *et al.*, 1982), as well as in the anterior lobe of the pituitary (Giannattasio *et al.*, 1981; De Camilli *et al.*, 1979) (see Fig. 2). In the nervous system, however, it has not been possible to detect dopamine-inhibited adenylate cyclase directly. Stoof and Kebabian (1981) have found indirect evidence for an interfering action of dopamine on adenylate cyclase in striatal slices. It has also been reported that dopamine inhibits guanylate cyclase from human and bovine caudate nucleus (Frey *et al.*, 1979).

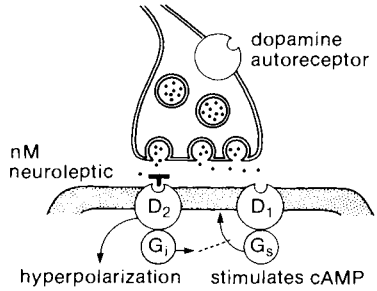


**Fig. 2.** The $D_2$ dopamine receptor interferes with the effect of $D_1$ stimulation by dopamine. Both the $D_1$ site and the $D_2$ dopamine receptor are postjunctional. The dopamine autoreceptor appears to have properties identical to the $D_2^{high}$ state of the $D_2$ receptor.

As indicated in Table I and illustrated in Table V, the $D_2^{high}$ site may be defined as one that is sensitive to nanomolar concentrations of dopamine (i.e., 10–35 n*M*) and picomolar concentrations of butyrophenones (60 p*M* spiperone). Sibley *et al.* (1981), however, have described a site in the pituitary with a dissociation constant of 200 n*M* for dopamine, using either [$^3$H]spiperone or *N*-[$^3$H]propylnorapomorphine.

It is well established that guanine nucleotides can reduce the $D_2$ receptor's affinity for dopamine (Zahniser and Molinoff *et al.*, 1978; Creese *et al.*, 1979; Sibley and Creese, 1979; Makman *et al.*, 1980), although occasionally the effect has not been detected (Andorn *et al.*, 1979). Thus, a scheme for the various dopaminergic sites is that given in Fig. 1.

It is known that lesions of the nigral dopamine neurons result (after 1 to 3 weeks) in elevated numbers of $D_2$ dopamine receptors in the striatum, indicating that the majority (if not all) of these $D_2$ receptors are postsynaptic to the dopamine terminals (see references in Seeman, 1980). $D_2^{high}$ sites are also located on the presynaptic terminals of the dopamine neurons. This conclusion comes from work on striatal slices wherein n*M* concentrations of apomorphine (generally 10–100 n*M*) inhibited the release of [$^3$H]dopamine, while nanomolar concentrations of butyrophenones (5–50 n*M* generally) enhanced the release (Table V) (Kelly, 1981; Perkins and Westfall, 1976; Miller and Friedhoff, 1979; Starke *et al.*, 1978; Kamal *et al.*, 1981; Jackisch *et al.*, 1980; Stoof *et al.*, 1980; Reimann *et al.*, 1979; Arbilla and Langer, 1981; Arbilla *et al.*, 1978).

There is one study that reported that there was a 20% fall in the striatal binding of [$^3$H]haloperidol 4 days after lesioning the nigral dopamine neurons (Mishra *et al.*, 1980). This reduction indicates that some of the $D_2$ binding sites for [$^3$H]haloperidol may be presynaptic in location. This interpretation of such lesion studies has shortcomings, since transynaptic degeneration occurs as soon as 3 days after a lesion (Hattori *et al.*, 1982).

The $D_2^{high}$ sites may be the functional state of $D_2$ that is associated with

TABLE V

$IC_{50}$ (n*M*) Values at $D_2^{high}$ Sites

| | Rat pit. cells prolactin release | Bovine ant. pit. [$^3$H]APO | Rat interm pit. DA-inhib. cyclase | Rat striat. [$^3$H]APO | Rat striat. [$^3$H]APO | Rat striat. [$^3$H]dopamine efflux |
|---|---|---|---|---|---|---|
| Bromocryptine | 2.9 | | | 13 | ~8 | |
| Apomorphine | 3 | 2 | 9 | 1.3 | 4 | 9 |
| (±)-ADTN | ~2 | | | 2.1 | 4 | |
| Dopamine | 35 | 32 | 20 | 19 | 10 | |
| Noradrenaline | 540 | 500 | 200 | | | |
| Spiperone | <0.7 | 0.7 | <1 | 0.37 | 0.7 | 15(H)[b] |
| (±)-Sulpiride | *S*[a] | 70 | | 182 | 25 | |
| References | Caron *et al.* (1978); Rick *et al.* (1979) | Ferland *et al.* (1982) | Munemura *et al.* (1980) | Sokoloff *et al.* (1980) | List *et al.* (1982) | Kelly (1981) |

[a] *S* indicates that *S*(−)-sulpiride base (Ravizza) was more potent than *R*(+)-sulpiride base (Müller *et al.*, 1979; Hofmann *et al.*, 1979).
[b] H indicates haloperidol (not spiperone) tested.

dopamine-inhibited adenylate cyclase, since the $IC_{50}$ values for both sites (in the pituitary) (see Table V) are virtually identical.

## D. Similarity between Central $D_2^{high}$ Sites and Peripheral $DA_2$ sites

Certain adrenergic nerve terminals in the peripheral nervous system contain dopamine receptors that inhibit the release of noradrenaline. These dopamine receptors, termed *$DA_2$ receptors* (Goldberg and Kohli, 1979), have sensitivities to dopamine agonists and antagonists that are virtually identical to those for the $D_2^{high}$ dopaminergic sites in the central nervous system, as summarized in Table VI. This similarity suggests that the central $D_2^{high}$ sites and the peripheral $DA_2$ sites may be identical.

## E. The $D_3$ Site

The $D_3$ site is defined as the site that has nanomolar affinity for dopamine but micromolar affinity for neuroleptics (Table I). This site has been routinely de-

TABE VI

*n*M Potencies[a] at Peripheral $DA_2$ Sites (= Central $D_2^{high}$ Sites)

| Nerve | Rabbit ear | Cat spleen | Other |
|---|---|---|---|
| Agonists | | | |
| Apomorphine | 44 | 80 | |
| (±)-ADTN | 1.2 | | |
| Dopamine | 37 | 50 | 30–200 |
| Antagonists | | | |
| Spiperone | 0.2 | | |
| (S)[b]-Sulpiride | 10[c] | <1,000 *S*[b] | *S* |
| References | Steinsland and Hieble (1978); Steinsland *et al.* (1979); Hope *et al.* (1978) | Dobocovich and Langer (1980) | Massingham *et al.* (1980); Tayo (1977); Fuder and Muscholl (1978); Enero and Langer (1975) |

[a]Concentrations of dopamine agonists that 50 percent inhibited the release of neurotransmitter from nerve terminals; usually done in the presence of cocaine (3–30 μ*M*) to preclude re-uptake into nerve terminals.

[b]*S* indicates that *S*-sulpiride was more potent than *R*-sulpiride. It should be noted that the rotation of light (+ or −) is opposite for sulpiride base and sulpiride HCl for each enantiomer.

[c]Brown and O'Connor (1980).

TABLE VII

$IC_{50}$ Values (n*M*) at the $D_3$ Site (Rat Striatum)

| | [$^3$H]Dopamine | [$^3$H]ADTN |
|---|---|---|
| Agonists | | |
| Bromocryptine | 150 | 317 |
| Apomorphine | 2.6 | 2 |
| (±)-ADTN | 1.5 | 2 |
| Dopamine | 6 | 5 |
| Noradrenaline | 22 | |
| Antagonists | | |
| Spiperone | 1,000 | 5,000 |
| (±)-Sulpiride | 43,000 | 9,000 |
| References | List and Seeman (1982) | List *et al.* (1982) |

tected in this lab since 1974 (Seeman, 1974, 1980; Seeman *et al.*, 1974a, 1975, 1976; List *et al.*, 1980, 1982; List and Seeman, 1982; Titeler *et al.*, 1979). This $D_3$ site has also been identified by Sokoloff *et al.* (1980) and by Leff *et al.* (1981).

Although the biological role of the $D_3$ site is not established, the criteria for its nomenclature have been clear and consistent, such that there has generally been little confusion on its definition. Thus, most workers accept a definition of the $D_3$ site as any site that has a nanomolar affinity for dopamine and a micromolar affinity for neuroleptics. Maeno's group, however, have referred to such a site as a "$D_2$ site," on the basis that any dopaminergic site not associated with adenylate cyclase could be referred to as a "$D_2$ site" (Sano *et al.*, 1979).

The rank order of potencies of the dopaminergic congeners at the $D_3$ site generally follows that for the $D_1$ and $D_2$ sites, with one important exception: Bromocryptine is particularly weak at the $D_3$ site, as summarized in Table VII.

## II. METHOD FOR MEASURING THE $D_1$ SITE WITH [$^3$H]THIOXANTHENES

Hyttel has developed a method for labeling the $D_1$ site with [$^3$H]thioxanthenes, using either (*Z*)-*cis*-[$^3$H]flupenthixol (Hyttel, 1978a,b, 1979, 1980; see also Cross and Owen, 1980) or more recently [$^3$H]pitflutixol (Hyttel, 1981a,b; Moller-Nielsen *et al.*, 1977). In order for the [$^3$H]thioxanthene to label the $D_1$ site preferentially, it is best to use 30 n*M* spiperone to occlude the $D_2$ receptors [Hyttel, 1981b; see also List and Seeman (1981b) and List *et al.* (1982)

who used 30 n*M* spiperone to occlude sites with nanomolar affinity for neuroleptics].

### A. Tissue Preparation for Measurement of $D_1$ Sites

Rat striatum was homogenized in 100 vol (w/v) of buffer, using an Ultra Turrax (10 sec) or a Brinkmann Polytron (setting of 6 or 7 for 10 sec). The buffer was ice-cold 50 m*M* Tris–HCl buffer (pH 7.7 at 25°C). This author prefers using a glass–Teflon homogenizer (10 up-and-down storkes; piston rotating at 500 rpm with the piston inserted in a machine drill) for any of the homogenizations before the final assay. The Turrax and Polytron homogenizers tend to produce small membrane fragments, some of which may not centrifuge and would thus be lost during the subsequent washing step; the glass-Teflon homogenizer does not yield such small membrane fragments.

The homogenate was centrifuged at 40,000 *g* (10 min at 4°C), resuspended, and rehomogenized. The final pellet was homogenized in 400 vol (w/v) (125 vol for [$^3$H]flupenthixol) ice-cold freshly prepared 50 m*M* Tris–HCl buffer containing 0.1% ascorbic acid, 10 μ*M* pargyline, 120 m*M* NaCl, 5 m*M* KCl, 2 m*M* $CaCl_2$ and 1 m*M* $MgCl_2$, pH 7.1 (at 37°C). The tissue suspension was preincubated at 37°C for 10 min before storing on ice. Such a preincubation step tends to reduce monoamine oxidase, endogenous dopamine, and endogenous nucleotides.

### B. Binding of [$^3$H]Thioxanthene to $D_1$ Sites

(*Z*)-*cis*-[5-$^3$H]Flupentixol dihydrochloride (5.9 Ci/mmole) and (*Z*)-*cis*-[4-$^3$H]piflutixol hydrochloride (7.7 Ci/mmole) may be obtained from the Nuclear Research Center, Negev, Israel. Although Hyttel dissolved the isotopes in 0.1% ascorbic acid, we have usually used ethanol, since ascorbic acid can injure receptors in general and ascorbic acid should be buffered to neutral pH. It is also known that 10 μ*M* ascorbic acid almost completely inhibits dopamine-stimulated adenylate cyclase in rat striatum (Thomas and Zemp, 1977).

The binding of either (*Z*)-*cis*-[$^3$H]flupentixol or [$^3$H]piflutixol has been done by Hyttel as follows: Each incubation tube (on ice, and in triplicate) received 100 μl of buffer or drug at varying concentrations, 100 μl of either [$^3$H]flupentixol or [$^3$H]piflutixol, and 800 μl of ice-cold tissue homogenate. For competition-type experiments, the final concentration of [$^3$H]flupentixol was 2 n*M* while that for [$^3$H]piflutixol was 0.5 n*M*. For saturation-type experiments (i.e., for Scatchard analysis of the density of receptors), the range of final concentrations for [$^3$H]piflutixol was 0.15–2.8 n*M*, while that for [$^3$H]flupentixol was 2–18 n*M*. Hyttel recommends that 30 n*M* spiperone be present in all the incubation tubes in order to preclude [$^3$H]ligand binding to $D_2$ receptors (Hyttel, 1981a).

The tubes were incubated at 37°C for 30 min (10 min for [$^3$H]flupentixol) and rapidly filtered under vacuum through Whatman GF/B (glass fiber, B porosity) filters (25 mm diameter). The tubes were rinsed with 5 ml, and then each filter was rinsed with two 5 ml aliquots of 50 m*M* Tris buffer (pH 7.7 at 25°C. Radioactivity on the filters was measured by liquid scintillation spectrometry after adding Picofluor $TM_{15}$ (Packard Co., Chicago, Illinois).

Specific binding of [$^3$H]thioxanthene was defined as that which was inhibited by the presence of 1 μ*M* (+)-butaclamol.

## III. METHODS FOR MEASURING $D_2$ RECEPTORS WITH [$^3$H]LIGANDS

### A. [$^3$H]Ligand of Choice Should Have High Selectivity for $D_2$ and Low Nonspecific Binding

Since sulpiride has a very low affinity for $D_1$ sites, $D_3$ sites, and serotonin receptors, it appears that (±)-[$^3$H]sulpiride is the most selective [$^3$H]ligand for $D_2$ receptors (Theodorou *et al.*, 1979, 1980). A major technical disadvantage of (±)-[$^3$H]sulpiride, however, is that it is very fat-soluble, thus resulting in specific binding that is a low percentage of the total binding. A further drawback is that this [$^3$H]ligand has only been made in the racemic form. Since it is known that (*S*)-sulpiride is far more potent than (*R*)-sulpiride on $D_2$ receptors, the synthesis of (*S*)-[$^3$H]sulpiride would enhance the specific binding by eliminating the nonspecific binding of (*R*)-[$^3$H]sulpiride.

[$^3$H]Domperidone also has high selectivity for $D_2$ receptors (Martres *et al.*, 1978; Baudry *et al.*, 1979; Sokoloff *et al.*, 1980). In this laboratory, however, we have found that [$^3$H]domperidone is very surface-active and has a very high amount of nonspecific binding (List and Seeman, unpublished).

[$^3$H]Haloperidol has a very high selectivity for $D_2$ receptors (Seeman *et al.*, 1975). To obtain consistent and reproducible results with [$^3$H]haloperidol, however, it generally requires high concentrations (2–5 n*M*) of this [$^3$H]ligand.

Of all [$^3$H]ligands for $D_2$ receptors, [$^3$H]spiperone gives the most consistently reproducible results. This [$^3$H]ligand labels not only $D_2$ receptors (Leysen *et al.*, 1978a; Fields *et al.*, 1977), however, but also serotonin receptors (Leysen *et al.*, 1978b, 1979; see also List and Seeman, 1981b).

Despite the fact that [$^3$H]spiperone also binds to serotonergic sites, [$^3$H]spiperone continues to be commonly used to label $D_2$ receptors. The advantageous features of [$^3$H]spiperone are that it does not wash off readily, thus enhancing reproducibility, and that it can be used with very small quantities of tissue because of its extremely high affinity for $D_2$ receptors ($K_D$ = 60 p*M*).

## B. Preparation of Tissue Homogenates for $D_2$ Measurements; Effects of Washing

The dissected brain regions are weighed, and 10 ml of buffer medium (see next section) are added for each gram of original wet tissue. This suspension is homogenized in a glass homogenizer with a Teflon piston rotating at 800 rpm, using 8 to 10 up-and-down strokes.

Hitherto, all studies of $D_2$ receptors have measured the density of these receptors on homogenates that had been washed by repeated centrifugation and resuspension in fresh medium. Recently, however, we have found that such washing consistently results in a loss of about 20% of all the original $D_2$ receptors in the original wet tissue (Seeman *et al.*, 1984). For example, in those experiments where the homogenates were washed, they were centrifuged (44,000 *g*) and resuspended with 10 vol (per gram of original wet tissue) three times. In general, the density (in fmoles/mg final protein) would be enhanced by about 10%. However, since the homgenate generally lost about 30% of its protein during the wash, this resulted in a net loss of about 20% of the total number of receptors per gram of original wet tissue.

The lost receptors could have been located in the cytosol or may have been inadequately centrifuged by the spin of 40,000 *g* (for 15 min). In either case, it was clear that receptors had been discarded during the wash procedure. Owen *et al.* (1979) found that the $B_{max}$ values after one wash and after three washes were the same; those workers had not measured, however, the $B_{max}$ value for the nonwashed tissue. Thus, since different workers wash the tissues in slightly different ways, it is difficult to compare the data from different laboratories as to the absolute density of receptors per gram of original wet tissue (Seeman, 1981). It is here recommended that nonwashed but highly diluted tissues be used (especially for studies on human brain diseases).

It is known that the supernatant of a brain homogenate contains an endogenous protein that nonselectively inhibits the binding of [$^3$H]spiperone to dopamine receptors (Leysen *et al.*, 1978c). This endogenous factor, when diluted about 50-fold, inhibits [$^3$H]spiperone binding by about 50%, and, when diluted by about 500-fold, results in only approximately 15% inhibition (Leysen *et al.*, 1978c). In the present method (see following sections) the final dilution of the brain tissue is 1400-fold. This high dilution, therefore, would be expected to be sufficient to preclude any interference or inhibition of [$^3$H]spiperone binding by the endogenous factor.

Thus, the current procedure in this laboratory is not to wash the homogenate, but simply to store it frozen (−20 or −70°C) at the concentration of 1 g (original weight) per 11 ml final volume (2 ml per freezing vial). Two washes are here only done on human postmortem brain tissues from schizophrenics, since virtually all of such patients had been medicated.

### C. Buffer for $D_2$ Measurement with [$^3$H]Spiperone

We now use a Tris–ion medium of the following composition: 50 m*M* Tris–HCl (pH 7.4 at 21°C), 120 m*M* NaCl, 5 m*M* KCl, 1 m*M* $MgCl_2$, and 2 m*M* $CaCl_2$. This medium differs from that used previously by either Creese *et al.* (1977) or ourselves (Seeman, 1980). Since we now define the specific binding of [$^3$H]spiperone as that inhibited by 10 μ*M* (−)-sulpiride (List and Seeman, 1981b), the high concentration of 120 m*M* NaCl in the medium is chosen to ensure the full action of (−)-sulpiride at the $D_2$ dopamine receptor (Stefanini *et al.*, 1980, 1981; Theodorou *et al.*, 1980). It is also known that isotonic NaCl is essential for the biological effects of dopamine on prolactin secretion from pituicytes (Marchisio *et al.*, 1981).

Although the medium previously used in this laboratory had also contained 5 m*M* $Na_2EDTA$, 0.02% ascorbic acid, and 12 μ*M* nialamide (Seeman *et al.*, 1975; Seeman, 1980), we found that these three ingredients had no effect on the density of [$^3$H]spiperone binding sites (by Scatchard analysis). Additional considerations on the effects of ascorbic acid are in the next section (Section III,D) and in Section VI.

### D. Ascorbate Injury and EDTA Protection of $D_2$ Receptors

Ascorbic acid and other antoxidants injure many types of receptors, including dopamine receptors (Leslie *et al.*, 1980). Hence, ascorbic acid should not be ordinarily added when measuring the binding of [$^3$H]butyrophenones. Ascorbic acid should be added only when oxidizable drugs are used (e.g., dopamine, apomorphine, catecholamines). EDTA and/or ions should be present when the ascorbic acid is present, since the inhibitory effect of ascorbic acid on the specific binding of [$^3$H]spiperone or [$^3$H]haloperidol is prevented by either EDTA or ions (Usdin *et al.*, 1980; Coughenour, 1981; Leslie *et al.*, 1980; Chan *et al.*, 1982). In other words, ascorbic acid may be omitted when examining the $K_D$ and $B_{max}$ (density) and other properties of [$^3$H-butyrophenone binding. The ascorbic acid need be added only for competition experiments between dopamine agonists and the [$^3$H]butyrophenone.

It is interesting to note that ascorbate *in vivo* at 2 g/kg can reduce amphetamine stereotype (Tolbert *et al.*, 1979), but at 1 g/kg ascorbate may (Zemp *et al.*, 1977; Heikkila *et al.*, 1981) or may not (Wilcox *et al.*, 1980) reduce dopaminomimetic-induced behavior.

### E. Incubation Conditions for [$^3$H]Spiperone Binding

In order to measure the binding of [$^3$H]spiperone to the hemogenate, the frozen samples were thawed, diluted to an appropriate concentration of protein

(see next section), and then Polytron-homogenized (Brinkmann PT-10; 20 sec; setting at 7, full power being 10).

The true values for the $K_D$ and the density of receptors ($B_{max}$) are only obtained when the final concentration of tissue protein is kept as low as possible, below 100 μg protein per final milliliter of incubate. This low protein concentration necessitates using larger incubation volumes, ranging from 0.6 to 20 ml, in order to obtain a sufficient number of [$^3$H]spiperone disintegrations per minute (dpm) on each filter.

Thus, the most suitable final incubation volume is 1.8 ml. The [$^3$H]spiperone binding assays are done in glass test tubes (12 × 75 mm) in triplicate, each of which receives the following aliquots in the order listed and delivered using Brinkmann Eppendorf polypropylene pipette tips (disposable): 0.6 ml buffer medium (with or without competing nonradioactive drug), 0.6 ml of [$^3$H]spiperone (31–34 Ci/mmole; New England Nuclear Corp., Boston, Massachusetts), and 0.6 ml of the Polytron-homogenized tissue suspension. The range of final concentrations for [$^3$H]spiperone is from 20 p*M* to 800 p*M*. The final concentration of protein should be less than 100 μg per ml of final incubate in accordance with the considerations given in the next section (Section III,F).

It is essential to ensure that the tubes are incubated for a sufficient length of time such that equilibrium is achieved. Dilute concentrations of tissue (below 100 μg protein per milliliter final) generally require longer periods to equilibrate. After incubating the tubes for 1.5 hr at room temperature (20–21°C), the contents are filtered (12 tubes simultaneously) by means of a Titertek Cell Harvester (Skatron AS Instruments, Lier, Norway; using a glass fiber filter mat and a vacuum of 400 to 500 mm Hg. The filter mat is then rinsed with a 15 sec rinse (10 ml) of 50 m*M* Tris–HCl (pH 7.4 at 20°C). The damp filters are removed from the mat and placed in liquid scintillation minivials to which 4 ml of scintillation fluid (Aquasol, New England Nuclear Corp., or ACS, Amersham, Arlington Heights, Illinois) is added. The vials should be rocked or shaken overnight to extract an additional 5% of the disintegrations per minute from the filters (which become translucent after 6 hr). The vials are then monitored by scintillation spectrometry at 30 to 47% efficiency, preferably in a spectrometer that has refrigeration (4°C).

The specific binding of [$^3$H]spiperone is best defined as that which is inhibited by the presence of 10 μ*M* (−)-sulpiride base (Ravizza, Milan, Italy) or 100 n*M* (+)-butaclamol (Ayerst Research Laboratories, Montreal, Canada).

The specific binding of [$^3$H]spiperone is about 15%–20% greater when defined by (+)-butaclamol, presumably because (+)-butaclamol inhibits both dopamine and serotonin receptors, the latter accounting for about 20% of the [$^3$H[spiperone receptor sites in the striatum (List and Seeman, 1981b). Thus, the specific binding of [$^3$H]spiperone to $D_2$ dopamine receptors is best defined by $10^{-5}$*M* (−)-sulpiride, this drug having little action on serotonin receptors (List and Seeman, 1981b).

