INFORMATION TO USERS

This reproduction was made from a copy of a manuscript sent to us for publication and microfilming. While the most advanced technology has been used to photograph and reproduce this manuscript, the quality of the reproduction is heavily dependent upon the quality of the material submitted. Pages in any manuscript may have indistinct print. In all cases the best available copy has been filmed.

The following explanation of techniques is provided to help clarify notations which may appear on this reproduction.

1. Manuscripts may not always be complete. When it is not possible to obtain missing pages, a note appears to indicate this.

2. When copyrighted materials are removed from the manuscript, a note appears to indicate this.

3. Oversize materials (maps, drawings, and charts) are photographed by sectioning the original, beginning at the upper left hand corner and continuing from left to right in equal sections with small overlaps. Each oversize page is also filmed as one exposure and is available, for an additional charge, as a standard 35mm slide or in black and white paper format.*

4. Most photographs reproduce acceptably on positive microfilm or microfiche but lack clarity on xerographic copies made from the microfilm. For an additional charge, all photographs are available in black and white standard 35mm slide format.*

***For more information about black and white slides or enlarged paper reproductions, please contact the Dissertations Customer Services Department.**

UMI University Microfilms International

8611383

Silva Ortiz, Walter I.

PHARMACOLOGICAL CHARACTERIZATION OF THE 3':5' CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY AND MUSCARINIC ACETYLCHOLINE RECEPTORS OF BRAIN COATED VESICLES

City University of New York PH.D. 1986

University
Microfilms
International 300 N. Zeeb Road, Ann Arbor, MI 48106

Copyright 1986
by
Silva Ortiz, Walter I.
All Rights Reserved

PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark __√__.

1. Glossy photographs or pages __✓__
2. Colored illustrations, paper or print ______
3. Photographs with dark background __✓__
4. Illustrations are poor copy ______
5. Pages with black marks, not original copy ______
6. Print shows through as there is text on both sides of page ______
7. Indistinct, broken or small print on several pages __✓__
8. Print exceeds margin requirements ______
9. Tightly bound copy with print lost in spine ______
10. Computer printout pages with indistinct print ______
11. Page(s) __________ lacking when material received, and not available from school or author.
12. Page(s) __________ seem to be missing in numbering only as text follows.
13. Two pages numbered ______. Text follows.
14. Curling and wrinkled pages ______
15. Dissertation contains pages with print at a slant, filmed as received __✓__
16. Other ______________________________

University
Microfilms
International

PHARMACOLOGICAL CHARACTERIZATION OF THE 3':5' CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY AND MUSCARINIC ACETYLCHOLINE RECEPTORS OF BRAIN COATED VESICLES

by

WALTER I. SILVA ORTIZ

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

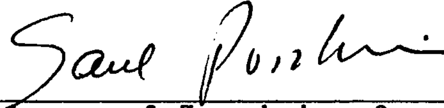
1986

COPYRIGHT BY

WALTER I. SILVA ORTIZ

1986

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy

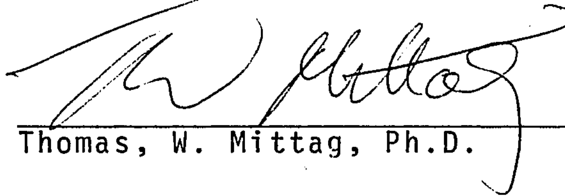


Date: 9/18/85

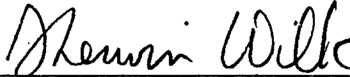
Chairman of Examining Committee
Saul Puszkin, Ph.D.

Executive Officer
Terry Krulwich, Ph.D.

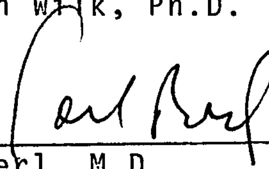
Alfred Stracher, Ph.D.



Thomas, W. Mittag, Ph.D.



Sherwin Wilk, Ph.D.



Soll Berl, M.D.

Abstract

PHARMACOLOGICAL CHARACTERIZATION OF THE 3':5'-CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITY AND MUSCARINIC ACETYLCHOLINE RECEPTOR OF BRAIN COATED VESICLES

by

Walter I. Silva Ortiz

Adviser: Professor Saul Puszkin

Bovine brain coated vesicles (CV) can display a high degree of microheterogeneity. This degree would be dependent on the contribution of diverse subcellular, cellular and neuroanatomical regions to the CV population. Two cargo molecules are described in this thesis with their respective pharmacological properties. These cargo molecules are 3':5'-cyclic nucleotide phosphodiesterase (PDE) and the muscarinic acetylcholine receptor (mAChR). The PDE activity is insensitive to calcium and stimulated by cyclic GMP. The basal hydrolysis has a broad alkaline pH optimum in contrast to the cGMP-stimulated hydrolytic activity which has a neutral pH optima. Analysis of the dose response curves for the hydrolysis of both cyclic cAMP and cGMP reveal very similar Kd and Vmax values. The kinetic behavior of the enzyme suggests positive cooperativity. Nonetheless, an alternative equillibrium kinetics model is proposed which can explain the kinetic behavior of the CV type II PDE activity. The inhibition profile of the CV PDE by papaverine, IBMX and theophylline can support partially the kinetic model, in addition to certain experimental observations. The presence of a type II PDE in coated vesicles could suggest a differential subcellular partitioning of the known PDE isozymes.

The mAChR had a Kd of 25 pM and a Bmax of 341 fmol/mg protein.

Competition experiments with both agonists and antagonists revealed heterogeneity of binding affinities. The binding site with high affinity for oxotremorine displayed poor sensitivity to GTP. The high and low affinity binding sites for carbachol could be interpreted in terms of distinct pre and postsynaptic receptors (Mash, et al., 1985). Nonetheless, the differences in the percentage of high affinity between both agonists, carbachol and oxotremorine leads to caution in the use of the latter approach in the interpretation of the data. Atropine did not display shallow inhibition or competition curves in contrast to the hydrophylic antagonist N-methyl-scopolamine. The heterogeneity of binding affinities for muscarinic drugs in CV as well as the PDE data is discussed in the text in the context of CV subpopulations (Pfeffer and Kelley, 1985 and Weidenmann et al., 1985).

Dedication

To my friend Isabelo "Saby" Reyes (1956-1974).

TABLE OF CONTENTS

PAGE

Abstract..iv

Dedication..vi

TABLE OF CONTENTS...vii

LIST OF TABLES..x

LIST OF FIGURES...xi

INTRODUCTION..1

1. General Overview and Goals of the Thesis Project............1

2. Coated Vesicles...2

a. Biochemistry..2

b. Recycling of clathrin.......................................4

c. Microheterogeneity..5

d. Biological functions..7

3. Muscarinic Cholinergic Receptors............................9

a. Cholinergic receptors and receptor theory...................9

b. Ligand binding studies......................................11

c. Antagonists...12

d. Agonists..14

e. Perturbations of ligand binding.............................15

f. Localization of muscarinic receptors........................17

g. Regulation of muscarinic receptors..........................18

4. 3':5'-Cyclic Nucleotide Phosphodiesterases..................21

a. Type I..21

PAGE

b. Type II 25

c. Type III 27

d. Type IV 28

e. A working hypothesis 29

MATERIALS AND METHODS 31

1. Materials 31

2. Coated Vesicle Preparation 31

3. Immunopurification of Coated Vesicles 32

4. Muscarinic Acetylocholine Receptors Binding Assays 33

5. Phosphodiesterase Assay 33

6. Data Analysis 34

7. Other Laboratory Procedures 36

RESULTS 37

1. Localization of cyclic nucleotide phosphodiesterase activity and muscarinic acetylcholine receptors in bovine brain coated vesicles 37

2. Equillibrium binding studies of the muscarinic acetylcholine receptors of coated vesicles 39

3. Competition binding experiments on the muscarinic acetylcholine receptor 40

4. Kinetic analysis of the cyclic nucleotide phosphodiesterase of brain coated vesicles 40

5. Inhibition of the cyclic nucleotide phosphodiesterase of brain coated vesicles 41

6. Equillibrium kinetics model for the cGMP-stimulated phosphodiesterase of brain coated vesicles 42

DISCUSSION 47

Muscarinic Acetylcholine Receptors of Brain Coated Vesicles 47

Equillibrium binding 47

Heterogeneity of binding affinities 47

PAGE

Clinical relevance..49

2. Cyclic Nucleotide PDE of Coated Vesicles...................50

a Pharmacological properties................................50

b. Subcellular location....................................53

c. Kinetic behavior..54

d. Evaluation of the orignal working hypothesis............55

CONCLUSIONS...57

Appendix A..59

Tables..60

Appendix B..66

Figure Legends..67

Figures...75

BIBLIOGRAPHY..114

LIST OF TABLES

Table 1. Analysis of the ^{3}H(-)QNB binding isotherms in coated vesicles using various equations.

Table II. Summary of the inhibition parameters of muscarinic agonists and antagonists.

Table III. Analysis of the competition curves for oxotremorine, carbachol and N-methyl-scopolamine using a two-site model.

Table IV. Summary of the kinetic parameters for the hydrolysis of cyclic nucleotides by the phosphodiesterase of coated vesicles.

Table V. Summary of the inhibition parameters of various phosphodiesterase inhibitors.

Table VI. Calculated kinetic parameters for the cGMP-stimulated phosphodiesterase of coated vesicles.

LIST OF FIGURES

Figure 1. Coated vesicles from the unicellular organism, Tetrahymena termophila according to the method of Fine et al. (1985).

Figure 2. Scheme for the recycling of clathrin triskelions in vivo.

Figure 3. List of the chemical structure of agonists and antagonists of the muscarinic acetylcholine receptor.

Figure 4. Working hypothesis proposing a role for cyclic nucleotide PDE as a proton generator which in turn modify coated vesicles dynamics.

Figure 5. Subcellular fraction scheme for the purification of brain coated vesicles.

Figure 6. Electron micrograph of coated (panel b) and decoated (panel d) vesicles. Pancel c displays their corresponding protein profiles.

Figure 7. Elution profile of brain coated vesicles and 3(-)QNB binding after chromatography over a Sephacryl-S-1000 column twice (panels a and b).

Figure 8. Elution profile of brain coated vesicles and cyclic nucleotide phosphodiesterase activity after chromatography over a Sephacryl-S-1000 column twice (2 panels, a and b).

Figure 9. Column purified coated vesicles and their phosphodiesterase activity in the absence and presence of cGMP or calcium.

Figure 10. Immunopurification of coated and the basal and cGMP-stimulated phosphodiesterase activity of brain coated vesicles using polyclonal antibodies to the coat proteins attached to Staphylococus aureus cells.

Figure 11. Immunotitration of coated vesicle ^{3}H(-)QNB binding (as in Figure 10).

Figure 12. Binding of ^{3}H(-)QNB to coated and decoated vesicles as a function of tissue concentration.

Figure 13. Kinetics of ^{3}H(-)QNB binding to coated vesicles.

Figure 14. Michaelis Menten plot of a QNB binding CV.

Figure 15. Schatchard plot of the data in Figure 13.

Figure 16. Analysis of the ^{3}H(-)QNB kinetic parameters. Variation in the apparent Kd as a function of the receptor's density (R+).

Figure 17. Competition of ^{3}H(-)QNB binding using the antagonists atropine and N-methyl-scopolamine.

Figure 18. Inhibition of ^{3}H(-)QNB binding by the gonist carbachol, and oxotremorine in the absence and presence of GTP.

Figure 19. Analysis of the competition curves for oxotremorine (in the absence of GTP) with a one and two-site models.

Figure 20. Analysis of the competition curves for oxotremorine (in the presence of GTP) with a one and two-site models.

Figure 21. Analysis of carbachol's competition curve using a one and two-site models.

Figure 22. Analysis of N-methyl-scopolamine's competition curve using a one and two-site models.

Figure 23. pH-dependence of the basal and cyclic GMP-stimulated cyclic AMP hydrolytic activity.

Figure 24. Dose response curves for the hydrolysis of cyclic AMP and cyclic GMP.

Figure 25. Dose response curves for the hydrolysis of cyclic AMP by coated and decoated vesicles.

Figure 26. Cyclic GMP's stimulation of cyclic AMP's hydrolysis by coated and decoated vesicles.

Figure 27. Loss of cyclic GMP's stimulation capacity after storage of the enzyme at 4°C.

Figure 28. Effects of activation and storage on the kinetics of cyclic AMP hydrolysis.

Figure 29. Inhibition of the cAMP hydrolytic activity of coated vesicles by papaverine, IBMX, and theophylline.

Figure 30. Inhibition of the cGPM-stimulated hydrolysis of cAMP by papaverine, IBMX,and theophylline.

Figure 31. Inhibition of the cyclic GMP hydrolytic activity by papaverine, IBMX, and theophylline.

Figure 32. Equillibrium kinetics model for the cyclic GMP-stimulated PDE of coated vesicles.

Figure 33. Simulation curves derived from the steady-state solution of the kinetic model for the CV PDE.

Figure 34. Variations in apparent Km as a function of the activation factor ( ) and the ratio of affinities of cyclic nucleotides for the regulatory and catalytic sites on the CV PDE.

Figure 35. Variations in the apparent Hill slope as a function of the activation factor and the K_C/K_R ratio for cyclic nucleotides in the CV PDE.

Figure 36. Normalized dose response curves obtained for cyclic nucleotides from the experimentally calculated kinetic constants inherent in the PDE model.

Figure 37. Scheme for the vesicular transport of muscarine receptors in pre and postsynaptic elements of cerebral cortical neurons.

Figure 38. Scheme for the vesicular and cytoplasmic transport of cyclic nucleotide phosphodiesterase in pre and postsynaptic elements of cerebral cortical neurons.

Figure 39. Evaluation of the original working hypothesis regarding PDE's.

INTRODUCTION

1. General Overview and Goals of the Thesis Project

In 1961, Gray described for the first time some specialized vesicles in synaptic terminals of cerebellar neurons, he called them complex vesicles. Similar vesicles were also seen in other early studies and given the names of bristle-coated, alveolate, densely-rimmed and coated vesicles (Nevorotin, 1980). These descriptive names alluded to the characteristic protein cage or coat surrounding the vesicles which is made of clathrin and its associated peptides. These "vesicles in a basket" or coated vesicles (CV) are ubiquitous structures observed from unicellular protozoans (see Figure 1) and algae to all cells of multicellular eukaryotic organisms (Blitz and Fine, 1980 and Nevorotin, 1980). Figure one displays an electron micrograph of CV obtained from the ciliated protozoan Tetrahymena termophila (prepared by W. Silva). Compare these to our bovine brain CV in Figure 6. These universal subcellular organelles are of primordial importance to the recycling of membranes, vesicles and their constitutive and transported (cargo) molecules. The recycling of membranes is instrumental in many developmental, homeostatic and pathological processes.

Pathological conditions correlated with deficiency in the expression of clathrin and/or its associated proteins have not been reported in the scientific literature. Due to their participation in development and homeostasis, mutations on their genes have high probabilities of resulting in lethality. Yet, some forms of hypercholesterolemia seem to result from low density lipoprotein (LDL) receptor mutants which are unable to interact with the coat proteins (Sudhof et al., 1985). This culminates in the expression of a mutant defective in the internalization of the LDL receptor molecule. Pharmacologically, CV can play a role in the development of tolerance and other pharmacodynamic phenomena observed during systemic drug therapy (Grahame-Smith, 1985 and Raffa, 1985). Thus, our

understanding of the biochemical machinery of coated vesicles should provide clues to the ethiology of certain pathological conditions. This knowledge may reveal potential targets for the treatment of certain disease states. An improvement of our pharmacotherapeutical regimes may also result from this understanding.

This thesis project's main contribution consists in the pharmacological characterization of two molecules transported by brain coated vesicles. One of these, phosphodiesterase (EC3.1.4.17), is the only known enzyme for the catabolism of cyclic nucleotides, and second a cholinergic receptor, the muscarinic type. We aimed at their localization in CV and at establishing their pharmacological properties in this subcellular organelle fraction. Until now very little research has been conducted related to cyclic nucleotides or muscarinic receptors in coated vesicles, this work constitutes one of the first efforts along these lines.

2. Coated Vesicles

a. Biochemistry. The coat. A coated vesicle (CV) can be subdivided into its coat and core (membrane) parts (Figure 2). The core proteins are those associated with the lipid vesicle, ranging from clathrin anchoring sites to transported (cargo) molecules. The coat proteins in turn are those responsible for the formation of the cage, basket or coat around the vesicles. The coat proteins can be obtained by alkaline extraction (pH = 8.0) from a CV preparation (Woodward and Roth, 1978 and Keen et al., 1979). This treatment leads to dissociation or disassembly of the coat which can now be separated from the stripped or decoated vesicles via differential centrifugation or chromatography over a Sepharose 4B gel filtration column. These biochemical studies have revealed that a 180,000 MW peptide, named clathrin, constitutes more than 60% of the coat proteins (Woodward and Roth, 1978 and Pearse, 1976). The word clathrin is derived from the greek clathi

which means lattice (Pearse, 1976). This name emulates its ability to form geometrical lattices and baskets during coated-pit and vesicles formation.

Clathrin is found tightly associated with peptides of 33,000 and 36,000 MW, known as clathrin associated proteins (CAP) or clathrin light chains (CLC) (Lisanti et al., 1982). Separation of the CAP from clathrin requires boiling or treatment with urea (Lisanti et al., 1982 and Ungewickell and Branton, 1981). A molecular complex composed of various 100,000 and 50,000 MW proteins is also associated with the coat (Zaremba and Keen, 1985). This molecular complex is separated from clathrin and its CAP by gel filtration chromatography over the Sepharose 4B column.

The basic unit of the coat and lattices is named a trimer or triskelion due to it being a composite of 3 clathrin molecules in a 1:1 ratio with its associated proteins (see Figure 2). Limited proteolytic digestion studies have shown that the distal components of the trimer are not essential for self-assembly. Purified clathrin can indeed be assembled into baskets in vitro by a pH shift of 8.0 to 6.5. During CV preparation, procedures involving the use of high sucrose concentrations will favor the isolation of empty coats (Nandi et al., 1982). The highly negative charge of the coats seems to govern their electrophoretic mobility, yet coated vesicles from different tissues, i.e. bovine brain versus rat liver, do migrate differently, the latter moving close to 25% slower (Goetlieb, et al., 1985). For still unknown reasons coats are seen in vitro to serve as better substrates for the enzyme, uncoating ATPase, which removes clathrin trimers (Schlossman et al., 1984 and Braell et al., 1984). In the electron microscope, preparations of CV are morphologically or geometrically distinct, being characterized by varying ratios of hexagonal and pentagonal arrays (Pearse, 1980). These have been described as barrel shaped, circular and others (Kanaseki and Kadota, 1965 and Pearse, 1980). Thermodynamic calculations show that

clathrin's coating of vesicles is the most optimal and economical.

b. Recycling of calthrin. At least three models have been proposed to explain CV formation (Lisanti et al., 1984). These models take into account a series of observations which can be used to construct a scheme for the in vivo recycling of clathrin (see Figure 2). The first phase (I) represents the event of clathrin's recruitment to membranes (plasmalemma, Golgi or intracellular cisternae). Indirect immunofluorescence studies suggest a role for calcium (Ca^{++}) and calmodulin (CAM) in the recruitment of clathrin to membranes (Salisbury et al., 1980, 1981). Due to the ability of CAPs to bind CAM they have been proposed to represent the target for the action of CAM (Lisanti et al., 1984). The activity of a protein kinase associated with coated vesicles is modulated by CAP and could also play a role in phase I or II events (Pauloin and Jolles, 1984). Due to the pH dependence of clathrin assembly a calcium-calmodulin dependent proton generator could putatively modulate these phases as well (see PDE section). Such a proton generator could also provide protons for the proton (H^{+})-ATPase present in CV and allow acidification of the vesicles' lumen (Xie et al., 1983 and Forgac et al., 1983). In addition, the alkaline extraction of coats yields a protein fraction containing several assembly polypeptides that promote reassembly of clathrin into vesicle-free coat structures (Zaremba and Keen, 1985 and Unanue et al., 1981). These may represent or interact with calthrin anchoring sites. Inhibitors of the enzyme transglutaminase (Haigler et al., 1980) block endocytosis, yet their effect at phase I or II has not been documented.

In phase II, the lattices initially composed of hexagons transform to allow pentagonal and heptagonal arrays (Kanaseki and Kadota, 1969, Heuser and Evans, 1980 and Lisanti et al., 1984). The appearance of the latter geometrical arrays coincides with the curvature of membranes required for coated pit/vesicle formation (Heuser, 1980). A tailoring or lattice transforming factor is proposed or

evoked to account for the latter observations. It is plausible that the uncoating ATPase could be this factor. The assembly peptides fraction has a family of at least six 100,000 MW polypeptides three of which exist as complexes with a strochiometric (2:2) amount of a 50,000 MW polypeptide (Pearse and Robinson, 1985). The latter complex is able to polymerize with low concentrations of clathrin to give a relatively homogeneous population of coats predominantly of the "barrel" size (Pearse and Robinson, 1985). In contrast, three other polypeptides of 100,000 MW lack the 50,000 MW proteins but polymerize with clathrin to yield coats of a wide range of sizes including "barrels", truncated icosahedra and particles of 100 nm diameter (Pearse and Robinson, 1985). These assembly polypeptides could thus function as transforming factors.

A clathrin trimer returns to a free state or cytosolic pool(s) after the action in phase III of the uncoating ATPase. This enzyme has a 70,000 MW and is found in the cytosol (Schlossman et al., 1984). At this point, the first derivative of a coated vesicle arises i.e. smooth transport vesicles for fast axoplasmic transport (Stone et al., 1984) or endosomes (Willingham and Pastan, 1980, 1983). This latter step involves fusion of various decoated vesicles. Recent studies in our laboratory have established in vitro conditons for the phosphorylation of the CAP and this apparently leads to the destabilization of the coat structure.

c. Microheterogeneity. Coated vesicles subpopulations, subsets or microheterogeneity can arise at both the coat and core levels. Clathrin iso-forms or tissue-variants have not been reported. Yet peptide mapping studies of liver versus brain clathrin show great similarities although they are not identical. In contrast, CAP seem to be of lower molecular weight in peripheral tissue (Brodsky et al., 1983). This observation of CAP variants can be taken as evidence suggestive of CV subsets. Recent work in our laboratory (S. Kohtz and J. Dutt) using monoclonal antibodies against CAP prepared from bovine brain CV has

revealed morphologically and biochemically distinct CV subsets. The CAP molecules, as well as the family of 100,000 MW peptides can be instrumental in the expression of CV subsets. This level of microheterogeneity due to coat elements can be manifested morphologically by both the size and subcellular location. Small CV of close to 75 nm are generally seen in relation to the Golgi region and those of 100 nm in connection to the plasmalemma (Farquhar and Palade, 1981 and Whyte and Ockleford, 1980). Yet, the relationship of the latter to specific CAP-variants is not known.

Nonetheless, probably the biggest source of diversity arises from the core elements. The core elements contributing to heterogeneity may be compartment and/or tissue specific "receptors" for clathrin trimers or intraluminal as well as intramembrane transported (cargo) moleclues. The studies of Rothman et al. (1980), suggested two distinct CV-subsets on their transport of vesicular stomatitis virus (VSV) proteins. These subsets apparently correspond to different stages of transport from ER to Golgi, and Golgi to plasmalemma (Rothman et al., 1980). In addition, the studies of Pfeffer and Kelly (1985) sugest distinct CV subpopulations from bovine brain differing in their content of two distinct synaptic plasma membrane proteins. These synaptic proteins are called p29 and the p38 peptides and the studies were performed using monoclonal AB's to the cytoplasmic domain of these proteins. The latter results also support the microheterogeneity of the CV preparation. It is indeed expected that these transport organelles will exhibit tissue specific cargo molecules. In brain, these cargo molecules could be from distinct neuroanatomical regions or cells i.e. glial cells. Subcellular microheterogeneity adds another level of diversity. Thus, we may expect CV transporting the same cargo molecules but in different states (newly synthesized receptor for hormones versus its endocytosed form). These various states could be reflected in different post-translational modifications like

phosphorylation/dephosphorylation reactions. These cargo molecules may thus express a particular state deeply influenced by their corresponding subcellular microenvironment.

d. Biological functions. Developmental. The early studies of Porter (1965) in mosquitoe oocytes showed how CV were involved in the uptake of yolk proteins. This suggested a role for this organelle in receptor-mediated endocytosis of proteins and oogenesis. Later on studies relating to synaptogenesis in both the cerebellum and pons were able to demonstrate a role for CV associated with the Golgi region in the provision of membranes for pre and postsynaptic sites (Bastiani and Goodman, 1984 and Blitz and Fine, 1980). This biochemical flow of materials carried on by CV has also been implicated in the events of erythrogenesis melanogenesis and liver regeneration (Nevorotin, 1980). Their involvement, CV, in all normal developmental phenomena is thus paramount to the continuity of life.

Homeostatic. Not only in late and early development are CV's functions manifested but they are of crucial importance to the homeostasis of cells and organisms. Secretion and uptake are basic cellular processes where CV play a role. An exocytic role for CV was already alluded in the developmental studies cited above. Yet, it was the work of Rothman et al. (1980) that established a definite role for these in exocytosis. This work also was able to show CV subsets involved in different stages of transport of a VSV core protein (Rothman et al., 1980). In addition, in the normal neuronal event of axoplasmic transport, CV's, have been shown by Stone et al. (1984) to represent temporary relay stations for those vesicular elements moving in the fast axoplasmic transport system. Clathrin, though, travels via the slow axoplasmic transport system (Stone et al., 1984).

Coated vesicles have recently gained importance and popularity due to their

prominent role in the process of receptor-mediated-endocytosis (RME). It is the ability of receptor or membrane molecules to interact with clathrin coats what provides the specificity of this process in contrast to the non-selective event of pinocytosis (micro or macro). The molecules bearing this role could be related to the intramembrane particles observed during freeze-etch replica electron micrographs of synaptic vesicles, synaptic membranes and coated vesicles (Miller and Heuser, 1984). It was indeed the application by Heuser and Reese (1973) of the findings in cellular biology regarding CV, to the neuromuscular junction (NMJ), what led them to propose the now famous vesicle recycling hypothesis in synaptic transmission (Heuser and Reese, 1973). This recycling is believed to occur in the CNS as well. Yet, the studies of Meshul and Pappas (1984) in identified NMJ's synapses seem to indicate that CV contribute to a minor extent or degree to the overall replenishment of the synaptic vesicle pool. The work of Goldstein et al. (1979), Brown et al. (1983) and Steinman et al. (1983) on the internalization of the LDL-receptor via clathrin coated pits and vesicles has emphasized the physiological relevance of this CV endocytic pathway. As stated before some cases of familial hypercholesterolemia could indeed be attributed to defects in endocytosis. Also signficant is the contribution by Schwartz and Lodish to our knowledge of general membrane recycling (Geuze et al., 1983 and Schwartz, 1984). They have proposed on the basis of both morphological and pharmacological evidence a complete scheme regarding the internalization and recycling of asialoglycoproteins (ASGP) and tranferrin, and their biological receptors (Geuze et al., 1983 and Schwartz, 1984). The list of molecules undergoing RME is vast and ranges from the classical hormone insulin to vitamins (Steinman et al., 1983).

Pathological. Helenius and colleagues (Helenius et al., 1980) has reviewed the role of RME in the entry of animal viruses into mammalian cells. Virus of

various families are competent for endocytosis, and their subsequent exposure to the acidic environment of CV and their derivatives, facilitates their fusion with the plasmalemma and translocation of these into the cell's cytosol. This allows them to escape from degradation and to exert their cytolytic actions. Indeed, chinese hamster ovary (CHO) cells mutants defective in acidification mechanisms are resistant to infection (Merion et al., 1983 and Klausner et al., 1984). Bacterial or animal toxins seem to behave in a similar fashion (Simpson et al., 1981). They all require a binding step and then an internalization step before cytolysis. Both viral and toxin entry can be safeguarded or delayed by the exposure of cells to basically charged molecules, inhibitors of endocytosis, like methylamine, ammonium chloride and others (Simpson, 1983). Finally, it has been reported that algal cells respond to cell injury with an enhanced formation of CV. This response is resemblant of the hypertrophied Golgi apparatus and increased number of its associated CV during liver injury and regeneration.

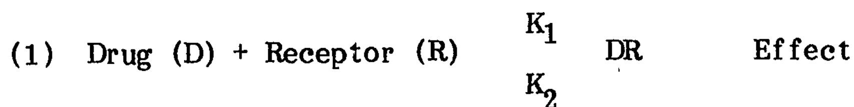
3. Muscarinic Cholinergic Receptors

a. Cholinergic Receptors and Receptor Theory. The pharmacological receptor concept was originally proposed by Erlich and Langley at the beginning of this century. Distinctive drug-sensitive areas were detected by both workers using dyes, and the drugs nicotine and curare, respectively. This led to the concept of specific receptive substances, or eventually receptors, which mediated the biological effects of the drugs. In 1914, Dale pharmacologically classified for the first time a biological receptor system, the cholinergic receptors (see Sokolovsky, 1984). Dale was able to show that the pharmacological action of ACh in skeletal muscle were mimicked by nicotine and blocked by curare. In contrast, the effects of ACh on heart tissue were unaffected by the latter drugs, while the alkaloid muscarine mimicked ACh and this effect was blocked by atropine. Since

then, the cholinergic receptors have been classified as nicotinic and muscarinic types.