## F. The Artifact of [$^3$H]Ligand Depletion and the Effect of Tissue Concentration of [$^3$H]Spiperone Binding Parameters

It is recommended that the $K_D$ and $B_{max}$ values for [$^3$H]spiperone be determined at low protein concentrations, ideally less than 100 μg protein per milliliter of final incubate. This recommendation is based on the following considerations.

It is known that the apparent $K_D$ value depends on the membrane receptor concentration for [$^3$H]ligands with either high fat solubility (e.g., [$^3$H]quinuclidinyl benzilate; [$^{125}$I]hydroxybenzylpindolol) (Fields *et al.*, 1978; Ludford and Talamo, 1980; Brown *et al.*, 1976) or high affinity for the receptor ([$^{125}$]insulin) (Chang *et al.*, 1975; Jacobs *et al.*, 1975); this dependence occurs as a result of the free concentration of the [$^3$H]ligand being depleted by the membrane.

Our earlier work on this problem (Hartley and Seeman, 1978) had shown that [$^3$H]spiperone was markedly depleted by the membrane and that this drug was highly membrane-soluble (membrane–buffer partition coefficient at 410).

Figure 3 illustrates that both the apparent dissociation constant ($K_D$) and the apparent density of binding sites for [$^3$H]spiperone ($B_{max}$) increases as the con-

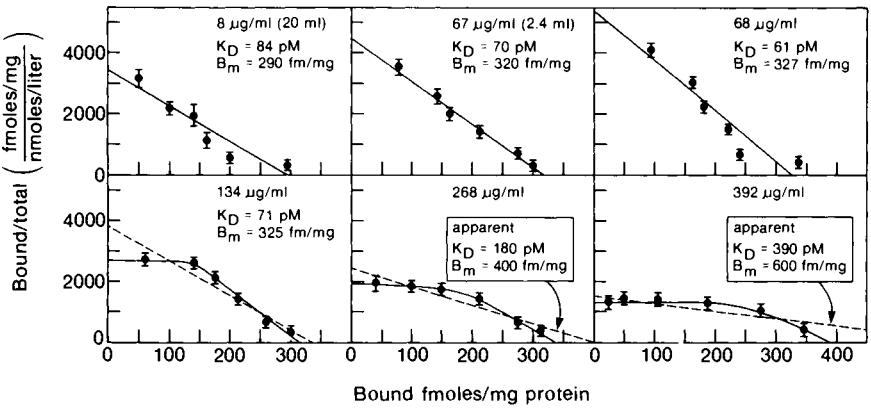


**Fig. 3.** The apparent dissociation constant ($K_D$) and apparent density of binding sites ($B_{max}$) for [$^3$H]spiperone increase as the concentration of membrane protein is increased. The protein concentration is given in μg/ml final incubate. The dashed lines are drawn assuming that the Scatchard relation is linear, which is not the case at the high protein concentrations. As predicted by Cuatrecasas and Hollenberg (1976), the length of the "flat" region (or horizontal region) of the Scatchard relation is directly dependent on the final concentration of receptor–membrane protein, and the $K_D$ values from the slope portion of each plot are all approximately the same (between 70 and 96 p*M*). The final incubation volume is 0.6 ml in all cases except that for 8 μg/ml (which is 20 ml) and that for 67 μg/ml (which is 2.4 ml); the incubation volume has little significant effect on the $K_D$ values. Washed human caudate nucleus (A136; patient had been 8 months of age). The specific binding of [$^3$H]spiperone is defined as that inhibited by 100 n*M* (+)-butaclamol in this experiment.

centration of membrane protein is increased. The Scatchard relation is linear when the membrane protein is below 100 μg/ml.

Thus, since different published studies employed different final protein concentrations, it might be expected that the $K_D$ values would also differ. As summarized in Fig. 4, there is a wide disparity among the values reported for the

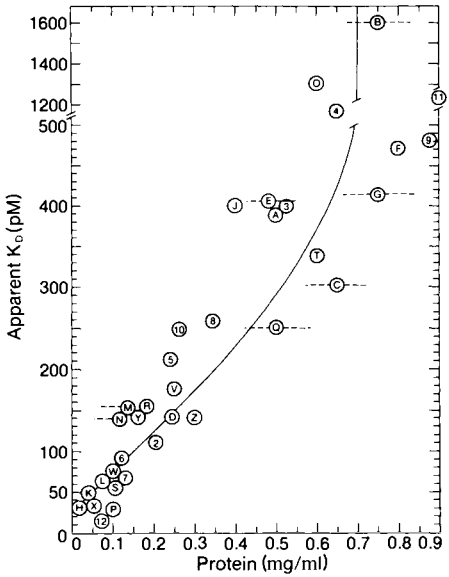


**Fig. 4.** A summary of the published apparent $K_D$ values, as measured by Scatchard analysis (from saturation-type experiments). The line illustrates the relation between $K_D$ and the final concentration of protein in the [$^3$H]spiperone-binding assay tube. All data are for rat striatum, unless stated otherwise. The horizontal dashed lines indicate that the final protein concentration had not been specified, so that it was necessary to estimate this value from the weight of the original tissue and the final volume in which the washed tissue pellet was finally suspended. The original wet tissue generally had 10–15% protein, an unspecified amount of which was lost during washing. A, Akiyama *et al.* (1981); B, Naber *et al.* (1980), 10% protein; C, Creese *et al.* (1977), calf, 10% protein; D, Di Paolo *et al.* (1979), also found low-affinity site (3 n*M*); E, Ebstein *et al.* (1979), 15% protein; F, Lin *et al.* (1980), calf; G, Lew and Goldstein (1979), bovine, 15% protein; H, Howlett and Nahorski (1980), a $K_D$ = 0.3 n*M* was also found; J, Kajiyama and Nomura (1981); K, Koide and Matsushita (1981); L, List and Seeman (1981); M, Marchais *et al.* (1980), rat med. prefrontal cortex, 15% protein; N, Nielsen *et al.* (1980), 15% protein; O, Memo *et al.* (1980a,b); P, Pedigo *et al.* (1978), a $K_D$ (low) of 0.34 n*M* was also found; Q, Quik *et al.* (1978), 10% protein; R, Howard *et al.* (1978); S, Staunton *et al.* (1981); T, Cross and Owen (1980), Owen *et al.* (1979), Cross and Waddington (1981); V, Hartley and Seeman (1978), calf; W, Withy *et al.* (1980,1981), bovine caudate microsomes; X, Reisine *et al.* (1978), human caudate nucleus; Y, Leysen *et al.* (1978), microsomes; Z, Howlett and Nahorski (1978); 2, Weinreich and Seeman (1980); 3, Madras *et al.* (1980), canine; 4, Memo *et al.* (1981); 5, Di Paolo *et al.* (1981); 6, Stefanini and Clement-Cormier (1981), area postrema; 7, Boehme and Ciaranello (1981), mouse; 8, Stefanini *et al.* (1981), dog anterior pituitary; 9, Meltzer and So (1979), bovine pituitary; 10, Helmeste *et al.* (1981); 11, Cronin and Weiner (1979), the $K_D$ was 850 p*M* for sheep pituitary at about 1.6 mg protein/ml final volume.

dissociation constant ($K_D$) of [$^3$H]spiperone at the $D_2$-type dopamine receptor (Seeman, 1980). For example, $K_D$ values range from 13 p*M* (Hruska and Silbergeld, 1980) to 1600 p*M* (Naber *et al.*, 1980).

This problem becomes particularly important when comparing conflicting data on dopamine receptor differences in schizophrenia (Lee and Seeman, 1980; Owen *et al.*, 1978; Mackay *et al.*, 1980; Reynolds *et al.*, 1980; Seeman, 1981) and drug-induced supersensitivity states (see Muller and Seeman, 1978 for references).

Figure 4 illustrates the relation between the published $K_D$ values and the concentration of protein in final incubate. These final protein concentrations were not always stated, since many studies simply reported the concentration of "original tissue," even though it had been washed several times and an unstated amount of protein had been discarded. The summary in Fig. 4 includes data for striatum, pituitary, and area postrema. The relation in Fig. 4 implies that the true $K_D$ value for [$^3$H]spiperone at the dopamine receptors in all these tissues may have been the same, namely about 50 p*M*, had the authors used very low concentrations of protein.

Thus, in studying the effects of various conditions on dopamine receptors, such as lesions, long-term medication, development, aging, or time of day, it would be appropriate to use low concentrations of tissue protein. This would minimize artifacts wherein changes in the $K_D$ or $B_{max}$ of the receptors could depend on the protein concentration.

Since [$^3$H]spiperone is highly fat-soluble (with a membrane–buffer partition coefficient of 410) (Hartley and Seeman, 1978), the [$^3$H]spiperone is presumably depleted by both the dopamine receptors and the hydrophobic constituents of the membrane (Seeman, 1972; Seeman *et al.*, 1974a).

## IV. METHOD FOR MEASURING $D_3$ DOPAMINERGIC SITES WITH [$^3$H]LIGANDS

The $D_3$ site, with its nanomolar affinity for dopamine and micromolar affinity for neuroleptics, is most selectively labeled by [$^3$H]dopamine in the presence of ascorbate and EDTA (List and Seeman, 1982). The $D_3$ sites can also be labeled by [$^3$H]apomorphine and [$^3$H]ADTN, but these [$^3$H]ligands are less selective, labeling $D_2^{high}$ sites as well.

The buffer used both in tissue preparation and [$^3$H]dopamine binding assays in TEAN [15 m*M* Tris–HCl, pH 7.4, 5 m*M* $Na_2EDTA$, 0.02% (1 m*M* ascorbate) and 12.5 μ*M* nialamide]. The effects of omitting EDTA, ascorbate, or nialamide are shown at the end of this section.

Dissected brain tissue is Teflon–glass homogenized (see earlier sections),

centrifuged (44,000 *g*) and suspended (15 vol) four times (in order to remove as much endogenous dopamine as possible), and finally stored frozen [−20°C, 3–5 ml aliquots of 180 to 300 mg (calf), 35 mg (rat), or 40 mg (human) original wet weight/ml]. Before final use, the thawed suspension is Polytron-homogenized (10 sec; setting of 7).

Assays are done in quadruplicate glass test tubes (12 × 75 mm), which receive in order: 200 μl buffer (with or without competing nonradioactive drug), 200 μl either of [$^3$H]dopamine (33–45 Ci/mmole, New England Nuclear, Boston, Massachusetts), [$^3$H]ADTN (25–35 Ci/mmole, New England Nuclear, Boston), or [$^3$H]apomorphine (39 Ci/mmole, New England Nuclear, Boston), and 200 μl tissue (0.2–0.4 mg protein for calf; 0.4–0.5 mg protein for rat; 0.4–0.5 mg protein for human). After incubation at 20 to 22°C for 30 min, 0.5 ml aliquots are vacuum-filtered through Whatman GF/B filters, followed by a 10 ml wash with buffer. Filters are inserted into liquid scintillation vials along with either 8 ml of Aquasol (New England Nuclear, Boston) or ACS (Amersham, Arlington Heights, Illinois) and equilibrated for at least 12 hr before determination of radioactivity.

Binding isotherms and Scatchard analyses are done using [$^3$H]dopamine, [$^3$H]ADTN, and [$^3$H]apomorphine in concentrations of 0.2 to 6.0 n*M*. Specific binding of [$^3$H]dopamine, [$^3$H]ADTN, and [$^3$H]apomorphine is defined as that binding displaceable by 5 μ*M* dopamine or by 1 μ*M* apomorphine.

Using a concentration of [$^3$H]dopamine of 0.7 n*M* results in 66% specific binding in the calf with total binding of 400 cpm/filter, 46% specific binding in the rat with total binding of 400 cpm/filter and 35–60% specific binding in the human with total binding of 300 to 700 cpm/filter.

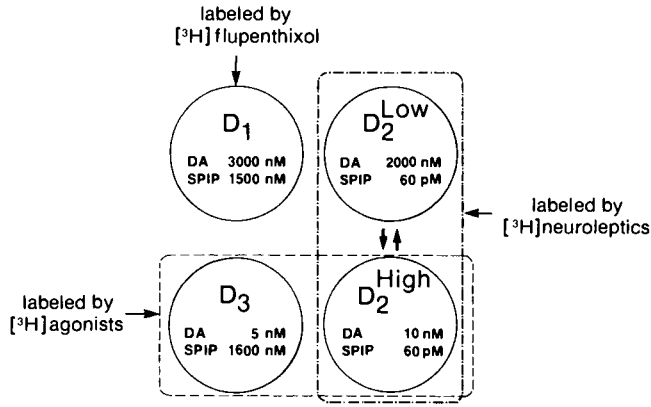


**Fig. 5.** Since [$^3$H]ligand binding methods are generally only practical in the nanomolar and subnanomolar concentration range, this diagram illustrates that nanomolar concentrations of [$^3$H]agonists will label both the $D_2^{high}$ and the $D_3$ sites. Nanomolar or picomolar concentrations of [$^3$H]spiperone, however, will only label both states of the $D_2$ receptors.

Omission of ascorbate from the standard buffer results in a reduction in specific binding (both in cpm and in percentage) for [$^3$H]dopamine. However, omission of ascorbate does not change the binding pharmacology of 0.7 n*M* [$^3$H]dopamine to rat striatum; competition data and drug $IC_{50}$ values in buffer without ascorbate are similar to those obtained in buffer with ascorbate. Scatchard analyses of [$^3$H]dopamine binding are difficult to do in the absence of ascorbate, due to the variability of the binding signal. $B_{max}$ values for [$^3$H]dopamine binding tend to be higher in the absence of ascorbate.

Omission of EDTA from the standard buffer results in a 50% increase in total [$^3$H]dopamine binding and a 100% increase in specific binding. The $IC_{50}$ value for dopamine also increases by four- to tenfold compared to that obtained in standard TEAN buffer conditions (i.e., the $IC_{50}$ value in absence of EDTA was 24 n*M*). Omission of both EDTA and ascorbate results in a further increase in total [$^3$H]dopamine binding and the dopamine $IC_{50}$ value. The binding, however, is extremely variable and difficult to characterize.

Omission of EDTA and ascorbate from the standard buffer results in a large increase in the binding of [$^3$H]ADTN and [$^3$H]apomorphine to rat striatum as well. Under these conditions, however, the data become extremely variable.

These [$^3$H]ligand binding methods are generally only practical at [$^3$H]ligand final concentrations below 5 n*M*. The lower the concentration of the [$^3$H]ligand, the more selective the results will be for a particular receptor. Concentrations above 10 n*M* [$^3$H]ligand generally give unacceptably high nonspecific binding, masking the selective data in the nanomolar and subnanomolar concentration ranges.

Thus, nanomolar concentrations of [$^3$H]agonists will label any dopaminergic site with nanomolar affinity for the agonist. This means that both the $D_3$ and the $D_2^{high}$ sites will be labeled by [$^3$H]agonists, as illustrated in Figs. 5 and 6. These

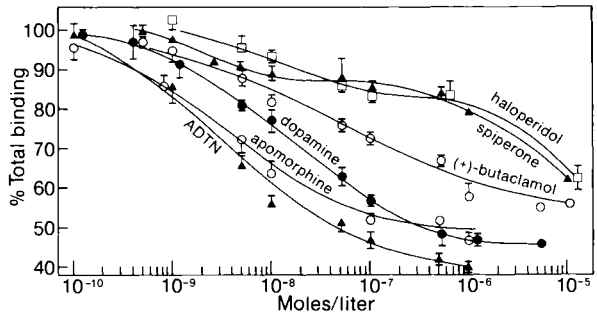


**Fig. 6.** Competition of dopaminergic catecholamines and neuroleptics for the binding of 0.75 n*M* [$^3$H]ADTN to human caudate. Spiperone and haloperidol both produced two distinctly separate phases of competition ($D_2^{high}$ on the left, $D_3$ on the right side). Homogenates of caudate nucleus were prepared from the brain of a 45-year-old male, 4 hr postmortem. Points are averages (± SEM) for 2 to 3 experiments (List *et al.*, 1982).

two sites can be discriminated by adding a neuroleptic, as shown in Fig. 6. Thus, the $D_2^{high}$ site will be occluded by 30 n*M* spiperone, leaving the $D_3$ sites alone to be occupied by [$^3$H]agonist.

## REFERENCES


Ahn, H. S., Gardner, E., and Makman, M. H. (1979). *Eur. J. Pharmacol.* **53,** 313–317.

Akiyama, K., Sato, M., and Otsuki, S. (1982). *Biol. Psychiat* **17,** 223–231.

Andorn, A. C., Cech, S. Y., Johnson, S., and Maguire, M. E. (1979). *Soc. Neurosci. Abstr.* **5,** 547.

Arbilla, S., and Langer, S. Z. (1981). *Eur. J. Pharmacol.* **76,** 345–351.

Arbilla, S., Briley, M. S., Dubocovich, M. L., and Langer, S. Z. (1978). *Life Sci.* **23,** 1775–1780.

Baudry, M., Martres, M. P., and Schwartz, J. C. (1979). *Naunyn-Schmiedeberg's Arch. Pharmacol.* **308,** 231–237.

Boehme, R. E., and Ciaranello, R. D. (1981). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 3255–3259.

Brown, E. M., Fedak, S. A., Woodard, C. J., Aurbach, G. D., and Rodbard, D. (1976). *J. Biol. Chem.* **251,** 1329–1246.

Brown, R. A., and O'Connor, S. E. (1980). *Br. J. Pharmacol.* **66,** 189P.

Caron, M.G., Beaulieu, M., Raymond, V., Gagné, B., Drouin, J., Lefkowitz, J., and Labrie, F. (1978). *J. Biol. Chem.* **253,** 2244–2253.

Chan, B., Seeman, P., Davis, A., and Madras, B. K. (1982). *Eur. J. Pharmacol.* **81,** 111–116.

Chang, K.-J., Jacobs, S., and Cautrecasas, P. (1975). *Biochim. Biophys. Acta* **406,** 294–303.

Coughenour, L. L. (1981). *Soc. Neurosci. Abstr.* **7,** 209.

Creese, I. (1981). *Proc. Am. Coll. Neuropsychopharmacol. (San Diego)* p. 34.

Creese, I., Schneider, R., and Snyder, S. H. (1977). *Eur. J. Pharmacol.* **46,** 377–381.

Creese, I., Usdin, T. B., and Snyder, S. H. (1979). *Mol. Pharmacol.* **16,** 69–76.

Cronin, M. J., and Weiner, R. I. (1979). *Endocrinology* **104,** 307–312.

Cross, A. J., and Owen, F. (1980). *Eur. J. Pharmacol.* **65,** 341–347.

Cross, A. J., and Waddington, J. L. (1981). *Eur. J. Pharmacol.* **71,** 327–332.

Cuatrecasas, P., and Hollenberg, M. D. (1976). *Adv. Protein Chem.* **30,** 251–451.

DeCamilli, P., Macconi, D., and Spada, A. (1979). *Nature (London)* **278,** 252–254.

Di Paolo, T., Carmichael, R., Labrie, F., and Raynaud, J.-P. (1979). *Mol. Cell. Endocrinol.* **16,** 99–112.

Di Paolo, T., Poyet, P., and Labrie, F. (1981). *Eur. J. Pharmacol.* **73,** 105–106.

Dubocovich, M. L., and Langer, S. Z. (1980). *J. Pharmacol. Exp. Ther.* **212,** 144–152.

Ebstein, R. P., Pickholz, D., and Belmaker, R. H. (1979). *J. Pharm. Pharmacol.* **31,** 558–559.

Enero, M. A., and Langer, S. Z. (1975). *Naunyn-Schmiedeberg's Arch. Pharmacol.* **289,** 179–203.

Ferland, L., Meunier, H., Di Paolo, T., Giguère, V., Veilleux, R., and Labrie, F. (1982). *In* "Psychoneuroendocrinology" (R. Collu, ed.), Vol. 12. Raven Press, New York (in press).

Fields, J. Z., Reisine, T. D., and Yamamura, H. I. (1977). *Brain Res.* **136,** 578–584.

Fields, J. Z., Roeske, W. R., Morkin, E., and Yamamura, H. I. (1978). *J. Biol. Chem.* **253,** 3251–3258.

Freedman, S. B., Poat, J. A., and Woodruff, G. N. (1981). *Eur. J. Pharmacol.* **69,** 117–118.

Frey, W. H., II, Senogles, S. E., Heston, L. L., Tuason, V. B., and Nicol, S. E. (1979). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **38,** 844.

Fuder, H., and Muscholl, E. (1978). *Naunyn-Schmiedeberg's Arch. Pharmacol.* **305,** 109–115.

George, S., Watanabe, M., and Seeman, P. (1983a). *ACS Symp. Ser.* **224,** 93–99.

George, S., Binkley, K., and Seeman, P. (1983b). *J. Neural Transm., Suppl.* **18,** 149–156.

Giannattasio, G., De Ferrari, M. E., and Spada, A. (1981). *Life Sci.* **28,** 1605–1612.
Goldberg, L. I., and Kohli, J. D. (1979). *Commun. Psychopharmacol.* **3,** 447–456.
Grigoriadis, D., and Seeman, P. (1983). *Soc. Neurosci. Abstr.* **9,** 31.
Hartley, E. J., and Seeman, P. (1978). *Life Sci.* **23,** 513–518.
Hattori, T., Weinreich, P., and Seeman, P. (1982). *Soc. Neurosci. Abstr.* **8,** 646.
Heikkila, R. L., Cabbat, F. S., and Manzino, L. (1981). *Res. Commun. Chem. Pathol. Pharmacol.* **34,** 409–421.
Helmeste, D. M., Seeman, P., and Coscina, D. V. (1981). *Eur. J. Pharmacol.* **69,** 465–470.
Hofmann, M., Jommi, G. C., Montefusco, I., Tonon, G. C., Spano, P. F., and Trabucchi, M. (1979). *J. Neurochem.* **32,** 1547–1550.
Hope, W., McCulloch, M. W., Rand, M. J., and Story, D. F. (1978). *Br. J. Pharmacol.* **64,** 527–537.
Howard, J. L., Large, B. T., Wedley, S., and Pullar, I. A. (1978). *Life Sci.* **23,** 599–604.
Howlett, D. R., and Nahorski, S. R. (1978). *FEBS Lett.* **87,** 152–156.
Howlett, D. R., and Nahorski, S. R. (1980). *Life Sci.* **26,** 511–517.
Hruska, R. E., and Silbergeld, E. K. (1980). *Science* **208,** 1466–1468.
Hyttel, J. (1978a). *Life Sci.* **23,** 551–556.
Hyttel, J. (1978b). *Prog. Neuro-Psychopharmacol.* **2,** 329–335.
Hyttel, J. (1979). "Neurochemical Effects of Neuroleptic Drugs." H. Lundbeck & Co., Valby, Denmark.
Hyttel, J. (1980). *Psychopharmacology* **67,** 107–109.
Hyttel, J. (1981a). *Life Sci.* **28,** 563–569.
Hyttel, J. (1981b). *In* "Dopamine," (M. Kohsaka, G. N. Woodruff, T. Shomuri, and Y. Tsukada, eds.), Pergamon, Oxford.
Jackisch, R., Zumstein, A., Hertting, G., and Starke, K. (1980). *Naunyn-Schmideberg's Arch. Pharmacol.* **314,** 129–133.
Jacobs, S., Chang, K.-J., and Cautrecasas, P. (1975). *Biochem. Biophys. Res. Commun.* **66,** 687–692.
Kajiyama, H., and Nomura, Y. (1981). *J. Pharm. Dyn.* **4,** 85–90.
Kamal, L. A., Arbilla, S., and Langer, S. Z. (1981). *J. Pharmacol. Exp. Ther.* **216,** 592–598.
Kebabian, J. W. (1978). *Life Sci.* **23,** 479–484.
Kebabian, J. W., and Calne, D. (1979). *Nature (London)* **277,** 93–96.
Kelly, M. J. (1981). *Arch. Int. Pharmacodyn. Ther.* **250,** 18–29.
Koide, T., and Matsushita, H. (1981). *Life Sci.* **28,** 1139–1145.
Lee, T., and Seeman, P. (1980). *Am. J. Psychiatry* **137,** 191–197.
Leff, S., Hamblin, M., Sibley, D. R., and Creese, I. (1981). *Soc. Neurosci. Abstr.* **7,** 126.
Leslie, F. M., Dunlap, C. E., III, and Cox, B. M. (1980). *J. Neurochem.* **34,** 219–221.
Lew, J. W., and Goldstein, M. (1979). *Eur. J. Pharmacol.* **55,** 429–430.
Leysen, J. E., Gommeren, W., and Laduron, P. M. (1978a). *Biochem. Pharmacol.* **27,** 307–316.
Leysen, J. E., Niemegeers, C. J. E., Tollenaere, J. P., and Laduron, P. M. (1978b). *Nature (London)* **272,** 168–171.
Leysen, J. E., Gommeren, W., and Laburon, P. M. (1978c). *Arch. Int. Pharmacodyn. Ther.* **236,** 310–314.
Leysen, J. E., Gommeren, W., and Laduron, P. M. (1979). *Biochem. Pharmacol.* **28,** 447–448.
Lin, C. W., Maayani, S., and Wilk, S. (1980). *J. Pharmacol. Exp. Ther.* **212,** 462–468.
List, S. J., and Seeman, P. (1981a). *Soc. Neurosci. Abstr.* **7,** 10.
List, S. J., and Seeman, P. (1981b). *Proc. Natl. Acad. Sci. U.S.A.* **78,** 2620–2624.
List, S. J., and Seeman, P. (1982). *J. Neurochem.* **39,** 1363–1373.
List, S., Titeler, M., and Seeman, P. (1980). *Biochem. Pharmacol.* **29,** 1621–1622.
List, S. J., Wreggett, K. A., and Seeman, P. (1982). *J. Neurosci.* **2,** 895–906.
Ludford, J. M., and Talamo, B. R. (1980). *J. Biol. Chem.* **255,** 4619–4627.

MacKay, A. V. P., Bird, O., Bird, E. D., Spokes, E. G., Rossor, M., Iversen, L. L., Creese, I., and Snyder, S. H. (1980). *Lancet* **2,** 916–918.

Madras, B. K., Davis, A., Kunashko, P., and Seeman, P. (1980). *In* "Psychopharmacology and Biochemistry of Neurotransmitter Receptors" (H. Yamamura, R. Olsen, and E. Usdin, eds.), pp. 411–419. Am. Elsevier, New York.

Makman, M. H., Dvorkin, B., Horowitz, S. G., and Thal, L. J. (1980). *Eur. J. Pharmacol.* **63,** 217–222.

Marchais, D., Tassin, J. P., and Bockaert, J. (1980). *Brain Res.* **183,** 235–240.

Marchisio, A. M., Eljarmak, D., and Collu, R. (1981). *Neuroendocrinol. Lett.* **3,** 110.

Martres, M. P., Baudry, M., and Schwartz, J. C. (1978). *Life Sci.* **23,** 1781–1784.

Massingham, R., Dubocovich, M. L., and Langer, S. Z. (1980). *Naunyn-Schmiedeberg's Arch. Pharmacol.* **314,** 17–28.

Meltzer, H. Y., and So, R. (1979). *Life Sci.* **25,** 531–536.

Memo, M., Lucchi, L., Spano, P. F., and Trabucchi, M. (1980a). *Brain Res.* **202,** 488–492.

Memo, M., Spano, P. F., and Trabucchi, M. (1980b). *Br. J. Pharmacol.* **66,** 124P–125P.

Memo, M., Battaini, F., Spano, P. F., and Trabucchi, M. (1981). *Acta Psychiatr. Scand.* **63,** 314–324.

Meunier, H., Giguère, V., and Labrie, F. (1980). *Proc. Int. Conf. Cyclic Nucleotides, 4th,* 1980 Abstr. THA9.

Miller, J. C., and Friedhoff, A. J. (1979). *Biochem. Pharmacol.* **28,** 688–690.

Mishra, R. K., Marshall, A. M., and Varmuza, S. L. (1980). *Brain Res.* **200,** 47–57.

Moller-Nielsen, I., Boeck, V., Christensen, A. V., Danneskiold-Samsoe, ■■, Hyttel, J., Langeland, J., Pedersen, V., and Svendsen, O. (1977). *Acta Pharmacol. Toxicol.* **41,** 369–383.

Müller, E. E., Stefanini, E., Camanni, F., Lacotelli, V., Massara, F., Spano, P. F., and Cocchi, D. (1979). *J. Neural Transm.* **46,** 205–214.

Muller, P., and Seeman, P. (1978). *Psychopharmacology* **60,** 1–11.

Munemura, M., Cote, T. E., Tsuruta, K., Eskay, R. L., Kebabian, J. W. (1980). *Endocrinology* **107,** 1676–1683.

Naber, D., Wirz-Justice, A., Kafka, M. S., and Wehr, T. A. (1980). *Psychopharmacology* **68,** 1–5.

Nielsen, E. B., Nielsen, M., Ellison, G., and Braestrup, C. (1980). *Eur. J. Pharmacol.* **66,** 149–154.

Owen, F., Crow, T. J., Poulter, M., Cross, A. J., Longden, A., and Riley, G. J. (1978). *Lancet* **2,** 223–226.

Owen, F., Cross, A. J., Poulter, M., and Waddington, J. L. (1979). *Life Sci.* **25,** 385–390.

Pedigo, N. W., Reisine, T. D., Fields, J. Z., and Yamamura, H. I. (1978). *Eur. J. Pharmacol.* **50,** 451–453.

Perkins, N. A., and Westfall, T. C. (1976). *Soc. Neurosci. Abstr.* **2,** 499.

Quik, M., Iversen, L. L., Larder, A., and MacKay, A. V. P. (1978). *Nature (London)* **274,** 513–514.

Reimann, W., Zumstein, A., Jackisch, R., Starke, K., and Hertting, G. (1979). *Naunyn-Schmiedeberg's Arch. Pharmacol.* **306,** 53–60.

Reisine, T. D., Fields, J. Z., Bird, E. D., Spokes, E., and Yamamura, H. I. (1978). *Commun. Psychopharmacol.* **2,** 79–84.

Reynolds, G. P., Reynolds, L. M., Riederer, P., Jellinger, K., and Gabriel, E. (1980). *Lancet* **2,** 1251.

Rick, J., Szabo, H., Payne, P., Kovathana, N., Cannon, J. G., and Frohman, L. A. (1979). *Endocrinology* **104,** 1234–1242.

Roufogalis, B. D., Thornton, M., and Wade, D. N. (1976). *Life Sci.* **19,** 927–934.

Rupniak, N. M. J., Jenner, P., and Marsden, C. D. (1981). *J. Pharm. Pharmacol.* **33,** 602–603.

Sano, K., Noshiro, O., Katsuda, K., Nishikori, K., and Maeno, H. (1979). *Biochem. Pharmacol.* **28,** 3617–3627.

Schmidt, M. J., and Hill, L. E. (1977). *Life Sci.* **20,** 789–798.
Seeman, P. (1972). *Pharmacol. Rev.* **24,** 583–655.
Seeman, P. (1974). *J. Pharmacol.* **5,** Suppl.2, 91.
Seeman, P. (1980). *Pharmacol. Rev.* **32,** 229–313.
Seeman, P. (1981). *Lancet* **1,** 1103.
Seeman, P., Staiman, A., and Chau-Wong, M. (1974a). *J. Pharmacol. Exp. Ther.* **190,** 123–130.
Seeman, P., Wong, M., and Lee, T. (1974b). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **33,** 246.
Seeman, P., Chau-Wong, M., Tedesco, J., and Wong, K. (1975). *Proc. Natl. Acad. Sci. U.S.A.* **72,** 4376–4380.
Seeman, P., Lee, T., Chau-Wong, M., Tedesco,J., and Wong, K. (1976). *Proc. Natl. Acad. Sci. U.S.A.* **73,** 4354–4358.
Seeman, P., Ulpian, C., Wreggett, K. A., and Wells, J. (1984). *J. Neurochem.* **43,** (in press).
Sibley, D. R., and Creese, I. (1979). *Eur. J. Pharmacol.* **55,** 341–343.
Sibley, D. R., Mahan, L., and Creese, I. (1981). *Soc. Neurosci. Abstr.* **7,** 126.
Sokoloff, P., Martres, M. P., and Schwartz, J. C. (1980). *Naunyn-Schmiedeberg's Arch. Pharmacol.* **315,** 89–102.
Starke, K., Reimann, W., Zumstein, A., and Hertting, G. (1978). *Naunyn-Schmiedeberg's Arch. Pharmacol.* **305,** 27–36.
Staunton, D. A., Wolfe, B. B., Groves, P. M., and Molinoff, P. B. (1981). *Brain Res.* **211,** 315–327.
Stefanini, E., and Clement-Cormier, Y. (1981). *Eur. J. Pharmacol.* **74,** 257–260.
Stefanini, E., Marchisio, A. M., Devoto, P., Vernaleone, F., Collu, R., and Spano, P. F. (1980). *Brain Res.* **198,** 229–233.
Stefanini, E., Clement-Cormier, Y., Vernaleone, F., Devoto, P., Marchisio, A. M., and Collu, R. (1981). *Neuroendocrinology* **32,** 103–107.
Steinsland, O. S., and Hieble, J. P. (1978). *Science* **199,** 443–445.
Steinsland, O. S., Hieble, J. P., and Laubach, D. J. (1979). *Adv. Pharmacol. Ther., Proc. Int. Congr. Pharmacol. 7th, 1978* Abstr. 2368.
Stoof, J. C., and Kebabian, J. W. (1981). *Nature (London)* **294,** 366–368.
Stoof, J. C., Horn, A. S., and Mulder, A. H. (1980). *Brain Res.* **196,** 276–281.
Tayo, F. M. (1977). *Br. J. Pharmacol.* **59,** 511P–512P.
Theodorou, A. E., Crockett, M., Jenner, P., and Marsden, C. D. (1979). *J. Pharm. Pharmacol.* **31,** 424–426.
Theodorou, A. E., Hall, M. D., Jenner, P., and Marsden, C. D. (1980). *J. Pharm. Pharmacol.* **32,** 441–444.
Thomas, T. N., and Zemp, J. W. (1977). *J. Neurochem.* **28,** 663–665.
Titeler, M., List, S., and Seeman, P. (1979). *Commun. Psychopharmacol.* **3,** 411–420.
Tolbert, L. C., Thomas, T. N., Middaugh, L. D., and Zemp, J. W. (1979). *Life Sci.* **25,** 2189–2195.
Usdin, T. B., Creese, I., and Snyder, S. H. (1980). *J. Neurochem.* **34,** 669–676.
Watanabe, M., George, S. R., and Seeman, P. (1983). *Soc. Neurosci. Abstr.* **9,** 994.
Weinreich, P., and Seeman, P. (1980). *Brain Res.* **198,** 491–496.
Wilcox, R. E., Humphrey, D. W., Riffee, W. H., and Smith, R. V. (1980). *J. Pharm. Sci.* **69,** 974–976.
Withy, R. M., Mayer, R. J., and Strange, P. G. (1980). *FEBS Lett.* **112,** 293–295.
Withy, R. M., Mayer, R. J., and Strange, P. F. (1981). *J. Neurochem.* **37,** 1144–1154.
Wreggett, K. A., and Seeman, P. (1983). *Acta Pharm. Suec., Suppl.* **1,** 30–40.
Zahniser, N. R., and Molinoff, P. B. (1978). *Nature (London)* **275,** 453–455.
Zemp, J. W., Tolbert, L., Thomas, T. N., and Middaugh, L. D. (1977). *Soc. Neurosci. Abstr.* **3,** 264.

# *Chapter 17*

# *RADIOACTIVE LIGAND BINDING STUDIES: IDENTIFICATION OF CENTRAL SEROTONIN RECEPTORS*

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## I. INTRODUCTION

Since the discovery of serotonin (5-hydroxytryptamine or 5-HT) in the central nervous system (CNS) about 30 years ago, an abundant amount of literature has been devoted to its neurotransmitter role in so-called serotoninergic neurons. All the characteristics typical of neurotransmitters, that is, synthesis and storage in specific neurons, $Ca^{2+}$-dependent release on the arrival of action potentials to nerve terminals, local inactivation by specific mechanisms in the synaptic cleft, apply perfectly to 5-HT. The best demonstration has been presented for invertebrates in which the functional properties of identified serotoninergic neurons can



ISBN 0-12-470350-X

be studied (Gerschenfeld *et al.*, 1978). With this preparation, Gerschenfeld and Paupardin-Tritsch (1974) have shown that 5-HT released in the synaptic cleft alters the electrical activity of postsynaptic neurons by interacting with specific sites in their membrane: the postsynaptic receptors. Similarly, the first studies on 5-HT receptors in the mammalian brain consisted of looking for possible changes in the electrical activity of target neurons during the iontophoretic application of 5-HT in their close vicinity. Both excitatory and inhibitory effects were observed, suggesting that different types of specific 5-HT receptors may exist in the CNS (Bloom *et al.*, 1972; Haigler and Aghajanian, 1977). Further studies on the sensitivity of target cells to various 5-HT agonists and antagonists indicated that different drugs are acting on the 5-HT-induced inhibition and excitation, therefore confirming the heterogeneity of 5-HT receptors mediating these responses in the CNS (Haigler and Aghajanian, 1977). However, iontophoretic techniques have serious limitations (the local concentration of applied neurotransmitters and drugs cannot be determined precisely; it is generally not known whether the recorded neuron is actually the primary target cell), which preclude their use for thorough investigations of central neurotransmitter receptors.

Marked progress in the study of central 5-HT receptors coincided with the introduction of biochemical methods. Such methods allow the selective labeling of neurotransmitter binding sites on receptors and, in some cases, the measurement of biological events induced by the neurotransmitter–receptor interaction at the postsynaptic level. These may consist of adenylate cyclase activation, ion fluxes through neuronal membrane, alterations in phospholipid metabolism, etc.

The present review will focus on the methods currently available for labeling 5-HT receptors using radioactive ligands. Such methods are extremely useful for directly investigating the characteristics of central 5-HT receptors under various experimental or pathological conditions.

## II. BASIC TECHNIQUES FOR *IN VITRO* BINDING STUDIES

Because a receptor is characterized by its ability to selectively interact with a given molecule (neurotransmitter or hormone), it possesses a site at which such interaction takes place, that is, a site with a high affinity for this molecule. Therefore, the incubation of biological material containing receptors (brain membranes) in the presence of a radioactive agonist or antagonist should result in the retention of radioactivity on these specific sites. The main problem consists of measuring the radioactivity selectively bound to these receptors. Filtration and centrifugation procedures associated with rapid washings are generally appropriate for collecting membranes without eliminating the radioactive ligand selectively bound to the receptors.

## A. Choice of the Labeled Ligand

Theoretically, the best ligand would be a molecule exhibiting an absolute specificity for the receptor to be studied. A reversible competitive ligand instead of an irreversible ligand is preferable for exploring the characteristics of the binding site. In addition, this ligand must be perfectly stable during the binding assays.

Radiolabeling must be achieved with isotopes such as $^{3}H$ or $^{125}I$, ensuring high specific radioactivity and therefore high levels of radioactivity selectively associated with receptor sites in membranes. Due to steric restrictions, however, iodinated derivatives of agonists or antagonists are often inactive. This explains why, in spite of very high specific radioactivities, the $^{125}I$-labeled molecules are less commonly used for studying neurotransmitter receptors.

In the case of 5-HT receptors, several molecules are currently used as labeled ligands (see Hamon *et al.*, 1984, for a review): *two agonists* ([$^{3}H$]5-HT itself and [$^{3}H$]lisuride); *a mixed agonist–antagonist* *d*-[$^{3}H$]LSD; *and four antagonists* ([$^{3}H$]spiperone, [$^{3}H$]ketanserin, [$^{3}H$]metergoline, and [$^{3}H$]mianserin).

Other labeled agonists and antagonists have been synthesized but appeared unsuitable for binding studies. For instance, [$^{3}H$]methiothepin (a potent 5-HT antagonist) binds mostly to nonspecific sites in membranes (i.e., to sites unrelated to neurotransmitter receptors) and only a small percentage of [$^{3}H$]methiothepin entrapped into membranes is associated with 5-HT receptors (Nelson *et al.*, 1979). Studies with [$^{3}H$]quipazine indicated that this putative 5-HT agonist binds to a low-affinity site ($K_D$ = 0.1 m*M*) in hippocampal and striatal membranes. This site is not a 5-HT receptor but has some properties expected for the 5-HT uptake mechanism (M. Hamon, unpublished observations).

## B. Preparation of Brain Membranes

### *1. Crude Membranes*

If the relative proportion of specific (i.e., onto receptors) to nonspecific (i.e., outside receptors) binding in synaptic membranes is high ($\geq$4), binding assays can be carried out with crude membranes. This is notably the case with [$^{3}H$]5-HT, [$^{3}H$]LSD, [$^{3}H$]ketanserin, and, to a lesser extent, [$^{3}H$]spiperone and [$^{3}H$]mianserin as the labeled ligands. In contrast, crude membranes are not suitable for measuring the specific binding of [$^{3}H$]metergoline. In the latter case, the relative proportion of specific binding reaches a value compatible with reliable quantitative studies only when microsomal or synaptic plasma membranes are used.

For the preparation of crude membranes, brain tissues are homogenized in hypotonic buffer (usually 10 vol of 0.05 *M* Tris–HCl, pH 7.4, v/w) using a

Polytron (PT 10 OD) disrupter at a low speed. After centrifugation at 40,000 *g* for 20 min at 4°C, the resulting pellet is suspended in 20 vol (v/w) of the same ice-cold buffer and membranes are collected by centrifugation as before. This washing procedure has to be repeated twice. The membrane pellet is then gently homogenized in 20 vol of the Tris buffer and incubated for 10 min at 37°C in a shaking water bath. During this incubation, 5-HT still bound to membranes rapidly dissociates and is partly degraded by monoamine oxidase type A (Nelson *et al.*, 1978). The incubated suspension is then centrifuged (40,000 *g*, 4°C, 20 min) and the pellet is washed three times as above. The sedimented material is finally gently homogenized in 10 vol of 0.05 *M* Tris–HCl, pH 7.4. Aliquots of this suspension (0.2 ml containing 0.8–1.0 mg of protein) can be used directly for binding assays.

### 2. *Membranes Obtained by Differential Centrifugations*

As compared to the above procedure, a first improvement may consist of a rapid fractionation of brain issues in order to eliminate cell debris and nuclei.

Tissue are homogenized into 10 vol (v/w) of ice-cold 0.32 *M* sucrose using a Potter-Elvehjem apparatus fitted with a Teflon pestle. The homogenate is centrifuged at 750 *g* for 10 min and the supernatant is carefully decanted. The pellet is rehomogenized in the same volume of 0.32 *M* sucrose and centrifuged as before. The two supernatants are pooled and centrifuged at 10,000 *g* for 30 min at 4°C. The sedimenting fraction ($P_2$) is resuspended in ice-cold distilled water (10 vol of original wet weight) and maintained for 60 min at 4°C with gentle stirring to allow lysis of synaptosomal components. After lysis, the samples are centrifuged at 40,000 *g* for 20 min, and the pellet is incubated (10 min 37°C), washed, and spun down as described above for crude membranes. The final suspension in 0.05 *M* Tris–HCl buffer can be used directly for binding assays (Nelson *et al.*, 1978).

When the nonspecific binding of the [$^3$H]ligand corresponds to about 2/3 of total binding to crude membranes, it is necessary to prepare membrane fractions enriched in receptors. The method of Laduron *et al.* (1975), based on differential centrifugations of brain homogenates in isotonic sucrose, is perfectly adapted to obtain such fractions.

Brain tissues are homogenized in 5 vol (v/w) of 0.25 *M* sucrose using a Potter–Elvehjem apparatus fitted with a Teflon pestle. The homogenate is first centrifuged at 600 *g* for 10 min at 4°C. The resulting pellet is rehomogenized and centrifuged as before to give the sedimenting nuclear fraction (N) containing cell debris, nuclei, etc. The pooled supernatants are then centrifuged at 10,000 *g* for 15 min. The material spun down under this condition is rehomogenized in 5 vol of ice-cold 0.25 *M* sucrose and centrifuged at 10,000 *g*. The final pellet from this centrifugation corresponds to the heavy mitochondrial fraction (M). The light

mitochondrial fraction (L) is obtained by centrifuging the supernatants from the 10,000 *g* centrifugation at 20,000 *g* for 15 min at 4°C. The membrane material still present in this supernatant is finally spun down at 82,000 *g* for 100 min at 4°C; it corresponds to the microsomal fraction (P). All the sedimented fractions (N,M,L,P) are then washed in 20 vol (v/w) of ice-cold 0.05 *M* Tris–HCl, pH 7.4, before being incubated (10 min, 37°C) and treated as crude membranes. The final pellets are suspended in 10 vol of 0.05 *M* Tris–HCl, pH 7.4, and aliquots of this suspension can be used directly for binding assays.

Using [$^3$H]5-HT, [$^3$H]spiperone, or [$^3$H]metergoline as the labeled ligand, the highest density of specific binding sites occurs in the microsomal P membranes (Nelson *et al.*, 1980a; Ilien *et al.*, 1980; Hamon *et al.*, 1981a). The microsomal fraction P in fact contains fragments of plasma membranes (Laduron and Ilien, 1982).

The whole procedure takes about 6 hr from the brain dissection (using 10–15 rats) to the final suspension of microsomal membranes. About half of total membrane proteins are recovered in this microsomal fraction; indeed, when starting with 1 g of fresh tissue, 25 mg of protein are finally collected in the microsomal fraction, whereas total "crude membranes" account for 50 mg of protein. These two characteristics, (relative) rapidity and a good yield, explain why the differential centrifugation technique can be used routinely for preparing membrane material for binding assays. For all the ligands selected for studying 5-HT receptors, microsomal membranes are undoubtedly much better than crude membranes, notably because the proportion of nonspecific binding is significantly lower with the former than with the latter material.

### *3. Synaptic Plasma Membranes*

In contrast to the differential centrifugation technique, the method for preparing purified synaptic plasma membranes (Cotman and Matthews, 1971) is time-consuming and usually not adapted for routine experiments. The preparation of purified synaptic membranes is, however, a necessary step for demonstrating the preferential location of specific binding sites in the synapses.