These receptor types not only differ in their pharmacological properties but also in their coupling to effector systems, and the latency of their biological actions. The nicotinic cholinergic receptor (nAChR) is a pentamer whose subunits contain the effector system or the Na^+ (sodium) channel. In the meantime, muscarinic receptors have been shown to exist coupled to various effector systems. Muscarinic receptors are coupled positively to the turnover of phosphatidylinositol and negatively to the activity of adenylate cyclase (Jones et al., 1982 , Michell, R.H., 1975, McKinney and Richelson, 1984 and Brown et al., 1984). In addition, they seem to affect guanylate cyclase, phosphodiesterase and K^+-channels in various cells and tissues (Matsuzawa and Nirenberg, 1975; Gross and Clark, 1976; Hanley and Iversen, 1977; Kayoso and Ui, 1983; Sokolovsky and Barfai, 1981; Meeker and Harden, 1982; Fisher et al., 1983 and Delhaye et al., 1984). The latency of nicotinic responses are in the low millisecond range while the muscarinic responses have longer latencies in the high msecond to seconds range (Krnjevic et al., 1971, 1974).

Both cholinergic receptor systems have contributed significantly to the evolution of receptor theory. Classical receptor theory has one of its landmarks in the works of Clark (1926) and Ariens (1954) who initiated the quantitative analysis of dose response relationships (see Stephenson, 1956). The quantification of the receptor concept attempted by Clark consisted in the application of the adsorption isotherms of Langmuir, which are in turn derived from the application of the law of mass action to the adsorption of gases on metal surfaces. This mathematical treatment is also analogous to the one used for enzyme substrate interactions. The scheme can be represented as follow.



The equilibrium kinetics solution of this equation is well known and is often referred to as the Michaelis-Menten equation.

$$\text{Effect} = \frac{R + D}{D + Kd}$$

where D = drug concentration, R_t = total receptor concentration and K_d = the equilibrium dissociation constant of the drug. In the elaboration of his analysis, Clark (1926) used two cholinergic responses, the contraction of the isolated frog rectus abdominus and the inhibition of the contraction of strips of electrically stimulated frog ventricle. Yet, experimental observations of drugs having only partially complete responses compared to others, partial agonists, led to the invocation of an ad hoc parameter called, intrinsic activity (a) (Ariens, 1954). The equation 2 now became

$$(3) \qquad \text{Effect} = \frac{R + D}{D + Kd} .a$$

The intrinsic activity term is often found interchanged with the term efficacy (e) of Stephenson (1956). Stephenson's (1956) modification of the classical receptor theory also tried to account for the fact that some agonists (full) are able to achieve maximal effects with the occupancy of just a fraction of the available receptor pool. The latter phenomena has been referred to as "spare receptors". Furchgott (1967) also modified the classical theory and proposed an intrinsic efficacy term (E). Again in the elaboration of these modifications of receptor theory, the biological responses under consideration were cholinergic.

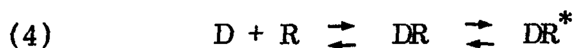
b. Ligand Binding Studies. The development of radiochemical ligand binding techniques has allowed the study of agonist and antagonist interactions with muscarinic receptors. These techniques have also led to the localization and

determination of the density of these cholinergic receptors. They have permitted the establishment of accurate occupancy-response relationships and served as tools to dissect the mechanisms of receptor regulation. These techniques together with quantum mechanical calculations in structure activity relationship (SAR) studies have led to the concept of the muscarinic pharmacophore (Weinstein et al., 1975).

c. Antagonists. Currently both agonist and antagonist (see figure 3) of the mAChR are available as labeling ligands. Agonist binding, as well as antagonist, has been measured directly, when the drug itself is the labeling ligand, or indirectly when used as competing or displacing ligands. All muscarinic labeling ligands are reversible with the exception of the antagonist propyl benzilyl choline mustard (PBCM) (Alberts and Bartfai, 1976). Also irreversibly inactivating the mAChR are the classical alkylating antagonists like dibenamine (El-Fakahany and Richelson, 1981).

The first antagonist used to label the muscarinic receptor was atropine. Other reversibly binding ligands available in their tritiated forms are scopolamine, quiniclidinylbenzilate (QNB), benzoloylcholine and N-methyl-piperidyl benzilate (4NMPB) (Sokolovsky, 1984 and Kloog et al., 1979b). Early studies with these antagonists revealed that their binding at equilibrium followed a simple law of mass action behavior (see equations 1 and 2). Yet, careful kinetic experiments soon contradicted this original observation. Three main empirical observations accounted for the contradiction. First, the dissociation constant (K_d) values obtained by equilibrium binding experiments were different from those of conditions short of equilibrium. Secondly, the dissociation kinetics for antagonist were multiexponential, and, third, the observed rates of association (Kobs) were not a linear function of ligand concentration, but were rather hyperbolic. These and other observations led to the proposition of an isomerization model for the

binding of antagonist to the muscarinic receptor (Kloog et al., 1978, Galpe et al., 1977 and Jarv et al., 1979).

(4) 

$$D + R \rightleftharpoons DR \rightleftharpoons DR^{*}$$

Thus, the initial drug-receptor (DR) complex is able to undergo a conformational change to a higher affinity drug-receptor complex (DR^{*}). A series of thermodynamic and kinetic data support this isomerization model.

Still though, regional variations on the binding of muscarinic antagonists in brain are observed particularly in the hypothalamus, medulla-pons and cerebral cortex (Kloog et al., 1978). These variations are reflected in a faster dissociation rate constant for antagonists such as ^{3}H(-)QNB and ^{3}H-4NMPB. The most significant variation has probably been documented for the muscarinic antagonist pirenzepine (Hammer and Giachetti, 1982, 1984). For this ligand, as well as for N-methylscopolamine (both hydrophylic ligands), heterogeneity of binding affinities has been observed in different preparations. Indeed, the proposed selectivity of pirenzepine has been used as a basis for the classification of muscarinic receptors into subtypes (Hammer and Giachetti, 1984). The muscarinic subtype M1 displays higher affinity for pirenzepine and seems to be localized in distinct tissues such as sympathetic ganglia, corpus striatum, and stomach. In constrast, the M2 subtype has a lower affinity for pirenzepine and is concentrated in hind brain, cerebellum and heart (Hammer and Giachetti, 1984). This selectivity of pirenzepine is supported by reports on its clinical efficacy as antiulcer compound controlling gastric secretion (see review by Konturek, 1983). Gallamine, a classical nicotinic antagonist used clinically as a muscle relaxant has in turn been claimed to selectively interact with the M2 site (Hammer and Giachetti, 1984). The M1 and M2 subtypes are coupled positively to phosphotodylinostol (PI) turnover and

negatively to adenylate cyclase, respectively (Delhaye, 1984, Kayoso and Ui, 1983 and Fisher et al., 1983).

The validity of this classification scheme for muscarinic sites is though questionable due to some pieces of biochemical and pharmacological information. In vitro binding studies revealed no differences in the affinity of pirenzepine for the receptors in dog stomach and striatum (Laduron et al., 1981). Moreover, pirenzepine has similar pA2 values for pre and postsynaptic muscarinic receptors of the guinea pig myenteric plexus-longitudinal muscle preparation (Halim et al., 1981) and the isolated rabbit and rat hearts (Fuder, 1982). Another pharmacological study (Szelenyi, 1982) also reported similar pA2 values for pirenzepine in the antagonism of betanechol-evoked responses in the guinea pig ileum and atrium, and on acid secretion from the isolated stomach of the mouse. Furthermore, the differential coupling of muscarinic receptors to its effector systems has also been challenged since both excitatory (i.e. muscle contraction), as well as inhibitory (i.e. slowing of heart rate) muscarinic responses seem to be coupled to the turnover of phosphatidylinostol (Heller Brown and Brown Masters, 1984). Nevertheless, in mammlian brain, the claim and evidence supports a presynaptic location for M2 receptors and a postsynaptic location for M1 receptors. M2 receptors function in the autoinhibition of acetylcholine's release (Kilbinger, 1984).

d. Agonists. Direct binding studies with agonists have been limited due to their availability and technical difficulties. Labeled N-methyl-oxotremorine, cis-methyldioxolane and pilocarpine have been used in direct ligand binding determinations (Ehlert et al., 1980 and Hedlund and Bartfai, 1981). Otherwise, agonist binding measurements have been mostly indirect based on their competing or displacing abilities of labeled reversible muscarinic antagonists. These indirect measurements have produced inhibition or competition curves which deviate from

the predictions of the law of mass action. This deviation is mainly manifested by shallow inhibition curves with Hill slopes significantly less than one for all agonist tested (Birdsall et al., 1978 and Ehlert et al., 1982). These results have been interpreted in terms of heterogenous binding sites having different affinities for agonists. Usually two distinct high (H) and low (L) affinity binding sites have been invoked (Ehlert et al., 1982). Yet, Birdsall et al. (1978) reported that their non-linear least square analysis of the binding data revealed the existence of a third super-high (SH) affinity binding site for agonist in several rat brain regions.

In direct agonist binding measurements with N-methyl-oxotremorine and cis-methyldioxolane, the low affinity sites are undetectable by the common techniques of filtration or centrifugation due to their generally faster dissociation rate constants. In contrast, studies with partial agonist ^{3}H pilocarpine have allowed the detection of two binding sites with densities higher than those of antagonists (Hedlund and Bartfai, 1981). Interestingly, the binding data of muscarinic ligands meets the predictions of Paton's Receptor Rate Theory (Sokolovsky, 1984). It states that the differences in efficacy between agonist, partial agonist and antagoist could be accounted for by their dissociation rate constants, these being higher for full agonists.

Heterogeneity of binding affinities for agonist in cerebral cortex and striatum has been shown to be related to different subtypes (Mash et al., 1985 and references therein). The form with high affinity for agonists is the M2 receptor while the M1 postsynaptic receptor has low affinity for agonists. This way the ratio of low to high affinity sites is an index of M1:M2 receptors. Otherwise the ratio of K_L/K_H is indicative of the agonists efficacies (Fisher et al., 1983 and Ehlert et al., 1982).

e. Perturbations of Ligand Binding. A spectrum of chemical agents have been reported to alter the binding of muscarinic ligands. Guanine nucleotides and

their non-hydrolyzable analoques have received the most attention. Due to their interaction with the GTP-binding protein of the adenylate cyclase complex, and in the presence of sodium, they are able to interconvert the high affinity agonist binding sites into low affinity ones (Ehlert et al., 1980, 1981b, Harden et al., 1982, and Waelbroeck, 1982). There is a regionalization of this effect, it being particularly seen in regions rich in H forms of the receptor like striatum, heart, cerebellum, medulla-pons and the female adenohypophysis (see Sokolovsky, 1984). These H forms can also be irreversibly blocked by drugs affecting voltage dependent-Na^{+}-channels like veratridine and tetrodotoxin (Sokolovsky and Bartfai, 1981). Depolarization with high potassium, valinomycin and gramicidin also block the H state (Sokolovsky and Bartfai, 1981). Two experimental conditions which also are able to abolish the H state reversibly and irreversibly are heating (50°C) and freeze-thawing cycles, respectively.

The conversion from L to H has also been achieved with n-ethylmaleimide (NEM) and divalent transition metal ions (Harden et al., 1982, Hedlund and Bartfai, 1978, Aranstrom et al., 1978, Aranstrom and Eldefrawi, 1979, and Ehlert et al., 1981b). These effect again displays regionalization, being most prominent in areas rich in L forms of the receptor. It is indeed this interconversion capacity, together with the fact that both H and L forms are expressed in a single homogeneous cell type (NE115), are evidences which may neglect the presence of isoreceptors. They indeed seem to point to a single receptor molecule capable of existing in various possible functional states. Supporting this concept of functional heterogeneity is the recent evidence that monoclonal antibodies raised against the mammalian muscarinic receptor do not recognize subtypes and are also capable of cross reacting with muscarinic receptors from organisms like the fruitfly, Drosophila melanogaster (Venter et al., 1984 and Venter, 1983).

Antagonists are also affected by guanine nucleotides in heart and

adenohypophysis (Mattera et al., 1985, Burgisser et al., 1982 and Sokolosky and Bartfai, 1981). The effect on the adenohypohysis is related to the hormonal state of the animal as well as to sex (Sokolovsky, 1984). The effect is mainly manifested as an increase in antagonist binding, although additional efects on their binding isotherms have recently been detected and interpreted in terms of cooperative interactions (Mattera et al., 1985).

f. Localization of Muscarinic Receptors. Most of the parasympathetic responses in the body are controlled by acetylcholine acting on muscarinic receptors, pointing to their physiological significance. They stimulate secretion in a series of exocrine tissues such as pancreas, parotid, submandibular, lacrimal and sweat glands (Michell, 1975, Engelhorn, 1981 and Hootman, 1982). Parasympathetic innervations also regulate the tone of various muscles like heart, iris, longitudinal muscle of the ileum, vascular, bladder sphincter, and vas deferens (Higuchi et al., 1983, Hammer and Giachetti, 1981 and Furchgott, 1984). With the sensitive ligand binding techniques mAChR's have been detected in other cells and tissues such as the retina, anterior pituitary, epithelial cells, red blood cells, lymphocytes, mast cells and oocytes (Sugiyama et al., 1977, Wojcikiwicz et al., 1984, Massini et al., 1983 and Sokolovsky, 1984). Their physiological function in the latter cells is not completely understood.

In neuronal tissue they are prominently present. In the peripheral nervous system, they are present in both the soma and nerve terminal of ganglionic cells. In the sympathetic ganglion, muscarinic agonist cause a slow postsynaptic depolarization resulting from a calcium-independent reduction in a potassium conductance (M-current) (Gardier et al., 1978 and review by McKinney and Richelson, 1984). In the superior cervical ganglia, there is modulation via preganglionic muscarinic receptors, of the serotonin-positive small intensely fluorescent cells located at the poles of the ganglion near blood vessels

(Hadjiconstantinou et al., 1982). From studies on depolarizing and hyperpolarizing responses to muscarinic agonist in this ganglion, distinct muscarinic receptor subtypes were proposed. In addition, presynaptic muscarinic autoreceptors have been found to diminish the release of ACh and other excitatory neurotransmitters from the myenteric plexus, parasympathetic nerves of heart and iris (Kilbinger, 1984 and Kilbinger and Wessler, 1983). In nicotinic synapses of lower vertebrates, i.e. Torpedo, muscarinic presynaptic receptors also inhibit the release of acetylcholine (Kilbinger, 1984). Nonetheless, as stated in the section on muscarinic antagonists, the pharmacological profile of pre and postsynaptic receptors seems to be similar in peripheral but not central tissue of higher vertebrates.

The main type of cholinergic receptor in the central nervous system is the muscarinic (Sokolovsky, 1984). As mentioned before, the striatum, cerebellum, medulla-pons and female adenohypophysis are rich in H forms of the receptor. The L form is in turn mainly seen in cortex and hippocampus (Mash et al., 1985, Ehlert et al., 1981 and Birdsall et al., 1978). In cortex and hippocampus the muscarinic activity is mainly excitatory, whereas in the hypothalamus and medulla-pons it is inhibitory (Krnjevic, 1974). Autoreceptors inhibiting ACh release have been detected in cortex, hippocampus and striatum (Weiler et al., 1984, and Kilbinger, 1984). Cortex is indeed very heterogeneous and the H (M2) and L (M1) sites seem to concentrate on distinct cortical layers (Wamsely et al., 1981).

g. Regulation of Muscarinic Receptors. The decreased responsiveness of a tissue to an agonist after exposure to an agonist, has received the name of desensitization or inactivation. These terms are usually reserved for the phenomena observed after relatively short exposure times. Long term desensitization has, in turn, been named subsensitivity or down regulation.

Desensitization can be specific (homologous), that is induced by the agonist in question, or nonspecific (heterologous) induced by another agonist. The first report on nonspecific desensitization by Cantoni and Eastman (1946) documented how the contractile responses of the guinea pig ileum to acetylcholine (ACh) or histamine (HA) were abolished by preincubation with high concentrations of either of the two (see Richelson and El-Fakahany, 1981). Specific desensitization was first observed by Barsown and Gaddum (1935), who showed that after preincubation of fowl rectal muscle with HA the tissue became insensitive to HA, while the responses to ACh and other agonists was only slightly reduced (see Richelson and El-Fakahany). Since then specific desensitization has been demonstrated for alpha and B adrenergic, nicotinic, insulin, muscarinic, histamine (H_1 and H_2) and prostaglandin receptors (see review by Richelson and El-Fakahany, 1981).

Desensitization may well be a result of post receptor events and may involve posttranslational modifications like phosphorylation. The latter has indeed been recently shown for the B-adrenoreceptor, rhodopsin and insulin receptors (Liebman and Pugh, 1981 and Shia et al., 1983). The latter phenomena may be more widespread since a series of hormone receptor systems, i.e. epidermal growth factor receptors, have been shown to possess intrinsic protein kinase activity (Shia et al., 1983, and Cochet et al., 1984). The latter mechanisms have not been reported for the muscarinic receptor. Nonetheless, in the case of the stimulation of cGMP synthesis by muscarinic agonists in the neuroblastoma cell line N1E115, the role of a postreceptor event involving cGMP has been ruled out. In this system, the desensitization has been shown to be independent of cGMP synthesis (Richelson and El-Fakahany, 1980). The phenomenon is observed after 30 minutes of exposure to agonists and the rate of desensitization is proportional to the efficacy and concentration of the agonist (El-Fakahany and Richelson, 1980). In

addition, it does not seem to involve a loss of receptors or a change in the affinity. Resensitization of this system has a half-life of 13 minutes (El-Fakahany and Richelson, 1980). In contrast, short term incubation of cultured heart cells with muscarinic agonists lead to a loss of a fraction of the receptors with a small change in the affinity of the receptor for the agonist. The lost binding sites can be recovered by GTP treatment (Galper and Smith, 1980).

Receptor down-regulation has also been studied with muscarinic receptor systems. Incubation of the neuroblastoma cell clone N1E115 for 1 hour with high concentrations of agonist (1 mM) results in a gradual decrease in the number and affinity of the muscarinic receptors, with a half-time for disappearance of 4 hours (El-Fakahany and Richelson, 1980). The return of the receptor sites (T 1/2 = 6 hr) precedes that of the receptor function (T 1/2 = 16 hr) and are dependent on protein synthesis. In cultured heart cells, the slow phase of receptor loss induced by agonists indicates that after wash out of agonist recovery of receptors had a 3 hour lag and reached 95% recovery after 9 hours (Galper and Smith, 1980). The loss of receptors in heart cells has also been shown to be dependent on the cytoskeletal system and protein synthesis (Galper and Smith, 1980). It has also been shown for the heart muscarinic receptors that the internalization of the receptors results in different states of the receptor differing in their ability to bind hydrophobic and hydrophylic muscarinic antagonist (Galper et al., 1982).

In the guinea pig ileum preparation desensitization induced by muscarinic agonists can be abolished by concanavalin A, dansyl cadaverine and bacitracin (Siegel and Triggle, 1983). The latter two are well known inhibitors of the enzyme transglutaminase, which has been claimed to be involved in the event of receptor-mediated endocytosis (Haigler et al., 1981). Other systems where down regulation of muscarinic receptors has been reported are the embryonic chick heart and brain, cultured thyroid cells, vas deferens smooth muscle, submaxillary glands and

brain (Meyer et al., 1982, Halvorsen and Nathanson, 1981, Ehlert et al., 1980, Higuchi et al., 1983, Champion and Mauchamp, 1982, dePeusner et al., 1984, Hootman, 1982, and Overstreet et al., 1984). The latter two were observed under chronic treatments with alcohol and acetylcholinesterase-inhibitors, respectively. The desensitization reported in rat striatum has been correlated with a diminished capacity of ACh to stimulate a high-affinity GTPase activity present in striatal membranes (Olianas et al., 1984). In cortex, the selective loss of the presynaptic (H form) M2 receptor subtype has been reported and proposed as one of the receptor mechanisms degenerated during impairment of memory functions during Alzheimer's disease (Mash et al., 1985). If endocytic mechanisms are involved in the latter phenomena remains to be shown.

4. 3':5'-Cyclic Nucleotide Phosphodiesterases

Various isoenzymes, or multiple forms of cyclic nucleotide phosphodiesterase (PDE E.C.3.1.4.17) exist (Wells and Hardman, 1977). Initial biochemical and pharmacological data obtained early after their discovery in 1958 by Rall and Sutherland (see Butcher, 1984) is to be interpreted carefully due to the presence in homogenates and microsomal preparations of various PDE types. Thus, early structure activity relationship (SAR) studies of the enzymes revealed an apparent poor selectivity of inhibitors, which ranged from the classical xanthine-derivatives to tricyclic antidepressants. Better purification procedures allow today the detailed study of distinct forms of the enzyme and the development of selective inhibitors for the various isoenzymes. A preliminary nomenclature and classification system for the various PDE subtypes has been proposed by a committee of workers in this field. The various forms have been classified according to their substrate preference and regulatory properties rather than any physical criterion or separation methodology.

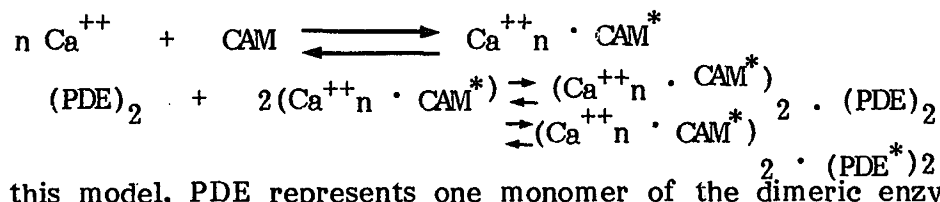
a. Type I. In 1970, Kakiuchi and Yamazaki, studied calcium-dependent and

independent forms of PDE in rat brain cytosolic extracts. They observed the presence in cytosol of an unidentified factor which shifted the calcium dose response curves to the left. Working with bovine brain cytosolic fractions, Cheung (1970) detected a similar factor which also shifted the cAMP hydrolysis dose response curve to the left and increased the Vmax for the reaction. The lowering of the Km for the Ca^{++} and cAMP responses meant that this factor enabled the PDE to function in physiological, low micromolar levels of calcium and cyclic nucleotides. This calcium dependent activating factor was latter identified as a low molecular weight (MW 18,000), heat-stable, acidic peptide now known as calmodulin (CAM). Indeed, many calcium-dependent biological processes were subsequently shown to involve the action of calmodulin. The calcium-calmodulin stimulated forms of PDE are named type I PDE's.

The index of stimulation of the purified enzyme varies widely (2-50 fold). This variability can be attributed to factors such as limited proteolysis, although loss in CAM stimulation in purified preparations can occur in the absence of detectable proteolysis or change in the basal activity (Kincaid et al., 1981; Shenolikar et al., 1985). Limited proteolysis eliminates CAM activation and increases the basal catalytic activity to that characteristic for the enzyme-calmodulin complex (Tucker et al., 1981 and Krinks et al., 1984). While the activation by Ca^{++}-calmodulin is reversible the one by limited proteolysis is irreversible (Krinks et al., 1984). Limited proteolysis of the purified enzyme with trypsin suggests that this PDE is composed of at least two functional domains: a catalytic domain, which binds the cyclic nucleotides, and a regularoty CAM-binding domain, which exerts an inhibitory influence on the catalytic domain in the absence but not in the presence of calmodulin (Krinks et al., 1984).

No cooperativity has been detected during either calmodulin activation or catalysis. Yet, the Ca^{++}-dependent activation of the enzyme exhibits a

pronounced cooperativity evidencing the requirement of more than one Ca^{++}/mole of CAM to promote interaction (Huang et al., 1981). Several laboratories have proposed a two-step model for CAM's interactions with CAM-binding proteins wherein Ca^{++} first binds to CAM and then the $CAM \cdot Ca^{++}$ complex binds to PDE or other CAM binding proteins to form an active enzyme complex as depicted below:



$$n\,Ca^{++} + CAM \rightleftharpoons Ca^{++}n \cdot CAM^{*}$$

$$(PDE)_2 + 2(Ca^{++}n \cdot CAM^{*}) \rightleftharpoons (Ca^{++}n \cdot CAM^{*})_2 \cdot (PDE)_2 \rightleftharpoons (Ca^{++}n \cdot CAM^{*})_2 \cdot (PDE^{*})2$$

In this model, PDE represents one monomer of the dimeric enzyme and CaM^{*} and PDE^{*} refer to the activated forms of the respective proteins. Each monomeric subunit size varies from MW 61,000 to MW 57,000, depending on the tissue, i.e. brain and heart (Klee et al., 1979).

The CAM-stimulated PDE (type I) is the predominant isoenzyme in mammalian brain (Kakiuchi and Yamazaki, 1970). Subtypes of CAM-stimulated PDE exist within brain tissue (Klee et al., 1979 and Shenolikar et al., 1985). The most characterized subtype displays higher affinity for cGMP than cAMP, Km's of 10 uM and 310 uM, respectively (Klee et al., 1979). Calmodulin's activation of this PDE subtype will lower the Km for cAMP to 100 uM and increase its Vmax. The newly characterized subtype (Shenolikar et al., 1985) responds to CAM's activation by increasing the Vmax for the hydrolysis of both cyclic nucleotides plus decreasing and increasing the Km for cGMP and cAMP, respectively (Shenolikar et al., 1985). The ratio of Vmax for both cylic nucleotides between these two subtypes also varies between them, with higher Vmax values for cGMP, cGMP-specific, seen in the recently purified and characterized subtype (Shenolikar et al., 1985). Both subtypes are usually regarded as high Km PDE's, nonetheless other CAM-stimulated low Km and/or cGMP-specific PDE's have been

reported in rat testis (Purvis and Hanson, 1980), hamster liver (Smoake et al., 1981) and rat erythocytes (Clayberger et al., 1981).

Type I enzymes are mainly cytosolic but they have been found in association with particulate preparations from rat erythocytes (Clayberger et al., 1981) and rat liver membranes (Smoake et al., 1981). While the rat erthrocytes PDE is responstive to B-adrenergic agonists, the cytosolic type I enzyme of WI-38 fibroblasts (Barber and Butcher, 1980 and Butcher, 1984), astrocytoma cells (Meeker and Harden, 1982) and thyroid (Miot et al., 1984 and Dumont et al., 1984) are responsive to muscarinic agonist. In coronary arteries type I PDE activity is affected by hormones and factors regulating vascular contractility (Saitoh et al., 1985). Type I PDE is also detectable in postsynaptic densities of canine and rat cerebral cortex and corpus striatum (Grab et al., 1981; Therien and Mushynski, 1979; Ariano and Appleman, 1979; Florendo et al., 1971). It is also seen in rat and cat axodendritic synapses of the caudate nucleus and neocortex (Ariano and Adinolfi, 1977). Fractionation studies of bovine brain preparations of microtubules and neurofilaments have shown the presence in these of type I PDE (Runge et al., 1979).

In addition to xanthine-derivatives (Dumont et al., 1984 and Miot et al., 1984) other inhibitors of type I PDE's have been reported. A number of endogenous proteins inhibit the activation of PDE by CAM; i.e. calcineurin, myelin basic protein and histone (see Sellinger-Barnette and Weiss, 1984 and Wallace et al., 1978). Opiod peptides (B-endorphin), dynorphin, polylysines and insect venom peptides (i.e. mellitin) will also inhibit CAM's activation of PDE (see Selliger-Barnette and Weiss, 1984 and Giedroc et al., 1983, 1985). The first drugs reported to inhibit CAM-stimulated enzymes were the phenothiazine antipyschotic drugs, which were also shown to inhibit CAM's stimulation of PDE (Levin and Weiss, 1976). A common feature of the inhibitory peptides listed above is that

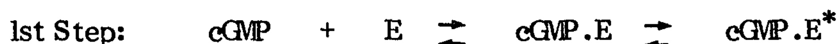
they are known or predicted amphipatic alpha-helices (Sellinger-Barnette and Weiss, 1984). In addition, B-endorphin-derived peptides and chlorpromazine binding domains on CAM possess some commonality (Giedroc et al., 1985). Finally, K^+ ions will inhibit basal activity without affecting the CAM-stimulation of the enzyme (Strada et al., 1984).

b. Type II. In 1970 Beavo et al. reported for the first time in cytosolic fractions from liver the presence of a PDE whose cAMP-hydrolytic activity was stimulated by cyclic GMP (Type II PDE). A year later, Beavo et al. (1971) reported the presence of this PDE type in both soluble and particulate fractions from various tissues, i.e., liver, thymus, brain, kidney and heart. Since the discovery of this type of PDE it has been detected in most tissues examined.

Indeed, immunological analysis using monoclonal antibodies against Type II enzymes has revealed that it constitutes one of the predominant forms of PDE in the cytosol of most tissues (Hurwitz et al., 1984). In cultured dog kidney cells the expression of type II PDE can be induced by treatment with butyrate (Manganiello et al., 1983). In contrast, its presence is diminished during transformation of 3T3-L1 preadipocytes or dexamethasone treatment of culture HTC hepatoma cells (Manganiello et al., 1983). It has been purified from bovine adrenal, heart and liver (Martins et al., 1982 and Yamamoto et al., 1983a). This type II PDE is larger than any other form, having an apparent MW of 102-107,000. It apparently exists in vivo as a dimer of MW 202,000 to 240,000 (Martins et al., 1982 and Yamamoto et al., 1983a).