The whole procedure is carried out in the cold (4°C). Tissues are homogenized in 10 vol of 0.32 *M* sucrose using a Potter–Elvehjem apparatus fitted with a Teflon pestle. The homogenate is centrifuged at 750 *g* for 10 min, and the resulting supernatant is spun down at 10,000 *g* for 30 min. The 10,000 *g* pellet ($P_2$) is resuspended in 10% sucrose (w/w) and applied onto a two-step discontinuous Ficoll (Pharmacia) gradient [10 ml of 13% Ficoll (w/v) in 0.32 *M* sucrose + 10 ml of 7.5% Ficoll (w/v) in 0.32 *M* sucrose]. After a centrifugation in a Beckman SW27 rotor for 45 min at 63,500 *g*, the crude synaptosomal fraction (B) is collected at the interface of the two Ficoll layers. This fraction is diluted with 4 vol of 10% sucrose (w/w) and centrifuged for 30 min at 65,000 *g*

using a Beckman type 30 rotor. The pellet is resuspended in a small volume (5 ml) of 10% sucrose and mixed with 5 vol (25 ml) of 6 m*M* Tris–HCl, pH 8.1, for osmotic lysis. After 90 min of gentle stirring, the suspension is centrifuged at 65,000 *g* for 15 min, and the pellet is gently homogenized in 10% sucrose. This homogenate is applied on to a discontinuous gradient composed of 5 ml layers of sucrose at the following concentrations (w/w in %): 38, 35, 32.5, and 25. The gradients are centrifuged at 63,000 *g* for 90 min in a Beckman SW27 rotor, after which the fraction $F_2$, which sediments at the interface of 25 and 32.5% sucrose layers, is saved. After a dilution with double-distilled water to reach 10% sucrose, the $F_2$ fraction is then centrifuged at 65,000 *g* for 30 min. This pellet is

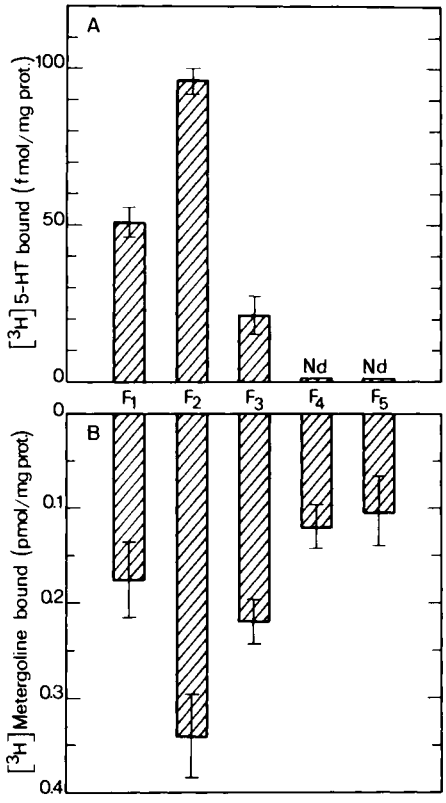


**Fig. 1.** [$^3$H]5-HT (A) and [$^3$H]metergoline (B) binding in various fractions obtained by subcellular fractionation of synaptosomes from the rat forebrain. After osmotic lysis of synaptosomes, fractionation on the second (sucrose) gradient gave five bands [$F_1$–$F_5$ according to Cotman and Matthews (1971)], which were assayed for [$^3$H]5-HT and [$^3$H]metergoline binding. The concentrations of [$^3$H]5-HT and [$^3$H]metergoline were 1.07 n*M* and 0.53 n*M*, respectively. Each bar represents the mean ± SEM ($n$ = 4) of [$^3$H]ligand specifically bound to membranes in each fraction. Nd, Not detectable.

suspended in 0.05 *M* Tris–HCl, pH 7.4, incubated (10 min, 37°C) and washed as described for crude membranes.

Using [$^3$H]5-HT or [$^3$H]metergoline as the labeled ligand, this $F_2$ fraction, containing the selective markers of postsynaptic plasma membranes (Cotman and Matthews, 1971), exhibits the highest density of specific binding sites (Fig. 1).

At least 10 hr are necessary from the brain dissection to the final suspension of $F_2$ fraction. Usually, binding assays cannot be carried out the same day as the membrane preparation, and $F_2$ membranes have to be kept at −30°C before being used. In our hands, brain membrane suspensions can be kept for about 1 week in 0.05 *M* Tris–HCl, pH 7.4, at −30°C without any detectable alteration of their [$^3$H]5-HT binding capacities. The major limitation of this procedure lies in its poor yield: 10 g of rat forebrain give finally only 10–15 mg of synaptic plasma membrane protein.

## III. BINDING ASSAYS FOR STUDYING CENTRAL 5-HT RECEPTORS

### A. *In Vitro* Procedures

In this section, I will describe briefly the methods designed for each ligand. Attention will be paid only to particularities in each case.

#### *1. [$^3$H]5-HT (G-[$^3$H]5-HT, 8–15 Ci/mmol, Amersham International, United Kingdom)*

The first step consists in fact of purifying the labeled molecule. Two techniques can be used; either ion-exchange chromatography on a weak cationic resin or HPLC. The first one consists of pouring the aqueous solution of [$^3$H]5-HT (1 mCi) adjusted to pH 6–6.5, on to a column of Amberlite CG50, 200–400 mesh (0.4 cm diameter, 2.5 cm high) buffered at pH 6.1 with 0.2 *M* Na–$K_2$ phosphate buffer. After a washing with 10 ml of double-distilled water, [$^3$H]5-HT is eluted with 5 ml of 0.2 *N* $CH_3COOH$ in a vial containing 20 μl of 5% ascorbic acid. Aliquots of the eluate are finally diluted with 0.05 *M* Tris–HCl, pH 7.4, to obtain the appropriate solution of [$^3$H]5-HT for binding assays. HPLC consists of injecting [$^3$H]5-HT into an Ultrasphere ODS column (Altex, 5μm, 25 cm length, 0.46 cm diameter). The elution is achieved with a mixture of 0.1 *M* $K_2HPO_4$, 0.1 m*M* EDTA, and methanol (7.5% v/v), final pH= 4.7. At a flow rate of 1.3 ml per min, [$^3$H]5-HT comes off the column 8.5 min after its injection. Aliquots of the eluate are then diluted with a large excess of 0.05 *M* Tris–HCl, pH 7.4, to prepare the final [$^3$H]5-HT solution for binding assays.

In all cases, binding assays with pure [$^3$H]5-HT are absolutely required to

determine kinetic parameters such as the apparent dissociation constant ($K_D$) and $B_{max}$. Discrepancies in the literature regarding $K_D$ values (from 1 to 15 n*M*) probably result, at least partly, from the use of more or less pure [$^3$H]5-HT. As a rule, the purification of [$^3$H]5-HT must be performed just before binding assays.

Membranes (0.2–1.5 mg protein) are incubated at 37°C in 2 ml of 0.05 *M* Tris–HCl containing [$^3$H]5-HT (0.3–15 n*M*), 1% ascorbic acid (i.e., 5.7 m*M*) and 10 μ*M* pargyline (final pH = 7.4). In the presence of the reducing agent and the monoamine oxidase inhibitor (MAOI), the labeled ligand remains stable for at least 20 min. Usually, binding assays are stopped 7–10 min after the addition of [$^3$H]5-HT to the incubating mixture; indeed, binding equilibrium occurs in less than 4 min under such conditions (Bennett and Snyder, 1976). Incubated membranes are collected by filtering samples through Whatman GF/B filters under slight vacuum. Filters are then washed three times with 5 ml of ice-cold Tris buffer. After drying, they are vigorously shaken in scintillation fluid (Aquasol®, New England Nuclear) for radioactivity counting. Nonspecific binding is defined as that persisting in the presence of a large excess of 5-HT (10 μ*M*).

Under these conditions, the specific and nonspecific binding increase linearly as a function of membrane concentration up to 1.25 mg prot/ml. With 1 n*M* of [$^3$H]5-HT, about 5% of the labeled ligand binds to 1 mg of membrane protein (from the rat hippocampus). An excess of 5-HT (10 μ*M*) reduces this binding to about 1.2% of total radioactivity indicating that the specific component accounts for 75% of total binding. Since divalent cations such as $Mg^{2+}$, $Ca^{2+}$ and $Mn^{2+}$ increase the specific binding and reduce the nonspecific binding (Mallat and Hamon, 1982), their addition to the assay mixture can facilitate the identification of [$^3$H]5-HT high-affinity binding sites in a given preparation. Originally, Bennett and Snyder (1976) recommended the inclusion of 4 m*M* $CaCl_2$ in the assay mixture. Under such conditions, almost 90% of total binding can be ascribed to specific sites. Similar results are obtained with much lower concentrations of $Mn^{2+}$ (0.1–0.2 m*M*) (M.H., unpublished observations).

Investigations of the pH effect on [$^3$H]5-HT specific binding on to membranes revealed an optimum at pH 7.4. Reducing the pH to 6.5 induces a 45% decrease in [$^3$H]5-HT binding; similarly, a shift towards alkaline pH also results in a significant reduction of [$^3$H]5-HT binding (−90% at pH 10).

### 2. *[$^3$H]lisuride ([$^3$H]lisuride hydrogen maleate, 15–30 Ci/mmol, Schering AG)*

Binding assays with the other 5-HT agonist, $^3$H-lisuride, consist of incubating brain membranes with the labeled ligand for 45 min at room temperature in 0.05 *M* Tris–HCl, pH 7.4 (Battaglia and Titeler, 1981). Binding assays are also stopped by filtration through Whatman GF/B filters as described for [$^3$H]5-HT.

Since [$^3$H]lisuride binds not only to 5-HT sites but also to dopamine and $\alpha_2$-

specific sites, the selective study of 5-HT sites with this ligand is possible only (1) using membranes from a region poor in dopaminergic sites such as the cerebral cortex, and (2) if 0.1 μ*M* clonidine is included in the assay mixture to saturate the $\alpha_2$ sites (Battaglia and Titeler, 1981). Under such conditions, however, only 20% of total binding can be ascribed to 5-HT specific sites in membranes of the bovine frontal cortex. Therefore, this ligand is far less appropriate than [$^3$H]5-HT for investigating 5-HT receptors in the CNS.

*3.* d-*[$^3$H]LSD ([2-$^3$H]lysergic acid diethylamide, 10–30 Ci/mmol, Amersham International, United Kingdom, or* N-*methyl-[$^3$H]lysergic acid diethylamide, 30–50 Ci/mmol, New England Nuclear)*

Binding assays with [$^3$H]LSD are usually carried out under conditions close to those used for [$^3$H]5-HT. Using nanomolar concentrations of [$^3$H]LSD, equilibrium is reached within 5 min at 37°C with 70–80% of total binding being displaceable by 1 μ*M d*-LSD (specific binding) (see Bennett and Snyder, 1975). Like [$^3$H]lisuride, [$^3$H]LSD also binds to dopamine-related sites, notably in the striatum (Bennett and Snyder, 1976), and it is necessary to include an appropriate concentration of a given dopamine agonist or antagonist to saturate these sites. Whitaker and Seeman (1978) recommend inclusion of 50 n*M* of both apomorphine and spiperone for this purpose; in addition, they also add 50 n*M* of phentolamine to prevent the possible interaction of [$^3$H]-LSD with α-adrenoceptors. Although this cocktail appears quite suitable to mask dopamine and noradrenaline related sites, it may also, at least partly, occlude 5-HT sites. Indeed, spiperone exerts strong 5-HT antagonist properties (see next section).

*4. [$^3$H]Spiperone ([*phenyl-*4-$^3$H]spiroperidol, 15–30 Ci/mmol, Amersham International, United Kingdom or [*benzene ring-*$^3$H]spiroperidol, 20–40 Ci/mmol, New England Nuclear)*

First known as a potent dopamine antagonist, spiperone has been subsequently shown to exhibit strong anti-5-HT properties in the cerebral cortex (Leysen *et al.*, 1978). Binding assays consist of incubating brain membranes with nanomolar concentrations of [$^3$H]spiperone in 0.05 *M* Tris–HCl containing pargyline (10 μ*M*), ascorbic acid (0.1%), and salts at the following concentrations: NaCl, 120 m*M;* KCl, 5 m*M;* $CaCl_2$, 2 m*M,* and $MgCl_2$, 1 m*M;* the final pH is 7.4. Salts reduce preferentially the nonspecific rather than the specific binding, so that the latter represents 65% of total binding in their presence but only 50–55% in their absence. Incubations usually proceed for 15 to 30 min at 37°C. They are stopped by filtration through Whatman GF/B filters as described for [$^3$H]5-HT.

Nonspecific binding is defined as that persisting in the presence of a saturating concentration (1 $\mu M$) of a specific 5-HT antagonist such as pipamperone or cinanserin (Leysen *et al.*, 1978).

Although the pharmacological properties of [$^3$H]spiperone-specific binding sites in the cerebral cortex and the hippocampus clearly confirm their serotoninergic nature, these sites are markedly different from those binding [$^3$H]5-HT (Hamon *et al.*, 1980b). This led Peroutka and Snyder (1979) to propose the existence of two distinct classes of binding sites for 5-HT in brain: the *5-HT$_1$ site*, binding [$^3$H]5-HT and having a higher affinity for 5-HT agonists than for antagonists, and the *5-HT$_2$ site*, binding [$^3$H]spiperone and exhibiting a higher affinity for 5-HT antagonists than for agonists. As expected for a mixed agonist–antagonist, *d*-LSD has been shown to bind equally to 5-HT$_1$ and 5-HT$_2$ sites (Peroutka and Snyder, 1979).

### 5. *[$^3$H]Ketanserin (3-(2-(4-(4-[2-$^3$H]Fluorobenzoyl-1-piperidinyl)-ethyl)-2,4(1H,3H)-quinazolinedione, 15–20 Ci/mmol, Janssen Pharmaceutica, Beerse, Belgium)*

Ketanserin is a rather selective 5-HT antagonist recently investigated by Leysen *et al.* (1981). Its tritium derivative is a good tool for labeling 5-HT$_2$ sites since 70% of the binding of this ligand to membranes from the rat frontal cortex involves these particular sites (Leysen *et al.*, 1982). [$^3$H]Ketanserin also binds to a limited extent to $\alpha_1$-adrenergic sites in the prefrontal cortex, but this can be easily included in the nonspecific binding, which persists in the presence of a saturating concentration of a pure 5-HT antagonist (1 $\mu M$ methysergide) (see Leysen *et al.*, 1982).

Binding assays are carried out in 0.05 *M* Tris–HCl, pH 7.4, in the absence of any added compound. Indeed, salts, pargyline, and ascorbic acid included in the assay mixture for studies on the interaction of [$^3$H]spiperone with 5-HT$_2$ sites have been shown to reduce the specific binding of [$^3$H]ketanserin to brain membranes (Leysen *et al.*, 1982). Incubation time is 15 min at 37°C, and filtration through Whatman GF/B filters associated with washing (3 × 5 ml of ice-cold Tris buffer) is used to stop the assay.

### 6. *[$^3$H]Metergoline ([9,10-$^3$H]metergoline, 10–20 Ci/mmol, CEA, Saclay, France)*

Metergoline is a rather specific 5-HT antagonist in the CNS and its tritiated derivative binds, at least partly, to 5-HT$_2$ sites in the rat brain (Hamon *et al.*, 1981a). Care must be taken, however, to prevent selectively its binding to dopamine sites when studying 5-HT receptors in dopamine-rich areas such as the striatum. This can be achieved by including a saturating concentration of a

dopamine agonist like ADTN (10 μ*M* of 2-amino-6,7-dihydroxytetralin) in the assay mixture.

[$^3$H]Metergoline is not a suitable ligand for labeling 5-$HT_2$ sites in crude membranes since 70% of total binding to this material is nonspecific (i.e., not displaceable by 10 μ*M* of cold metergoline). Reliable binding assays can be carried out with microsomal (P fraction) or synaptic plasma membranes ($F_2$ fraction). Nonspecific binding accounts for only 35–45% and 25–30% of total binding, respectively, with these membrane preparations (from the rat forebrain).

Standard conditions consist of incubating membranes (usually microsomal membranes equivalent to 0.3–0.8 mg protein) in 2 ml of 0.05 *M* Tris–HCl, pH 7.4, containing nanomolar concentrations of [$^3$H]metergoline for 10 min at 37°C. Binding assays are stopped by rapid filtration through GF/B filters. However, before filtering samples, GF/B filters are washed with 5 ml of ice-cold 0.05 *M* Tris–HCl, pH 7.4, containing 0.01% bovine serum albumin. This treatment greatly reduces the nonspecific binding of [$^3$H]metergoline to the glass fiber filters (Hamon *et al.*, 1981a). After the sample has passed through the filter, a further wash is performed with 3 × 5 ml of the serum albumin-containing buffer. Dried filters are then vigorously shaken in 10 ml of Aquasol® for counting.

As in the case of [$^3$H]ketanserin, salts exert an inhibitory effect on the specific binding of [$^3$H]metergoline to 5-$HT_2$ sites and must be omitted in the assay medium (Hamon *et al.*, 1981a).

### 7. *[$^3$H]Mianserin ([8-$^3$H]mianserin, 10–30 Ci/mmol Amersham International, United Kingdom or [N-methyl-$^3$H]mianserin, 50–80 Ci/mmol, New England Nuclear)*

In addition to its potent 5-HT antagonist properties, mianserin exerts strong antihistaminic effects (Peroutka and Snyder, 1981a). Nevertheless, $^3$H-mianserin can be used as a selective ligand of 5-$HT_2$ sites provided that histamine ($H_1$)-related sites are totally occupied by triptolidine (0.3 μ*M*) (Peroutka and Snyder, 1981a) or mepyramine (0.3 μ*M*) (Brunello *et al.*, 1982). Under such conditions, the proportion of [$^3$H]mianserin specifically bound to 5-$HT_2$ sites in brain membranes corresponds to that displaced by an excess of cinanserin (1 μ*M*) or spiperone (30 n*M*).

The standard assay consists of incubating brain membranes with nanomolar concentrations of [$^3$H]mianserin for 15 to 30 min at 37°C in 0.05 *M* Tris–HCl, pH 7.4, supplemented with 0.1% ascorbic acid. Assays are terminated by filtration under vacuum through Whatman GF/B filters and washing with 3 × 5 ml of the ice-cold Tris buffer (Peroutka and Snyder, 1981a; Brunello *et al.*, 1982).

Whereas only one ligand, [$^3$H]5-HT, is suitable for labeling 5-$HT_1$ sites, four

molecules ([$^3$H]spiperone, [$^3$H]ketanserin, [$^3$H]metergoline, and [$^3$H]mianserin) can be used for investigating 5-$HT_2$ sites in brain membranes. Unfortunately, none of these four compounds can be considered a pure 5-HT antagonist. The development of a new ligand with selective anti-5-HT properties would be of considerable interest to explore further the characteristics of 5-$HT_2$ sites in the CNS.

## B. *In Vivo* Procedures

Before examining the properties of serotoninergic sites, it must be mentioned that measurements of ligand binding can be also performed *in vivo*. Briefly, a labeled ligand is injected intravenously into rats or mice and the radioactivity accumulated in several brain regions is estimated at various times thereafter. The specific binding corresponds to the fraction of bound radioactivity which disappears when an excess of a cold drug is administered simultaneously with the labeled ligand.

In 1979, Duchemin *et al.* described marked regional differences in the accumulation of tritium in the mouse brain 0.5 and 1 hr after an iv injection of *d*-[$^3$H]LSD. Maximal binding occurred in the cerebral cortex, followed by the striatum and the hippocampus; the lowest binding was found in the cerebellum. Potent 5-HT antagonists (methysergide, cyproheptadine, cinanserin, mianserin, and methiothepin) markedly reduced the accumulation of tritium in cerebral cortex but not in the cerebellum, indicating that *d*-[$^3$H]LSD occupied a 5-HT site only in the former region.

Similar studies were conducted in the rat with [$^3$H]metergoline as the labeled ligand. As shown in Fig. 2, maximal specific binding (i.e., that displaceable by an excess of cold metergoline) was detected in the cerebral cortex, followed by the olfactory tubercle, the striatum, and the hippocampus. Subcellular fractionation of brain tissues from rats treated with [$^3$H]metergoline revealed that the specific binding was maximal to microsomal membranes (M.H., unpublished observations). Comparison of the regional distribution of *in vivo* specific binding with that established *in vitro* with membranes from the same regions confirmed that the *in vivo* approach is valuable for exploring 5-$HT_2$ sites. As shown in the insert of Fig. 2, a good correlation was observed between the two sets of data.

*In vivo* investigations were also carried out with [$^3$H]spiperone in rats (Leysen *et al.*, 1978) and in mice (Clements-Jewery and Robson, 1980). In both cases, low doses of 5-HT antagonists were sufficient to reduce the accumulation of tritium in the frontal cortex but not in the striatum and other dopamine-rich areas. Accordingly, as already noted *in vitro* (Leysen *et al.*, 1978), [$^3$H]spiperone probably binds *in vivo* to 5-$HT_2$ sites in the frontal cortex.

The last example, which can be briefly mentioned, is that of [$^3$H]methio-

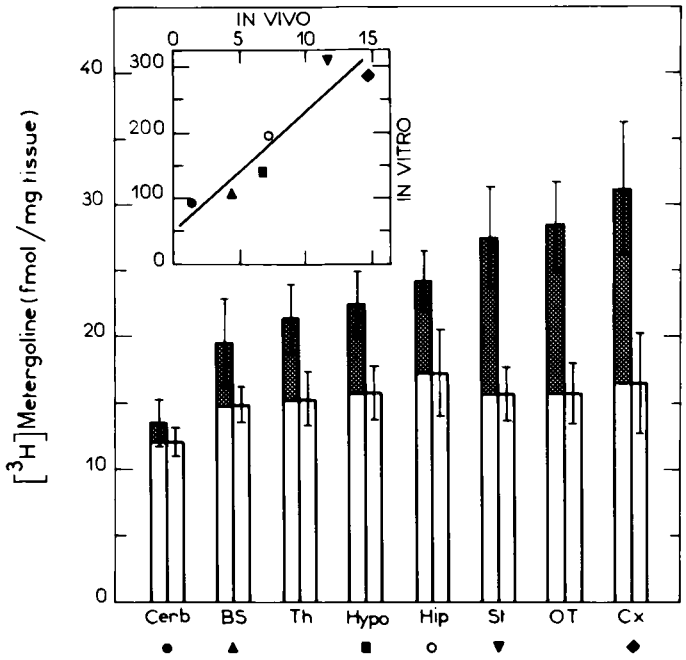


**Fig. 2.** Saturable accumulation of [$^3$H]metergoline in various regions of the rat brain. Groups of six rats were killed 1 hr following the intravenous administration of [$^3$H]metergoline (120 μCi–8.45 nmol, i.e., 11.4 μg/kg) either alone (left bar) or simultaneously with 10 mg/kg (ip) of metergoline (right bar). The shaded area represents the difference ("specific *in vivo* accumulation") between [$^3$H] levels in the two groups of animals. The graph in the inset illustrates the positive correlation ($r$ = 0.93) existing between the *in vitro* specific binding of [$^3$H]metergoline (ordinate, measured with 0.96 n*M* of the labeled ligand) and the *in vivo* specific accumulation of radioactivity (abscissa) in six different brain regions. *In vivo* accumulation is expressed as fmol/mg tissue; *in vitro* binding is expressed as fmol/mg membrane protein. Each point represents the mean of at least six separate determinations. Cerb (●), Cerebellum; BS (▲); brainstem; Th, thalamus; Hypo (■), hypothalamus; Hip (○), hippocampus; St (▼), striatum; O.T., olfactory tubercle; Cx (◆), cerebral cortex.

thepin. Although this molecule is unsuitable for *in vitro* binding assays (see Section II,A and Nelson *et al.*, 1979), a specific accumulation of tritium (i.e., one that can be prevented by the simultaneous administration of 20 mg/kg of cold methiothepin) was observed in several brain regions following the iv injection of [$^3$H]methiothepin to rats (Hamon *et al.*, 1984). As noted with [$^3$H]metergoline (Fig. 2), the regional distribution of [$^3$H]methiothepin-specific binding closely resembles that of 5-HT$_2$ sites in the rat brain (Hamon *et al.*, 1984). This example illustrates that results obtained *in vitro* with a given ligand can be of poor significance for *in vivo* experiments.