As type I PDE, the stimulation index of type II enzymes varies widely (0.5 to 60). The stimulation by cyclic GMP seems dependent on proteolysis, degree of purity, pH and storage (Klotz and Stock, 1972; Russell et al., 1973; Rhoads et al., 1976; Yamamoto et al., 1983a and Martins et al., 1982). The stimulation index is generally lower for particulate forms of the enzyme (Franks and Macmanus, 1971;

Egrie and Siegel, 1977 and Sakai et al., 1974). The optimal concentration of cyclic GMP stimulating hydrolysis is usually higher for particulate forms also (Moss et al., 1977). The enzyme hydrolyses both cyclic nucleotides with Km's of 10 uM and 30 uM for cGMP and cAMP, respectively. The kinetics of hydrolysis are suggestive of positive cooperativity, as reflected in Hill slopes higher than one for both cyclic nucleotides. Activation of cAMP hydrolysis by cGMP and other analogues results in a shift of the dose response curve to the left, a return to unity of the Hill slopes values, no significant changes in Vmax, and an enhanced sensitivity to proteolysis in a domain relevant to catalytic function (Moss et al., 1977, Beavo et al., 1971 and Erneux et al., 1984). A two-step model has been proposed for the Type II PDE of rat liver (Erneux et al., 1982: where the first step (activation process) is rather specific for cGMP, in contrast to the second one (hydrolytic process):



where E and E^* stand for the enzyme in a free state and after cGMP binding, respectively. Pharmacological studies revealed distinct activation and catalytic sites on the mammalian enzyme (E) and the type II PDE of the slime mold Dictyostelium discoideum; yet, the latter one is a cGMP-specific enzyme which does not recognize cAMP at either site (Van Haarstet and Van Lookeren Campagne, 1984 and Erneux et al, 1981, 1984).

Inhibition studies with rat liver type II enzymes have revealed paradoxical stimulatory effects of otherwise competitive inhibitors of PDE, i.e. isobutyl-methyl-xanthine (IBMX) (Erneux et al., 1981, 1984 and Yamamoto et al., 1983b),

papaverine and dipyridamole (Yamamoto et al., 1983b). They activate at low concentrations and inhibit hydrolysis at higher concentrations reflecting their capacity to interact at both activating and catalytic sites on the enzyme. The classical inhibitor theophylline and the calmodulin antagonist MY-5445 are not effective inhibitors (Yamamoto et al., 1983b). Cyclic GMP analogues are more potent activators than cyclic AMP analogues; this is probably related to a better hydrogen-binding capacity or the dipolar character of guanine (Erneux et al., 1984). The catalytic site of type II enzymes is also pharmacologically distinct from those of the other PDE types (Erneux et al., 1984 and Miot et al., 1984).

c. Type III. Miki et al., (1973) first isolated a light-activated, cyclic-GMP-specifc PDE of rod outer segments (ROS) from frog retina, a type III PDE. Light activation requires the interaction of three major proteins; the photoreceptor rhodopsin, a GTP-binding protein and the phosphodiesterase (Yamazaki et al., 1984 and Fung et al., 1981). This molecular complex is analogous to membrane-associated adenylate cyclase activity with the GTP-binding protein of ROS (transducin) displaying homology to the GTP-binding proteins of adenylate cyclase (Gilman, 1984). The PDE has been purified from bovine retina and characterized as a dimer having a MW of 84,000 and 88,000 and an inhibitory subunit of MW 11,000 (Baehr et al., 1979) According to peptide mapping techniques the retinal PDE appears unrelated to calmodulin and insulin-sensitive PDE's, to the exception of a single peptide (Takemoto et al., 1984). This PDE is highly conserved and present in the retina of all vertebrates.

The PDE of rod outer segments, as well as that of adrenal, lung and heart tissues, possess high-affinity, non-catalytic cyclic-GMP specific binding sites, an unusual feature of calcium-calmodulin-insensitive PDE's (Yamazaki et al., 1982 and Martins et al., 1983). Binding of cGMP to these sites is stimulated by the inhibitory factor purified from bovine retina PDE (Yamazaki et al., 1982).

Nonetheless, the physiological relevance of these cGMP-binding sites remains unknown. Activation of type III PDE by GTP/GTP-binding protein complex results in the release of the inhibitory protein to the cytosol while hydrolysis of GTP results in its reassociation with and concomitant inhibition of disc membrane PDE (Yamazaki et al., 1984). A more complex cycle of activation and deactivation of type III PDE has been proposed where GTPase activity is not sufficient to account for the inactivationof the enzyme (Liebman and Pugh, 1980). Phosphorylation of the bleached photoreceptor rhodopsin via rhodopsin kinase is the mechanism by which ATP quenches the ROS type III PDE (Sitaramayga and Liebman, 1983). These integrated phenomena will thus constitute the biochemical basis for the processess of light transduction and adaptation. Interestingly, treatment with an inhibitor directed to the catalytic site of type III PDE will result in experimental retinal degeneration (Lolley et al., 1977).

Other PDE preparations coupled to GTP-binding proteins are those for adenosine in rat brain membranes and insulin in rat liver members (Mazancourt and Giudicelli, 1984 and Heyworth et al., 1983). Nonetheless, these have a low-Km catalytic PDE coupled to them, which may make them suitable to categorize among type IV enzymes. It is also very probable that many of the membrane bound particulate PDE which have low Km's and are responsive to hormones will be found coupled to GTP-binding proteins.

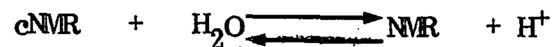
d. Type IV. Information concerning purification and characterization of the high-affinity form of PDE (type IV) is not as extensive as that of the lower-affinity forms (Thompson et al., 1984). The low Km, type IV PDE, most extensively studied have been derived from soluble fractions of dog kidney (Thompson et al., 1984), human lung (Moore and Schroedter, 1982), and calf liver (Yamamoto et al, 1984). In the type IV PDE of canine tracheal smooth muscle the rank order of potency of its inhibitors directly correlates with the rank order

displayed by them in the relaxation of the airway muscle (Polson et al., 1982). From calf liver cytosol, two pharmacologically distinct type IV PDE's have been detected (Yamamoto et al., 1984). The latter two differ in their sensitivity to inhibition by cGMP. The lowest Km form (Km 0.5 uM) is particularly sensitive to cGMP. The dog kidney PDE, in turn, exhibits negative cooperativity or heterogeneity which is eliminated after extensive purification (Thompson et al., 1984). It has a MW of 60,000, a broad pH optima (8.0 to 9.5), and has a considerably low affinity for cGMP (Thompson et al, 1979, 1984). Papaverine and etazolates (Squibb compounds) are the most potent inhibitors of the dog kidney enzyme (Thompson et al., 1984).

Membrane bound type IV PDE's have already been alluded to in the previous section. The rat liver membranes PDE is responsive to insulin, such is also the case for mammary acini and rat adipocytes (Aitchison et al, 1984 and Makino and Kono, 1980). This type IV PDE has been shown to be GTP-sensitive (Heyworth et al., 1983) and insulin's activation seems to involve phosphorylation of a peripheral membrane PDE of MW 53,000 (Houslay et al, 1984). An integral enzyme, not phosphorylated, is also activated by insulin. Adenosine via its interaction with its R-external receptors activate the type IV PDE activity of rat adiopocyte and brain membranes (Mazancourt and Giudicelli, 1984). Although type IV membrane bound PDE's have generally lower Vmax values, their physiological relevance is pinpointed by their responsiveness to hormones and their high affinity for cAMP, allowing it to work at low submicromolar levels of the nucleotide. Fell (1980) has estimated that particulate PDE may account for 60-95% of the total hydrolytic activity of a cell.

e. A working hypothesis. A model for endocytosis may be proposed considering two main observations. They are the pH-dependence of clathrin's assembly-disassembly and the requirement of calcium-calmodulin for the

recruitment of clathrin to the membranes (Salisbury et al., 1980, 1981 and Pearse, 1976). Due to the favoring of clathrin's assembly at the acidic pH's of less than 7.0, a calcium-calmodulin-dependent proton generator could be postulated to modulate cycles of clathrin assembly (see Figure 2). This activity could be provided by cyclic nucleotide PDE which generate protons (H^+) via their hydrolytic actions:



Calcium calmodulin dependent PDE exist as well, thus Type I PDE (or others) may represent the target for the action of calmodulin in endocytosis. Other PDE types may act as proton generators and suggest a role for certain modulators, i.e. cyclic GMP in the process of endocytosis. Interestingly, theophylline will produce delays in the onset of paralysis elicited by clostridial bacterial toxins, an effect also seen with inhibitors of endocytosis (Simpson, 1981). The PDE activity of CV may exist functioning in concert with other CV-proteins, i.e. calcium-magnesium ATPase and H^+-pump (ATPase). The PDE may also provide a pool of protons for the H^+-pump of endocytic organelles (see Figures 4 and 39).

MATERIALS AND METHODS

1. Materials

All reagents were of analytical grade I. Cyclic-AMP, cGMP, bovine serum albumin, papaverine, theophylline, 1-methyl-3-isobutylxanthine (IBMX), trifluoperazine, N-tris (hydroxymetheyl) methyl-2-aminoethane sulfonic acid, morpholino-ethane-sulfonic acid, 5' nucleotidase were from Sigma Co., St. Louis, MO. Tritiated cAMP and cGMP were from New England Nuclear, Boston, MA. The AG1-X8 resin and electrophoresis reagents were from BioRad Co., Rockville Centre, NY. Methanol, dimethyl sulfoxide and Scintiverse were from Fisher Co., Springfield, NJ. The Sephacryl S-1000 gel filtration column was Pharmacia (Sweden). Staphylococcus aureus cells were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN. Inhibitors of PDE (IBMX, theophyllline and papaverine) were prepared in water. Trifluoperazine was dissolved in dimethylsulfoxide and diluted in water.

2. Coated vesicle preparation.

Our CV from bovine brain were prepared by modifying the method of Keen et al. (1979, 1984) (Figure 5). Beef brains were obtained from a slaughterhouse, the meninges removed and the gray matter separated from the white by suction. Everything was done at $4^{o}C$ in the presence of a cocktail of proteolytic inhibitors. The gray matter was resuspended in 0.1 M morpholino-ethane-sulfonic acid buffer, pH 6.5, 1 mM ethylene glycol-bis-(B-amino ethyl ether) N,N'-tetraacetic acid, 0.5 mM $MgCl_2$, 0.02% sodium azide plus 0.3 mM phenylmethyl sulfonyl fluoride, 1 mM benzamidine, 5 uM leupeptin and 2 ug/ml of soybean trypsin inhibitor. The tissue was homogenized in a Waring blendor and the homogenate spun down at 17,000 rpm for 15 minutes in a Sorvall RC2B centrifuge with a SS34 rotor. This pellet was discarded and the supernatant spun down at 24,000 rpm in a Beckman ultracentrifuge for one hour with a type 30 rotor. The

resulting pellet was resuspended in homogenization media and loaded onto the first discontinuous sucrose density gradient of the following compositions: 7 ml of 50%, 8 ml of 40%, 10 ml of 30% and 5 ml of 20% sucrose (w/v), in homogenization buffer. The material from this first gradient was concentrated and loaded onto a second gradient of the following compositions: 3 ml of 50%, 4 ml of 40%, 7 ml of 30%, 8 ml of 20%, and 10 ml of 10% sucrose. These gradient-purified CV were passed twice over a Sephacryl S-1000 gel filtration column (Forgac et al., 1983). Optical density at 280 nm of the material eluting from the column was determined using a Beckman spectrophotometer (Figure 7-9). The main peak of the absorbance profile was more than 90% pure CV (Figure 6). Decoated vesicles were prepared by resuspending the CV pellet in either 5 mM N-tris(hydroxymethyl)methyl-2-amino ethane sulfonic acid or 0.5 M Tris-HC1, pH 8.1 (Keen et al., 1979, 1984). After one hour at room temperature decoated membranes were spun down at 100,000 x g in a Beckman ultracentrifuge for one hour using a Ti 50 rotor. Coated vesicles were used immediately for assay, or, when indicated, stored at $4^{o}C$ for 48-72 hours.

3. Immunopurification of Coated Vesicles

Coated vesicles were purified further using polyclonal antibodies against clathrin (Bloom et al., 1980) coupled to heat-inactivated, formaldehyde-fixed Staphylococcus aureus cells Merisko et al., 1982. Routine, CV (approximately 25-50 ug) in morpholino-ethane-sulfonic acid buffer purified over the Sephacryl S-1000 column were incubated overnight at $4^{o}C$ with 10-50 ul of sera (dissolved in immunotitration buffer of 10 mM Tris-HCl-5 mM $MgCl_2$ 1% bovine serum albumin pH 7.0) in a final volume of 200-250 ul. At the end of the incubation period 25 ul of a Staphylococcus aureus suspension in immunotitration buffer was added to each tube. The tubes were incubated at room temperature for one hour with periodic shaking, following which the tubes were spun down for 10 seconds in a

Beckman table-top microfuge. The pellets were washed twice and resuspended in 250 ul of PDE or binding assay buffer; 100 ul of the pellets were directly used in the enzymatic assays.

4. Muscarinic Acetylcholine Receptors Binding Assays

Muscarinic receptor binding assays were performed using ^3H(-)QNB (specific activity = 33.1 mCi/mmol) as the labeling ligand (500-30,000 cpm/ml and 5 uM atropine as the masking ligand to define specific binding (Yamamura and Snyder, 1974). Coated vesicles or immunopurified coated vesicles were incubated at room temperature or 30°C for 60-90 minutes (see Figures 12 and 13). The assays were done in 10 mM Tris - 5 mM $MgCl_2$ pH 7.2-7.4. Routinely 0.05-0.3 mg of coated or decoated vesicles were used (Figure 12). Under these conditions, binding was 80% or more specific in the region of 0.1 - 0.2x Kd. Bound and free ligands were separated by filtration over GF/B filters. Filters were dried and mixed with 4-5 ml Scintiverse and counted at a 40% efficacy.

The material eluted from the Sephacryl-S-1000 column was directly assayed for its binding activity (500 ul) in a final assay volume of 1-2 ml using 50 mM phosphate buffer pH 7.4 (Figures 7). To obtain a Kd value dose to the true dissociation constant of ^3H(-)QNB for the muscarinic receptor(s), the apparent K values obtained at various receptor densities were plotted against each other (Figure 16). In the competition experiments, the final assay volume was 5 ml and the ^3H(-)QNB concentration equal to about 800 pM. These experiments were peformed on affinity purified coated vesicles.

5. Phosphodiesterase Assay

We used an improved version of the isotopic method involving the column-separation procedure (Thompson et al, 1979). The final reaction mixture consisted of 40 mM Tris-HCl, pH 7.4, 5 mM 2-mercaptoethanol, 5 mM $MgCl_2$ and about 10^5 counts per minute of ^3H-cAMP per assay tube. For cAMP-hydrolytic dose-

response curves, isotopic dilutions were performed using 0.5-200 uM cAMP. Non-specific hydrolysis was determined on parallel blanks containing 10 mM theophylline or 1 mM IBMX or by boiling the tissue prior to assay. Final assay volume was 400 ul and contained 5-25 ug of tissue protein, a range wherein hydrolysis was a linear function of tissue with cAMP added. Twenty percent or less of the total counts added were usually hydrolyzed. Reactions were started by mixing all reagents in an ice bath, rapidly equilibrating them to 30°C and incubating them for 10 minutes. The reaction was stopped by placing the tubes in boiling water for one minute. Tubes were cooled immediately in an ice bath; 100 ul of 5'nucleotidase from Crotalus atrox (Sigma, Co. (2.5 units/ml)) prepared in distilled water was added. The tubes were mixed and incubated for 10 minutes at 30°C, at which time they were placed in an ice bath and 1 ml of methanol added to each tube. Tube contents were separated over an AG-1 x 8 anion-exchange resin. One ml of ethanol was added and columns were run to dryness. The column eluate was collected in scintillation vials and 10 ml of Scintiverse added to each. Counting was performed at 40% efficiency.

6. Data Analysis

Analysis of ^{3}H(-)QNB binding isotherms was done using the Scatchard equation (1949).

$$B/F = B(.1/Kd) + \frac{R_T}{Kd}$$

The linearity of the data was evaluated using a simple linear regression analysis with least squares solution available as the Fit Line program in the Prophet computer. The data was also fitted to the Michaelis-Menter equation using the hyperbolic program in Prophet. The binding data as well as the cyclic nucleotide hydrolysis dose response curves were fitted to the Hill equation using the LOGISTIC program available also in PROPHET;

$$B = \frac{B\,max}{1 + \left[\frac{(K_Q)}{(Q)}\right]^N}$$

where R = ^{3}H(-)QNB bound (or cyclic nucleotide hydrolyzed. Rmax = maximal bound (maximal hydrolysis), K_Q = apparent dissociation constant for QNB (or cyclic nucleotide), (Q) = concentration of ^{3}H(-)QNB (cyclic nucleotide or competing ligand), and N = Hill slope.

Competition experiments were also analyzed according to the Hill equation to obtain the IC_{50} and Hill slope values using the Logistic program. A similar approach was used in the analysis of the PDE inhibition profiles. Competition experiments where heterogeneity of binding affinities was apparent for the drugs tested, were further analyzed according to a two site model:

$$\%B + \frac{f_H}{1 + \frac{KH}{X}} + \frac{100-f_H}{1 + \frac{KL}{X}}$$

where %B = percent bound, f_H = percent of high affinity sites, X = concentration of competing ligand, K_H = affinity for the high affinite site and K_L= affinity for the low affinity site (Figures 19-22). The analysis of the data was done using an iterative non-linear regression analysis program available in PHOPHET as the FIT FUNCTION program. FIT FUNCTION was also used in the fitting of the cyclic nucleotide dose response curves to the equillibrium kinetics solution for the scheme presented in Section 6 of Results:

$$\frac{v}{Vmax} = \frac{1}{1 + \frac{Kc}{S} \cdot \frac{(1 + S/K_R)}{(1 + S/\alpha K_R)}}$$

7. Other laboratory procedures.

Sodium dodecyl sulfate (SDS) polyacrylamide slab gel electrophoretic analysis was done using Laemmli's method (1970). Protein concentration was determined with Lowry method (1951) using bovine serum albumin as a standard.

RESULTS

1. Localization of cyclic nucleotide phosphodiesterase activity and muscarinic acetylcholine receptors in bovine brain coated vesicles.

The final step in the purification of our CV requires their chromatography over a Sepharcyl-S-1000 column. Any CV associated molecule or activity must co-purify with CV during this step. In order to obtain the purest CV preparation possible we have passed our CV twice over this column, this approach has previously been utilized by Branton (Forgac et al., 1983) and co-workers in their studies on the proton (H^+) pump associated with coated vesicles. Figures 7 to 9 demonstrate that both the PDE and mAChR activities copurify with CV during both runs over the Sephacryl-S-1000 column. The CV peak is the peak of material retained by the column and determined by the absorbance at 280 nanometers (nm). The assay of 3':5'-cyclic nucleotide PDE activity in the presence of $CaCl_2$ (1 mM) or cGMP (5uM) revealed that after a second run the cGMP stimulated activity also copurified with CV (see Figure 9). This activity seems to be Ca^{++} independent. As stated before, this approach was seemingly enough to establish the presence of the H^+-pump in coated vesicles. This approach seems suitable since in our hands CV obtained through this procedure are more than 90% pure (see Figure 6). Yet, the presence of smooth vesicles is still a main concern, thus, additional purification steps are considered as more definite proofs or criteria for the direct demonstration of molecular association to CV. Amongst these additional purification steps are agarose-gel electrophoresis (Goetlib et al., 1985) and immunoprecipitation procedures (Merisko, et al., 1982). We have considered both techniques and present here the results of immunoprecipitation studies.

Using polyclonal antibodies against clathrin, coupled to formaldehyde-fixed, heat-killed Staphylococus aureus cells, Merisko et al. (1982) have been able to obtain a very efficient CV purification from fractions as heterogeneous as

microsomes. The smooth membrane vesicles are infinitesimally reduced using this procedure. We have obtained similar results as shown in Figure 10. The purity of these affinity purified CV was evaluted biochemically and its protein profile is charateristic of CV (Figure 6). Both morphological and biochemical information support the specificity of this immunoprecipitation assay. The specificity of our clathrin antisera has also been documented in the literature (Bloom et al., 1980). It has been reported to immunoreact with clathrin cages and produce immunofluorescent staining patterns characteristic of clathrins' distribution in neuronal and non-neuronal tissues. Not only qualitative but also quantitative immunoprecipitation of both the PDE activity and mAChR were obtained (see Figures 10 and 11). The histograms in Figure 10 shows that both the basal and stimulated phosphodiesterase activity can be selectively and completely immunotitrated. The stimulation index in both preparations was 241 and 212% for the control and immunotitrated CV. A similar result is shown for the mAChR binding activity in Figure 11. As seen in Figure 11, at least 80% of mAChR binding activity of the S-1000 purified CV is associated with CV. Maximal precipitation values are obtained at a range of 1-5 ul of sera per ug of coated vesicle (see Methods).

The quantitative and specific immunotitration of both the PDE and mAChR activities strengthens the evidence regarding their location in coated vesicles. Both of these molecules are associated with the membranes of coated vesicles, not the coat. That is to say that both are core proteins. The evidence supporting this observation is that both the receptor and enzymatic activity sediment with the stripped or decoated membranes after decoating with alkaline treatment (Figures 12 and 25). In addition, the Vmax of the PDE and the density of mAChR are increased by 50-60% after removal of the coat proteins (see Figures 12 and 25). In the case of the PDE activity, the stimulation of cAMP hydrolysis by cGMP

is also retained by the decoated membranes (see Figure 26).

2. Equilibrium binding studies of the muscarinic acetylcholine receptors of coated vesicles.

In our binding studies for the mAChR (-)Quinuclidynylbenzilate (not the racemate (±)) was used as the tritiated labelling ligand. Use of the racemate mixture can yield spurious kinetic information. QNB is known as a hydrophobic muscarinic antagonist in constrast, for instance, to the hydrophylic antagonist, N-methylscopolamine (Galper, 1983). For the equilibrium binding experiments and competition experiments 10 mM Tris-HCl-5 mM $MgCl_2$ pH 7.2-7.3 was used as the binding media. This low ionic strength, hypotonic media is used for two main reaons. First, it has been shown to be optimal to observe the effects of guanine nucleotides in other tissues (Luthin and Wolfe, 1983). And secondly, this media has allowed the expression of psuedo-Hill slopes less than unity for the antagonist N-methylscopolamine (Ehlert et al., 1981).

As seen in Figure 12, ^{3}H(-)QNB binding was linear as a function of coated or decoated vesicle population in the range of 10-300 ug of tissue protein. Equillibrium was achieved after 30-45 minutes at 32°-34°C and remained stable for the next 90 minutes (Figure 13). Thus, the equillibrium binding of ^{3}(-)QNB was done at 32-34°C for 75-90 minutes. Equillibrium binding isotherms for ^{3}H(-) QNB were obtained and anlyzed using the Michaelis-Menten, Hill and Scatchard equations (see Table I). The Michaelis-Menten plot (Figure 14) Shows saturability while the Scatchard plot (Figure 15) shows no evidence of heterogeneity of sites. The average Kd value for the receptor using these equations was of 81 pM (N = 4) and Bmax = 245 fmol/mg. Nonetheless, plotting of the apparent Kd values obtained in these experiments as a function of receptor density allows estimation of an affinity value close to the real Kd (Figure 16). Using this approach the true Kd can be extrapolated to be of 24.6 pM (R = 0.93).

3. <u>Competition binding experiments on the muscarinic acetylcholine receptor</u>.

The rank order of potencies of several muscarinic drugs were determined. Two antagonists (atropine and n-methyl-scopolamine) and two agonists (carbachol and oxotremorine) were used for this purpose. Their ranking as depicted from Figures 17 and 18 can be seen to be typical of muscarinic receptors with atropine $>$ n-methylscopolamine (N-MS) $>$ oxotremorine $>$ carbachol (Table II). The competition curves for all drugs, except atropine displayed Hill slopes different from unit (Table III). In addition, the presence of 500 uM GTP did not affect significantly the competition curve for oxotremorine.

The heterogeneity of binding affinities for oxotremorine, carbachol and N-MS was further analyzed using a two site model (see Methods). Figures 19 and 20 display the fits to the competition curves for oxotremorine in the absence and presence of GTP. The analysis reveals only 13% of high affinity sites for oxotremorine which are diminished to a minor 9% value in the presence of GTP. The high affinity site for oxotremorine had a value of 1 nM while the low affinity site had a value of 827 nM. Similar values were obtained for K_H and K_L in the presence of GTP (Table III). In contrast, the two-site analysis for carbachol revealed the presence of 67% high affinity sites with a K_H value of 100 uM and a K_L value of 3.17 mM (see Figure 21 and Table III). Curiously, the antagonist N-MS also displayed 62% of high affinity sites (Table III and Figure 22).

4. <u>Kinetic analysis of the cyclic nucleotide phosphodiesterase of brain coated vesicles</u>.

Hydrolysis of cAMP had a broad alkaline pH optima (8-10) and the stimulation of cyclic AMP hydrolysis by cGMP shows a neutral pH optima between 7.0-7.4 (Figure 23). thus, the pH of the assay was set to 7.4 for all experiments. The cyclic nucleotide hydrolytic activity is calcium and calmodulin independent

(Figure 9). Additon of EGTA or KCl was also without effect (data not shown), further supporting the observation that the main form of PDE activity associated with the CV fraction is calcium indepedent and stimulated by cGMP.

The enzyme hydrolyzes both cyclic nucleotides (Figures 24). The apparent Km obtained by the fit of the data to the logistic function or Hill equation (Segel, 1975) were of 11 uM and 22 uM for cGMP and cAMP, respectively. The Vmax for cGMP was 3.8 nmol/min/mg protein and the one of cAMP 3.5 nmol/mg (Table IV). Interestingly, the Hill coefficients for cAMP (N_H = 1.5) and cGMP (N_H = 1.2) were reproducibly different from one. Decoating experiments showed how the cAMP hydrolytic activity (Figure 25) and its stimulation by cGMP (Figure 26) sedimented with the decoated membranes. Decoating resulted in an increase in the Vmax of the enzyme (Table IV).

The stimulation indexes for cGMP ranged between 50-600% (Figure 26). We have observed similar stimulation by cGMP in CV prepared for synaptosome-enriched fractions and from rat liver (data not shown). The stimulation capacity is lost considerably (Figure 27) during storage. This inactivation as well as the activation of the enzyme by cGMP had particular effect on the dose response curves for the hydrolysis of cAMP (Figure 28). It can be seen that in both cases the Hill slopes relax back to one. For the case of activation, the curve is shifted to the left (22 to 10 uM) and to the right when it is almost inactive (22 to 38 uM). Although the Hill slopes for the hydrolysis of cGMP are close to unity the same behavior during storage as with cAMP, is noted in its dose response curves (Table IV). this set of observations are considered in another section in the development of a kinetic model (see section 6, Results).

5. Inhibition of the cyclic nucleotide phosphodiesterase of brain coated vesicles.

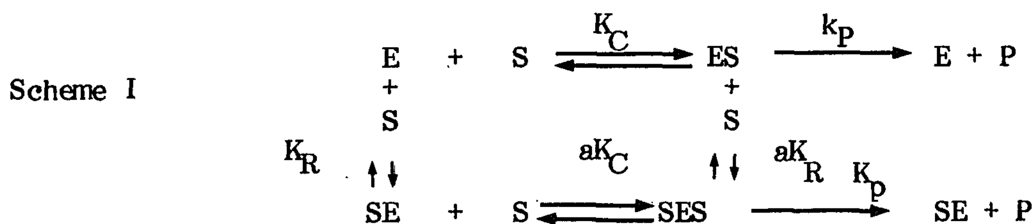
Phosphodiesterase stimulated by cGMP display complex inhibition profiles

for compunds like IBMX and papaverine (Erneux et al., 1981 and Yamamoto et al., 1983). These have been reported to paradoxically stimulate cAMP hydrolysis at low concentrations. The CV PDE was mildy stimulated by papaverine only (Figure 23). Otherwise, the rank order of potency of the PDE inhibitors using 5 uM cAMP as substrate was papaverine > IBMX > theophylline (Figure 29, Table IV). This rank order of potency was again displayed when tested for the inhibition of cGMP hydrolysis and cGMP-stimulated cAMP hydrolysis (Figures 30 and 31). Table IV which summarizes the IC_{50} for these inhibitors also shows the deviation from unity of the inhibiton Hill slopes for all compounds tested. These deviation from unity is most likely reflecting their interaction at the catalytic and/or regulatory sites of the enzyme.