Although these *in vivo* investigations are obviously not as precise as *in vitro* binding studies, they seem very promising for the exploration of neurotransmitter receptors in humans. Indeed, using neurotransmitter agonists or antagonists labeled with positron-emitting short-lived isotopes (like carbon-11), it will be possible to visualize these receptors by following the distribution and kinetics of the label with external positron emission tomography. Such studies have been performed already in the baboon with [$^{11}$C]-flunitrazepam, a specific ligand of benzodiazepine receptors (Comar *et al.*, 1981). Current efforts are devoted to the synthesis of *d*-[$^{11}$C]LSD, which should be of great interest for studying central 5-HT receptors *in vivo* in man.

## IV. PROPERTIES OF THE CENTRAL 5-HT RECEPTORS IDENTIFIED BY BINDING STUDIES

### A. Characteristics of the 5-HT$_1$ Site

Kinetic analyses of the association and dissociation of [$^3$H]5-HT from its specific binding sites as well as Scatchard plots of the ligand binding under equilibrium conditions revealed that brain membranes contain a class of noninteracting sites with a high affinity for [$^3$H]5-HT ($K_D$ = 1.5–5.0 n*M*) (Hamon *et al.*, 1980a,b; Nelson *et al.*, 1978; Peroutka and Snyder, 1979). These 5-HT$_1$ sites probably involve a proteolipid structure, since proteolytic enzymes like trypsin, and chymotrypsin, and phospholipase A markedly reduce the specific binding of [$^3$H]5-HT to brain membranes (Bennett and Snyder, 1976). As illustrated in Fig. 3, membrane exposure to *N*-ethylmaleimide results in a marked decrease in the number of [$^3$H]5-HT-specific binding sites, indicating that SH group(s) is (are) also necessary for the ligand binding to 5-HT$_1$ receptors.

Only one team has attempted so far to identify the protein(s) which bind [$^3$H]5-HT with a high affinity in brain membranes. For this purpose, Cheng and Shih (1979) have synthesized a photosensitive arylazide derivative of 5-HT: nitroarylazidophenylserotonin (NAP-5-HT). Upon irradiation with ultraviolet light, [$^3$H]NAP-5-HT covalently attaches to protein components of brain membranes. Solubilization of proteins with 0.05 *M* sodium phosphate, pH 7.6, containing 1% sodium dodecylsulfate (SDS) followed by SDS–polyacrylamide gel electrophoresis revealed that three proteins (MW of 80,000, 49,000, and 38,000) were specifically labeled with the ligand (Cheng and Shih, 1979). Whether one of these proteins corresponds to the 5-HT$_1$ site is still an unanswered question at present.

The specific high-affinity binding of [$^3$H]5-HT in brain exhibits marked regional and interspecies differences. In all cases, these differences concern only the total number ($B_{max}$) of 5-HT$_1$ sites; their affinity for [$^3$H]5-HT remains equal

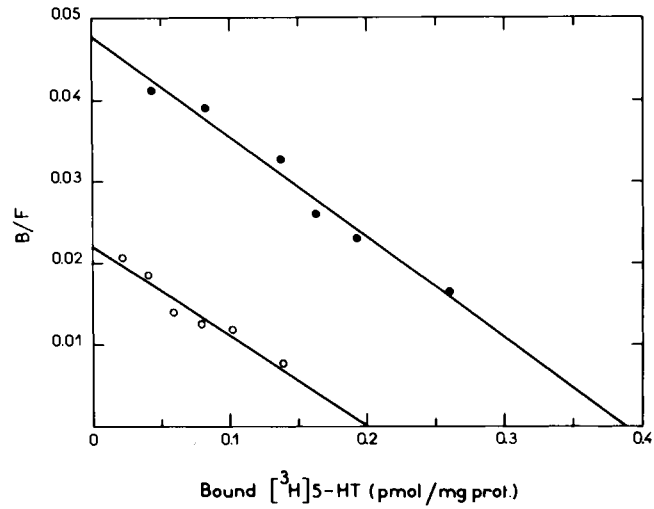


**Fig. 3.** Effect of *N*-ethylmaleimide on the specific binding of [$^3$H]5-HT to rat hippocampal membranes. Hippocampal membranes were incubated in 0.05 *M* Tris–HCl, pH 7.4, for 10 min at 37°C in the absence (●, control) or the presence (○) of 0.5 m*M* *N*-ethylmaleimide (NEM). They were collected by centrifugation, washed, and then incubated with various concentrations of [$^3$H]5-HT for binding assays. Scatchard plots indicate that NEM markedly reduced the $B_{max}$ without affecting the apparent $K_D$ of the 5-HT$_1$ site. Control conditions: $K_D$ = 3.23 n*M*, $B_{max}$ = 0.388 pmol/mg protein. NEM: $K_D$ = 3.63 *nM*, $B_{max}$ = 0.201 pmol/mg protein.

to 1.5–5 n*M*. In the rat, the density of [$^3$H]5-HT binding sites is highest in the hippocampus and decreases in the following order: hippocampus > striatum > cerebral cortex (Bennett and Snyder, 1976; Nelson *et al.*, 1978; Peroutka and Snyder, 1981b). In mice, the same number of 5-HT$_1$ sites is present in these three regions (Hamon *et al.*, 1984), and in man the order is inverted: frontal cortex > caudate–putamen > hippocampus (Heltzel *et al.*, 1981). However, in all species, the lowest binding capacity is found in the cerebellum. Recently, two groups investigated the regional distribution of 5-HT$_1$ sites in the rat brain using an autoradiographic procedure with either [$^3$H]5-HT (Biegon *et al.*, 1982) or *d*-[$^3$H]LSD (Meibach *et al.*, 1980) as the labeled ligand. Brain sections were incubated with the labeled molecule using the same medium as that selected for the optimal specific binding of [$^3$H]compounds to 5-HT$_1$ sites (see Section III,A,1). After a washing in ice-cold buffer, brain sections were dried and then placed against a tritium-sensitive film for 2 to 8 weeks before development. The data reported by Meibach *et al.* (1980) and Biegon *et al.* (1982) not only confirm but also extend those based on binding assays with membranes from various brain regions. As illustrated by their observations in the rat hippocampus, autoradiography is extremely useful for studying the relative concentrations of 5-HT$_1$ sites in discrete brain regions: indeed, the density of 5-HT$_1$ sites is not uniformly

high in this structure, since area $CA_3$ contains about 13 times fewer binding sites than do the subiculum or the dentate gyrus (Meibach *et al.*, 1980; Biegon *et al.*, 1982).

Autoradiographic as well as biochemical investigations confirmed that no correlation exists between the density of 5-$HT_1$ sites and the degree of serotoninergic innervation of a given brain area (Nelson *et al.*, 1978; Biegon *et al.*, 1982). Although this can be considered as a first argument against the presynaptic localization of 5-$HT_1$ sites, more direct support for this conclusion is derived from lesion experiments. Thus, electrolytical (Bennett and Snyder, 1976) as well as chemical (with an intracerebral injection of 5,7-dihydroxytryptamine, Nelson *et al.*, 1978) lesions of serotoninergic neurons do not reduce the number of high-affinity binding sites for [$^3$H]5-HT in any brain region examined. In the rat striatum, about 50% of 5-$HT_1$ sites disappear after the local injection of kainic acid, suggesting that these sites are situated on the postsynaptic intrinsic neurons (which degenerate after the treatment) (Hamon *et al.*, 1981a; Mallat and Hamon, 1982). The postsynaptic localization of 5-$HT_1$ sites is further confirmed by subcellular fractionation studies that show that the high-affinity binding of [$^3$H]5-HT is maximum in membrane fractions enriched in postsynaptic elements (Hamon *et al.*, 1981a).

## B. Is the 5-$HT_1$ Site a Receptor for 5-HT in the CNS?

At least two series of investigations must be performed in order to answer this question: (1) an analysis of the possible interaction of known 5-HT agonists and antagonists with the high-affinity binding of [$^3$H]5-HT and (2) a comparison of the affinity of 5-HT agonists and antagonists for the 5-$HT_1$ site with their relative efficacy in biological tests involving 5-HT receptors.

As shown in Table I, known 5-HT agonists and antagonists displace [$^3$H]5-HT from 5-$HT_1$ sites. Other pharmacological agents acting on various targets (MAO inhibitors, re-uptake blockers, depletors, etc.) are poorly active or inactive (Bennett and Snyder, 1976; Nelson *et al.*, 1978), strongly suggesting that the 5-$HT_1$ site in fact corresponds to a receptor. Analysis of the inhibition of [$^3$H]5-HT binding by various agonists and antagonists, using logit-log inhibition plots or Scatchard plots, indicate, however, that the interaction does not always involve a simple competition between a given drug and [$^3$H]5-HT (Nelson *et al.*, 1978; Fillion *et al.*, 1978). Such anomalies may result from the existence of several subclasses of 5-$HT_1$ sites with the same affinity for the labeled neurotransmitter but different affinities for other agonists and antagonists (Pedigo *et al.*, 1981). Alternatively, it can be proposed that allosteric changes in 5-$HT_1$ sites are induced by some (but not all) agonists and antagonists. Finally, due to large variations in the lipophilic character of drugs, surface phenomena may also complicate the analysis of drug–[$^3$H]5-HT interactions; in particular, a lipophilic

TABLE I

Displacement of Specifically Bound [$^3$H]5-HT and [$^3$H]Spiperone by Various Drugs[a]

| | $IC_{50}$ (n*M*) | | |
|---|---|---|---|
| | [$^3$H]5-HT (5-$HT_1$) | [$^3$H]Spiperone (5-$HT_2$) | log $R^b$ |
| Agonists[c] | | | |
| 5-HT | 4.0 | 7,240 | −3.26 |
| RU 24969 | 20.2 | 4,000 | −2.30 |
| 5-MDMT | 21.8 | 2,540 | −2.10 |
| Bufotenine | 25.1 | 1,330 | −1.72 |
| 8-OH-*N,N*-DPAT | 74.8 | 62,500 | −2.92 |
| TFMPP | 153 | — | — |
| MK-212 | 2,180 | — | — |
| Antagonists | | | |
| Metergoline | 7.8 | 9.0 | −0.06 |
| Methysergide | 89.0 | 31.5 | 0.45 |
| Methiothepin | 170 | 9.2 | 1.27 |
| Spiperone | 340 | 1.3 | 2.42 |
| Pizotifen | 387 | 25.0 | 1.19 |
| Mianserin | 447 | 20.0 | 1.35 |
| Cinanserin | 674 | 38.1 | 1.25 |
| Quipazine | 1,920 | 1,744 | 0.04 |
| Cyproheptadine | 2,170 | 28.3 | 1.88 |
| Mixed agonist–antagonist | | | |
| *d*-LSD | 8.2 | 12.5 | −0.18 |
| Indoles | | | |
| Iprindole | 12,800 | 1,585 | 0.91 |
| Harmaline | 24,000 | — | — |
| Harmalol | 29,500 | — | — |
| Other compounds | | | |
| Clozapine | 822 | 52.5 | 1.19 |
| Domperidone | 2,420 | 426 | 0.75 |
| Fluphenazine | 2,510 | 98.7 | 1.41 |
| Haloperidol | 16,000 | 128 | 2.10 |
| Fluoxetine | 18,190 | 2,100 | 0.94 |
| Dopamine | 19,500 | 234,000 | −1.08 |
| Norepinephrine | 180,000 | >1 m*M* | — |

[a]The specific binding of [$^3$H]5-HT was measured using crude membranes from the rat hippocampus and 2 n*M* of the labeled ligand. Crude membranes from the rat cerebral cortex were incubated with 1 n*M* of [$^3$H]spiperone to label 5-$HT_2$ sites. The concentration of each drug giving half maximal displacement of each [$^3$H]ligand ($IC_{50}$ in n*M*) was determined by log probit analysis. Values are the means of at least three determinations. The following compounds produced less than 20% displacement of either ligand at 10 μ*M*: harmine, tabernanthine, vincamine, morphine, fenfluramine, ticlopidine, reserpine.

[b]*R* is the ratio of the $IC_{50}$ value against [$^3$H]5-HT binding to that against [$^3$H]spiperone binding.

[c]5-MDMT, 5-methoxy-*N,N*-dimethyltryptamine; RU 24969, 5-methoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl) 1H indole; 8-OH-*N,N*-DPAT, 8-hydroxy-*N,N*-dipropyl-2-aminotetralin; TFMPP, trifluoromethylphenylpiperazine.

drug accumulates in membranes, so its concentration in the vicinity of 5-$HT_1$ sites is probably much higher than that in the assay medium.

Several biological responses evoked by 5-HT are currently measured for assessing the pharmacological profile of drugs on 5-HT receptors; they consist of the bilateral clonic seizures induced by tryptamine, the head twitches evoked by 5-hydroxytryptophan (5-HTP) or 5-methoxy-*N*,*N*-dimethyl tryptamine, and the 5-HT–induced vasoconstriction of rat caudal arteries (see Leysen *et al.*, 1982). Because no correlation exists between the affinity of drugs for 5-$HT_1$ sites and their ability to reproduce or to prevent any of these 5-HT–evoked responses, it can be concluded that 5-$HT_1$ sites do not correspond to the receptors which mediate these responses (Peroutka *et al.*, 1981; Leysen, 1981). This lack of correlation led even Laduron and Ilien (1982) to postulate that the 5-$HT_1$ site is more likely to represent a recognition site for indol-like compounds than a true receptor. However, indoles such as iprindole and harmala alkaloids (Table I) and even 5-hydroxyindoles, for instance, 5-hydroxytryptophol and 5-hydroxyindoleacetic acid (Bennett and Snyder, 1976), are almost completely inactive with regard to the high-affinity binding of [$^3$H]5-HT to brain membranes. In any case, conclusions based on the existence or the absence of correlations between *in vitro* binding data and the effects of drugs *in vivo* are often questionable. It can be recalled, for instance, that Young *et al.* (1974) observed that benzodiazepine potency *in vivo* is positively correlated to the ability to inhibit [$^3$H]strychnine binding to glycine receptors *in vitro* in spite of clear-cut evidence showing that *in vivo* effects of benzodiazepines do not involve these receptors. Presently, biological responses relatively far from the interaction of 5-HT with its receptor(s) are probably not appropriate for assessing the receptor function of the 5-$HT_1$ site.

Among the possible immediate cellular responses triggered by the interaction of 5-HT with the 5-$HT_1$ site, those dealing with adenylate cyclase activation and ionic fluxes have been particularly studied. As expected for a coupling of the 5-$HT_1$ site with adenylate cyclase (Rodbell, 1980), guanine nucleotides (GTP, GppNHp) have been shown to reduce its binding affinity for 5-HT and other agonists but not that for antagonists (Fig. 4, Peroutka *et al.*, 1979; Hamon *et al.*, 1980a,b; Mallat and Hamon, 1982). However, the comparison of the relative efficacy of agonists and antagonists on 5-HT–sensitive adenylate cyclase activity and on [$^3$H]5-HT high-affinity binding demonstrated that the 5-$HT_1$ site does not correspond to the 5-HT receptor associated with adenylate cyclase in brain membranes (Hamon *et al.*, 1980a,b; Nelson *et al.*, 1980a). Subsequent studies on the ontogenetic development, subcellular distribution (Nelson *et al.*, 1980b), and adaptive changes of 5-HT–sensitive adenylate cyclase activity and [$^3$H]5-HT high-affinity binding following 5,7-dihydroxytryptamine treatment (Hamon *et al.*, 1981b) confirmed that these two markers belong, in fact, to two distinct classes of central 5-HT receptors. In molluscs, several 5-HT receptors are functionally associated with ionophores controlling the membrane fluxes of various

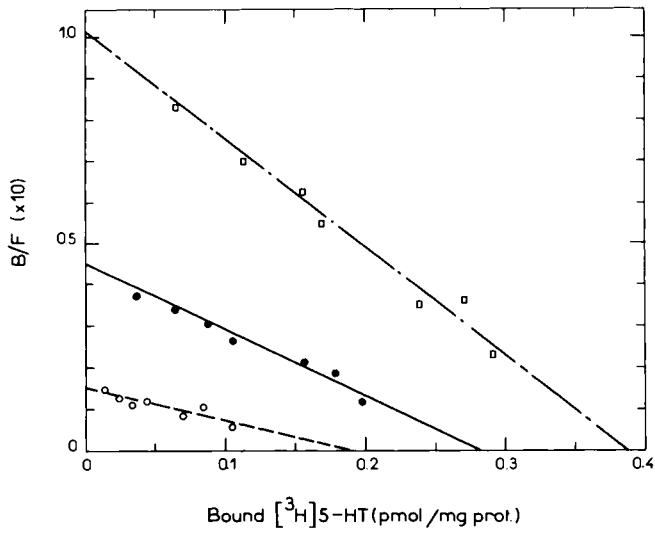


**Fig. 4.** Scatchard plots of [$^3$H]5-HT binding to rat hippocampal membranes in the absence (●, control) or the presence of 5 m*M* $CaCl_2$ (□) or 0.1 m*M* GTP (○). B/F corresponds to the ratio of specifically bound [$^3$H]5-HT (in pmol/mg membrane protein) to the quantity of labeled ligand remaining free in the assay mixture. Control conditions: $K_D$ = 3.18 n*M*, $B_{max}$ = 0.284 pmol/mg protein. GTP: $K_D$ = 6.28 n*M*, $B_{max}$ = 0.189 pmol/mg protein. $Ca^{2+}$: $K_D$ = 1.76 n*M*, $B_{max}$ = 0.387 pmol/mg protein.

ions (Gerschenfeld and Paupardin-Tritsch, 1974). The same situation probably occurs in vertebrates, since Segal and Gutnick (1980) reported that 5-HT evokes an efflux of $K^+$ in the rat hippocampus. This 5-HT–induced response was observed in hippocampal areas containing 5-$HT_1$ sites but not in the $CA_3$ stratum pyramidale, where these sites are almost absent (Meibach *et al.*, 1980; Biegon *et al.*, 1982). Further studies will be necessary, however, to demonstrate conclusively that the 5-HT receptor controlling the membrane flux of $K^+$ corresponds to the 5-$HT_1$ site.

Extensive studies on the *in vitro* and *in vivo* modulations of [$^3$H]5-HT high-affinity binding also support the idea that 5-$HT_1$ sites are, in fact, specific receptors for 5-HT in the CNS. Like receptor types (see Mallat and Hamon, 1982, for references), 5-$HT_1$ sites are affected in opposite directions by guanine nucleotides and di- or trivalent cations. In contrast to guanine nucleotides, di- and trivalent cations increase the affinity of 5-$HT_1$ sites for agonists (including 5-HT, see Fig. 4 for $Ca^{2+}$) without affecting their affinity for antagonists. These selective effects are very useful for assessing the possible agonist properties of a given compound (Hamon *et al.*, 1984): Using [$^3$H]5-HT as the labeled ligand, comparative measurements of $IC_{50}$ values of a given drug in the presence of GTP or of a divalent cation (for instance $Mn^{2+}$) allow one to detect whether this drug

TABLE II

$IC_{50}$ Values of Various 5-HT Agonists and Antagonists on the Specific Binding of [$^3$H]5-HT to Hippocampal Membranes: Influence of GTP (0.1 m*M*) and $Mn^{2+}$ (1 m*M*)[a]

| Compound | $IC_{50}$ GTP (n*M*) | $IC_{50}$ $Mn^{2+}$ (n*M*) | *R* |
|---|---|---|---|
| Agonists | | | |
| 5-HT | 22.3 | 1.5 | 14.9 |
| 5-MDMT | 71.9 | 8.1 | 8.9 |
| Bufotenine | 75.9 | 3.2 | 23.7 |
| RU 24969 | 130.2 | 5.4 | 24.1 |
| 8-OH-*N,N*-DPAT | 369.2 | 7.9 | 46.7 |
| TFMPP | 406.0 | 40.0 | 10.2 |
| Antagonists | | | |
| Metergoline | 12.4 | 17.4 | 0.7 |
| Methysergide | 102.6 | 61.7 | 1.7 |
| Methiothepin | 243.0 | 162.0 | 1.5 |
| Pizotifen | 366.0 | 516.0 | 0.7 |
| Quipazine | 1832 | 2094 | 0.9 |
| Other drugs | | | |
| *d*-LSD | 59.5 | 2.2 | 27.0 |

[a]Binding assays were carried out using 2 n*M* of [$^3$H]5-HT. $IC_{50}$ GTP refers to the concentration of a given compound reducing by half the specific [$^3$H]5-HT binding in the presence of 0.1 m*M* GTP; $IC_{50}$ $Mn^{2+}$ corresponds to the same parameter when 1 m*M* $MnCl_2$ replaced 0.1 m*M* GTP in the assay mixture. Each value (in m*M*) is the mean of at least three separate determinations. *R* is the ratio $IC_{50}$ GTP/$IC_{50}$ $Mn^{2+}$ for each compound. This ratio is $\geq 10$ when the displacing compound is an agonist. It does not significantly differ from 1.0 with antagonists. The determination of *R* is therefore a simple means for identifying the agonist properties of a new compound.

is an agonist or an antagonist; indeed, the ratio of $IC_{50}$ values determined in the presence of GTP ($IC_{50}$GTP) or $Mn^{2+}$ ($IC_{50}Mn^{2+}$) is $\geq 10$ with agonists, whereas it does not significantly differ from 1 with antagonists (Table II). In particular, this test confirms that quipazine is a 5-HT antagonist (Wallis, 1981). The agonist properties of quipazine *in vivo* do not involve a direct interaction with postsynaptic receptors but result from various presynaptic effects, all of which contribute to increasing the concentration of 5-HT in the synaptic cleft (Hamon *et al.*, 1976).

Since behavioral studies clearly indicate that 5-HT depletion can be associated with hypersensitivity to 5-HT agonists (Trulson *et al.*, 1976), attempts have been made to look for possible changes in 5-$HT_1$ sites under similar conditions. Bennett and Snyder (1976) observed that 5-HT depletion resulting from raphe lesion, reserpine, or *para*-chlorophenylalanine treatment promotes [$^3$H]5-HT binding by increasing the apparent affinity of 5-$HT_1$ sites for the labeled ligand. Such a change may well be explained by the persistence in brain membranes of

endogenous 5-HT interfering with [$^3$H]5-HT binding (see Nelson *et al.*, 1978). Thus, a reduction of this contaminant would produce a reduced competitive inhibition of [$^3$H]5-HT binding and therefore an apparent increase in the affinity of 5-$HT_1$ sites for the labeled ligand. Accordingly, reliable studies require the use of extensively washed membranes from which all compounds (including endogenous 5-HT) able to interfere with [$^3$H]5-HT binding are eliminated. Using such membranes, Nelson *et al.* (1978) and Seeman *et al.* (1980) observed a higher density of 5-$HT_1$ sites in the hippocampus after central serotoninergic neurons have degenerated. These findings indicate that 5-$HT_1$ sites comply with the general phenomenon of denervation supersensitivity, as do other functional receptors in brain.

The chronic blockade of 5-HT receptors by potent antagonists can also result in a marked increase in the number of [$^3$H]5-HT binding sites in brain membranes (Hamon *et al.*, 1980a; Samanin *et al.*, 1980). Behavioral supersensitivity to 5-HT agonists has been described under similar experimental conditions (Stolz and Marsden, 1982).

Conversely, the chronic administration of *d*-fenfluramine, a 5-HT releaser, decreases the $B_{max}$ of [$^3$H]5-HT high-affinity binding to brain membranes (Samanin *et al.*, 1980). Whether this simply results from the persistence of higher amounts of endogenous 5-HT firmly bound to membranes or from an actual reduction of the number of 5-$HT_1$ sites remains to be explored.

In conclusion, 5-$HT_1$ sites undoubtedly exhibit properties typical of true receptors. However, as long as the biological response induced by 5-HT interacting with these sites is not known, it cannot be definitively asserted that they are functional 5-HT receptors in the CNS.

## C. Characteristics of the 5-$HT_2$ Site

Whereas [$^3$H]spiperone binding to striatal membranes is more sensitive to dopamine-related drugs than to any other class of pharmacological agents, that observed using membranes from the frontal cortex or the hippocampus is even more easily displaced by 5-HT–related drugs than by dopamine agonists and antagonists (Leysen *et al.*, 1978). The first observation led to the proposal that [$^3$H]spiperone labels a 5-HT site in these two regions (see Hamon *et al.*, 1982, for references). In contrast to 5-$HT_1$ sites, which exhibit a high affinity for 5-HT and related agonists, those labeled by [$^3$H]spiperone have an apparent affinity only in the micromolar range for 5-HT and related agonists (Table I). Detailed analysis of the respective pharmacological characteristics of [$^3$H]5-HT and [$^3$H]spiperone binding sites clearly indicated that they are, in fact, distinct (Table I; Hamon *et al.*, 1980b; Leysen, 1981). Peroutka and Snyder (1979) proposed that [$^3$H]spiperone binding sites be designated the 5-$HT_2$ sites.

Comparison of the 5-HT sites labeled by [$^3$H]spiperone with those labeled by other 5-HT antagonists like [$^3$H]mianserin, [$^3$H]ketanserin, and [$^3$H]metergoline indicated that they probably correspond to the same 5-$HT_2$ site (Peroutka and Snyder, 1981a; Hamon *et al.*, 1982; Leysen *et al.*, 1982). The characteristics of *d*-[$^3$H]LSD binding to brain membranes are compatible with the involvement of both 5-$HT_1$ and 5-$HT_2$ sites. Indeed, like the labeled antagonists mentioned above, *d*-[$^3$H]LSD can be used as a specific ligand of 5-$HT_2$ sites provided that 5-$HT_1$ sites are occupied by a saturating concentration of 5-HT (0.3 $\mu M$) (Peroutka and Snyder, 1979).

Like the 5-$HT_1$ sites, 5-$HT_2$ sites are particularly concentrated in synaptic membranes (Hamon *et al.*, 1982). The selective degeneration of serotoninergic neurons does not reduce the number of [$^3$H]spiperone, [$^3$H]metergoline, and [$^3$H]mianserin binding sites in various brain regions, therefore demonstrating that 5-$HT_2$ sites are located in postsynaptic membranes (Seeman *et al.*, 1980; Hamon *et al.*, 1981a; Dumbrille-Ross *et al.*, 1981; Brunello *et al.*, 1982). Further comparison between 5-$HT_1$ and 5-$HT_2$ sites, however, reveals striking differences. For instance, *N*-ethylmaleimide exerts only discrete effects on [$^3$H]spiperone binding to membranes from the cerebral cortex (M.H., unpublished observation), whereas it dramatically reduces the density of 5-$HT_1$ sites in the same membrane preparation. In contrast to the binding mechanisms for 5-$HT_1$ sites, SH groups are, therefore, little (if at all) involved in the binding of [$^3$H]spiperone to 5-$HT_2$ sites in the cerebral cortex. However, this does not apply to all brain regions, since the specific binding of [$^3$H]spiperone to striatal membranes is markedly reduced following *N*-ethylmaleimide exposure (M.H., unpublished observation). Such regional differences further confirm that [$^3$H]spiperone binds to distinct receptors in the cerebral cortex (5-$HT_2$ site) and the striatum (mainly the dopamine $D_2$ site).

The distinction between 5-$HT_1$ and 5-$HT_2$ sites concerns also their location in brain. In the rat, for instance, the highest density of 5-$HT_2$ sites is found in the cerebral cortex, followed by the striatum and the hippocampus (Peroutka and Snyder, 1981b). This order is the reverse of that noted for 5-$HT_1$ sites (see Section IV,A). Like the regional distribution of 5-$HT_1$ sites, that of 5-$HT_2$ sites is not correlated with the topography of serotoninergic terminals in the CNS (Peroutka and Snyder, 1981b).

Successful solubilization of 5-$HT_2$ binding sites has been reported recently. Ilien *et al.* (1980) mentioned that the microsomal fraction of frontal cortex treated with 0.25% lysolecithin for 15 min at 0°C releases material that cannot be sedimented at 182,000 *g* for 60 min but exhibits nanomolar affinity for [$^3$H]spiperone. The relative potency of 5-HT agonists and antagonists in inhibiting [$^3$H]spiperone binding to this solubilized material corresponds exactly to that previously noted on 5-$HT_2$ sites in membranes (Ilien *et al.*, 1980). Although these observations confirm that the 5-$HT_2$ site is an intrinsic protein included in

membranes (Laduron and Ilien, 1982), no data concerning the physicochemical properties of this protein have been reported so far.

### D. Is the 5-$HT_2$ Site a Receptor for 5-HT in the CNS?

As is the case for 5-$HT_1$ sites, the drugs displacing [$^3$H]ligands from the 5-$HT_2$ sites are those exhibiting 5-HT agonist and/or antagonist properties in various *in vivo* tests. However, in contrast to 5-$HT_1$ sites, which have higher affinity for agonists, the 5-$HT_2$ sites preferentially bind the antagonists (Table I). Logit-log inhibition plots indicate that 5-HT agonists generally displace bound [$^3$H]spiperone in a complex fashion with Hill coefficients lower than one (Hamon *et al.*, 1980b). As for 5-$HT_1$ sites, such anomalies may reflect the existence in cortical membranes of several [$^3$H]spiperone binding sites having the same affinity for the labeled ligand but different affinities for 5-HT agonists. Alternatively, they may be ascribed to allosteric changes or surface phenomena. Indeed, agonists are generally much more hydrophilic than [$^3$H]spiperone (and other antagonists) so that their respective interactions with biological membranes exhibit marked differences.

Whereas the pharmacological characteristics of 5-$HT_1$ sites do not correspond to those established for 5-HT receptors mediating various *in vivo* responses (see Section IV,B), positive correlations are found between the relative efficacy of 5-HT agonists or antagonists to displace bound [$^3$H]spiperone and their ability to reproduce or prevent 5-HT–evoked responses such as the contractions of isolated rat caudal arteries and fundus strip, clonic seizures, and head-twitches in rats (Leysen, 1981; Peroutka *et al.*, 1981). Such correlations strongly suggest, but do not demonstrate, that 5-$HT_2$ sites probably correspond to some kind of central and peripheral 5-HT Receptors.

Although numerous findings indicate that 5-$HT_2$ sites are not coupled to adenylate cyclase in brain membranes (Hamon *et al.*, 1980b; Peroutka *et al.*, 1981), several authors observed that guanine nucleotides can reduce their affinity for 5-HT agonists (Hamon *et al.*, 1980b; Rosenfeld and Makman, 1981). This is illustrated in Fig. 5, which shows that GTP significantly decreases the ability of 5-HT to displace [$^3$H]metergoline from 5-$HT_2$ sites in the rat forebrain. However, the effect of GTP is generally less pronounced on 5-$HT_2$ than on 5-$HT_1$ sites; this is probably the reason that Peroutka *et al.* (1979) failed to detect any significant reduction of the affinity of 5-$HT_2$ sites for agonists in the presence of GTP. In any case, this modulation by GTP cannot be considered as a proof of the possible coupling of 5-$HT_2$ sites with adenylate cyclase, since the guanine nucleotide-induced reduction in the site affinity is maximum for an agonist, RU 24969, which does not interact with 5-HT–sensitive adenylate cyclase (Euvrard and Boissier, 1980).

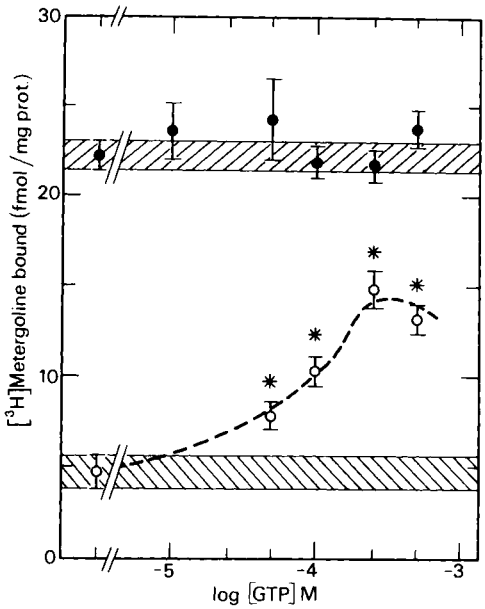


**Fig. 5.** Effects of GTP on the 5-HT-induced inhibition of [$^3$H]metergoline binding to microsomal forebrain membranes. Microsomal membranes from the rat forebrain were incubated with 0.15 n*M* of [$^3$H]metergoline and various concentrations of GTP in the absence (●) or the presence (○) of 8 μ*M* 5-HT (dashed line). Although GTP does not affect the specific binding of [$^3$H]metergoline, it significantly reduces the inhibitory effect of 5-HT on this binding. Asterisk (*) indicates $p < .05$ when compared to [$^3$H]metergoline specifically bound in the presence of 8 μ*M* 5-HT alone. The ranges of [$^3$H]metergoline specifically bound in the absence (●) and in the presence (○) of 8 μ*M* 5-HT are indicated by hatched areas.

If 5-HT$_2$ sites correspond to functional receptors, *in vivo* modulations of their characteristics should take place following alterations in central serotoninergic synapses. Indeed, the selective degeneration of serotoninergic neurons following chemical or electrolytical raphe lesions produces in rats a significant increase in the density of specific sites labeled by [$^3$H]mianserin (Brunello *et al.*, 1982) and [$^3$H]metergoline (Hamon *et al.*, 1981a) in the hippocampus. These findings indicate that 5-HT$_2$ sites comply with the general phenomenon of denervation supersensitivity. Conversely, chronic treatments with drugs promoting 5-HT neurotransmission provoke a significant reduction in the number of 5-HT$_2$ sites in brain membranes, particularly in the frontal cortex (Peroutka and Snyder, 1980; Enna *et al.*, 1981; Blackshear and Sanders-Bush, 1982). Since these drugs are either 5-HT uptake blockers or MAO inhibitors, that is, antidepressants, it is generally proposed that antidepressant therapy might be associated with down regulation of 5-HT$_2$ sites in the frontal cortex (Peroutka and Snyder, 1980; Enna *et al.*, 1981). Several observations, however, contradict this hypothesis. First,

another potent antidepressant treatment, electroconvulsive shock, actually increases the density of 5-$HT_2$ sites in the cerebral cortex (Kellar *et al.*, 1981; Vetulani *et al.*, 1981). Second, therapeutic effects of antidepressants generally occur only after 2 weeks of treatment, but similar apparent down regulation of 5-$HT_2$ sites can be observed after acute or chronic administration of mianserin, for instance (Blackshear and Sanders-Bush, 1982). In most cases, antidepressants that decrease the number of 5-$HT_2$ sites are also those that interfere directly with the binding of the labeled ligand to these sites (mianserin, amitriptyline, desipramine) (see Dumbrille-Ross *et al.*, 1981; Peroutka *et al.*, 1981). Accordingly, the reduction in the number of 5-$HT_2$ sites might well result simply from the accumulation of these drugs in brain membranes following chronic administration and not from a true down regulation (see Hamon *et al.*, 1984). Recent observations by Blackshear and Sanders-Bush (1982) strongly suggest that the so-called down regulation of 5-$HT_2$ sites following mianserin treatment is, in fact, the consequence of the direct inhibition of [$^3$H]spiperone binding by mianserin accumulated in brain membranes. Therefore, antidepressants like mianserin (and amitriptyline) can be considered as slowly reversible 5-HT antagonists, and the recovery of 5-$HT_2$ sites after the cessation of *in vivo* administration probably depends on the turnover rate of these sites. Calculations from data reported by Blackshear and Sanders-Bush (1982), using mianserin as the *in vivo* blocker, give a half life of about 3.3 days for 5-$HT_2$ sites in the rat frontal cortex. Similarly, the rate of recovery of 5-$HT_2$ sites following the chronic blockade by amitriptyline (Peroutka and Snyder, 1980) is compatible with a half life of 2.8 days. Both values are very close to that (3 days) calculated by following the reappearance of [$^3$H]spiperone binding to cortical membranes after the administration of a slowly reversible 5-HT antagonist (with no antidepressant action): methiothepin (Nelson *et al.*, 1979; Hamon *et al.*, 1982).

5-$HT_2$ sites undoubtedly exhibit properties of typical receptors in the CNS. However, some of their *vivo* modulations, which have been taken as proof of their functional significance seem artifactual.

## V. ADVANTAGES AND PITFALLS OF BINDING MEASUREMENTS FOR STUDYING CENTRAL 5-HT RECEPTORS

Owing to the simplicity of binding assays, an abundant literature has been devoted to studies on central 5-HT receptors during the last 6 years. One of the most important findings so far is the discovery of at least two separate binding sites for 5-HT, 5-$HT_1$, and 5-$HT_2$, with different regional distributions and pharmacological properties. However, neither of these sites apparently corre-

sponds to the receptor coupled to adenylate cyclase. Furthermore, no clear correspondence can be found between these binding sites and the receptors involved in the feedback control of raphe neurons (Haigler and Aghajanian, 1977). Finally, binding studies have not yet allowed the identification of presynaptic 5-HT receptors, although numerous lines of evidence support their functional implication in the control of 5-HT release (Hamon *et al.*, 1984). For technical reasons, binding studies are only useful for receptors with a very high affinity for the ligand ($K_D$ in the nanomolar range). In most cases, receptors with $K_D$ in the micromolar range cannot be investigated because specific binding is generally too low relative to the nonspecific binding, if micromolar concentrations of a labeled ligand are used. This explains why the 5-HT receptor involved in the activation of adenylate cyclase by micromolar concentrations of the indoleamine (Nelson *et al.*, 1980a) cannot be detected in binding assays with [$^3$H]5-HT. Binding studies are, therefore, extremely useful for defining the biochemical and pharmacological characteristics of some but not all receptors.

In addition to *in vitro* studies devoted to the identification of 5-$HT_1$ and 5-$HT_2$ sites and the analysis of their pharmacological properties, binding assays are also extremely useful for exploring the possible *in vivo* modulations of receptors after lesion and/or drug administration. However, numerous conditions must be fulfilled in order to make reliable observations in this respect. For instance, 5-HT remains firmly bound to brain membranes (Nelson *et al.*, 1978), and care must be taken to remove all endogenous 5-HT contaminating the membranes when comparing [$^3$H]5-HT binding to 5-$HT_1$ sites in tissues with different levels of the indoleamine (notably after the lesion of serotoninergic neurons). Numerous drugs administered *in vivo* slowly dissociate from brain membranes and can affect directly the ligand binding *in vitro*. In all cases, control experiments must be carried out to check for the possible persistence in brain membranes of endogenous and exogenous compounds able to interfere with the ligand binding *in vitro*. Discrepancies in the literature can very often be explained by differences in the techniques used for preparing the membranes. Depending on the washing procedure, more or less 5-HT or drugs persist in membranes, thus affecting ligand binding to various extents.

The major pitfall of binding studies concerns their lack of immediate physiological significance. Indeed, almost nothing is known of the biological consequence(s) of 5-HT binding to 5-$HT_1$ or 5-$HT_2$ sites and, indeed, only indirect evidence suggests that these sites really correspond to receptors. New approaches must be developed for studying 5-HT receptors and identifying the biological responses in cells bearing these 5-$HT_1$ and 5-$HT_2$ sites. Investigations of the effects of 5-HT agonists and antagonists on various parameters including ion fluxes through membranes, phospholipid metabolism, glycogenolysis, etc., should be of decisive interest to confirm that binding assays are really relevant for exploring central 5-HT *receptors*.

To conclude, several positive points clearly demonstrate that ligand binding studies were, are, and will be particularly fruitful for studying receptors. First, binding studies markedly simplify the search for new agonists and antagonists. Indeed, a rapid *in vitro* test consisting of measuring the displacement of bound [$^3$H]5-HT in the presence of either GTP or $Mn^{2+}$ was sufficient to establish that 8-hydroxy-*N*,*N*-dipropyl-2-amino tetralin is a potent central 5-HT agonist (Hamon *et al.*, 1984). In addition, the measurement of a bound radioactive ligand is undoubtedly the simplest way to follow 5-HT receptors in the course of solubilization and purification procedures. Therefore, this technique should permit the extensive study of the physicochemical characteristics of central 5-HT receptors in the future. Finally, experiments with radioactive ligands injected into rats and mice revealed that *in vivo* binding assays can be used to study central 5-HT receptors. Positron emission by [$^{11}$C]ligands has been used recently to investigate central receptors by external tomography in the baboon (Comar *et al.*, 1981). Such *in vivo* binding measurements are of great potential utility for exploring cerebral neurotransmitter (including 5-HT) receptors in cases of mental and neurological diseases in man.

## REFERENCES


Battaglia, G., and Titeler, M. (1981). *Life Sci.* **29,** 909–916.

Bennett, J. P., Jr., and Snyder, S. H. (1975). *Brain Res.* **94,** 523–544.

Bennett, J. P., Jr., and Snyder, S. H. (1976). *Mol. Pharmacol.* **12,** 373–389.

Biegon, A., Rainbow, T. C., and McEwen, B. S. (1982). *Brain Res.* **242,** 197–204.

Blackshear, M. A., and Sanders-Bush, E. (1982). *J. Pharmacol. Exp. Ther.* **221,** 303–308.

Bloom, F. E., Hoffer, B. J., Siggins, G. R., Barker, J. L., and Nicoll, R. A. (1972). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **31,** 97–106.

Brunello, N., Chuang, D. M., and Costa, E. (1982). *Science* **215,** 1112–1115.

Cheng, S. H., and Shih, J. C. (1979). *Life Sci.* **25,** 2197–2203.

Clements-Jewery, S., and Robson, P. A. (1980). *Neuropharmacology* **19,** 657–661.

Comar, D., Mazière, M., Cepeda, C., Godot, J-M., Menini, C., and Naquet, R. (1981). *Eur. J. Pharmacol.* **75,** 21–26.

Cotman, C. W., and Matthews, D. A. (1971). *Biochim. Biophys. Acta* **249,** 380–394.

Duchemin, A.-M., Quach, T. T., Rose, C., and Schwartz, J.-C. (1979). *Life Sci.* **24,** 401–410.

Dumbrille-Ross, A., Tang, S. W., and Coscina, D. V. (1981). *Life Sci.* **29,** 2049–2058.

Enna, S. J., Mann, E., Kendall, D., and Stancel. G. M. (1981). *In* "Antidepressants: Neurochemical, Behavioral and Clinical Perspectives" (S. J. Enna, J. B. Malick, and E. Richelson, eds.), pp. 91–105. Raven Press, New York.

Euvrard, C., and Boissier, J. R. (1980). *Eur. J. Pharmacol.* **63,** 65–72.

Fillion, G. M. B., Rousselle, J.-C., Fillion, M.-P., Beaudoin, D. M., Goiny, M. R., Deniau, J. M., and Jacob, J. J. (1978). *Mol. Pharmacol.* **14,** 50–59.

Gerschenfeld, H. M., and Paupardin-Tritsch, D. (1974). *J. Physiol. (London)* **243,** 427–456.

Gerschenfeld, H. M., Hamon, M., and Paupardin-Tritsch, D. (1978). *J. Physiol. (London)* **274,** 265–278.

Haigler, H. J., and Aghajanian, G. K. (1977). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **36,** 2159–2164.
Hamon, M., Bourgoin, S., Enjalbert, A., Bockaert, J., Héry, F., Ternaux, J. P., and Glowinski, J. (1976). *Naunyn-Schmiedeberg's Arch. Pharmacol.* **294,** 99–108.
Hamon, M., Nelson, D. L., Herbet, A., Bockaert, J., and Glowinski, J. (1980a). *Monogr. Neural Sci.* **7,** 161–175.
Hamon, M., Nelson, D. L., Herbet, A., and Glowinski, J. (1980b). *In* "Receptors for Neurotransmitters and Peptide Hormones" (G. Pepeu, M. J. Kuhar, and S. J. Enna, eds.), pp. 223–233. Raven Press, New York.
Hamon, M., Mallat, M., Herbet, A., Nelson, D. L., Audinot, M., Pichat, L., and Glowinski, J. (1981a). *J. Neurochem.* **36,** 613–626.
Hamon, M., Nelson, D. L., Mallat, M., and Bourgoin, S. (1981b). *Neurochem. Int.* **3,** 69–79.
Hamon, M., Bourgoin, S., El Mestikawy, S., and Goetz, C. (1984). *In* "Handbook of Neurochemisty" (A. Lajtha, ed.). Plenum, New York (in press).
Heltzel, J. A., Boehme, D. H., and Vogel, W. H. (1981). *Brain Res.* **204,** 451–454.
Ilien, B., Gorissen, H., and Laduron, P. (1980). *Biochem. Pharmacol.* **29,** 3341–3344.
Kellar, K. J., Cascio, C. S., Butler, J. A., and Kurtzke, R. N. (1981). *Eur. J. Pharmacol.* **69,** 515–518.
Laduron, P. M., and Ilien, B. (1982). *Biochem. Pharmacol.* **31,** 2145–2151.
Laduron, P. M., Verwimp, M. F., Janssen, P. F. M., and Gommeren, W. R. (1975). *Biochimie* **57,** 253–260.
Leysen, J. E. (1981). *J. Physiol. (Paris)* **77,** 351–362.
Leysen, J. E., Niemegeers, C. J. E., Tollenaere, J. P., and Laduron, P. M. (1978). *Nature (London)* **272,** 168–171.
Leysen, J. E., Awouters, F., Kennis, L., Laduron, P. M., Vandenberk, J., and Janssen, P. A. J. (1981). *Life Sci.* **28,** 1015–1022.
Leysen, J. E., Niemegeers, C. J. E., Van Nueten, J. M., and Laduron, P. M. (1982). *Mol. Pharmacol.* **21,** 301–314.
Mallat, M., and Hamon, M. (1982). *J. Neurochem.* **38,** 151–161.
Meibach, R. C., Maayani, S., and Green, J. P. (1980). *Eur. J. Pharmacol.* **67,** 371–382.
Nelson, D. L., Herbet, A., Bourgoin, S., Glowinski, J., and Hamon, M. (1978). *Mol. Pharmacol.* **14,** 983–995.
Nelson, D. L., Herbet, A., Pichat, L., Glowinski, J., and Hamon, M. (1979). *Naunyn-Schmiedeberg's Arch. Pharmacol.* **310,** 25–33.
Nelson, D. L., Herbet, A., Enjalbert, A., Bockaert, J., and Hamon, M. (1980a). *Biochem. Pharmacol.* **29,** 2445–2453.
Nelson, D. L., Herbet, A., Adrien, J., Bockaert, J., and Hamon, M. (1980b). *Biochem. Pharmacol.* **29,** 2455–2463.
Pedigo, N. W., Yamamura, H. I., and Nelson, D. L. (1981). *J. Neurochem.* **36,** 220–226.
Peroutka, S. J., and Snyder, S. H. (1979). *Mol. Pharmacol.* **16,** 687–699.
Peroutka, S. J., and Snyder, S. H. (1980). *J. Pharmacol. Exp. Ther.* **215,** 582–587.
Peroutka, S. J., and Snyder, S. H. (1981a). *J. Pharmacol. Exp. Ther.* **216,** 142–148.
Peroutka, S. J., and Snyder, S. H. (1981b). *Brain Res.* **208,** 339–347.
Peroutka, S. J., Lebovitz, R. M., and Snyder, S. H. (1979). *Mol. Pharmacol.* **16,** 700–708.
Peroutka, S. J., Lebovitz, R. M., and Snyder, S. H. (1981). *Science* **212,** 827–829.
Rodbell, M. (1980). *Nature (London)* **284,** 17–22.
Rosenfeld, M. R., and Makman, M. H. (1981). *J. Pharmacol. Exp. Ther.* **216,** 526–531.
Samanin, R., Mennini, T., Ferraris, A., Bendotti, C., and Borsini, F. (1980). *Brain Res.* **189,** 449–457.
Seeman, P., Westman, K., Coscina, D., and Warsh, J. J. (1980). *Eur. J. Pharmacol.* **66,** 179–191.
Segal, M., and Gutnick, M. J. (1980). *Brain Res.* **195,** 389–401.