6. Equilibrium kinetics model for the cGMP-stimulated phosphodiesterase of brain coated vesicles.

As discussed by Segel (1975) various kinetic schemes can account for Hill slopes higher than one, in addition to the classical models of positive cooperativity. Schemes regarding substrate activation are examples of these and are directly pertinent to the case of type II enzymes where a substrate cGMP is an activator also. Activation by the substrate could be a requirement for catalysis or not, essential versus non-essential activation (Segel, 1975). We can observe catalysis of either cyclic nucleotide after considerable loss (> 200%) of the cGMP-activation capacity seen after prolonged storage of CV in the PDE assay buffer at 4°C (see Figures 27 and 28). Thus, the CV enzyme fits a case of nonessential activation. It can also be observed , as seen in Figure 28 and Table IV, that this loss is also accompanied by a change in the kinetic behavior of the enzyme. This change culminates for both cyclic nucleotides in an increase in the apparent Km and a return of the Hill slope to a value not significantly different from unity (Table VI). A return of the slope to a value not significantly different

from unity (Table VI). A return of the slope to a value not significantly different from unity is also observed for the cAMP dose response curves, in the CV PDE as well as in other cGMP-stimulated enzymes, when done in the presence of cGMP (Figure 18, Beavo, et al., 1971) and other activators. In all cases the Vmax is not significantly changed and the apparent Km is now decreased (Erneux et al., 1984). Thus two situations can reflect the behavior of the enzyme in two predominant states the unactive and activated states respectively. These are considered in the following kinetic scheme as the species ES and SES, respectively, contributing to the observed catalysis (see Figure 32).

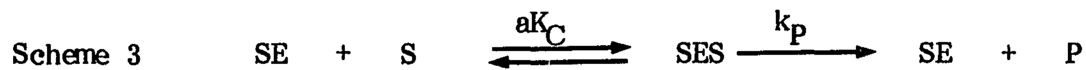


where, E = enzyme with a regulatory (R) and catalytic (C) site, S = substrate (cAMP or cGMP), ES = enzyme - substrate complex at the catalytic site, SE = substrate-enzyme complex at the regulatory site, SES = ternary complex where substrate is bound to the regulatory and catalytic sites, K_C = intrinsic dissociation constant of the substrate for the catalytic site (unactivated state), K_R = intrinsic dissociation constant of the substrate for the regulatory-activation site, k_p = catalytic rate constant, P = product (AMP or GMP) and = factor by which the apparent affinity of the catalytic site for the substrate is changed after occupancy of the regulatory-activation site (<u>activation factor</u>).

It can visually be depicted in the kinetic scheme that when the enzyme is inactivatable the scheme reduces to

This model will thus predict that under this condition the slopes and curves be of simple Michaelis-Menten kinetics behavior. A simple kinetic behavior will also be predicted for the experimental situation where saturating amounts of activators

are present in the cAMP dose response curves. Again the equilibrium will be pulled to make the kinetic scheme reduced to



Scheme 3 $\quad SE + S \underset{}{\overset{aK_C}{\rightleftharpoons}} SES \xrightarrow{k_P} SE + P$

Both predictions are approached by cGMP-stimulated or type II cyclic nucleotide phosphodiesterases of rat liver cytosol and CV (Figure 28) (Beavo et al., 1971 and Erneux et al., 1984).

This relationship is also expressed mathematically in the solution under equilibrium assumptions of the above presented kinetic scheme.

$$(1)\quad V = K_p\ (ES + SES)$$

$$(2)\quad E_T = E + ES + SE + SES$$

$$(3)\quad V_{max} = K_P \cdot E_T$$

$$(4)\quad \frac{V}{Vmax} = \frac{ES + SES}{E + ES + SE + SES}$$

$$(5)\quad \frac{V}{Vmax} = \frac{\frac{S}{Kc} + \frac{S^2}{KcKr}}{1 + \frac{S}{KC} + \frac{S}{KR} + \frac{S^2}{KCKR}}$$

$$(6)\quad \frac{V}{Vmax} = \frac{1}{\frac{KC}{S}\frac{\left(1 + \frac{S}{KR}\right)}{\left(1 + \frac{S}{-K_R}\right)} + 1}$$

$$(7)\quad \text{apparent } Km = Kc \cdot \left(\frac{1 + S/K_R}{1 + S/K_R}\right.$$

$$(8)\quad \text{apparent } Vmax = V\ max$$

From equations 6 and 7 it stands that when a = 1 they will reduce to the simple Michaelis Menten equation.

An increase in the alpha value reflected as a shift of the hydrolysis dose response curve to the right, may be what is occurring during the loss of activation capacity. The experimental conditions of aged CV enzymes thus may provide a Km value approaching K_C not truly K_C because in the fractions analyzed some still displayed 10-50% residual activation capacities. This value is of near 40 uM (38 uM)and thus this was the fixed value of K_C arbitrarily chosen for the simulation analysis seen in Figures 33, 34 and 35. Meanwhile, when saturating amounts of activator(s) are present (as in Figure 28)

$$\text{Apparent Km} = aK_C$$

and the apparent K_M is now equal to aK_C. The experiments in the presence of activators will provide a close approximation of a , when their Km are divided by those of stored tissues (K_C). From Table III it can be seen that the aK_C value for the CV enzyme is of 10 uM. But, this value must be corrected for the possible amount of cGMP (5 uM) binding to the catalytic site. Now the apparent K_m for this experiment is

$$\text{apparent Km} = K_C \left(1 + \frac{G}{K_G}\right)$$

Using K_C (for cAMP) = 40 uM, ((cGMP)) = 5 uM, K_C for cGMP = 10 uM, and an apparent K_m of 10 uM (see Table IV), we can estimate a tentative value for cyclic AMP of 0.16. This low value of a for cAMP is also consistent with its dose response curves having Hill slopes in the vecinity of 1.5 (Figure 24). From the stimulation analysis it can be seen that only alpha values lower than 0.25 will yield Hill slopes close to 1.5 (Figure 35).

The other factor contributing to changes in the observed slopes and Km

values is the relative affinity of each substrate for the catalytic and regulatory sites of the enzyme, the K_C/K_R ratio. For the case of cAMP and the CV PDE the K_R value for cAMP can be calculated to be of 137 uM, and an approximate K_C/K_R of 0.28 (Table VI). From Figures 33 and 34 it can be depicted that the lower the K_C/K_R ratio the farther to the right they will be. This together with the fact that the dose response curve for cAMP lies to the right of the curve for cGMP may suggest a larger K_C/K_R ratio for the latter. This implies a higher affinity for the regulatory site for cGMP which may explain why cGMP activates cAMP dydrolysis and not the converse occurs. Indeed, similar calculations for cGMP can be attempted. Table VI now resumes the estimated and K_C/K_R ratios of 0.38 and 13, respectively for cGMP. These relatively high values of alpha and K_C/K_R for cGMP are consistent and may explain the lower apparent K_m and Hill slope values for this cyclic nucleotide. The calculated values can indeed be used to generate dose response curves for both cyclic nucleotides which closely fit the experimental (fresh) dose response curves (Figure 36).

DISCUSSION

4. Muscarinic acetylcholine receptors of brain coated vesicles.

a. Equilibrium binding. Direct biochemical assays of muscarinic acetylcholine receptors in subcellular fractions have usually been restricted to crude homogenates, synaptosomes and synaptic membranes (Aguilar et al., 1982, Sokolovsky, 1985 and Yamamura and Snyder, 1974). Nerve ligature experiments of rat vagus, nerve sciatic nerve and brain (Wamsely et al., 1981, 1983; Zarbin et al., 1982; and Laduron, 1980), have tackled the receptor in its vesicular fast anterograde and retrograde axoplasmic transport. The work presented here represents one of the first attempts to detect and characterize the mAChR-binding activity in purified subcellular preparations of an internalization and/or transport organelle, CV. Its congener, the nicotinic receptor has been visualized in CV's (Burstajn and Fischbach, 1984). The experiments reported here support the presence of mAChR binding sites in bovine brain CV. The binding activity copurified with CV through all purification steps. Binding isotherms with ^{3}H(-)QNB reveal that the receptor has a Kd of 25 pM and a Bmax of 341 fmol/mg protein. The Kd value obtained is comparable to the values reported for ^{3}H(-)QNB in mammalian brain and other tissues (Ehlert et.al, 1981 and Sokolovsky, 1984). This low Kd values are belived to reflect the contribution of a mixed fraction of receptor binding states in accordance with the isomerization model proposed for ^{3}H(-)QNB binding (Galper et al., 1977 and Sokolovsky, 1984). Thus, the mAChR in this CV preparation seems to be able to isomerize during binding of QNB. The density of antagonist binding sites is also comparable to that reported for mammalian brain gray matter (McKinney & Coyle, 1982). This similarity may suggest a very balanced dynamic equillibrium between the mAChR on the plasmalemma and its intracellular pools.

b. Heterogeneity of binding affinities. Competition experiments with N-MS

in brain CV revealed the existence of high and low affinity binding sites. This heterogeneity of binding affinities for N-MS has been previously reported for brain and heart tissue (Elher et al., 1981 and Lee and El-Fakahany, 1985). The situation is reminiscent of the also hydrophylic antagonist pirenzepine. It has already been discussed how the heterogeneity of binding sites for pirenzepine may be related to distinct muscarinic receptor subtypes. A similar argument could be applied to the case of N-MS. If such were the case, the 38% low-affinity N-MS binding sites could be directly related to the 34% of low affinity carbachol binding sites (M1 receptors). The major 66% of high affinity binding sites for carbachol and N-MS would then represent the M2 subpopulation. Nonetheless, this classification scheme is only opposed by the observation that for another agonist, oxotremorine, the percent of high affinity agonist binding sites is less than 20%. This is a common observation in brain tissue (Sokolovsky, 1984) and has led to the proposition that the K_L/K_H ratio is more important and is an index of the agonists' efficacy.

The K_L/K_H ratio for carbachol in CV is within the range of values seen in brain and other tissues (Fisher et al., 1983). A minimal K_L/K_H ratio is required for full agonist activity ($\sim$40) (Fisher et al., 1983). For oxotremorine the ratio in CV is the highest (915) among all values reported for it in the literature. Curiously it has been reported that during down-regulation of mAChR the K_L/K_H ratio increases. The high CV K_L/K_H ratio value for agonists may suggest that these mAChR's are present as cargo molecules in endocytic CV. The putative sources of endocytic CV are depicted in Figure 37 as subsets 1 and 2. These may derive from presynaptic terminals or the soma of postsynaptic neurons.

According to the latest classification scheme, M2 receptors should be in presynaptic terminals and may also be present in the Golgi-associated CV en route to their fast axoplasmic transport (subsets 1 and 3 of Figure 37). The latter could

correspond to the 66% of high-affinity carbachol binding sites. The insensitivity of GTP could suggest that a significant portion of these are from endocytic (Zarbin et al., 1982) CV. The relative fraction of mAChR in CV which are internalized cargo molecules could be estimated from the low-affinity N-MS binding sites. This approach seems valid in heart and brain (Galper, 1983). If such were the case, then less than 38% of the M2 presynaptic receptors could be in endocytic CV's. This leaves us with at least 20% of the putative M2 receptors in CV's being related to the Golgi region of presynaptic cholinergic neurons. Thus, the postsynaptic M1 (low-affinity carbachol binding site) positive CV could thus also represent a mixture of endocytic and exocytic CV.

The microheterogeniety of CV preparations from bovine brain has already been reported on the basis of biochemical, immunochemical and morphological observations (Wiedenmann et al., 1985, Pfeffer and Kelly, 1985 and Ockleford and Whyte, 1981). The presence of specific cargo molecules with molecular weights of 38,000 and 29,000 has been used as the marker for distinct CV subpopulations. From the results presented here it can be concluded that the heterogeneity of binding affinities for agonists and antagonists of the mAChR suggests the presence of various CV subsets. At least 4 CV subpopulations can be inferred all carrying for mAChR. Various "functional" or affinity states of the receptor will be discriminant of specific CV subset. It can be envisioned that monoclonal antibodies against specific CV subsets may allow the purification of CV subpopulations enriched in mAChR with a particular affinity profile for muscarinic drugs.

c. Clinical relevance. It has recently been shown that human and rat cerebral cortex muscarinic M2 receptors are diminished in senile dementia of the Alzheimer's type (SDAT) (Mash et al., 1985). This state results as a consequence of eliminating the presynaptic cholinergic neurons, particularly the innervation

from the nucleus baslis of Meynert. This has led to some prospects in the pharmacotherapy of this disease and led to precaution in the use of drugs like atropine or possessing antimuscarinic side-effects in the treatment of disease states like Parkinsonism and depression. The pharmacotherapy of SDAT will move in the direction of developing M1 selective agonists or M2 selective antagonist (Hammer and Giachetti, 1984). In addition, SDAT could as well results from an imbalance of the recycling of M1:M2 receptors. An enhanced rate of endocytosis or defective exocytosis of M2 receptors will result in less autoinhibition of ACh release, acetycholine's interaction with the postsynaptic M2 receptor may result in its down regulation. Thus, subsequent down regulation of M1 receptors could indeed result in the onset of the disease. Alterations in the transport CV could also deplete the postsynaptic plasmalemma of M1 receptors. It can be visualized how enhanced rates of endocytosis of either M1 or M2 receptors, as well as defective transport of the newly-synthesized receptors may result in SDAT. In conclusion, imbalances in the levels of mAChR in the different CV subsets (1,2,3) of mammalian cerebral cortex could result in SDAT.

2. Cyclic Nucleotide PDE of Coated Vesicles.

a. Pharmacological properties. For all known types of PDE, both soluble and particulate forms have been reported. In brain tissue PDE activity is distributed differentially with postsynaptic and intermediate filaments densities, displaying a calmodulin-sensitive PDE (Grab et al., 1981). The other major PDE activity associated with particulate preparations from bovine and rat cerebra is Ca^{2+} independent (Davis, 1984; Grab et al., 1981, Therien & Mushyski, 1979; Vale et al., 1984). The biochemical assay of cAMP-PDE in the presence and absence of cGMP on the material eluted from the S-1000 column and further immunopurified demonstrated cAMP hydrolytic activity sensitive to cGMP copurifying with the CV. Indeed, purification of our Sephacryl S-1000-column-purified CV by an

agarose gell electrophoresis technique (Gottlieb et al., 1985) corroborates our finding that cyclic nucleotide PDE activity of CV was stimulated by cGMP, a type-II enzyme (data not shown). Both the hydrolytic activity and cGMP sensitivity were retained

by the membrane after removal of the coat, suggesting a particulate enzyme.

Particulate forms of type-II enzymes have been found in brain, liver, thymus (Beavo et al., 1970, 1971); T-cells (Franks & Macmanus, 1971); hepatoma cells (Turnbull & Hickie, 1984); and cultured 3T3L1 preadipocytes (Manganiello et al., 1983). The indexes of maximal stimulation by cGMP in the latter preparations are comparable to those of our CV preparation, ranging anywhere between 50-600% (Beavo et al., 1971; Egrie & Siegel, 1977; Franks & Macmanus, 1971; Klotz & Stock, 1972; Sakai et al., 1979). These stimulations indexes are lower than those from cytosolic forms of this enzyme type (Erneux et al., 1984), perhaps because cytosolic and particulate forms of the enzyme may have different pharmacological properties. In addition, studies on the cytosolic type-II PDE have shown that the stimulation index is a function of degree of enzyme purification (Martins et al., 1982). Otherwise, the relatively lower indexes of stimulation in these particulate preparations (including CV) may be due to the contribution of some low K_m low V_{max}, membrane-bound PDE from either smooth vesicles or CV subsets. We have observed that the sensitivity to cGMP and its allosteric behavior is lost during storage at 4^oC. Proteolysis as well as association with endogenous inhibitors may also expalin the low stimulation indexes. Thus, the values reported here for stimulation most likely are underestimates of _in vivo_ sensitivity to cGMP.

Kinetic parameters were obtained from the analysis of cAMP hydrolysis dose-response curves in the presence and absence of cGMP. The K_m (21 uM) for cAMP is close to that of other type-II enzymes (Martins et al., 1982; Yamamoto et al., 1983) and Ca^{2+}-independent forms from bovine tissues i.e. (Vale et al., 1984).

The slope index for cAMP is 1.5, suggesting positive cooperativity, for bovine brain CV-PDE. This allosteric behavior is typical of type-II PDE from rat and bovine liver cytosol; they also share modulation of this allosteric behavior by cGMP (Erneux et al., 1984 and Martins et al., 1982). This modulation by cGMP is believed to induce conformational changes which increase sensitivity to inactivation by chymotrypsin (Moss et al., 1977).

Phosphodiesterases differ beyond their kinetic behavior in the potency displayed by their inhibitors (Chasin & Harris, 1976; Yamamoto et al., 1983, 1984). Using a set of classical PDE inhibitors, competition or inhibition experiments of cAMP hydrolysis in the absence and presence of cGMP were performed. The rank order of potency of these antagonists in inhibiting the cGMP-stimulated cAMP hydrolysis is: papaverine $>$ IBMX $>$ theophylline. This rank order of potency is similar to the ranking they displayed against the cytosolic type-II enzyme or calf liver, including the ineffectiveness of trifluoperazine (Yamamoto et al., 1983). The rank order of potency in the inhibition of cAMP hydrolysis (no cGMP present) was the same (with trifluoperazine, again inactive at 100 uM). The IC_{50}s for IBMX and papaverine are in the micromolar range. This behavior is atypical for these compounds because in the cytosolic type-II enzyme they are very poor inhibitors of cAMP hydrolysis in the absence of cGMP (Yamamoto et al., 1983). Moreover, IBMX and papaverine also have been shown to stimulate cAMP hydrolysis by type-II cytosolic enzymes of rat and calf liver (Yamamoto et al., 1983). In CV, papaverine is a very poor activator of PDE activity, while IBMX is completely inactive in this respect. The latter differences in the behavior of these inhibitors between cytosolic type-II enzymes and the CV type-II PDE may be due to their being intrinsically distinct type-II isozymes or to the contribution of another PDE type to cAMP hydrolysis in the absence of cGMP. Various PDE conceivably may exist within a CV, or subpopulations of

these may carry different PDE types. The possibility that some or all of the PDE activity we detect in CV may represent contamination from the cytosol must be considered. We do not believe the latter to be the case due to the fact that type-II enzymatic activity sediments with the CV membrane under both hypotonic and hypertonic conditions; if such were the case, contamination would have been predominantly of the Ca^{2+}-calmodulin-sensitive PDE, which is the main cytosolic form in brain (Strada et al., 1984.

b. Subcellular location. In the receptor section, various CV subsets were invoked. These various subsets may as well carry the type II PDE of CV. The preferential expression of different PDE types in distinct subcellular structures may pinpoint to compartamentalization in the regulation of this enzymatic activity. Nonetheless, the type II PDE may coexist with type I PDE in postsynaptic densities. This possibility arises due to the fact that these subcellular structures (PSD) express also calcium-independent forms of PDE (Grab et al., 1981). In the latter preparations the sensitivity to cyclic GMP was not determined. In addition, the lack of expression of type I PDE activity in CV's may not be solely indicative of the poor degree of cytosolic contaminants but may suggest as well the means by which this PDE type is transported in the cell. Thus, type I PDE may be transported cytoplasmically (see Figure 38). A factor may exist in the cytosol which confers this isozyme its ability to anchor in postsynaptic densities or intermediate filaments. It is also entirely possible that other PDE types are present in our CV but in an inactivatable (desensitized) state in distinct or the same CV subsets. Inactivation of these enzyme(s) could be a function of proteolysis, aging (storage at 4°C) or association with endogenous inhibitors (Tucker et al., 1981). Specific allocation of this type II PDE to a distinct CV subset is difficult and further experimentation will be required. The eventual purification of CV subsets may reveal the coexistence of specific muscarinic

receptor binding activities with type II PDE.

c. Kinetic behavior. The 3':5' cyclic nucleotide PDE activity copurified, as the mAChR, with CV's. The main type of PDE activity detected under our assay conditions is stimulated by cyclic GMP, a type II PDE. Among type II PDE enzyme systems, the best characterized is the soluble form of the enzyme from rat liver (Erneux et al., 1984). The particulate type II PDE's have not been as thoroughly characterized (Beavo et al., 1971). The particulate nature of the type II PDE of CV's should lead to its consideration as a model system for the study of particulate type II PDE.

One of the idiosyncrasies of type II PDE are their Hill coefficients higher than one 1.2 - 1.8 (Erneux et al., 1984, Martins et al., 1983, and Yamamoto et al., 1983). This has usually been referred to as suggestive of positive cooperativity. But as discussed in Results other models may as well account for Hill slopes higher than one. Exemplifying these are the substrate activation models to which type II enzymes belong by definition. The presence of catalysis in the absence of a considerable amount of activation capacity suggests that the type II enzyme of CV fits a case of nonessential activation.

The absence of significant changes in the Vmax of the enzyme during activation or inactivation also provides insights into the mechanism of the enzyme. First, it means that activation of the enzyme by substrate only produces changes in the apparent affinity of the enzyme for the substrate at the catalytic site. This also means that no changes in the K_p of the reaction take place during substrate (cGMP) activation. This lack of changes in Vmax contrasts those seen during activation of type I enzymes by CAM. It can thus be inferred that activation of type II PDE by cGMP or its analogues (Erneux et al., 1981, 1984) does not release an inhibitory influence imposed by the regulatory domain on the catalytic function of the enzyme. These observations hold true for both cytosolic

and particulate type II PDE's. This type II PDE activity constitutes the only known example of nonessential-substrate activation.

Detailed analysis of the various kinetic parameters of the kinetic model also yielded valuable information. The differences in apparent affinities for both cyclic nucleotides (cAMP and cGMP) can be attributed to different K_C/K_R ratios or to the higher affinity of cyclic GMP for the regulatory site. In contrast, the higher Hill slope values for cAMP can be attributed to lower alpha (activation factor) values. This observation on the CV type II PDE can again apply to the cytosolic type II enzymes (Erneux et al., 1984). Finally, the kinetic analysis of the inhibition of the CV PDE shows Hill slopes for all drugs significantly different to one. This deviation from normal Michaelis-Menten behavior could be related to their abilities to interact with both regulatory and catalytic sites (or states) of the enzyme. The tendencies to have Hill slopes higher or lower than one could be related to the drugs abilities to inhibit or activate, respectively, via interaction at the regulatory site. Otherwise, the shallow slopes will be reflective of the heterogeneity of binding affinities of the drugs to these distinct sites on the enzyme.

d. Evaluation of the original working hypothesis. In the evaluation of the original working hypothesis one can still conceive PDE's as proton generators. Yet, the physiological activators of cyclic nucleotide PDE may vary. In the case of CV the PDE activity associated with these is stimulated by cGMP. Apparently, different PDE types are associated with different synaptic structures. The particulate type II PDE activity of bovine brain may exist in vivo preferentially associated with endocytic structures (see Figure 38, 39). This CV PDE would be maximally sensitive at pH values achieved intracellularly by the action of hormones (i.e. EGF) (see Figure 39). In addition, it would be responsive to sub and micro molar amounts of cGMP. Curiously, muscarinic acetylcholine receptors are

known to mediate increases in guanylate cyclase activity which could in turn activate the brain particulate type II or cGMP-stimulated PDE. Cyclic GMP has indeed been postulated to be involved in the anti-nociceptive and cognitive muscarinic response centrally (McKinley and Richelson, 1984) and Drosophila mutants in PDE lead to memory impairments (Kawar, 1982).

As proposed earlier the CV cyclic GMP-stimulated PDE could be part of an intrinsic regulatory cycle which controls the intracellular and intravesicular pH together with other enzymes, i.e. proton pump, Ca^{++}/Mg^{++} ATPase, others (see Figure 39) (Blitz et al., 1977 and Forgac et al., 1983). As a closing note, the first paper describing type II PDE activities (Beavo et al., 1970) has as its last sentence: "An in vivo stimulatory effect of cyclic GMP on cAMP hydrolysis could be restricted to a localized pool of the latter nucleotide since, in most tissues examined, this effect of cGMP was appreciable only with particulate PDE activity". I would like to propose that this localized pool is constituted by plasmalemma, Golgi and endocytic organelles. Ultimately, the CV PDE may function in terminating an unknown physiological response to cyclic nucleotides in these subcellular organelles. Nonetheless, the hydrolysis of cyclic nucleotides should not be regarded as the onlyp roton generator. The pH on the cytoplasmic leaflet of the plasma membrane can also be acidified by the ydrolysis of ATP and phospholipids, among others. Allowing this consideration one could view the pDE as only one of the elements regulating the intracellular pH and possible in a on-significant manner. Finally, it must also remain clear that no evidence has been presented in my work for the coexistence of PDE with the other molecules presented in the working hypothesis.

CONCLUSIONS

1. Coated vesilces have a particulate PDE which is insensitive to calcium and calmodulin, and stimulated by cyclic GMP, a type II PDE,

2. Cyclic GMP's stimulation of cyclic AMP hydrolysis was optimal at 5 uM cGMP, using 5 uM cAMP as substrate, and had a pH optima at about 7.4..

3. Basal PDE activity in CV had in turn a broad alkaline pH optima, 8.0-10.0,

4. The enzyme hydrolyzes both cyclic nucleotides with nonlinear kinetics suggestive of positive cooperativity,

5. An equillibrium kinetics model is proposed where differences in the apparent kinetic parameters for each cyclic nucleotide can be explained by their relative affinities for regulatory and catalytic sites on the enzyme (K_C/K_R ratio) and by their efficacies at the regulatory site,

6. Cyclic nucleotide hydrolysis dose response curves were modulated by the regulator cGMP and storage in a distinct manner allowing dissection of the various equillibrium constants and factors governing the behavior of the enzyme,

7. The proposed kinetic model for the CV vesicle type II PDE could be applied in the analysis of other type II enzymes,

8. Bovine brain coated vesicles also display specific muscarinic cholinergic binding activity, as determined by:

a) The use of ^{3}H(-)QNB as the labelling ligand, and

b) The use of a series of muscarinic agonists and antagonists, in competition experiments,

9. The coated vesicle's mAChR has a Kd = 25 pM and a Bmax = 341 fmol/mg protein,

10. Heterogeneity of binding affinities is observed for both agonists and the antagonist n-methyl-scopalamine in coated vesicles,

11. The heterogeneity of agonist and antagonist binding can be interpreted in terms of distinct functional states, receptor subtypes (M1 vs M2) or subcellular microheterogeneity.

12. Both enzymatic and receptor activities in bovine brain can be selectively expressed in distinct coated vesicles subsets.

APPENDIX A

Table I.

Kinetic Parameters of the Coated Vesicle PDE
Obtained Using Various Kinetic Equations

Equation	Kd (pM)	Bmax (fmol/mg)	N
Scatchard	87	247	(4)
M.M.	80	383	(5)
Hill Equa.	88	394	(4)
Ave:	85	341	

Table II.

Inhibition Parameters of Agonists and Antagonists of the CV Muscarinic Receptor

	IC_{50}	N_H	N
Agonists:			
Oxo-	671 nM	0.60	(6)
+ GTP	671 nM	0.67	(4)
CCh	254 uM	0.72	(3)
Antagonists:			
Atro	1.34 nM	0.88	(5)
NMS	3.96 nM	0.49	(5)

N = number of brain preparations assayed.

N_H = Hill slope

IC_{50} = concentration of competitor required to achieve 50% inhibition of $^3H(-)$QNB bound.

Table III

Analysis of the Inhibition of QNB Binding with a Two-site Model

Compound	% High Affinity Sites	K_H	K_L	N
Oxo - GTP	13	1 nM	827 nM	(6)
+ GTP	9	1 nM	784 nM	(4)
CCH -	66	100 uM	3.2 mM	(3)
NMS -	62	627 pM	203 nM	(5)

N = number of brain preparations considered.

Table IV. Summary of Kinetic Parameters of the CV PDE

Substrate	Apparent Km (uM)	Vmax	Slope	N
cAMP:				
Fresh coated vesicles	22	3.2	1.5	(5)
Stored coated vesicles	37	3.4	1.08	(4)
Decoated vesicles	35	8.5	1.08	(2)
Coated vesicles	20	2.1	1.5	(3)
Coated vesicles + 5 uM cGMP	11	2.3	1.05	(3)
cGMP:				
Fresh coated vesicles	11	3.8	1.3	(4)
Stored coated vesicles	26	4.5	1.08	(3)

Vmax = nmol/min/mg

Slope = Hill slope

N = number of experiments

Table V. Summary of Inhibition Parameters of the CV PDE

Drug	5 uM cAMP		5 uM cAMP+ 5 uM cGMP		5 uM GMP		N
	IC_{50}	Slope	IC_{50}	Slope	Ic_{50}	Slope	
Papaverine	15 uM	1.3	4 uM	0.8	3 uM	0.85	4
IBMX	56 uM	0.5	16 uM	0.8	8 uM	0.85	4
R0201724	48 uM	0.4	1 mM	0.2	—		5
Theophylline	1 uM	0.7	249 uM	1.4	273 uM	1.12	4
cAMP	—		—		17 uM	0.75	3
cGMP	1 mM	--	--		--	--	7

N = number of brain preparations considered in the analysis.

Table VI

Summary of the Calculated Kinetic Parameters for the CV PDE

	K_C	K_R	K_C/K_R	alpha (a)
cAMP	28 uM	137 uM	0.28	0.16
cGMP	26 uM	2 uM	13	0.38

APPENDIX B

FIGURE LEGENDS

Figure 1. Electron micrograph of coated vesicles from the ciliated protozoan Tetrahymena termophila obtained according to the method of Campbell et al. (1984) (prepared by W. Silva).