Stolz, J. F., and Marsden, C. A. (1982). *Eur. J. Pharmacol.* **79,** 17–22.
Trulson, M. E., Eubanks, E. E., and Jacobs, B. L. (1976). *J. Pharmacol. Exp. Ther.* **198,** 23–32.
Vetulani, J., Lebrecht, U., and Pilc, A. (1981). *Eur. J. Pharmacol.* **76,** 81–85.
Wallis, D. (1981). *Life Sci.* **29,** 2345–2355.
Whitaker, P. M., and Seeman, P. (1978). *Proc. Natl. Acad. Sci. U.S.A.* **75,** 5783–5787.
Young, A. B., Zukin, S. R., and Snyder, S. H. (1974). *Proc. Natl. Acad. Sci. U.S.A.* **71,** 2246–2250.

# *Chapter 18*

# *MUSCARINIC RECEPTOR [$^3$H]LIGAND BINDING METHODS*

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## I. INTRODUCTION

Initially, studies of the interaction of drugs with the muscarinic receptor were usually restricted to measuring the effects of cholinergic analogs on whole tissues. In recent years, the availability of radiolabeled cholinergic drugs of high specific activity has permitted the direct measurement of the binding of [$^3$H]ligands to the muscarinic receptor. Muscarinic receptor binding assays can be conveniently run on a variety of cholinergically innervated tissues including brain, secretory glands, and smooth and cardiac muscle, making the method suitable for a broad range of pharmacological studies. When ligand binding methods are used in conjunction with other pharmacological procedures, the information gained from such studies can be helpful in drawing specific conclu-

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ISBN 0-12-470350-X

sions about drug–receptor interactions. The realization of the full power and utility of [$^3$H]ligand binding assays requires an understanding of the basic principles governing ligand–receptor interactions and the nature of the binding sites that are likely to be encountered in various tissues during the course of pharmacological investigation. In the present chapter, we have outlined a few important considerations regarding the methodology of muscarinic receptor binding assays and have described the complex, heterogeneous nature of the muscarinic receptor. An understanding of the multisite nature of muscarinic receptor binding properties is required for an accurate interpretation of muscarinic receptor binding data. Where appropriate, we have also discussed the significance of the complex binding properties of the muscarinic receptor.

## II. IDENTIFICATION OF MUSCARINIC RECEPTORS USING [$^3$H]LIGANDS

In all receptor studies using radioligand binding methods, it is necessary to demonstrate that the specific binding site under investigation has characteristics of a pharmacologically relevant receptor. Thus, the investigator must show a good correlation between the values of the affinity constants of a series of agonists and antagonists for the specific binding site to which the radioligand binds and the affinity constants of the same series of drugs as measured by pharmacological methods. With regard to muscarinic receptors, the availability of convenient whole tissue assay systems has provided a means for such comparisons, so that the potency of drugs for influencing smooth muscle contraction in an intact tissue can be compared with the affinity constant as measured by radioligand binding in homogenates of the same tissue. In an early study of the binding of the muscarinic antagonist, 3-[$^3$H]quinuclidinyl benzilate ([$^3$H]QNB), to muscarinic receptors in the guinea pig ileum, Yamamura and Snyder (1974a) demonstrated good agreement between the potency of agonists and antagonists for inhibition of [$^3$H]QNB binding and the affinity constants of the same drugs as determined by pharmacological procedures. In another ligand binding study of rat brain muscarinic receptors (Birdsall *et al.*, 1977), the measured affinity constants of 18 antagonists were nearly identical to those values determined by pharmacological antagonism of smooth muscle contraction in the guinea pig ileum.

In addition to the drug potency characteristics described above, the binding sites for muscarinic [$^3$H]antagonists display other characteristics that are indicative of a pharmacological receptor. For example, the binding of [$^3$H]antagonists is saturable with increasing ligand concentration and, in most instances, it displays a remarkable consistency with the consequences of the law of mass action

for a single class of independent receptors (Birdsall and Hulme, 1976). The binding is stereospecific and sensitive to inhibition by drugs that are thought to interact with muscarinic receptors but not by drugs that lack direct muscarinic cholinergic effects (Paton and Rang, 1965; Beld and Ariens, 1974; Yamamura and Snyder, 1974a,b). The regional distribution of [$^3$H]antagonist binding is consistent with concepts of cholinergic innervation, and within the brain it generally parallels other elements of the cholinergic nervous system including acetylcholinesterase, choline acetyltransferase, and high-affinity choline uptake (Yamamura *et al.*, 1974; Snyder *et al.*, 1975; Hiley and Burgen, 1974). Thus, taken together, the results described above provide compelling evidence that the binding of muscarinic [$^3$H]antagonists represents a specific interaction with the muscarinic receptor.

## III. SOME COMMENTS ON MUSCARINIC RECEPTOR [$^3$H]LIGAND BINDING METHODS

### A. Introduction

The most common techniques for measuring [$^3$H]ligand binding to the muscarinic receptor are the filtration and centrifugation methods. Briefly, these methods involve incubation of tissue homogenates or membrane fractions thereof with labeled ligand, and membrane bound ligand is subsequently trapped by filtration or centrifugation of the membranes into a pellet. The competitive inhibition of [$^3$H]ligand binding and the extent of nonspecific binding can be assessed in the usual manner by coincubation of tissue and radioligand with the appropriate nonlabeled drug. In our hands, we find that the filtration method for [$^3$H]QNB is unsurpassed as a rapid technique that maintains accuracy and very low nonspecific binding. The method enjoys widespread use and detailed descriptions of the procedure occur in the literature (Fields *et al.*, 1978).

The procedure for measuring [$^3$H]QNB binding is not invariant but obviously depends on the experimental objective. The following description gives the general features of the assay. Aliquots of tissue homogenate (5–40 mg original wet tissue weight/ml 0.05 *M* $Na_2HPO_4$–$KH_2PO_4$ buffer) are incubated in triplicate tubes with [$^3$H]QNB in a final volume of 2 ml containing 0.05 *M* $Na_2HPO_4$–$KH_2PO_4$ buffer, pH 7.4, for 1 hr at 37°C. A parallel system of tubes are run containing, in addition, $10^{-6}$ *M* atropine. Following the incubation, bound [$^3$H]QNB is trapped by rapid vacuum filtration of the incubation mixture over Whatman glass fiber filters (GF/B). The filters are washed with three aliquots (5 ml of 0.05 *M* $Na_2HPO_4$–$KH_2PO_4$ buffer. Subsequently, the filters are placed in scintillation vials and scintillation cocktail is added. After allowing suitable time for the [$^3$H]QNB trapped on the filters to be extracted into the solvent, the

radioactivity in each sample is determined by liquid scintillation spectrophotometry. Specific [$^3$H]QNB binding is calculated as the difference between binding measurements made in the presence and absence of $10^{-6}$ *M* atropine.

When using such a highly potent ligand as [$^3$H]QNB, the experimenter must take certain precautions to insure that artifacts do not influence the data. Most of these precautions are directed to the slow approach to equilibrium and changes in the free [$^3$H]ligand concentration. Thus, in the following section, we have identified some possible sources of error that might be encountered in [$^3$H]QNB binding assays. We have also included a brief description of [$^3$H]agonist binding methods.

## B. Slow Approach to Equilibrium

Because [$^3$H]QNB is a highly potent [$^3$H]ligand having a very small rate constant for dissociation, the time required to achieve equilibrium binding can be relatively long. Usually, a 60 min incubation at 37°C is adequate for equilibration. It should be emphasized that the time required for equilibrium depends on the concentration of the [$^3$H]ligand. Figure 1 illustrates this relationship between [$^3$H]ligand binding and incubation time for a series of different [$^3$H]ligand concentrations. The theoretical curves were calculated assuming the simple law of mass action:

$$[D] + [R] \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} [DR] \tag{1}$$

If we restrict our attention to conditions where the drug concentration remains constant during the binding reaction ([D]>>[R]), then the equation which describes drug-bound receptor concentration ([$DR_t$]) as a function of time ($t$) is

$$[DR_t] = \frac{[D][R_T]}{[D] + K_D}(1 - e^{-(k_1[D] + k_{-1})t}) \tag{2}$$

in which [$R_T$] is the total receptor concentration ([R] + [DR]) and $K_D$ is the equilibrium dissociation constant ($K_D = k_{-1}/k_{+1}$). It can be seen in Fig. 1 that the time required to reach equilibrium increases as the concentration of the [$^3$H]ligand decreases. Thus, to decide upon adequate incubation conditions for equilibrium binding, the investigator must determine the time required to achieve equilibrium with the lowest [$^3$H]ligand concentration. If equilibrium is reached with the lowest ligand concentration, then it will certainly be reached with the higher concentrations, but not necessarily the converse. Figure 2 shows what happens to the Scatchard plot when equilibrium is not achieved. It is apparent

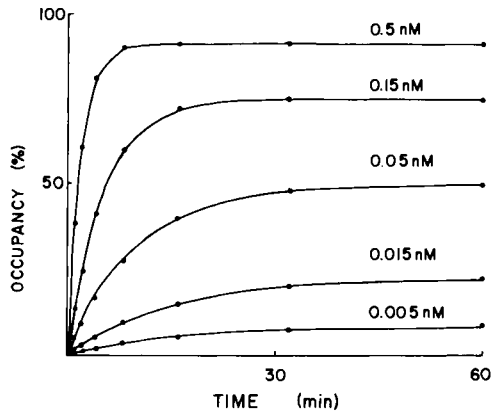


**Fig. 1.** Effect of incubation time on the binding of an [$^3$H]ligand to a hypothetical receptor. The theoretical curves were calculated for the indicated [$^3$H]ligand concentrations according to Eq. (2) in the text. [$^3$H]Ligand binding is expressed as a percentage of the total receptor concentration. For the computations, $k_1 = 10^9/M$ and $k_{-1} = 5 \times 10^{-2}$/min.

that an underestimation of affinity and a suggestion of positive cooperativity can result from nonequilibrium conditions.

Another situation in which incomplete attainment of equilibrium can lead to erroneous conclusions is in competitive inhibition experiments. This topic has been discussed by several investigators (Berson and Yalow, 1959; Rodbard *et al.*, 1971; Bouton and Raynaud, 1978), particularly with regard to radioimmunoassays. The behavior of the competition curve before equilibrium depends

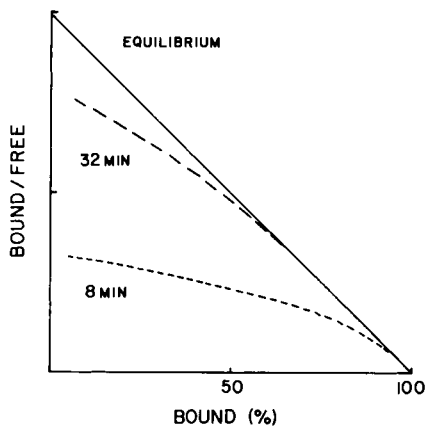


**Fig. 2.** Effect of incomplete equilibrium on Scatchard analysis of [$^3$H]ligand binding. The dashed lines represent Scatchard plots of preequilibrium binding isotherms, which were computed at the indicated times. The kinetic constants are the same as in the legend of Fig. 1.

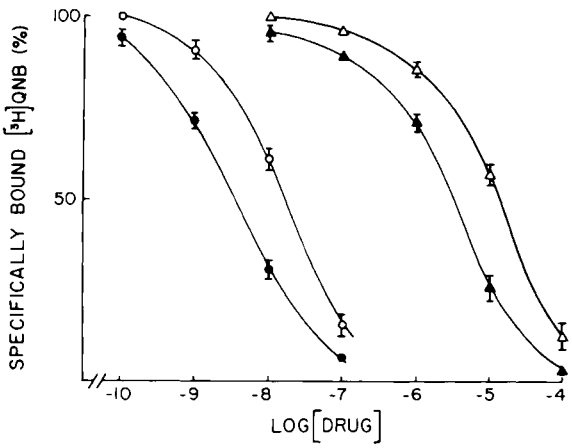


**Fig. 3.** Effect of incubation time on the competitive inhibition of [$^3$H]QNB binding by atropine (○) and oxotremorine (△). Incubations were carried out for 10 min (●,▲) and 60 min (○,△). Mean binding values ± SEM of four experiments are shown. The data are from Ehlert *et al.* (1981).

upon the initial assay conditions. During most [$^3$H]ligand binding experiments, the incubation is initiated by tissue addition, so that receptor, [$^3$H]ligand, and competitive inhibitor are brought together in unison. After such starting conditions, the competition curve shifts to the right or left, depending upon the ratio of dissociation rate constants ($k_{-1}/k_{-2}$) of the two competing ligands (Aranyi, 1980; Ehlert *et al.*, 1981). Since [$^3$H]QNB dissociates so slowly from the receptor, the dissocation rate constant of the competitive inhibitor is usually greater ($k_{-2} > k_{-1}$), and for such conditions, the kinetics of simple competitive inhibition predict that the competition curve should shift to the right as equilibrium is approached. Figure 3 shows the results of oxotremorine–[$^3$H]QNB and atropine–[$^3$H]QNB competition experiments under equilibrium and preequilibrium conditions corresponding to incubation times of 60 min and 10 min, respectively. It is apparent that the equilibrium curves have $IC_{50}$ values that are approximately five times greater than those measured before equilibrium. This kind of behavior is to be expected when the competitive inhibitor dissociates from the receptor more rapidly than the [$^3$H]ligand. If the dissociation rate constant of the competitive inhibitor is the same or smaller than that of the [$^3$H]ligand, then the competition curve can shift to the left as equilibrium is approached (Aranyi, 1980; Ehlert *et al.*, 1981). It is clear from the foregoing that an accurate estimate of the affinity of a competing ligand can only be made if the data are collected under equilibrium conditions.

## C. Depletion of the Free [$^3$H]Ligand Concentration

In all radioligand binding assays, consideration should be given to possible complications arising from depletion of the free [$^3$H]ligand concentration. This

concern is particularly important when highly potent [$^3$H]ligands like [$^3$H]QNB are used, since the dissociation constant of the [$^3$H]ligand is in the range of the receptor concentration normally encountered in the binding assay. Thus, when assays are carried out using [$^3$H]ligand concentrations below or in the range of the dissociation constant, a considerable portion of the total [$^3$H]ligand concentration may be bound. Theoretically, this depletion of the free [$^3$H]ligand concentration should not influence the parameters ($K_D$, $B_{max}$) of the [$^3$H]ligand binding isotherm at equilibrium as long as the calculations account for the depletion in the [$^3$H]ligand concentration. Nevertheless, when high receptor concentrations are used, problems do arise in accurately determining specific binding and the free [$^3$H]ligand concentration. The net result is that the dissociation constant may appear to increase with an increase in the receptor concentration. For these reasons, it is best to ensure that binding is a linear function of the receptor concentration over the working range of concentrations.

One possible source of error in estimating the free [$^3$H]ligand concentration may be encountered when racemic mixtures of [$^3$H]ligands are used. If only one of the enantiomers is pharmacologically active, then the data must be calculated assuming that only the free concentration of the active enantiomer is depleted by specific binding. Otherwise, a dependency of the dissociation constant on the receptor concentration might be construed. These considerations have obvious relevance to muscarinic receptor binding assays if racemic [$^3$H]QNB and *N*-[$^3$H]methylscopolamine are used. Currently, we recommend using the single stereoisomer, for example, (−)-[$^3$H]QNB.

Another source of error if high receptor concentrations are used is related to the estimation of nonspecific binding. A common way of calculating specific binding is to measure total binding and nonspecific binding at the same [$^3$H]ligand concentration. The difference between the two measurements is usually considered to be the specific component of binding. The problem with this computation is that the free [$^3$H]ligand concentration for the total binding determination can be less than that for the nonspecific binding determination since there is a greater depletion of the free [$^3$H]ligand during the total binding measurement. This can result in an overestimation of the nonspecific binding at the free [$^3$H]ligand concentration of the total binding determination. Typically, this correction is a trivial one unless the receptor concentration is high.

An "advantage" of the filtration method is that the membrane-bound [$^3$H]ligand trapped on the filter can be washed several times to reduce nonspecific binding. As long as the receptor bound [$^3$H]ligand does not dissociate during the washing procedure, repeated washing can increase the specific–nonspecific binding ratio. Although it is usually never considered as such, this "advantage" can be a problem if it is desirable to estimate the extent of nonspecific binding that occurred during the incubation. If the tissue concentration is high, then nonspecific binding can cause some depletion in the free [$^3$H]ligand concentration. Since there is no good way of estimating the nonspecific tissue binding after

most of it has been washed away, it may be difficult to calculate accurately the free [$^3$H]ligand concentration during the binding assay. The net result is that the observed dissociation constant may tend to increase with the tissue concentration.

### D. [$^3$H]Agonist Binding Methods

In most previous studies, the method used for studying the binding of muscarinic agonists has been an indirect one: that is, measuring the competitive inhibition of [$^3$H]antagonist binding by nonlabeled agonists. Typically, the agonist–[$^3$H]antagonist competition curves for efficacious agonists have flatter slopes than a Langmuir isotherm, so that [$^3$H]antagonist displacement occurs over 3 to 4 log concentration units of the agonist. Since such a large increase in the agonist concentration is required to go from low to high levels of receptor occupancy, it is difficult to measure the binding of [$^3$H]agonists directly at the high [$^3$H]ligand concentrations required to achieve moderate to high levels of receptor occupancy. Even in the most suitable tissues, the ratio of specific–nonspecific binding becomes unmanageably low at [$^3$H]agonist occupancy levels greater than 30%. Nevertheless, there are certain experimental paradigms in which the direct assessment of [$^3$H]agonist binding is desirable. The [$^3$H]agonists that have been used for this purpose include [$^3$H]oxotremorine-M, [$^3$H]acetylcholine (Birdsall *et al.*, 1978), *cis*-[$^3$H]methyldioxolane (Ehlert *et al.*, 1980), and [$^3$H]pilocarpine (Hedlund and Bartfai, 1981). The centrifugation method has been the most commonly used technique for measuring [$^3$H]agonist binding, since it allows measurement of the readily dissociating [$^3$H]agonist–receptor complex. Nevertheless, the high-affinity component of [$^3$H]oxotremorine-M binding in the myocardium has been measured successfully using the filtration method (Waelbroeck *et al.*, 1982).

An equilibrium method for separating free and bound [$^3$H]ligand, which should be useful for quantifying rapidly dissociating [$^3$H]ligand–receptor complexes is the ultracentrifugal filtration method of Freundlich and Taylor (1980, 1981). With this method, bound [$^3$H]ligand is trapped by ultracentrifugation of the membrane suspension through Nucleopore filters. Ultracentrifugation removes practically all of the incubation media and free [$^3$H]ligand, leaving the filters essentially dry except for 1 to 2 μl of fluid. Since the ultracentrifugal filtration procedure does not require that the filters be washed with buffer, the problem of the [$^3$H]ligand dissociating from the receptors during filtration is eliminated. This method should have practical application in muscarinic receptor studies of the binding of rapidly dissociating agonist [$^3$H]ligands. The methods should work best with quaternary ammonium [$^3$H]ligands, since these agents do not partition themselves nonspecifically into lipids and therefore would not be expected to bind nonspecifically to any great extent.

One special use of muscarinic [$^3$H]agonist ligands is the measurement of ACh by the radioreceptor method (Ehlert *et al.*, 1982). The high potency with which ACh inhibits the binding of [$^3$H]agonists ($IC_{50}$ = 5 n*M;* as measured by displacement of [$^3$H]CD binding) provides the basis for the assay. Depending upon the assay volume and the presence of interfering drugs and ions, the assay has a limiting sensitivity of about a picomole of ACh. An advantage of the method is that samples containing a huge excess of choline (choline/ACh = $10^3$) can be handled adequately.

## IV. MUSCARINIC RECEPTOR HETEROGENEITY

### A. Antagonist Receptor Heterogeneity

Most classical muscarinic antagonists interact with muscarinic receptors in a manner that is consistent with the consequences of the law of mass action. Thus, the equilibrium binding isotherms of several muscarinic [$^3$H]antagonists resemble Michaelis-Menten functions, and most antagonist–[$^3$H]antagonist competition curves have Hill coefficients close to one (Hulme *et al.*, 1978; Birdsall *et al.*, 1979). Moreover, the affinity constants of antagonists, as measured by ligand binding assays, show little tissue and species variation (Birdsall *et al.*, 1979). This uniformity of binding affinity is also manifest in pharmacological experiments. Atropine, for example, inhibits muscarinic responses in a variety of tissues with similar potency. Observations such as those cited above would suggest that muscarinic receptors in different anatomical and cellular locations are generally similar.

There are, however, some important differences among muscarinic receptors from different tissues. In a study of the antimuscarinic potency of 17 antagonists, Barlow *et al.* (1976) found that *N,N,*-dimethyl-4-piperidinyl diphenyl acetate was 20 times more potent at blocking ileal muscarinic receptors than atrial receptors. Thus, these data suggest that there are some structural differences between atrial and ileal muscarinic receptors. The *N,N,*-dimethyl-4-piperidinyl ester of diphenylacetic acid is unique among antimuscarinic esters in the sense that it is a dehydroxylated benzilic acid ester that still retains high affinity for muscarinic receptors in the rat cerebral cortex and guinea pig ileium (Hulme *et al.*, 1978). Typically, when substituted glycolic acid esters like QNB are dehydroxylated to yield the corresponding acetic acid ester derivatives, there is an approximate 100-fold reduction in anticholinergic potency (Long *et al.*, 1956).

An antagonist which shows rather selective antimuscarinic effects is the dibenzodiazepine, pirenzepine. At low doses, pirenzepine has selective inhibitory effects on gastric secretion, while at high doses, it blocks salivation yet has little influence on heart rate (Matsuo and Seki, 1979; Fink and Irwin, 1980). This

selectivity of antimuscarinic action can be rationalized on the basis of structural differences in muscarinic receptors. The potency of pirenzepine for inhibition of [$^3$H]NMS binding varies in different tissues with the receptors in the cerebral cortex displaying the highest affinity, whereas receptors in heart and smooth muscle have 30–40 times lower affinity for pirenzepine (Hammer *et al.*, 1980). In some tissues, particularly the salivary glands, the pirenzepine–[$^3$H]NMS competition curves have Hill coefficients of less than 1, suggesting that pirenzepine discriminates between different subclasses of muscarinic receptors (Hammer *et al.*, 1980). Apparently, the tissue variation in the potency of pirenzepine for inhibition of [$^3$H]ligand binding to the muscarinic receptor can be rationalized on the basis of variations in the proportion of subclasses of the muscarinic receptor that have different affinities for pirenzepine. The results of binding studies with [$^3$H]pirenzepine are consistent with this postulate. Watson *et al.* (1982) have shown that [$^3$H]pirenzepine, at concentrations in the range of $10^{-9}$ to $10^{-8}$ *M*, binds to a high-affinity population of muscarinic receptors in the cerebral cortex. In contrast, little or no specific [$^3$H]pirenzepine binding was detectable in the myocardium under similar assay conditions. These findings are consistent with the idea that the cortex contains a subpopulation of muscarinic receptors that have high affinity for pirenzepine, whereas the heart contains primarily lower-affinity pirenzepine sites.