Figure 2. Scheme for the recycling of clathrin triskelions (A) in vivo. Proton (H^+) production may facilitate the formation of clathrin lattices (B) in phase I. Phase II involves the lattice transformation and the genesis of coated vesicles (C). H^+ are also used in the lowering of the pH in the CV's lumen. In the final phase (III) CV lose their coat to yield their derivative structures, i.e. endosomes (D). These will in turn travel back to the plasmalemma, to lyzosomes, Golgi or nucleus (2).

Figure 3. Chemical structure of muscarinic drugs (from Brita Hedlunds thesis).

Figure 4. Working hypothesis scheme which postulates a dynamic interaction between various CV cargo molecules i.e. Ca^{++}/Mg^{++} ATPase, cyclic nucleotide PDE and H^+ pump. Cyclic nucleotide PDE in CV may be activated (+) by Mg^{++} ions provided by the Ca^{++}/Mg^{++} ATPase, PDE's may be activated by calcium-calmodulin and generate protons to facilitate the assembly of clathrin or provide acid protons (H^+) for the acidification of the coated vesicle's lumen.

Figure 5. Subcellular fractionation scheme routinely used for the isolation of bovine brain coated vesicles. The steps constitute an integration of the procedures of Keen et al. (1979) and Forgac et al. (1982).

Figure 6. Morphological and biochemical analysises of CV and decoated vesicles: Panel a is a decoated vesicle preparation. Panel b shows both SDS-polyacrylamide gradient (5-15%) slab-gel electrophoretic analyses. Lane 1 corresponds to the CV; lanes 2 and 3 are the cages extracted during the decoating

procedure; and lane 4 shows the corresponding protein profile of the decoated membrane. Protein samples (5-20 ug) were routinely loaded per lane. Panel _c_ is an electron micrograph of our bovine brain CV. Magnification: 50,000 x.

Figure 7. Analyses of the specific binding of ^{3}H(-)QNB (●) (1 nM) to brain CV eluting from a S-1000 gel filtration column. Atropine (5 uM) was used as a masking ligand. Specific binding was 70% or more using 0.5 ml aliquots of the material eluting from the column. The final pH was of 7.0 and the final assay volume of 2 ml in 50 mM phosphate buffer pH 7.4. Panel a is material after a first run which was concentrated and run for a second time (panel b).

Figure 8. Phosphodiesterase activity of the material eluted after a first (panel a) and second (panel b) runs over a Sephacryl S-1000 column. In both runs 1 uM ^{3}H cAMP was used as substrate, and 50 ul aliquots of material from the Sephacryl column was directly used in the assay. Specific hydrolysis (●) was determined by substracting the blank counts obtained using 1 mM IBMX or boiling of the tissue. The right ordinate represents the absorbance at 280 nm of the material eluting from the column. This peak of absorbance within the included volume of the column (or material retained by the column) is the CV peak. The results presented are representative of typical experiments and qualitatively and quantitatively similar results have been obtained in various (at least 5) preparations and using anywhere from 0.1 — 50 uM cAMP as substrate.

Figure 9. Biochemical assay of cAMP hydrolysis by CV purified and eluted from a Sephacryl S-1000 column: The main peak of absorbance at 280 nm represents the CV rich (> 90%) fraction. Fifty-ul aliquots of the indicated fractions were directly assayed using 5 uM cAMP as substrate in the absence (●) and presence of 5 uM cGMP (△) or 1 mM Ca^{2+} (■). These results are representative of a typical experiment and qualitatively and quantitatively similar results have been obtained in at least five different brain preparations.

Figure 10. Immunoprecipitation of coated vesicles and the PDE activity. Panel a. represents the material immunotitrated with Staphylococcus aureus cells coupled with human IgG. Panel b. represents coated vesicles immunoprecipitated with antibodies against clathrin. Panel c., the histogram shows the PDE activity of a CV sample purified over the Sephacryl-S-1000 column (control) and that of CV purified as in panel b (experimental). The ordinate on the right is in pmol/minsample.

Figure 11. Immunotitration of the muscarinic binding activity from column-purified CV's. The percentage of bound ^3H-QNB (ordinate) is plotted against the volume of sera (abscisa) containing polyclonal antibodies to clathrin, used to immunotitrate 125-250 ug of column-purified CV. Non-specific immunotitration was determined using commercially available human immunoglobulins (IgG).

Figure 12. Specific binding of ^3H-QNB (1 nM) as a function of the amount of tissue used, coated (■) or decoated (●) vesicles. Non-specific binding was determined using 5 uM atropine. The final assay volume was of 4-5 ml in 10 mM Tris HCl-10 uM $Mg^{++}Cl_2$ pH 7.2. The results are the average from 5 different preparations from bovine brain.

Figure 13. Kinetics of ^3H(-)QNB binding to coated vesicles. The concentration of ligand used was near 0.5 nM and the specific binding determined using 5 uM atropine in parallel blanks. In this particular experiment 225 ug of coated vesicles proteins were used.

Figure 14. Michaelis-Menten plot for the binding of ^3H(-)QNB to bovine brain coated vesicles. The results are representative of a typical experiment where 125 ug of CV were used in a binding media of 10 mM Tris HCl_2 10 mM $MgCl_2$ pH 7.2 and a final assay volume of 5 ml. Blanks were obtained using 5 uM atropine for each point. Specific binding was more than 90% between 0.1 - 10 x Kd.

Figure 15. Scatchard plot from the data presented in Figure 14. The correlation coefficient for the line was of 0.93 (n = 4). The kinetic parameters from these graphs are included in the analysis presented in Table I.

Figure 16. Analysis of the $^3H(-)$ QNB kinetic parameters. Variation of the apparent Kd as a function of the receptor's density (R_t). The correlation coefficient for this line was of 0.98. The intercept on the y-axis provides a Kd value approximating the true Kd of the reaction, 25 pM.

Figure 17. Inhibition of $^3H(-)$QNB binding to CV by the antagonist atropine (●) and N-Methyl-scopolamine (■). Control groups routinely bound near 800-1100 CPM's specifically (> 90%). See Table II for the analysis of these competition curves using the Hill-equation.

Figure 18. Inhibition of $^3H(-)$QNB by the agonists carbachol (■) and oxotremorine. Competition experiments for oxotremorine were done in the absence (●) or presence (○) of 500 uM GTP. Conditions for binding were as in Figure 17. Table II summarizes the kinetic parameters for these competition curves using the Hill equation.

Figure 19. Analysis of the competition curves for oxotremorine (Figure 18) in the absence of GTP by a one (---) and two-site (___) models. Table II and III summarize the various kinetic constants obtained by both approaches.

Figure 20. Analysis of the competition curves for oxotremorine (Figure 18) in the presence of GTP by a one (---) and two (___) sites model. Tables II and III summarize the kinetic parameters obtained by these approaches.

Figure 21. Analysis of carbachol's (no GTP) competition curve using a one and two-site model. Tables II and III summarize the kinetic parameters obtained using both equations.

Figure 22. Analysis of N-methyl-scopolamine's competition curves (Figure 17) using a one (---) and two site (___) models. Table's II and II provide the kinetic

parameters obtained using both equations.

Figure 23. Effect of pH on the basal (●) and cyclic GMP-stimulated (■) cAMP hydrolysis by coated vesicles. Maximal stimulation was obtained in these experiments (2) at pH 7.4 and was of 411%. Basal hydrolysis in turn displays a broad alkaline pH (pH 8-10) optima. Five micromolar cyclic AMP was used as substrate and 5 uM cGMP as the stimulator's concentration.

Figure 24. Dose response curves of the hydrolysis of cyclic AMP (●) and cyclic GMP (■) by coated vesicles. Analysis of these dose response curves with the Hill equation was done and the results displayed in Table IV.

Figure 25. Dose-response curves for the hydrolysis of cAMP by CV (●) and decoated vesicles (■). Non-specific hydrolysis was determined in parallel blanks using 10 mM theophylline. See Table 1 for a summary of the kinetic parameters obtained from these curves.

Figure 26. Effect of cGMP on the hydrolysis of 5 uM cAMP by CV (●) and decoated vesicles (■) from bovine brain: Non-specific hydrolysis was determined in parallel blanks using 1 mM IBMX. Maximal activation was achieved at approximately 5 uM cGMP.

Figure 27. Effect of cyclic GMP on the hydrolysis of cyclic AMP by fresh (●) and stored (■) coated vesicles. The maximal level of stimulation by cyclic GMP in the aged, stored preparations was less than 50%.

Figure 28. Effects of activation (■) and storage (◆) on the kinetics of cyclic AMP hydrolysis. Control dose response curves (●) displayed Hill slopes different from one (see Table IV). In the activation experiments, 5 uM cGMP was used as the activator's concentration. Non-specific hydrolysis was determined using 1 mM IBMX. See Table IV for the summary of the kinetic parameters of these various curves.

Figure 29. Inhibition of cAMP hydrolysis (5 uM) by various PDE inhibitors:

papaverine (●); IBMX (■); and theophylline (△). See Table V for a summary of the IC_{50} values for the inhibitors.

Figure 30. Inhibition of the cGMP-stimulated cAMP hydrolysis by various PDE inhibitors: The concentration of both cAMP and cGMP was 5 uM. Inhibitors: papaverine (●); IBMX (■); and theophylline (△). See Table V for the IC_{50} values of the inhibitors.

Figure 31. Inhibition of cyclic GMP hydrolyis. The concentrationof cGMP used was 5 uM. Inhibitors used were papaverine (●), IBMX (■), cyclic AMP (▲) and theophylline (◆). Table V summarizes the kinetic parameters from these inhibition curves.

Figure 32. Kinetic scheme proposed for the CV PDE. This equillibrium kinetics model could be classified as one of random, non-essential substrate activation model. This model for the cGMP-stimulated PDE of CV is developed in section 6 of Results.

Figure 33. Simulation curves obtained from the steady-state solution of the kinetic model in Figure 32. The value of alpha (a) was set to 1 (panel 1), 0.75 (panel b), 0.25 (panel c) and 0.1 (panel d). In each panel the curves from right to left represent increasing K_C/K_R ratios: 0.1, 0.5, 1, 2, 20, 800 and 400, respectively. They all represent normalized dose response curves since no significant changes are seen in the Vmax under the simulations or experimental data.

Figure 34. Variations in apparent Km as a function of alpha (a, activation factor) and the K_C/K_R ratio. The values are from the simulation curves in Figure 33. Various alpha values were used: 1 (a), 0.75 (b), 0.25 (c) and 0.1 (d).

Figure 35. Variations in the apparent Hill slope as a function of the activation factor, alpha, and the K_C/K_R ratio. Values were derived from the analysis of the simulation curves in Figure 33. The curves from top to bottom are

representative of increasing values of alpha, 0.1, 0.25, 0.75 and 1, respectively.

Figure 36. Normalized dose response curves obtained for both cyclic nucleotides using the experimentally determined kinetic constants for each of them (see Table VI). The points represent the experimental values for the control dose response curves of cyclic GMP (■) and cAMP (●) (see Figure 24).

Figure 37. Illustration of the putative vesicular transport of muscarinic receptors in neuronal tissue. The darkened areas represent postsynaptic densities, N.T. = nerve terminal and N = nucleus. According to the view of some workers (Mash et al., 1985) pre and postsynaptic elements carry distinct muscarinic receptor subtypes M2 and M1, respectively. Four putative sources of receptors in CV can be thought of, two of them may be endocytic CV's from either pre or postsynaptic tissue ((1) and (2), respectively). The third and fourth may be related to newly synthesized receptors in Golgi associated coated vesicles (3) en route to either the soma's plasmalemma (M1 receptors) or the nerve terminal (M2 receptors).

Figure 38. Illustration of the possible vesicular (V.T.) and cytoplasmic (C.T.) transport of cyclic nucleotide phosphodiesterases types 1 and 2. Type I is represented attached to the postsynaptic densities (shaded areas) and the intermediate filaments system of the axon in the nerve terminal (N.T.). As for the muscarinic cholinergic receptor various putative CV transport systems are proposed for the type II PDE of coated vesicles.

Figure 39. Evaluation of the original working hypothesis regarding phosphodiesterases as proton generators. As seen and discussed in the text, cyclic GMP stimulation could regulate proton production. It is particularly interesting that some culture cells respond to hormones, i.e. epidermal growth factor (EGF) with an increase in the cytoplasmic intracellular pH (pHi), usually reaching a value, 7.4 (top left insert) where the type II PDE of CV's is maximally sensitive to

activation by cGMP (bottom left insert). These event could recruit the type II PDE activity and lead to the reacidification of the cells cytosol seen also with i.e. EGF. These intrinsic regulatory cycle could be applicable to other intracellular vesicular systems i.e. receptosomes and lysosomes. Finally, the role of protein kinases, Na^+/H^+ exchangers, phospholipases and phosphoinositides remain to be incorporated into this scheme.

Figure 1.

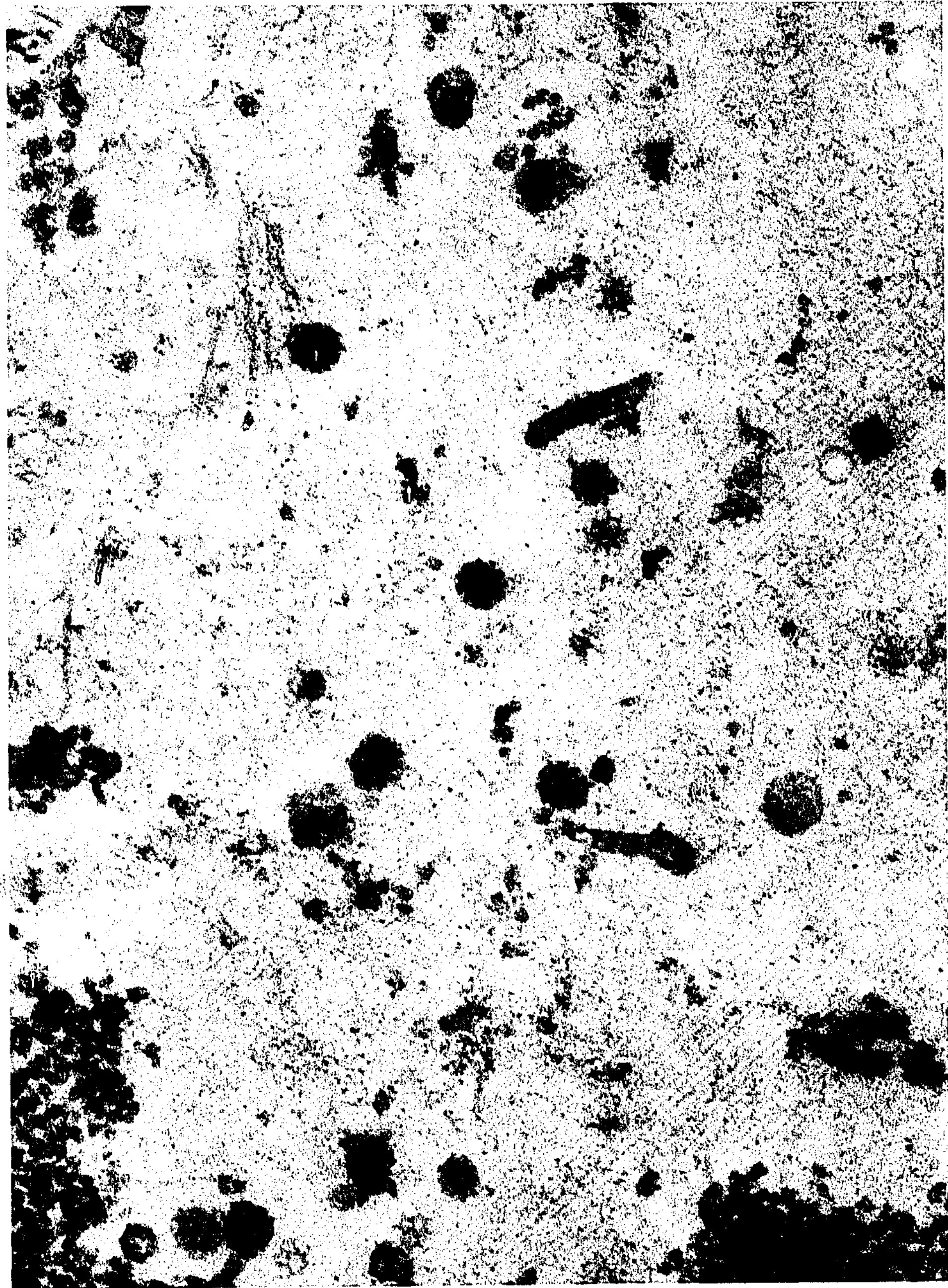


Figure 2.

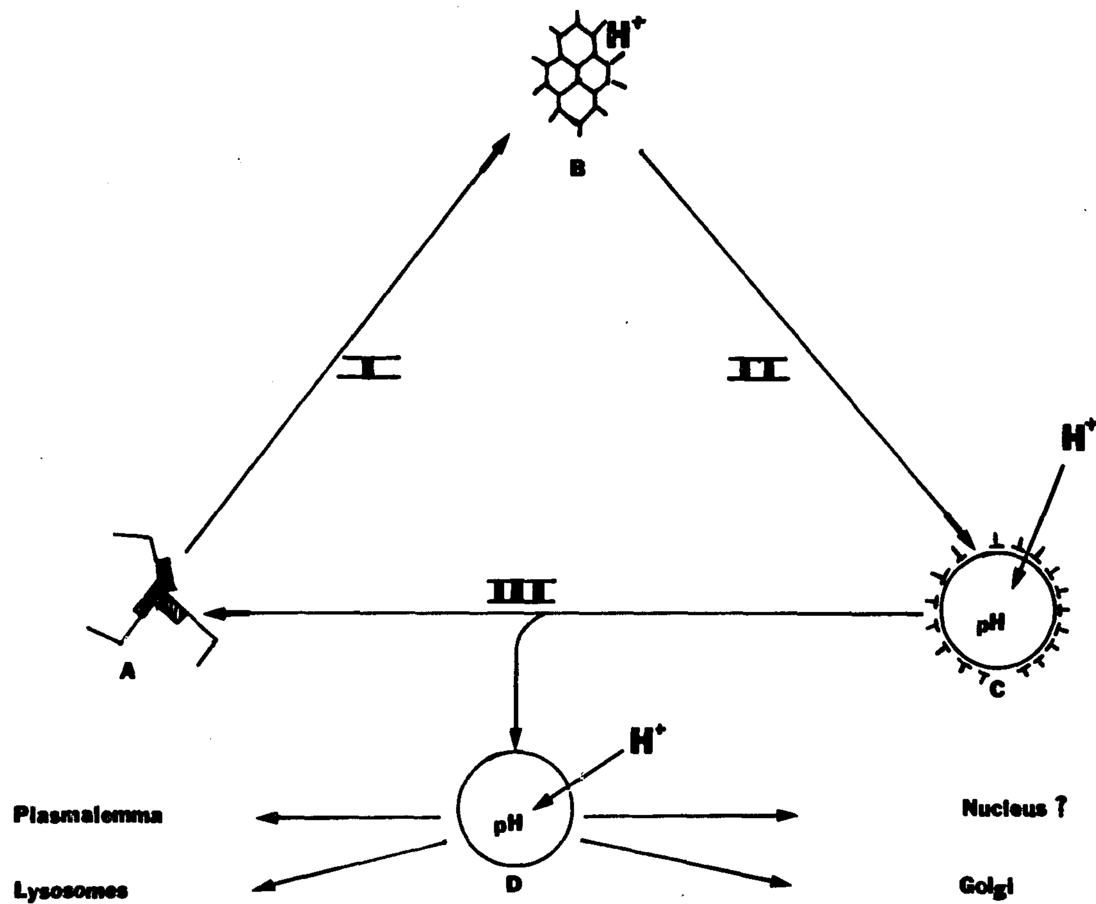


Figure 3.

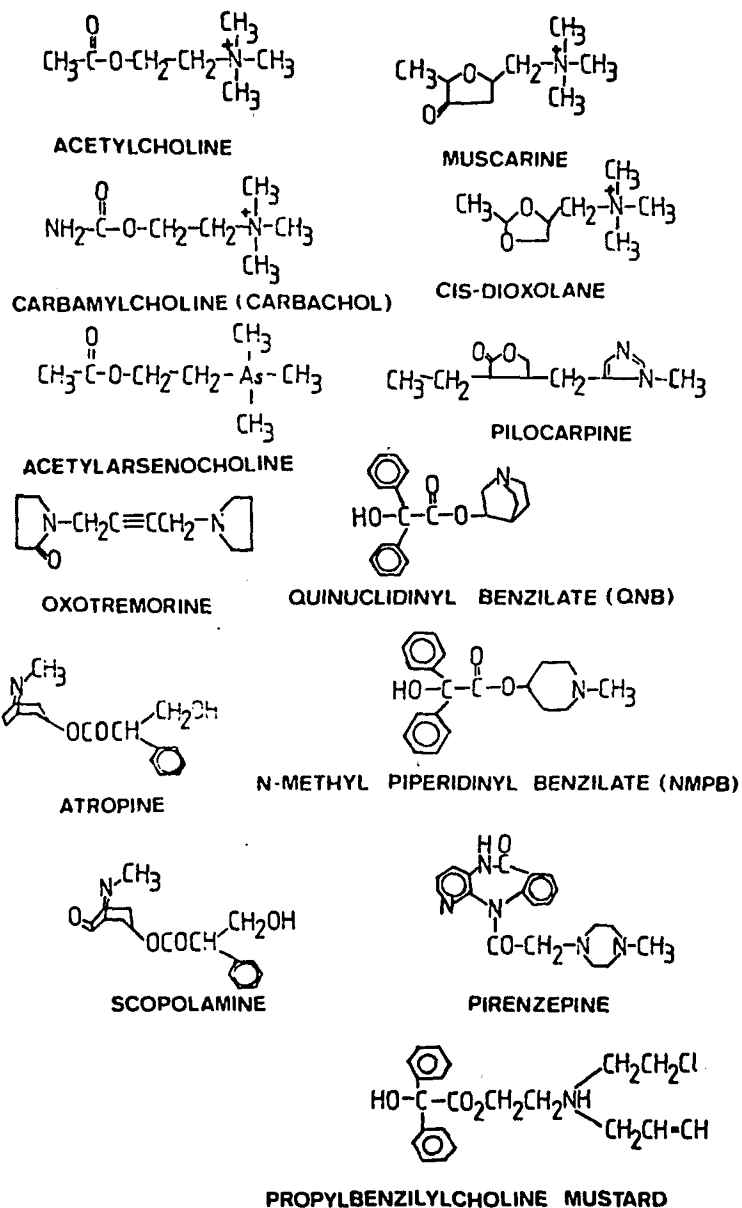
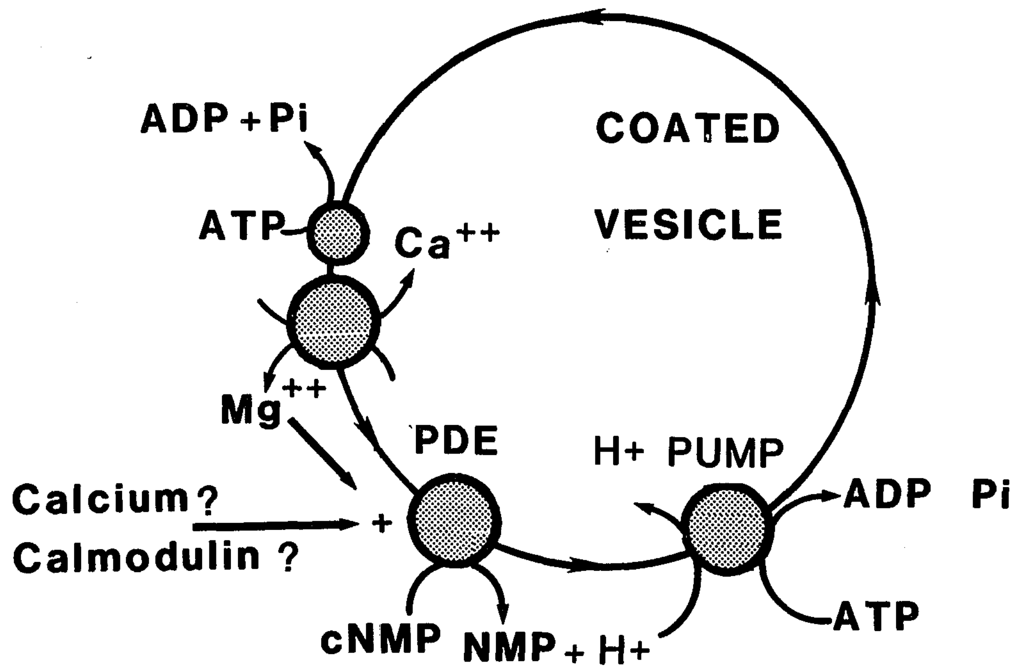


Figure 4.



Is PDE a Calmodulin-dependent proton generator ?

Figure 5.

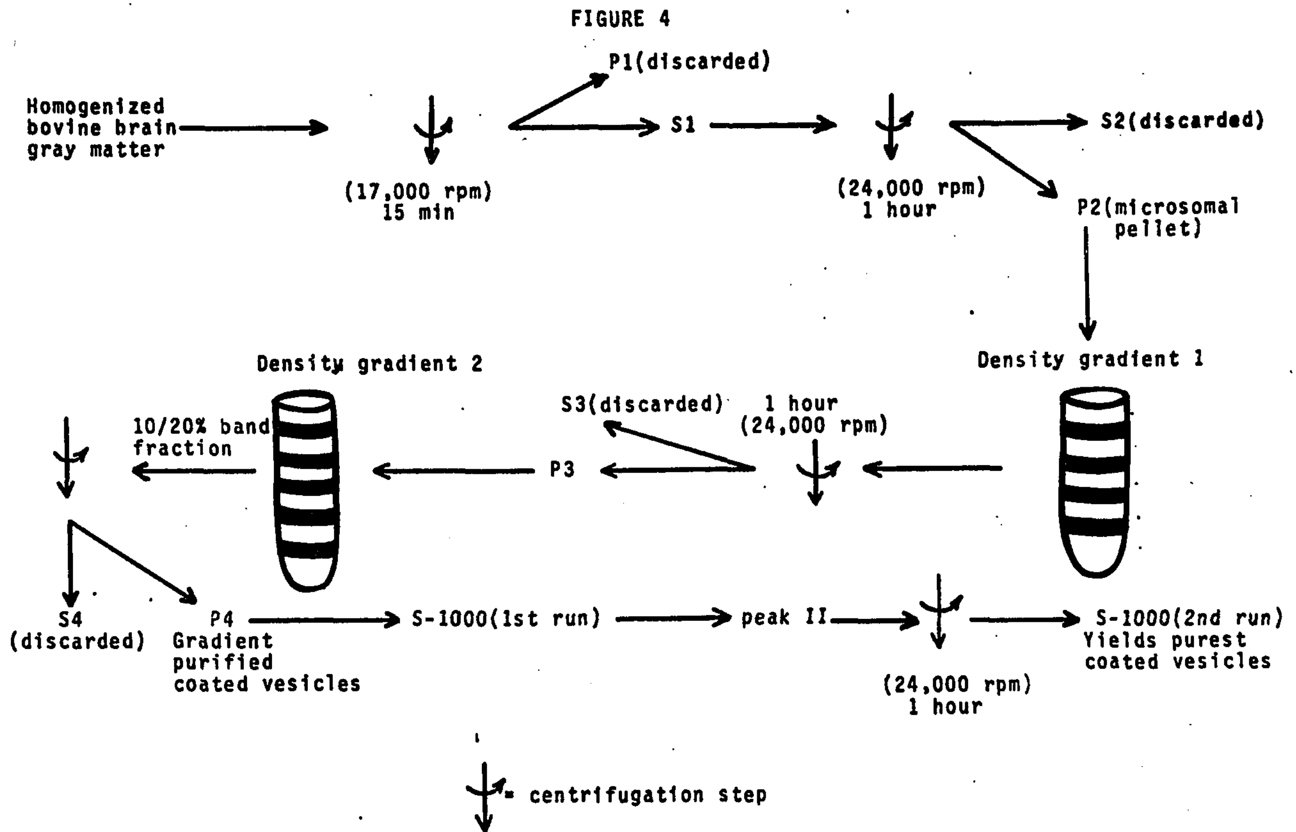


Figure 6.

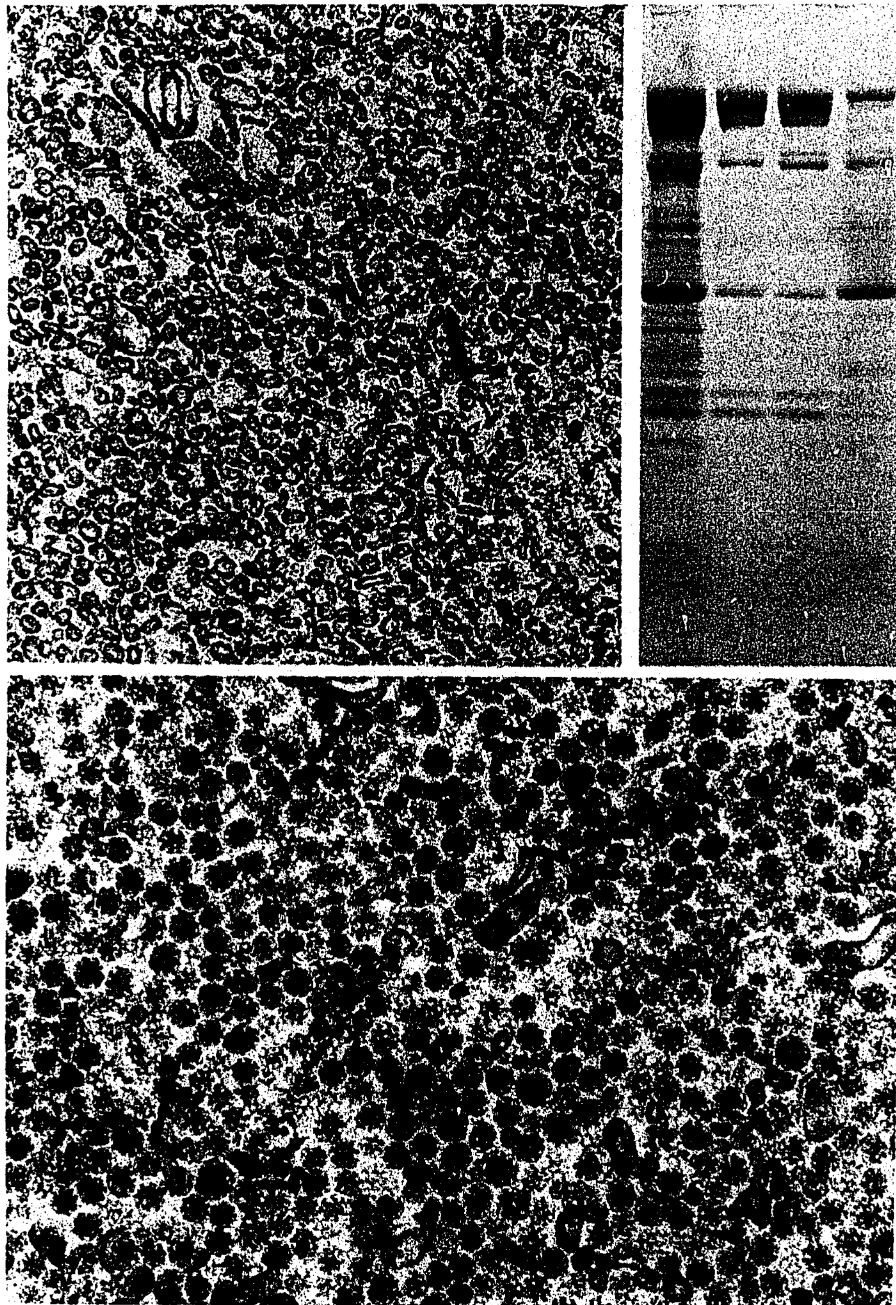


Figure 7.

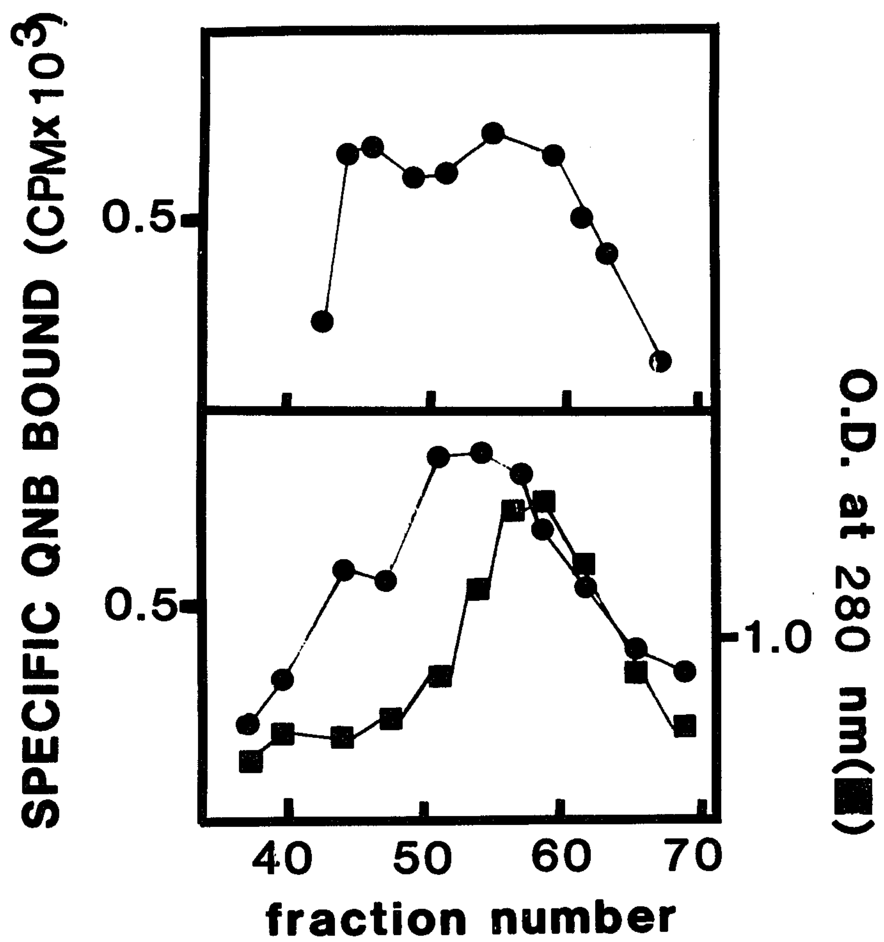


Figure 8.

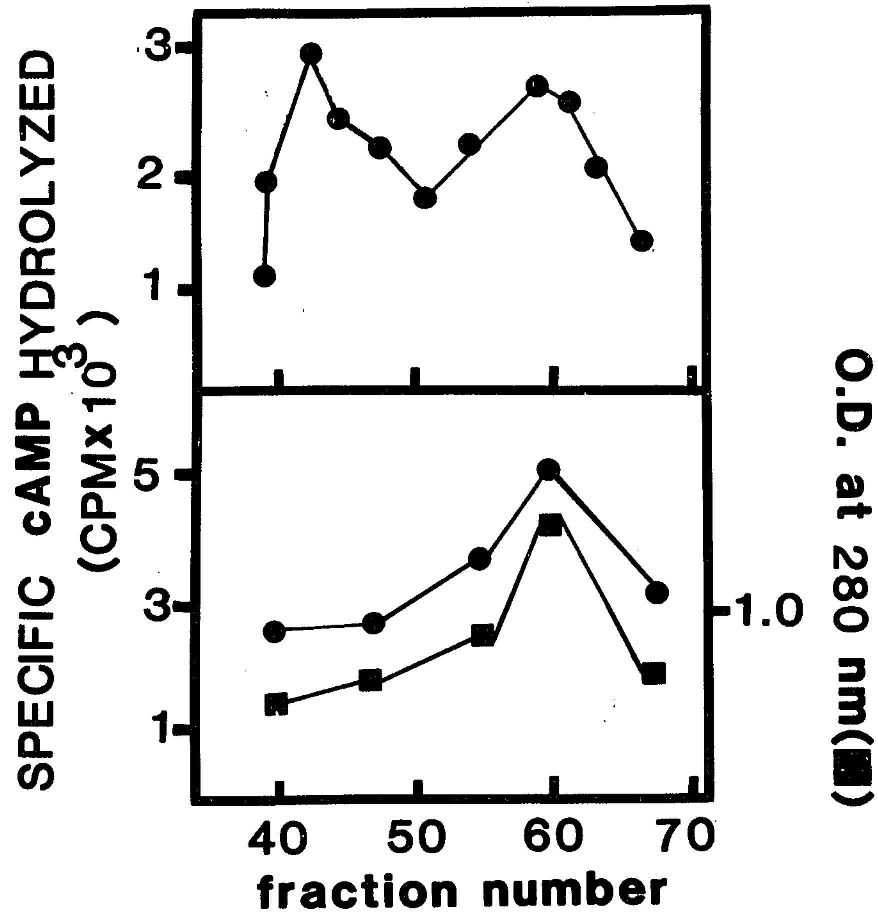


Figure 9.

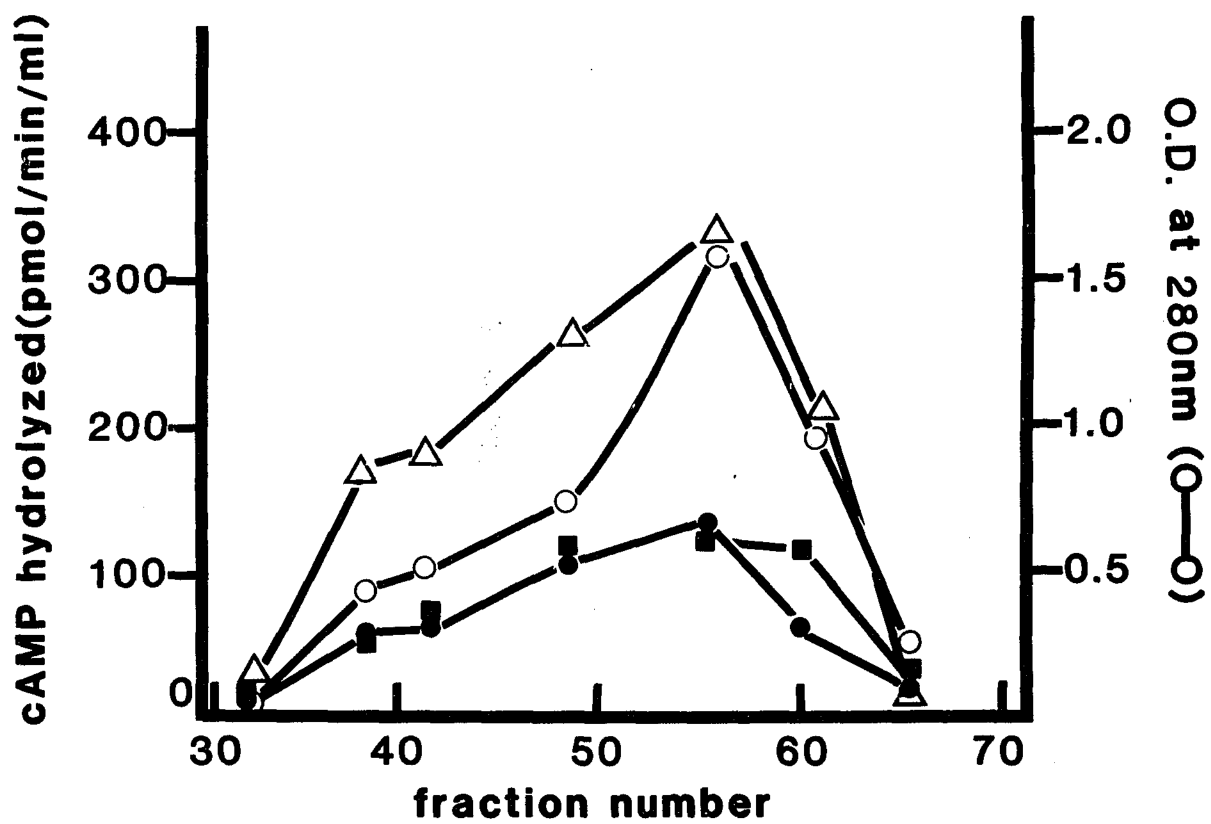
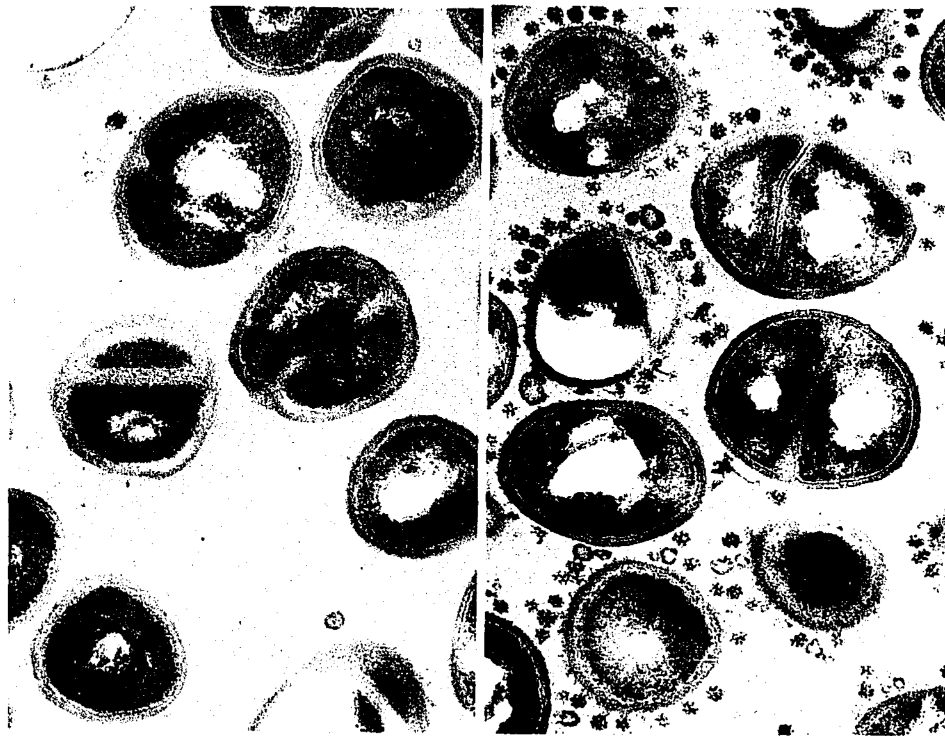


Figure 10.



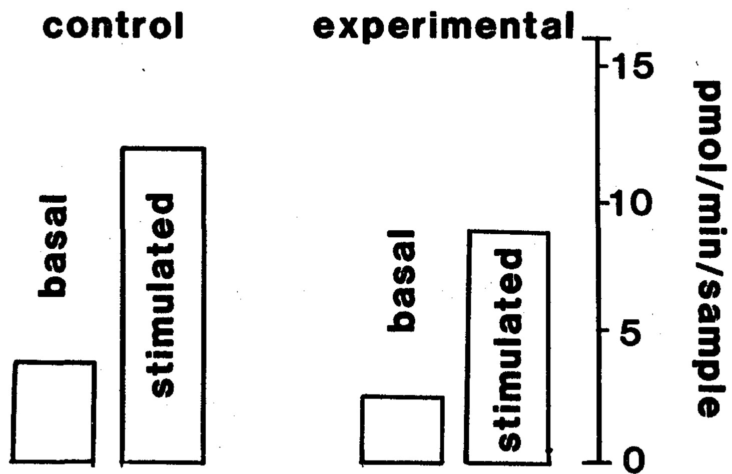


Figure 11.

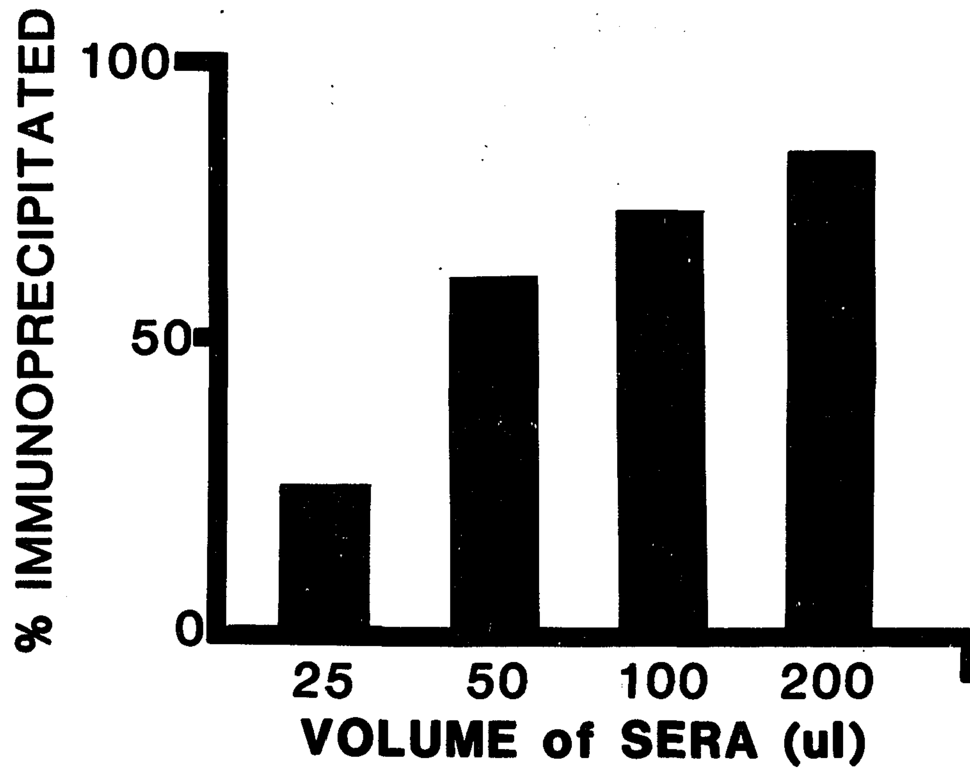


Figure 12.

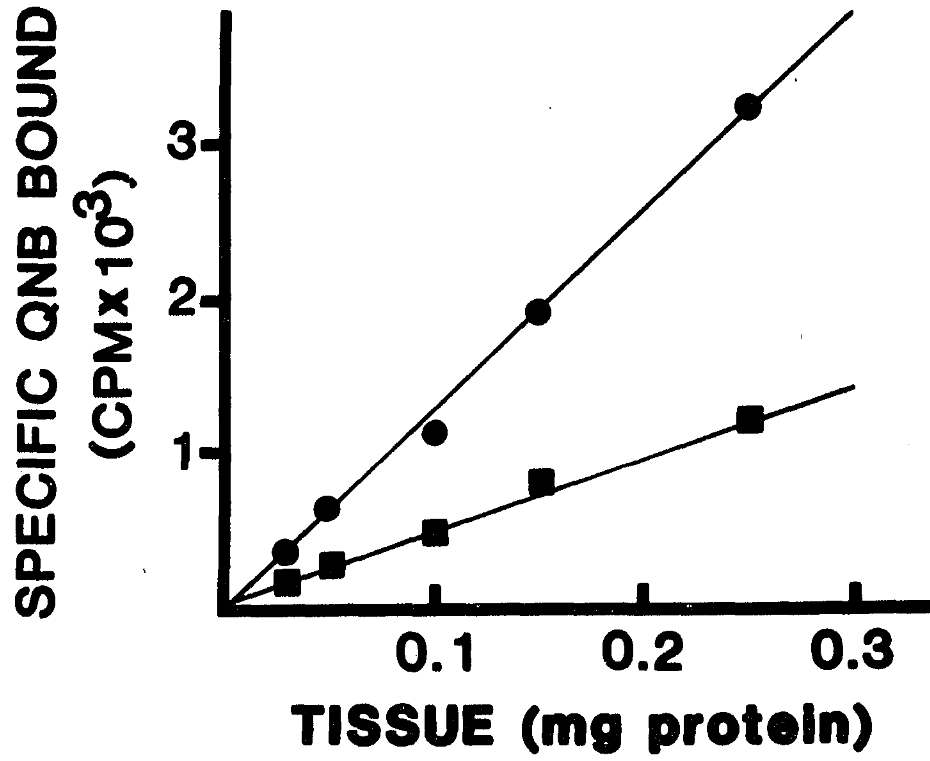


Figure 13.

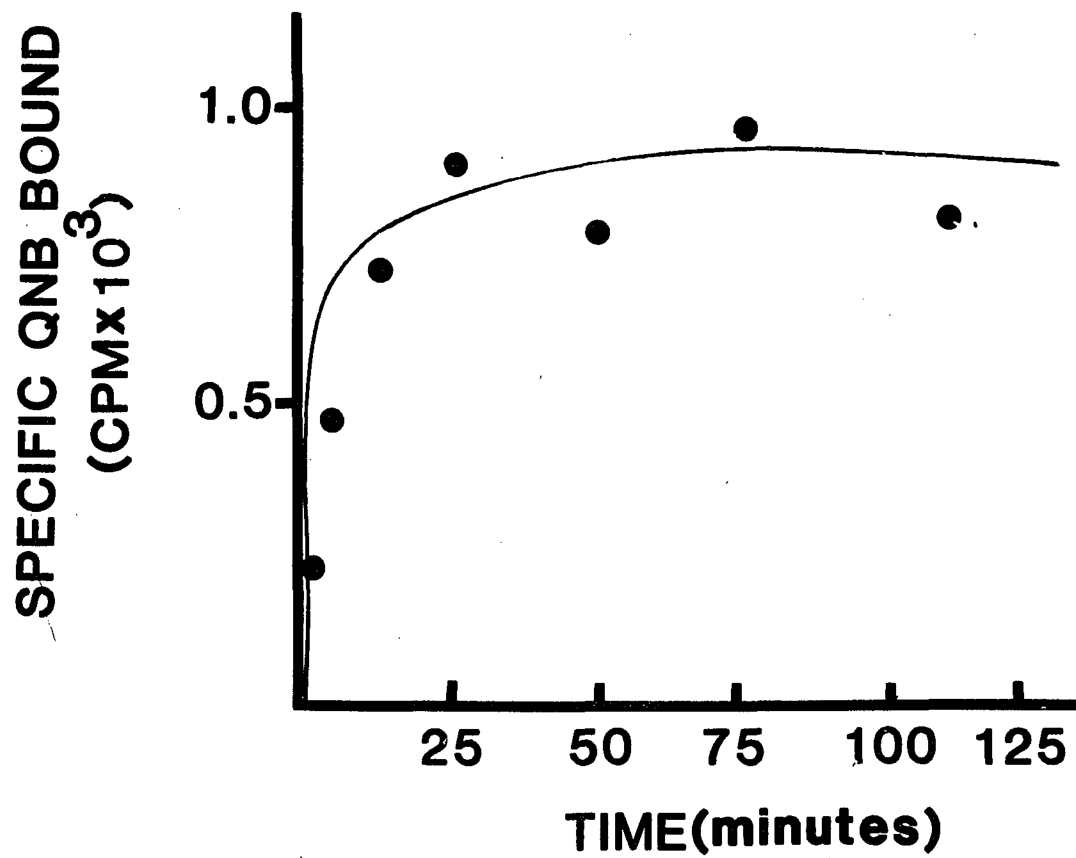


Figure 14.

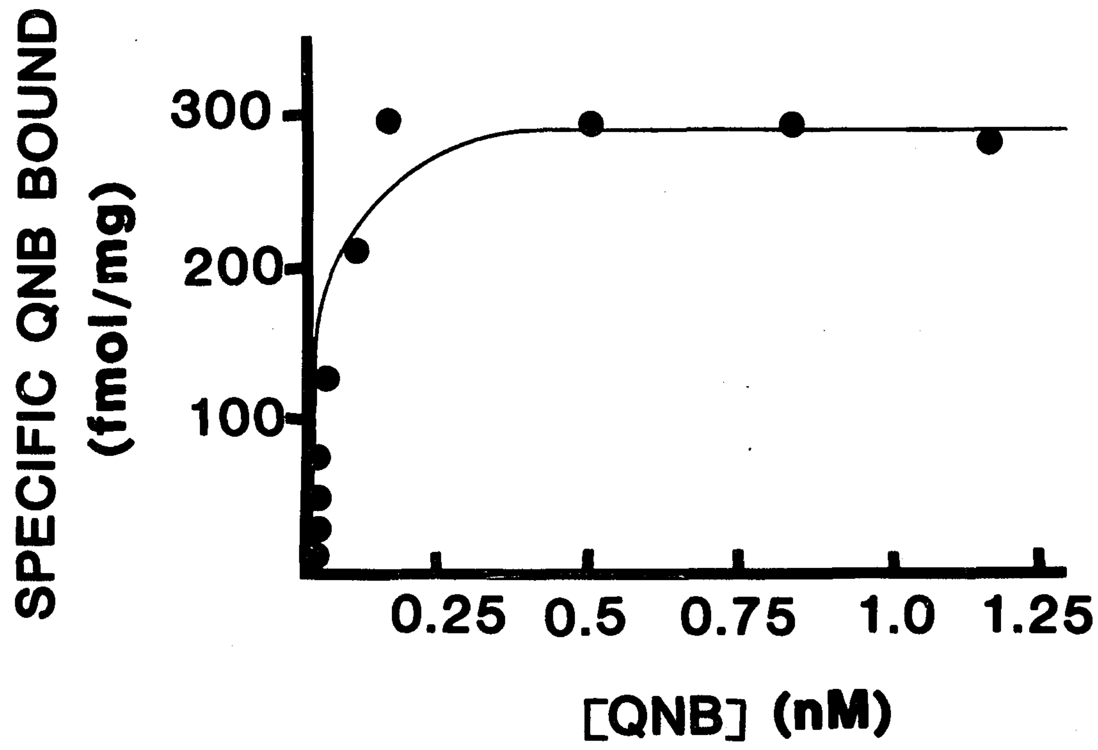


Figure 15.

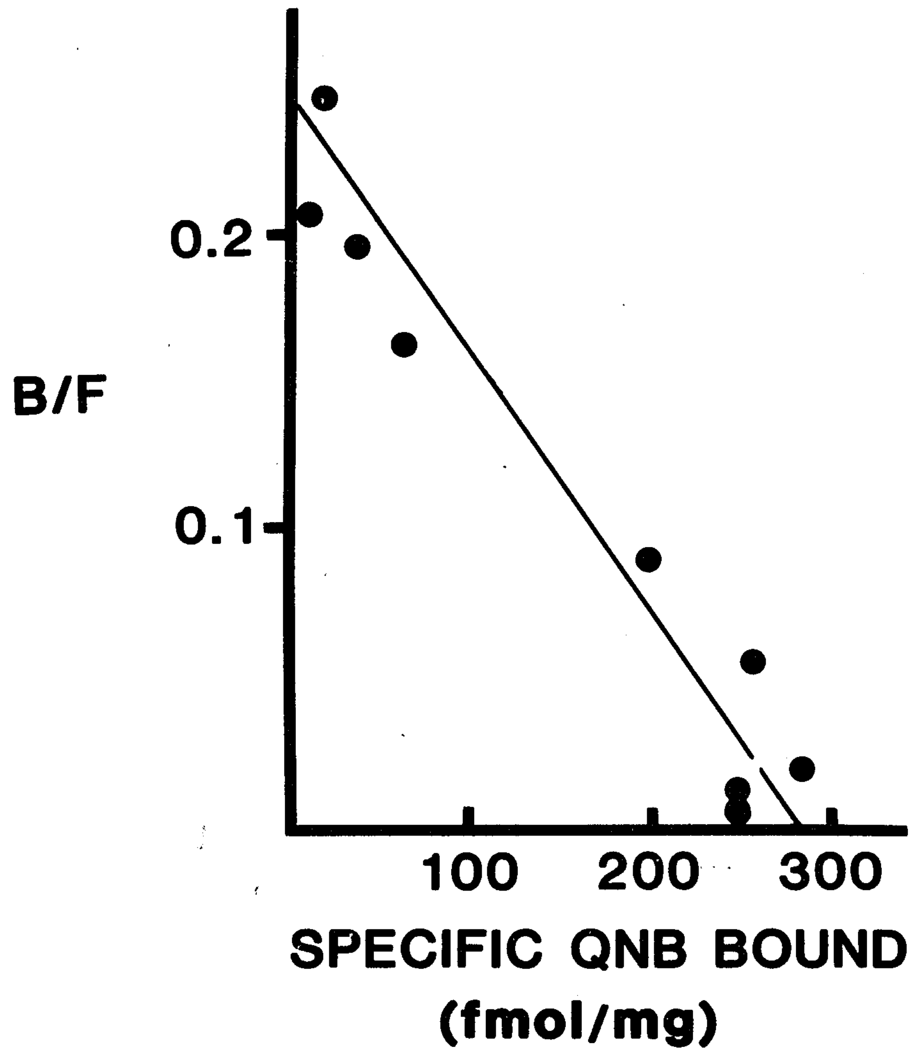


Figure 16.

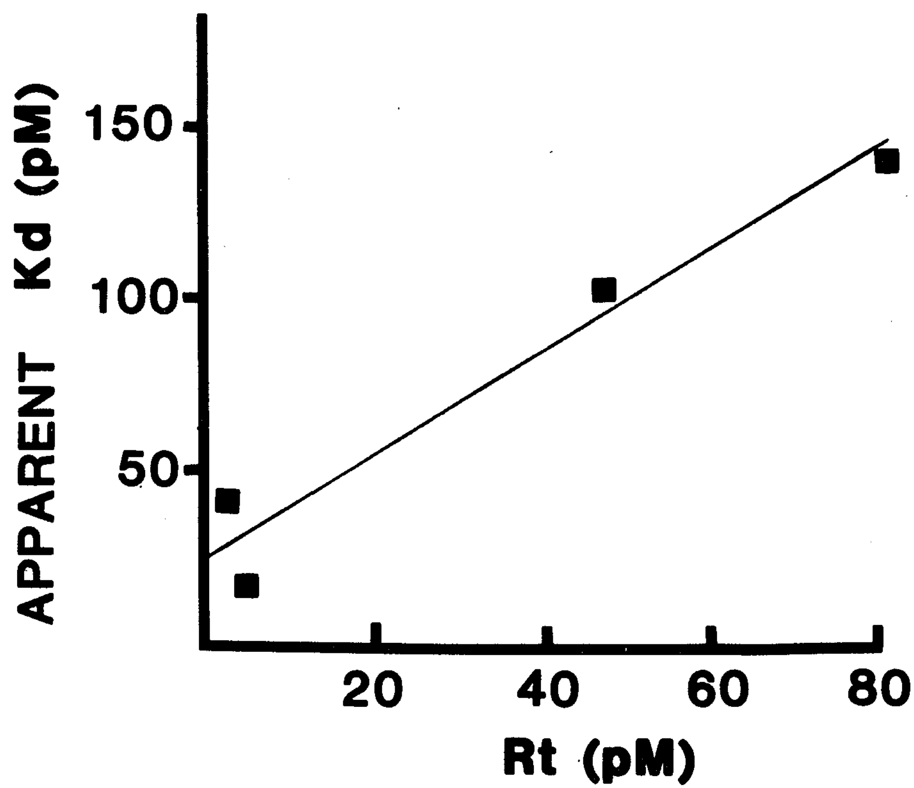


Figure 17.