Although the equilibrium binding isotherms of most classical muscarinic antagonists are consistent with mass action behavior, the kinetics of the binding of some muscarinic antagonists differ from a simple bimolecular interaction. The kinetics of the association of (±)-[$^3$H]QNB to the avian myocardium resembled a fast bimolecular interaction followed by a slower isomerization into a higher-affinity state (Galper *et al.*, 1977). Consistent with this model was the observation that the dissociation kinetics of (±)-[$^3$H]QNB varied depending upon the length of time allowed for (±)-[$^3$H]QNB to associate with the receptors prior to the initiation of dissociation. After a short preequilibrium incubation, the dissociation kinetics of (±)-[$^3$H]QNB were relatively fast, whereas after a longer equilibrium incubation, the dissociation of (±)-[$^3$H]QNB was relatively slow (Galper *et al.*, 1977). It has been suggested that this kind of behavior can result from the binding of both enantiomers of racemic (±)-[$^3$H]QNB (Burgiser *et al.*, 1981). However, this explanation is unsatisfactory because the isomerization kinetics have been observed with the pure active enantiomer of QNB, (−)-[$^3$H]QNB (Galper *et al.*, 1982) and also with an [$^3$H]antagonist that lacks a chiral center, *N*-[$^3$H]methyl-4-piperidinyl benzilate ([$^3$H]4-NMPB) (Kloog and Sokolovsky, 1978; Kloog *et al.*, 1978). It seems unlikely that the antagonist isomerization process is the same as that induced by agonists but rather that some antagonists are capable of stabilizing the receptor in an inactive conformation (ground state) that is distinct from the native and agonist-stimulated conformations. Thus, it might not be adequate to consider drug–receptor interactions solely within the constraints of a two-state

model (ground and activated states). It may be that there are different inactive conformations of the receptor that are populated when antagonists bind to the receptor. In any case, it is clear that hidden within the mass action-like equilibrium binding isotherms of most classical muscarinic antagonists are some complex kinetics that are not detectable from equilibrium binding measurements alone.

Another site on the muscarinic receptor has been detected when high concentrations (10–50 n*M*) of [$^3$H]QNB and [$^3$H]4-NMPB were used to measure binding (Hedlund *et al.*, 1980, 1982). The second binding site gave the saturation isotherm a double-humped appearance. It should be emphasized that such behavior cannot be explained as the result of the summation of two mass action isotherms with differing affinities, since the slope of the binding isotherm was too steep in the ligand concentration range of the lower affinity site. Thus, the data resembled those predicted by the summation of a high-affinity mass action site and a lower-affinity highly cooperative binding site. Apparently, the data were also consistent with a model that allows two or more ligands to bind to the same receptor complex (Hedlund *et al.*, 1982). The capacities of the two [$^3$H]antagonist binding sites were practically the same, which would be expected if the two sites were coupled together on the same macromolecule. The significance of the second, lower-affinity binding site is not readily apparent, since the binding of antagonists to this site does not correlate with antagonism of common muscarinic responses. However, it is an important consideration in attempting to understand the complex molecular interactions among ligands and the muscarinic receptor.

Further evidence for additional sites on the muscarinic receptor comes from the results of binding experiments with the nicotinic antagonist, gallamine. It has been demonstrated that gallamine causes an inhibition of muscarinic receptor binding in the heart and cerebral cortex and that the nature of the inhibition deviates from simple competitive inhibition (Birdsall *et al.*, 1981). The simplest explanation is that gallamine binds to the muscarinic receptor at a site distinct from the site at which ACh and classical muscarinic antagonists bind. Consistent with this postulate of negative heterotropic cooperativity is the observation that gallamine slows down the kinetics of [$^3$H]NMS binding to the muscarinic receptor (Birdsall *et al.*, 1981). It has been suggested that some of the anomalous effects of gallamine on the heart can be explained on the basis of a muscarinic blockade.

## B. Agonist Receptor Heterogeneity

One of the most striking differences between the nature of agonist and antagonist binding to the muscarinic receptor is the multisite nature of the agonist binding isotherm. If we restrict our attention to those muscarinic binding sites

that are saturated with low nanomolar concentrations of [$^3$H]QNB or [$^3$H]NMS, we find that efficacious muscarinic agonists cause considerable displacement of [$^3$H]antagonist binding over 3 to 4 log concentration units of the agonist (Birdsall *et al.*, 1978). In contrast, when the competitive inhibition of [$^3$H]antagonist binding by classical muscarinic antagonists is measured in buffered physiological media, the change in receptor occupancy from 9 to 91% occurs with a 100-fold variation in the concentration of the antagonist (Hulme *et al.*, 1978). This kind of behavior is typically mass action-like. Apparently, the shallow slopes of agonist–[$^3$H]antagonist competition curves are not the result of negatively cooperative, homotropic interactions, since alkylation of a large percentage of the sites with an irreversible antagonist does not steepen the slope of the agonist competition curve (Birdsall *et al.*, 1978). The deviations from mass action behavior seen in agonist–[$^3$H]antagonist competition experiments have been rationalized on the basis of three classes of agonist binding sites—superhigh, high and low affinity (SH, H, and L)—each having a different affinity for agonists (Birdsall *et al.*, 1978, 1979). Direct measurements of [$^3$H]agonist binding have yielded results consistent with this interpretation (Birdsall *et al.*, 1978, 1980b).

It is important to consider the results of agonist–[$^3$H]antagonist competition experiments within the context of a multisite model. Frequently, a variation in the potency ($IC_{50}$) with which an agonist inhibits the binding of an [$^3$H]antagonist in different tissues can be explained as the result of a variation in the proportion of agonist binding sites. Carbachol, for example, binds with much higher affinity to muscarinic receptors in the cerebellum than in the hippocampus, as shown by the much lower concentration of carbachol that is required to achieve half maximal occupation of muscarinic receptors in the cerebellum ($7.6 \times 10^{-7}$ *M*) as compared to the hippocampus ($9.1 \times 10^{-5}$ *M*) (Birdsall *et al.*, 1980b). Critical analysis of the carbachol binding curves shows that the hippocampus contains a large proportion of L sites, whereas the cerebellum contains predominately SH and H sites (Birdsall *et al.*, 1980b). Thus, the large observed difference in agonist binding characteristics between the two brain regions is due mainly to variations in the proportion of agonist binding sites and not to variation in the affinity of these sites.

The variation in the $IC_{50}$ value of an agonist that occurs depending upon whether an [$^3$H]agonist or an [$^3$H]antagonist probe is used can also be explained in terms of receptor heterogeneity. The $IC_{50}$ point of an agonist–[$^3$H]antagonist competition curve in the cortex, for example, depends primarily on the affinity of the agonist for the low-affinity site, since the cortex contains a majority of low-affinity sites (75%). In contrast, when the binding affinity of the agonist is estimated by an agonist–[$^3$H]agonist competition experiment, the observed potency of the agonist is much greater, since the [$^3$H]agonist probe selectively labels only the higher-affinity sites (SH and H). Thus, by considering the relative stoichiometric contribution of the SH, H, and L sites to the observed binding of

the [$^3$H]probe, it is possible to rationalize the differences in agonist potency noted between [$^3$H]agonist and [$^3$H]antagonist competition experiments.

If the various types of agonist binding sites represent independent receptors of varying structure, then the possibility of selective agonist ligands for each receptor type would seem likely. However, the results of numerous binding studies have shown that the rank order for the affinity constants of agonists for the various sites is invariant ($K_{SH} \geq K_H \geq K_L$). Moreover, the ratio of $K_H/K_L$ is highly correlated with efficacy and the ratio of $K_{SH}/K_H$ is nearly constant for pure and partial agonists (Birdsall *et al.*, 1977, 1980b). The probability that such correlations between pharmacological and binding parameters would exist for a group of three structurally distinct receptors is vanishingly small. Alternatively, Birdsall and co-workers (1977, 1980b) have postulated that the various agonist binding sites represent a single receptor subunit subject to different conformational constraints. Ultimately, this postulate can account for the observed correlations between the pharmacological activity and binding parameters of agonists and antagonists.

It might seem anomalous that agonists and antagonists can interact competitively at the same locus, yet antagonists are unable, apparently, to discriminate among the various agonist receptor subtypes. A consideration of the structure–activity relationships of agonists and antagonists helps to resolve this anomaly. This topic has been discussed in detail by Ariens and his colleagues (Ariens and Simonis, 1967; Ariens and Rodrigues de Miranda, 1979). Briefly, classical antimuscarinic esters have hydrophobic ring systems on the acyl moiety, which contribute to high-affinity binding by interacting with loci adjacent to the ACh binding region of the receptor (Ariens and Simonis, 1967). In contrast, most ACh analogs lack chemical groups that are complimentary to the peripheral receptor regions. The importance of the hydrophobic ring systems of antagonists is demonstrated by the large difference in the pharmacological activity of enantiomers of classical antagonists having an asymmetric carbon atom in the acyl moiety. This stereospecificity contrasts with the lack of selectivity of some antagonist enantiomers having an asymmetric carbon atom in the amino alcohol residue. An example of such an antagonist is benzilyl-β-methylcholine. However, the closely related agonist analog, acetyl-β-methylcholine, does show a large difference in the pharmacological activity of its enantiomers (Ellenbrock *et al.*, 1965). These relationships suggest that the critical moieties involved in the binding of agonists and antagonists are different and that classical antagonists derive considerable affinity by interacting with accessory receptor loci. When rationalized from this viewpoint, it is not surprising that antagonists are unable to discriminate between the various agonist states of the muscarinic receptor.

In the light of these concepts, the binding of nonclassical muscarinic analogs should provide some unique insight into the functional significance of agonist receptor heterogeneity. Among the compounds that fall into this category are a

large group of oxotremorine analogs including some agonists and antagonists that differ only in the presence or absence of a methyl group (Lindgren *et al.*, 1970, 1973; Dahlbom, 1981; Dahlbom *et al.*, 1982). This kind of relationship between structure and pharmacological activity is unique among muscarinic cholinergic analogs insofar as the efficacy of classical cholinergic analogs gradually diminishes as the number of carbon atoms in the acyl moiety increases.

Additional insight into the functional significance of the various agonist binding sites (SH, H, and L) can be achieved by comparing the dissociation constants of agonists ($K_{SH}$, $K_H$, and $K_L$) determined in ligand binding assays with $ED_{50}$ values for muscarinic responses. Ideally, there should be good agreement between the binding affinity and the $ED_{50}$ for the pharmacological response. However, quantitative agreement between the two parameters is often precluded by the unknown sequence of events between agonist receptor occupation and the measured pharmacological response. In the guinea pig ileum, for example, there is evidence that maximal smooth muscle contraction occurs at a very low level (<0.05%) of receptor occupancy by a highly efficacious agonist. When the large receptor reserve is taken into account by classical pharmacological procedures, contraction of the guinea pig ileum agrees with agonist occupancy of the low-affinity site (Birdsall *et al.*, 1977). A similar relationship has been observed for biochemical responses that are thought to be closely coupled to the muscarinic receptor. Stimulation of cyclic GMP formation in striatal brain slices (Hanley and Iversen, 1978) and murine neuroblastoma cells (Strange *et al.*, 1977) and inhibition of GTP-stimulated adenylate cyclase activity in cardiac homogenates (Birdsall *et al.*, 1980a) correlates generally with agonist occupancy of the low-affinity site. In human astrocytoma cells, oxotremorine and carbachol inhibit the accumulation of cyclic AMP caused by isoproterenol (Meeker and Harden, 1982), and there is good agreement between the $K_i$ for inhibition of the biochemical response and the $K_L$ of oxotremorine and carbachol that are usually estimated in binding experiments on most muscarinic receptor preparations. Interestingly, this effect of the muscarinic agonists was shown to be due to an activation of phosphodiesterase and not to an inhibition of adenylate cyclase (Meeker and Harden, 1982). Another response that is thought to be closely coupled to the muscarinic receptor is the breakdown of phosphatidyl inositol. In strips of the guinea pig ileum, there seems to be a correlation between agonist receptor occupancy of both high- and low-affinity sites and carbachol-stimulated incorporation of inorganic phosphate into phosphatidyl inositol (Jafferji and Michell, 1976).

Thus, from the few pieces of evidence cited above, there does not appear to be unequivocal evidence to link all muscarinic responses to any one class of agonist binding sites. However, when we consider the possible outcomes of a receptor–effector system for which the sequence of events between receptor occupation and pharmacological response are unknown, most available evidence would

point in the direction of the low-affinity site as being the pharmacologically relevant receptor. Thus, these relationships are consistent with the original postulate of Birdsall *et al.* (1977), which predicted that the low-affinity site was the coupled receptor.

For other receptor–effector systems, the idea is emerging that the high- and low-affinity agonist binding sites represent different coupling states of the same receptor subunit and that both states of the receptor are involved in the circuit of reactions that take place as the agonist–receptor complex activates the effector. In other words, the high- and low-affinity states of the receptor represent different intermediates in the process of receptor–effector activation. If we consider the life cycle of a receptor—beginning with the nascent polypeptide chain as it extrudes from the ribosomes and is ultimately transported to the plasma membrane where it associates with other intrinsic membrane proteins and perhaps later is engulfed into coated pits by the process of endocytosis—it seems probable that the ability of the receptor to bind with an agonist ligand at different stages in the cycle may vary depending upon the microenvironment of the receptor and the macromolecules with which it is coupled. In this regard it is interesting to note that the high- and low-affinity agonist forms of the muscarinic receptor travel in opposite directions down the rat vagus nerve (Zarbin *et al.*, 1982).

The influence of environmental factors on the binding affinity of agonists has important implications in the interpretation of agonist binding data. The binding affinity of a given agonist may vary in different tissues because of receptor coupling differences and not necessarily because of differences in the intrinsic affinity of the agonist for the receptor. Consequently, a simple positive correlation between binding affinity and pharmacological activity might not exist for an agonist if assays are run in different tissues. The binding of antagonists, on the other hand, is not as sensitive to the coupling constraints of the receptor, since antagonists do not isomerize the receptor into an active conformation. Consequently, the interpretation of antagonist binding data should be less complicated, and the binding affinity of an antagonist should correlate well with the potency for pharmacological antagonism in various tissues. Nevertheless, there are some agonists with unique properties that may be useful probes for identifying structural differences in muscarinic receptors. The trichloro derivatives of *cis*-methyldioxolane ($Cl_3CD$), for example, shows higher affinity for muscarinic receptors in the forebrain as compared to the medulla–pons (Ikeda *et al.*, 1980). Typically, muscarinic agonists have the reverse specificity for muscarinic receptors in the brain because of the large proportion of high-affinity sites in the brainstem. The highly rigid 2-methylspiro(1,3-dioxolane)-4,3′-quiniuclidine (MSDQ) compound of Fisher and his colleagues (1976) shows large tissue differences in its equipotent molar ratio for muscarinic responses when compared to ACh and aceclidine. It is possible that structural differences in muscarinic recep-

tors might be identified in [$^3$H]ligand binding assays using rigid structural analogues like MSDQ.

## V. CONCLUSION

The use of the radioligand binding procedure for the study of muscarinic receptors has expanded the field of muscarinic receptor pharmacology and has enabled the detection of complex ligand–receptor interactions that were not apparent in pharmacological studies on whole tissue assay systems like the guinea pig ileum. Moreover, evidence has accumulated suggesting that there are some differences in muscarinic receptors from different tissues. Muscarinic receptor binding methods should have application in the development of novel ligands which discriminate among muscarinic receptors in different tissues. Such drugs may have important therapeutic application.

## REFERENCES

Aranyi, P. (1980). *Biochim Biophys. Acta* **628,** 220–227.

Ariens, E. J., and Rodrigues de Miranda, J. F. (1979). *In* "Recent Advances in Receptor Chemistry" (F. Gualtieri, M. Giannella, and C. Melchiorre, eds.), pp. 1–36. Elsevier/North-Holland Biomedical Press, Amsterdam.

Ariens, E. J., and Simonis, A. M. (1967). *Ann. N.Y. Acad. Sci.* **144,** 842–867.

Barlow, R. B., Berry, K. J., Glentou, P. A. M., Nikolaou, N. M., and Soh, K. S. (1976). *Br. J. Pharmacol.* **58,** 613–620.

Beld, A. J., and Ariens, E. J. (1974). *Eur. J. Pharmacol.* **25,** 203–209.

Berson, S. A., and Yalow, R. S. (1959). *J. Clin. Invest.* **38,** 1996–2016.

Birdsall, N. J. M., and Hulme, E. C. (1976). *J. Neurochem.* **27,** 7–16.

Birdsall, N. J. M., Burgen, A. S. V., and Hulme, E. C. (1977). *In* "Cholinergic Mechanisms and Psychopharmacology" (D. J. Jenden, ed.), pp. 25–33. Plenum, New York.

Birdsall, N. J. M., Burgen, A. S. V., and Hulme, E. C. (1978). *Mol. Pharmacol.* **14,** 723–736.

Birdsall, N. J. M., Burgen, A. S. V., and Hulme, E. C. (1979). *In* "Recent Advances in Receptor Chemistry" (F. Gualtieri, M. Giannella, and C. Melchiorre, eds.), pp. 71–96. Elsevier/North-Holland Biomedical Press, Amsterdam.

Birdsall, N. J. M., Burgen, A. S. V., Hiley, C. R., and Hulme, E. C. (1980a). *In* "Receptors for Neurotransmitter and Peptide Hormones" (G. Pepeu, M. J. Kuhar, and S. J. Enna, eds.), pp. 107–116. Raven Press, New York.

Birdsall, N. J. M., Hulme, E. C., and Burgen, A. S. V. (1980b). *Proc. R. Soc. London* **207,** 1–12.

Birdsall, N. J. M., Burgen, A. S. V., Hulme, E. C., and Stockton, J. (1981). *Br. J. Pharmacol.* **74,** 798.

Bouton, N. M., and Raynaud, J. P. (1978). *J. Steroid Biochem.* **9,** 9–15.

Burgisser, E., Lefkowitz, R. J., and DeLean, A. (1981). *Mol. Pharmacol.* **19,** 509–512.

Dahlbom, R. (1981). *In* "Cholinergic Mechanisms, Phylogenetic Aspects, Central and Peripheral Synapses, and Clinical Significance" (G. Pepeu and H. Ladinsky, eds.), pp. 621–638. Plenum, New York.

Dahlbom, R., Jenden, D. J., Resul, B., and Ringdahl, B. (1982). *Br. J. Pharmacol.* **76,** 299–304.
Ehlert, F. J., Dumont, Y., Roeske, W. R., and Yamamura, H. I. (1980). *Life Sci.* **26,** 961–967.
Ehlert, F. J., Roeske, W. R., and Yamamura, H. I. (1981). *Mol. Pharmacol.* **19,** 367–371.
Ehlert, F. J., Roeske, W. R., and Yamamura, H. I. (1982). *Life Sci.* **31,** 347–354.
Ellenbrock, B. W. J., Nivard, R. J. F., Van Rossum, J. M., and Ariens, E. J. (1965). *J. Pharm. Pharacol.* **17,** 393–404.
Fields, J. Z., Roeske, W. R., Morkin, E., and Yamamura, H. I. (1978). *J. Biol. Chem.* **253,** 3251–3258.
Fink, M., and Irwin, P. (1980). *Scand. J. Gastroenterol., Suppl.* **15**(66), 39–46.
Fisher, A., Weinstock, M., Gitter, S., and Cohen, S. (1976). *Eur. J. Pharmacol.* **37,** 329–338.
Freundlich, R., and Taylor, D. B. (1980). *Anal. Biochem.* **105,** 268–273.
Freundlich, R., and Taylor, D. B. (1981). *Anal. Biochem.* **114,** 103–104.
Galper, J. B., Klein, W., and Catterall, W. A. (1977). *J. Biol. Chem.* **252,** 8692–8699.
Galper, J. B., Dziekan, L. C., O'Hara, D. S., and Smith, T. W. (1982). *J. Biol. Chem.* **257,** 10344–10356.
Hammer, R., Berrie, C. P., Birdsall, N. J. M., Burgen, A. S. V., and Hulme, E. C. (1980). *Nature (London)* **283,** 90–92.
Hanley, M. R., and Iverson, L. L. (1978). *Mol. Pharmacol.* **14,** 246–255.
Hedlund, B., and Bartfai, T. (1981). *Naunyn-Schmiedeberg's Arch. Pharmacol.* **317,** 126–130.
Hedlund, B., Unden, A., Grynfarb, M., and Bartfai, T. (1980). *Acta Chem. Scand., Ser. B* **B34,** 767–778.
Hedlund, B., Grynfarb, M., and Bartfai, T. (1982). *Naunyn-Schmiedeberg's Arch. Pharmacol.* **320,** 3–13.
Hiley, C. R., and Burgen, A. S. V. (1974). *J. Neurochem.* **22,** 159–162.
Hulme, E. C., Birdsall, N. J. M., Burgen, A. S. V., and Mehta, P. (1978). *Mol. Pharmacol.* **14,** 737–750.
Ikeda, S. R., Aronstam, R. S., and Elderawi, M. E. (1980). *Neuropharmacology* **19,** 575–585.
Jafferji, S. S., and Michell, R. H. (1976). *Biochem. J.* **154,** 653–657.
Kloog, Y., and Sokolovsky, M. (1978). *Brain Res.* **144,** 31–48.
Kloog, Y., Egozi, Y., and Sokolovsky, M. (1978). *Mol. Pharmacol.* **15,** 545–558.
Lindgren, S., Lindquist, A., Lindeke, B., Svensson, U., Karlen, B., Dahlbom, R., and Blair, M. R. (1970). *Experientia* **26,** 1232–1233.
Lindgren, S., Lindquist, A., Lindeke, B., Svensson, U., Karlen, B., and Dahlbom, R. (1973). *Acta Pharm. Suec.* **10,** 435–440.
Long, J. P., Luduena, F. P., Tullar, B. F., and Lands, A. M. (1956). *J. Pharmacol.* **117,** 29–38.
Matsuo, Y., and Seki, A. (1979). *Arzneim.-Forsch.* **29,** 1028–1035.
Meeker, R. B., and Harden, T. K. (1982). *Mol. Pharmacol.* **22,** 310–319.
Paton, W. D., and Rang, H. P. (1965). *Proc. R. Soc. London, Ser. B.* **163,** 1–44.
Rodbard, D., Ruder, H. J., Vaitukaitus, J., and Jacobs, H. S. (1971). *J. Clin. Endocrinol. Metab.* **33,** 343–355.
Snyder, S. H., Chang, K. J., Kuhar, M. J., and Yamamura, H. I. (1975). *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **34,** 1915–1921.
Strange, P. G., Birdsall, N. J. M., and Burgen, A. S. V. (1977). *Biochem. Soc. Trans.* **5,** 189–191.
Waelbroeck, M., Robberecht, P., Cuatelain, P., and Christophe, J. (1982). *Mol. Pharmacol.* **21,** 581–588.
Watson, M., Roeske, W. R., and Yamamura, H. I. (1982). *Life Sci.* **31,** 2019–2023.
Yamamura, H. I., and Snyder, S. H. (1974a). *Mol. Pharmacol.* **10,** 861–867.
Yamamura, H. I., and Snyder, S. H. (1974b). *Proc. Natl. Acad. Sci. U.S.A.* **71,** 1725–1729.
Yamamura, H. I., Kuhar, M. J., Greenberg, D., and Snyder, S. H. (1974). *Brain Res.* **66,** 541–546.
Zarbin, M. A., Wamsley, J. K., and Kuhar, M. J. (1982). *J. Neurosci.* **2,** 934–941.

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