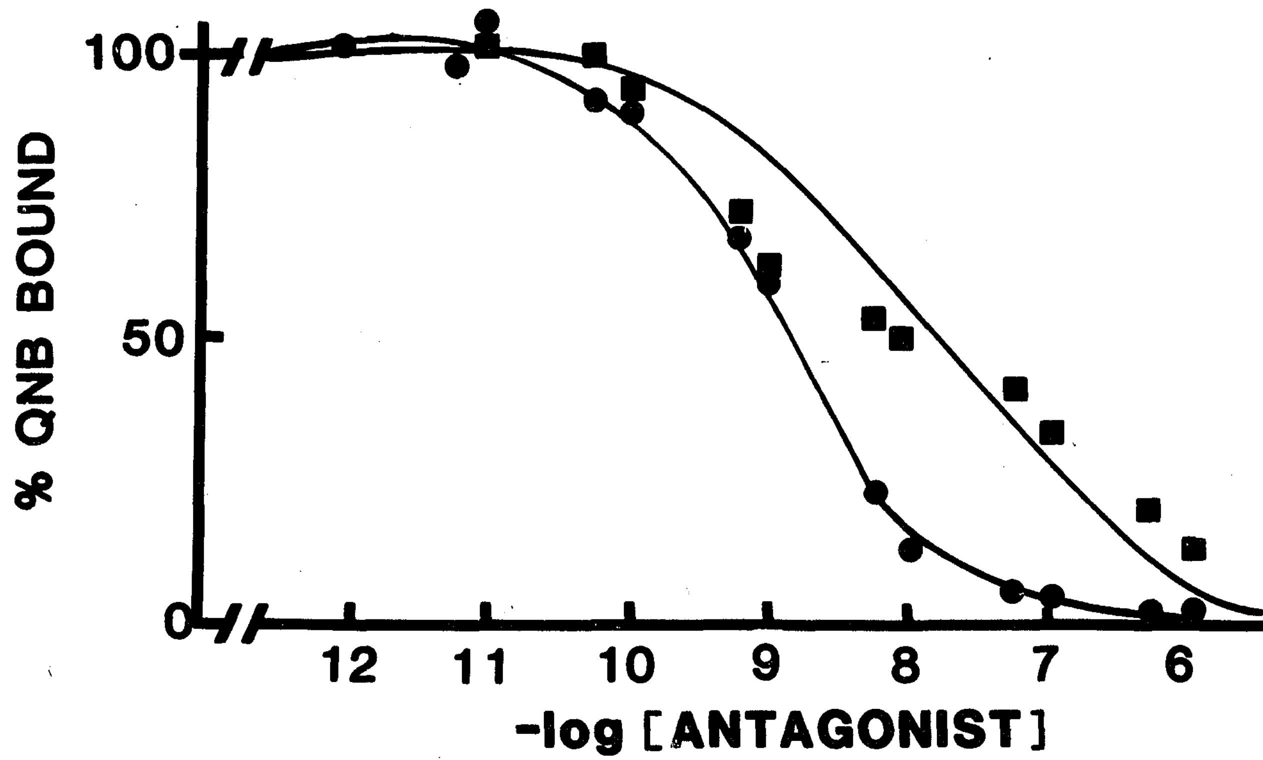


Figure 18.

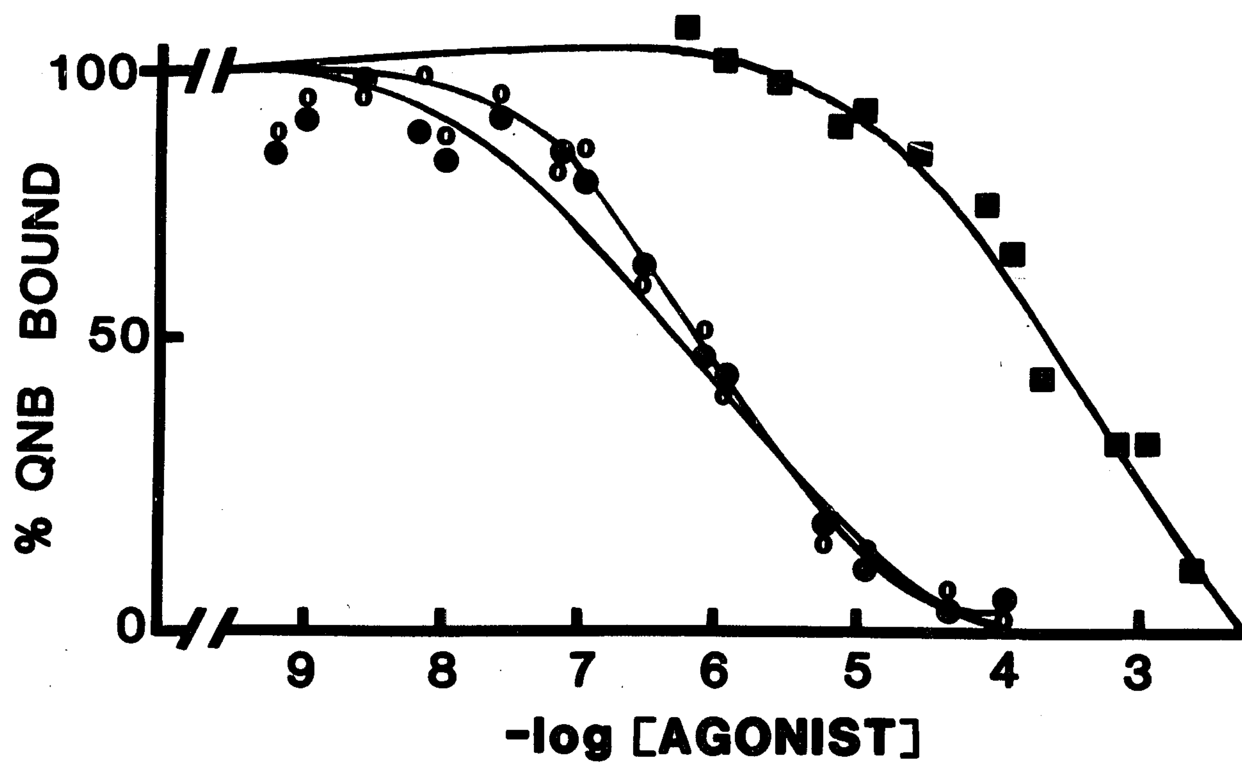


Figure 19.

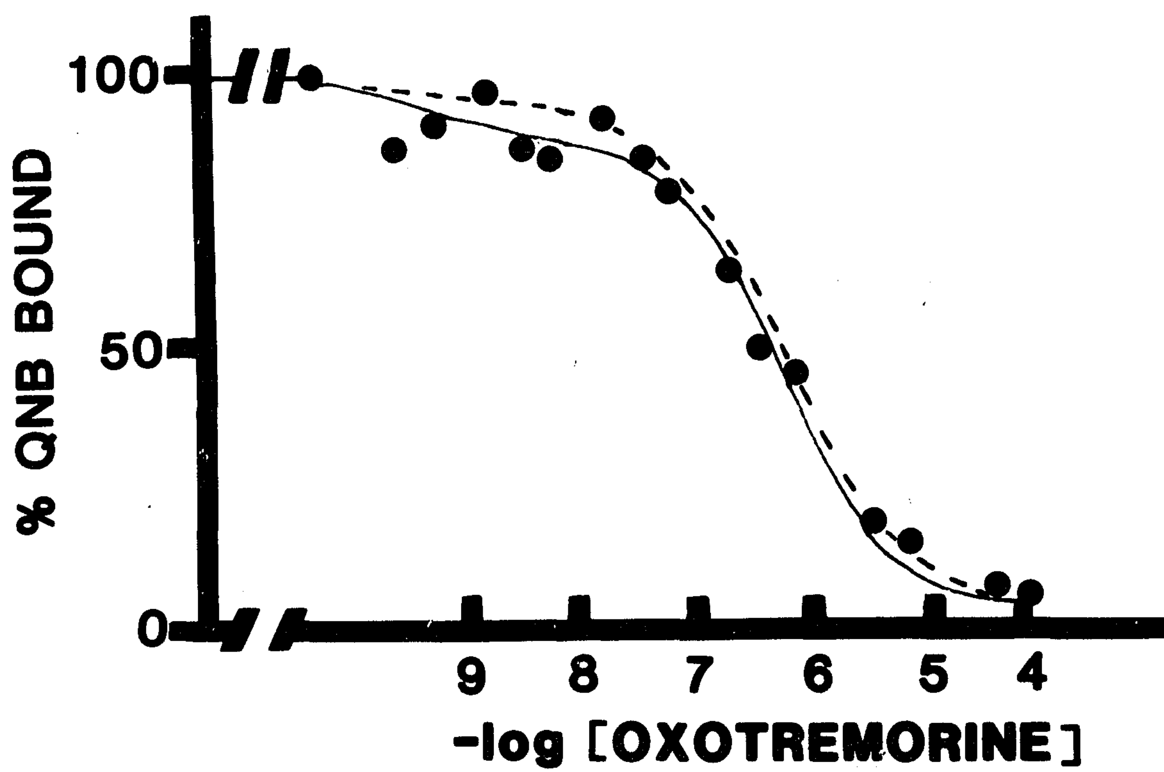


Figure 20.

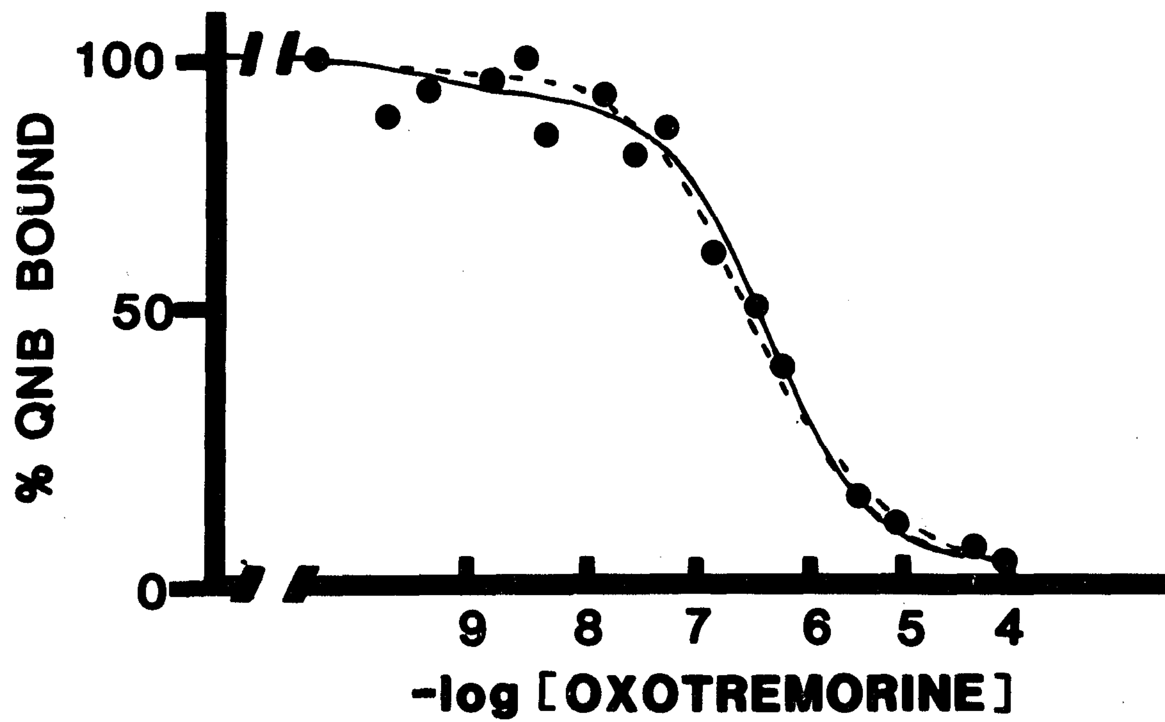


Figure 21.

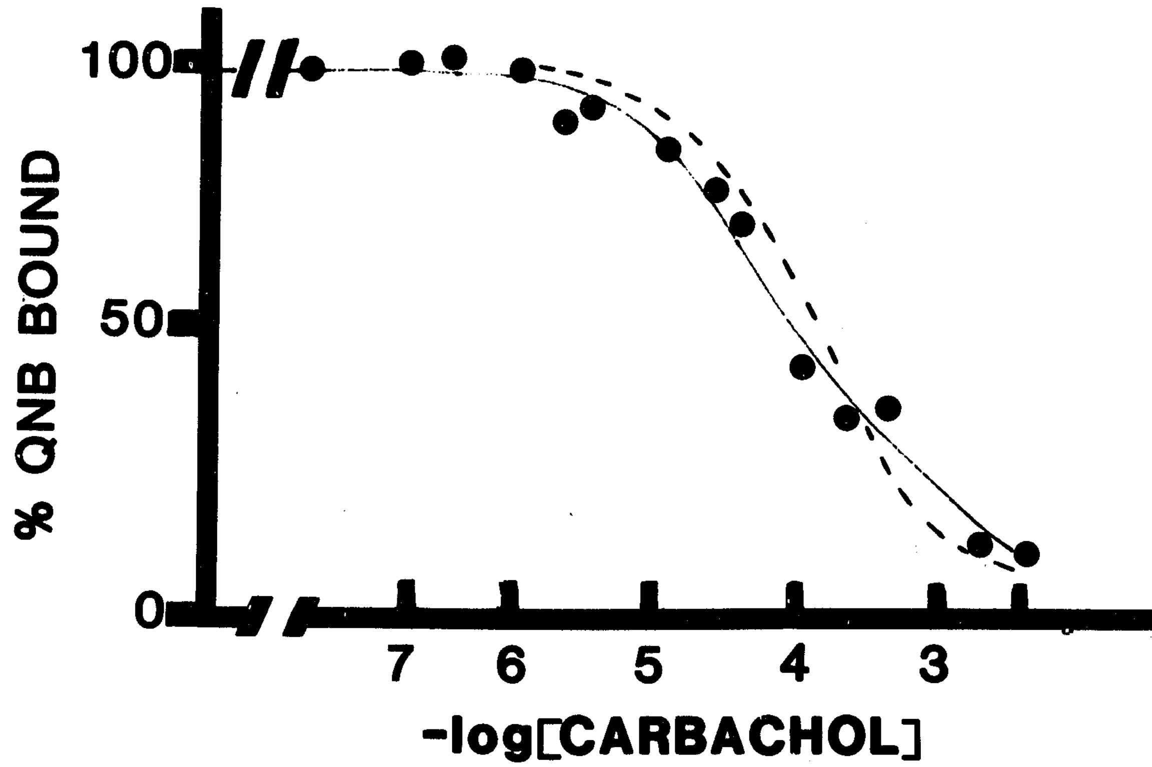


Figure 22.

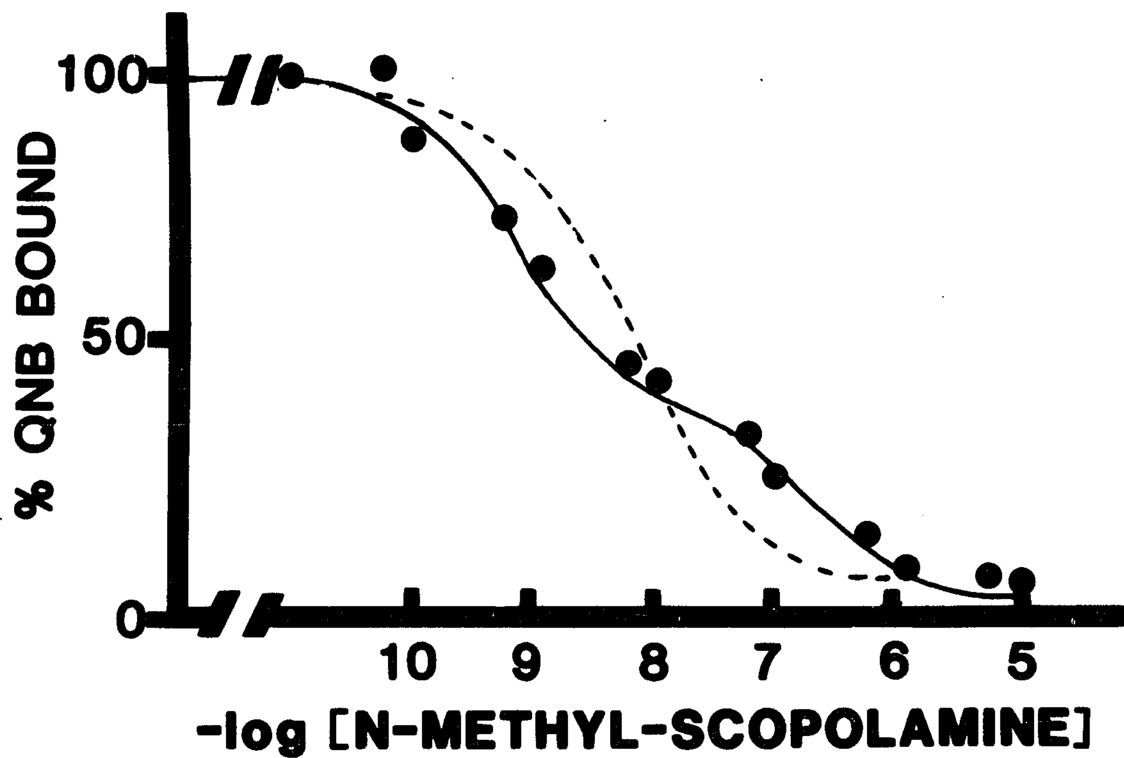


Figure 23.

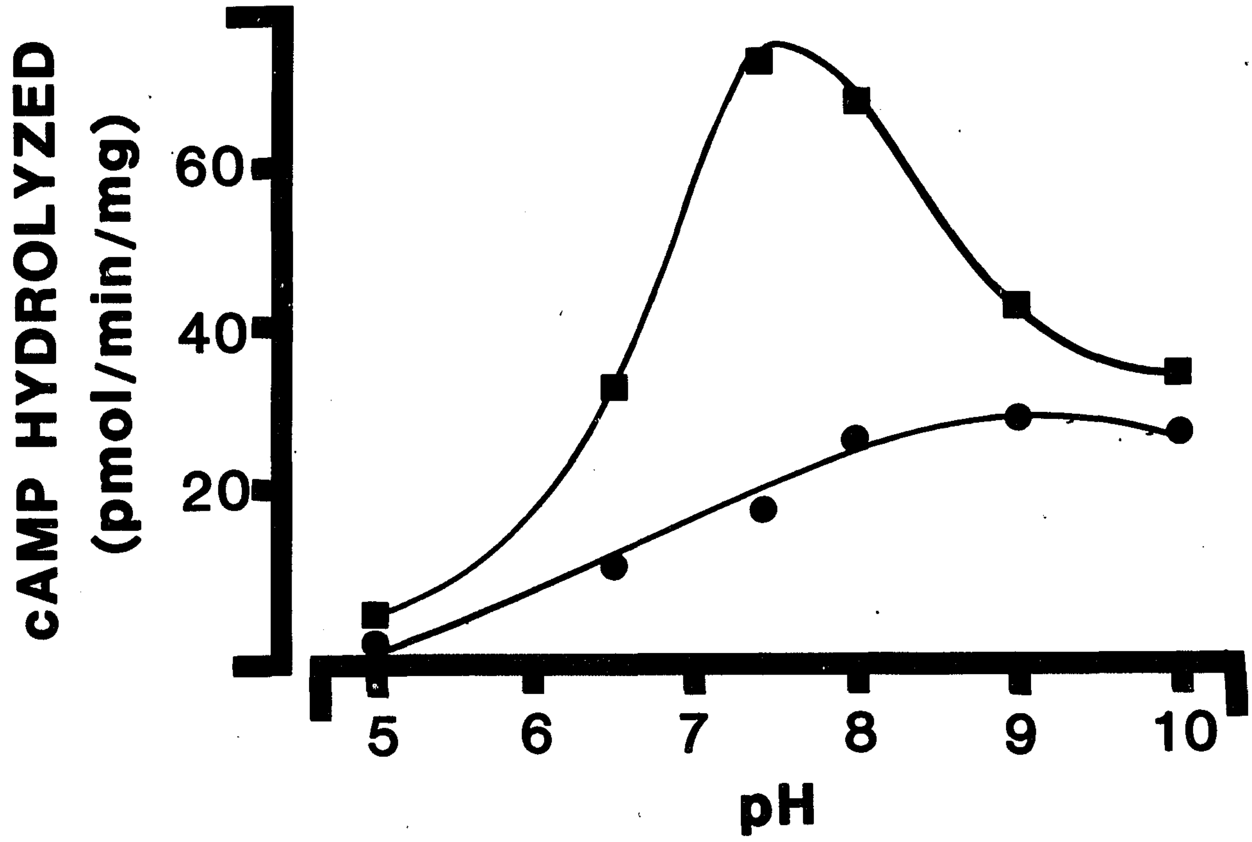


Figure 24.

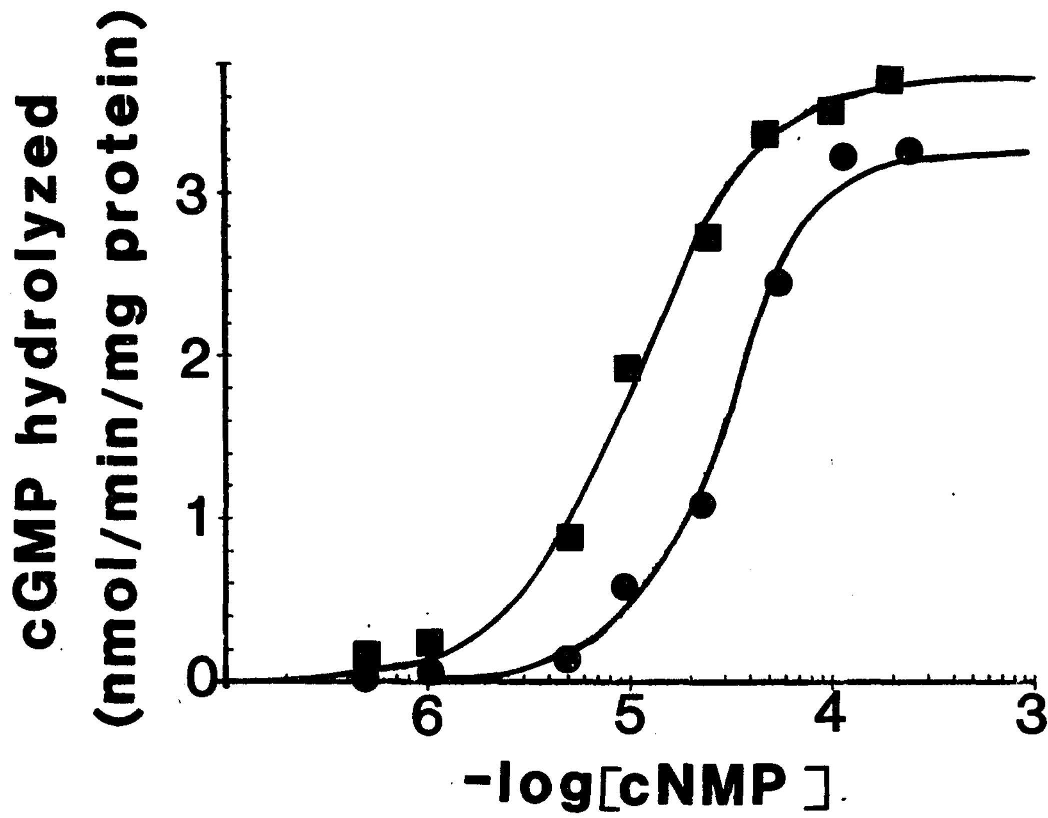


Figure 25.

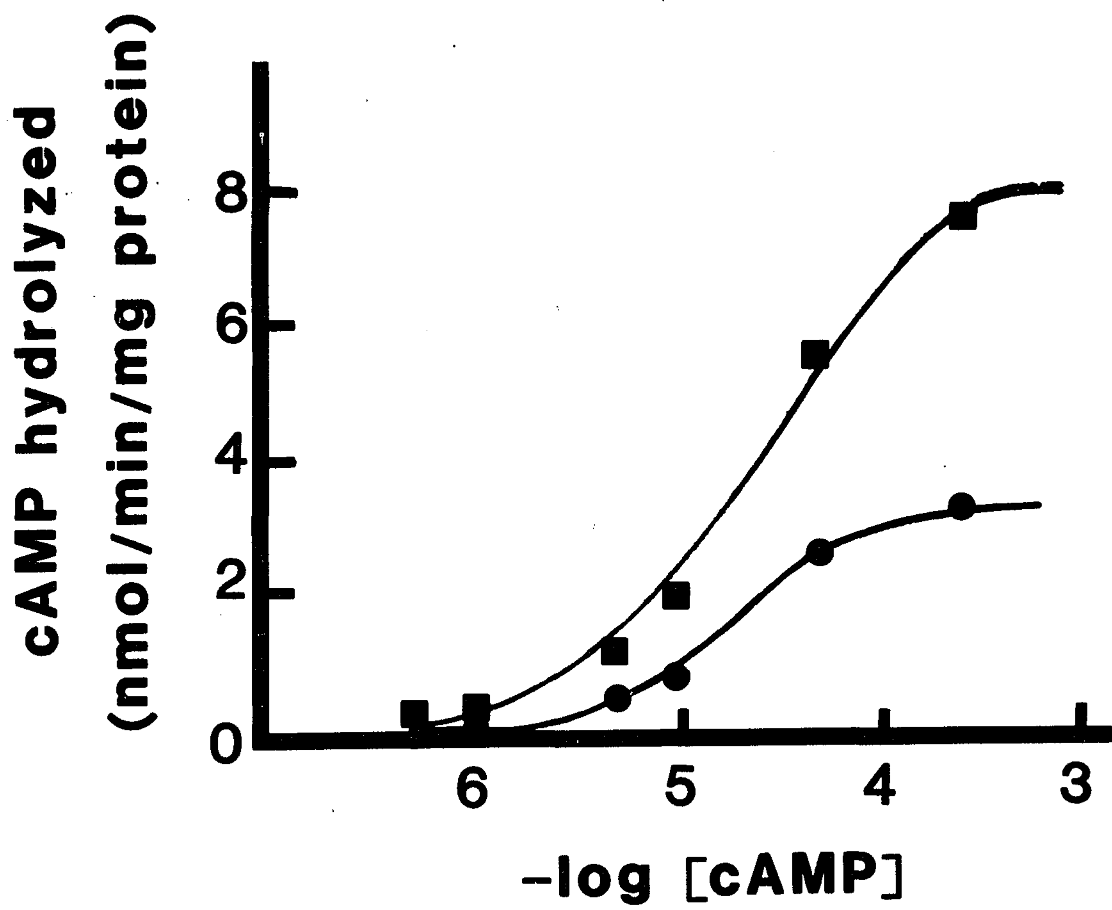


Figure 26.

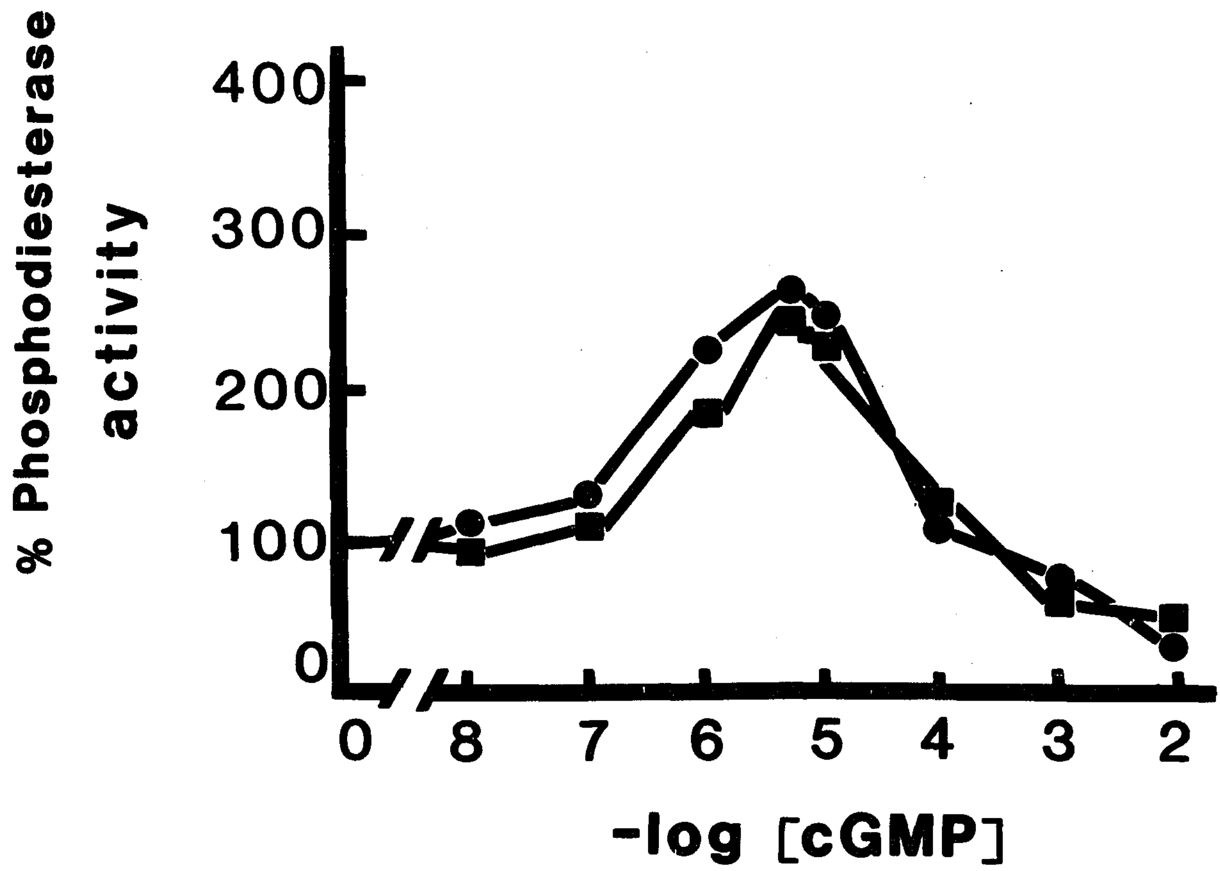


Figure 27.

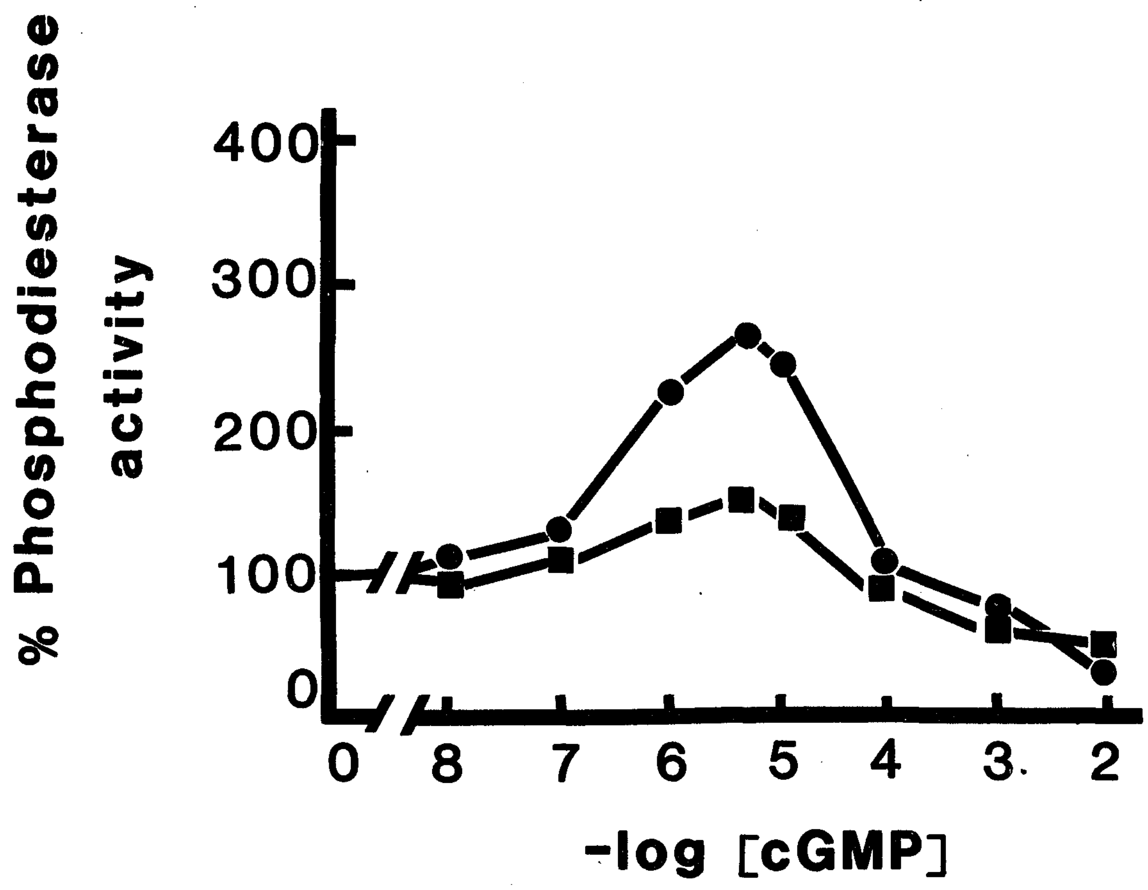


Figure 28.

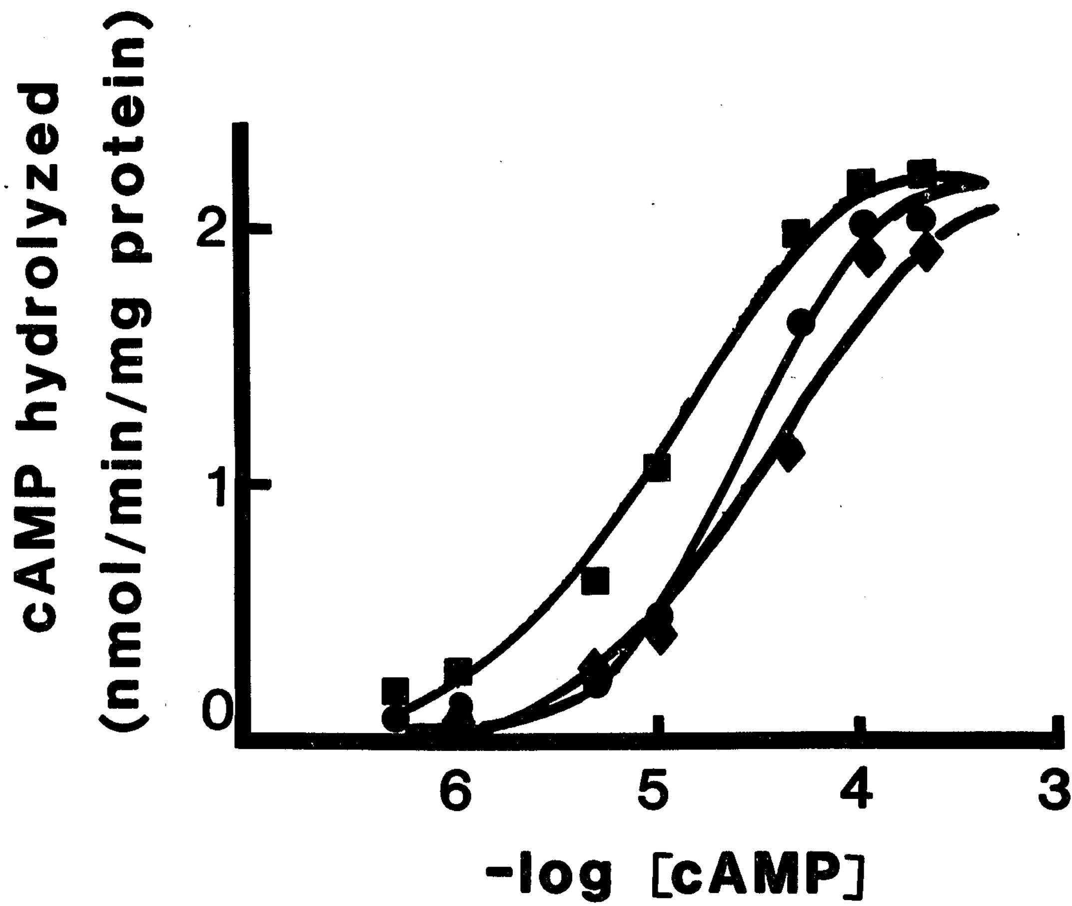


Figure 29.

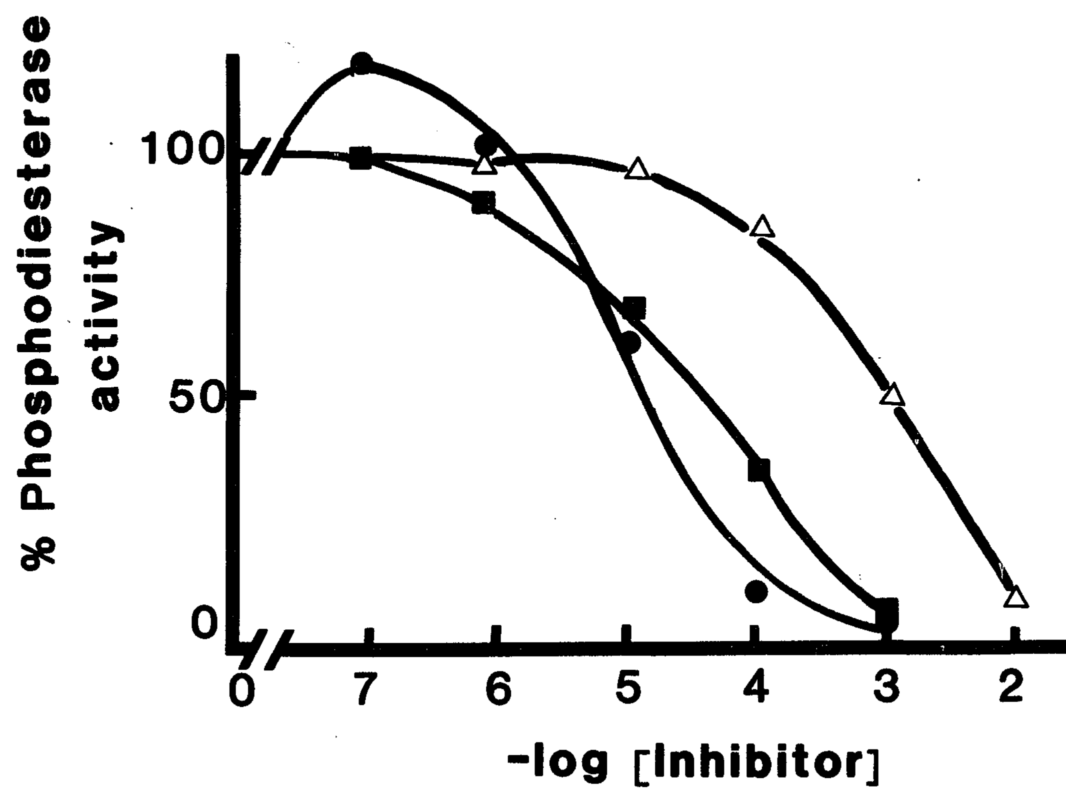


Figure 30.

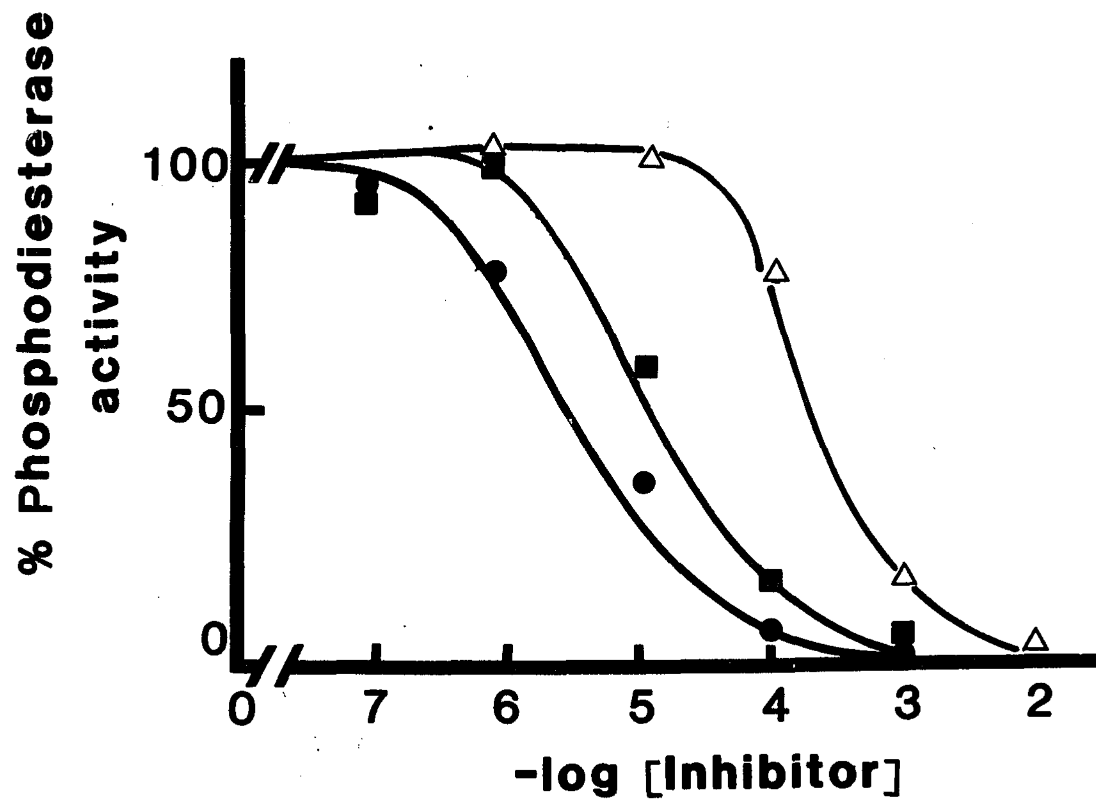


Figure 31.

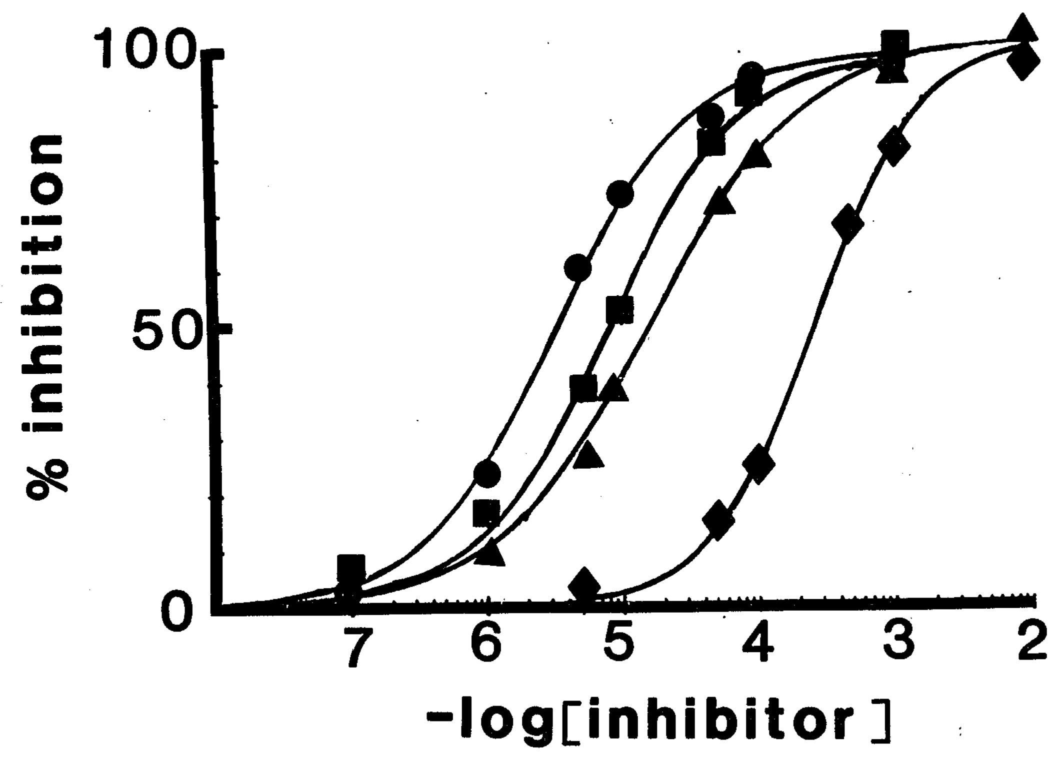


Figure 32.

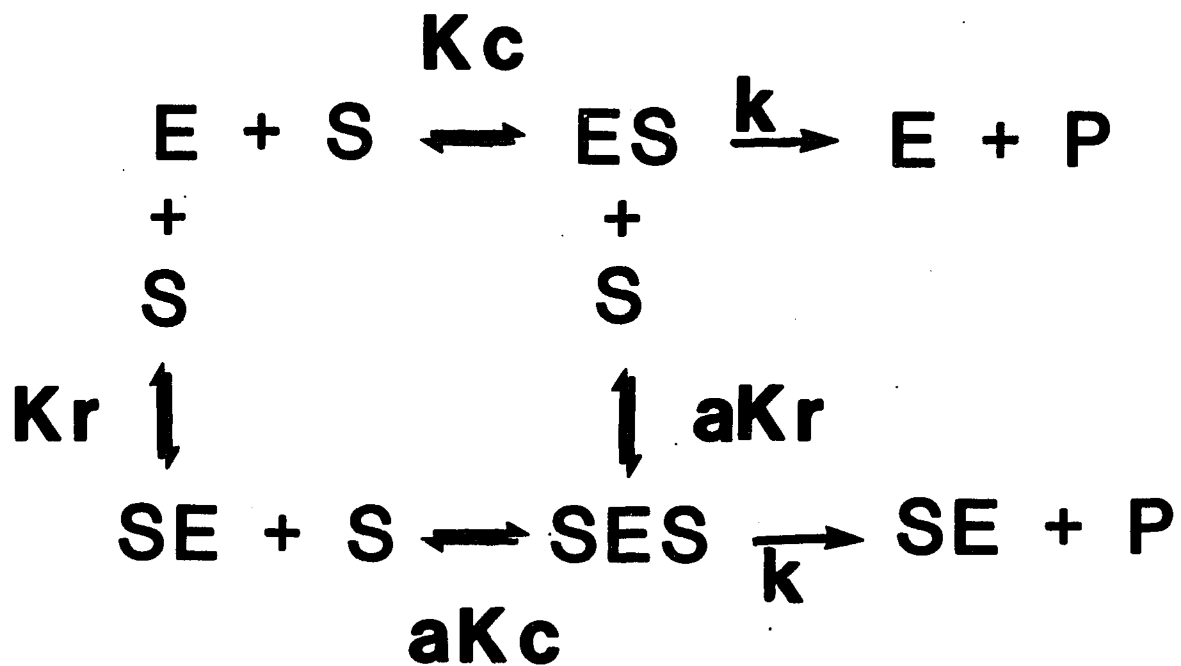


Figure 33.

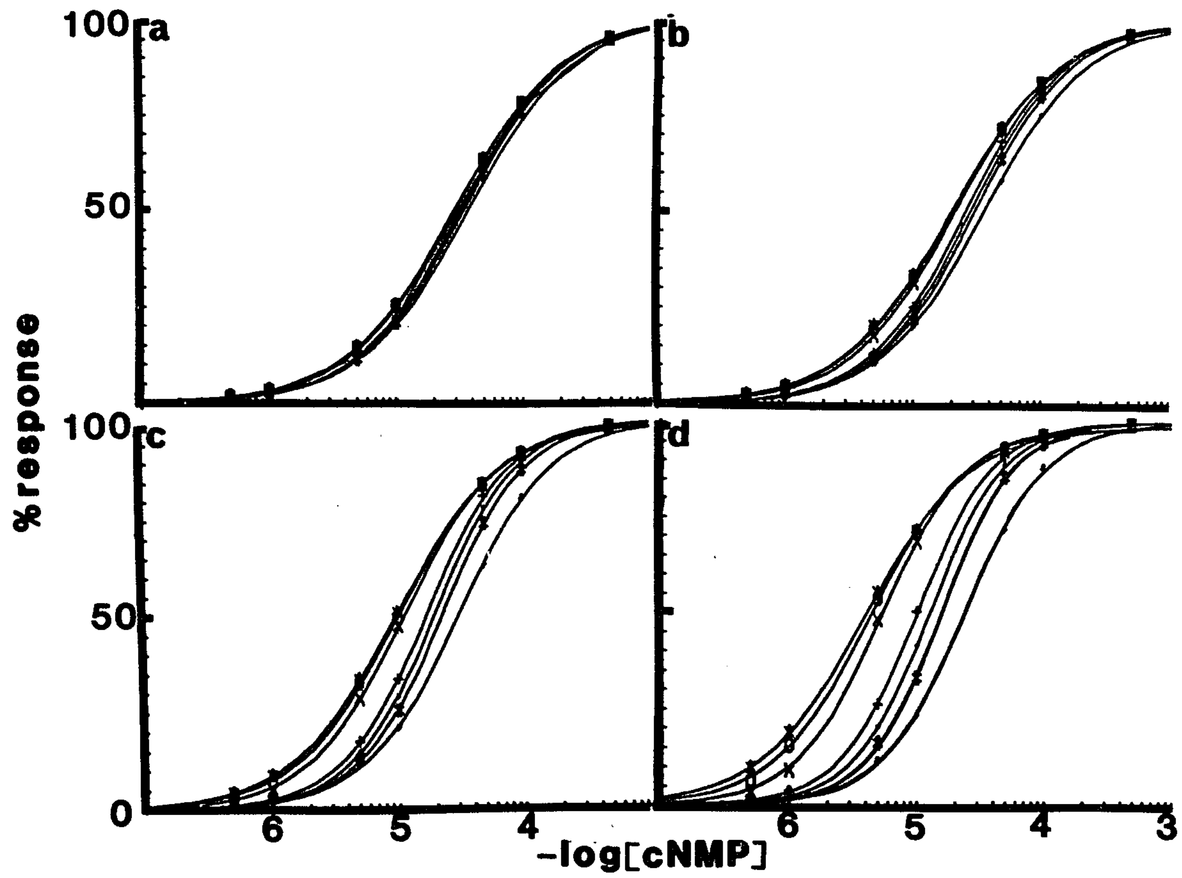


Figure 34.

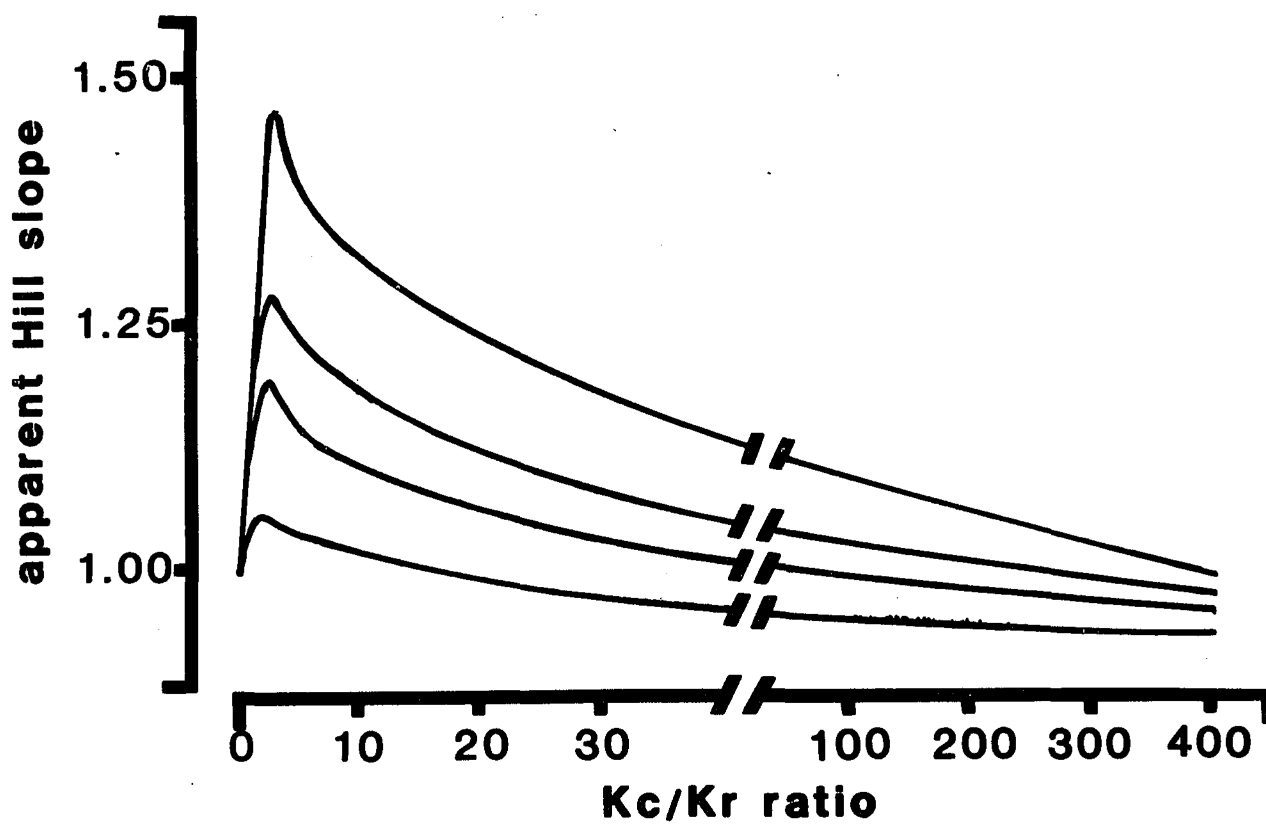


Figure 35.

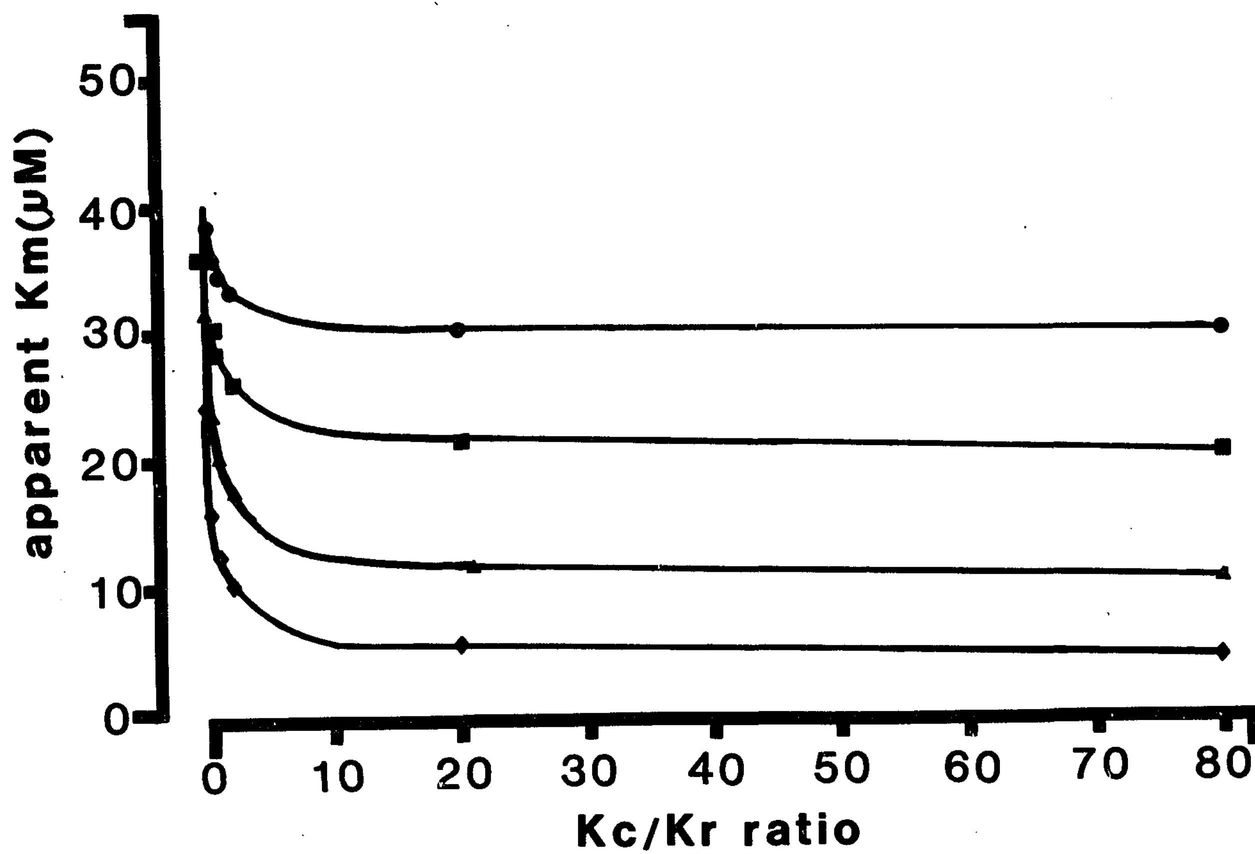


Figure 36.

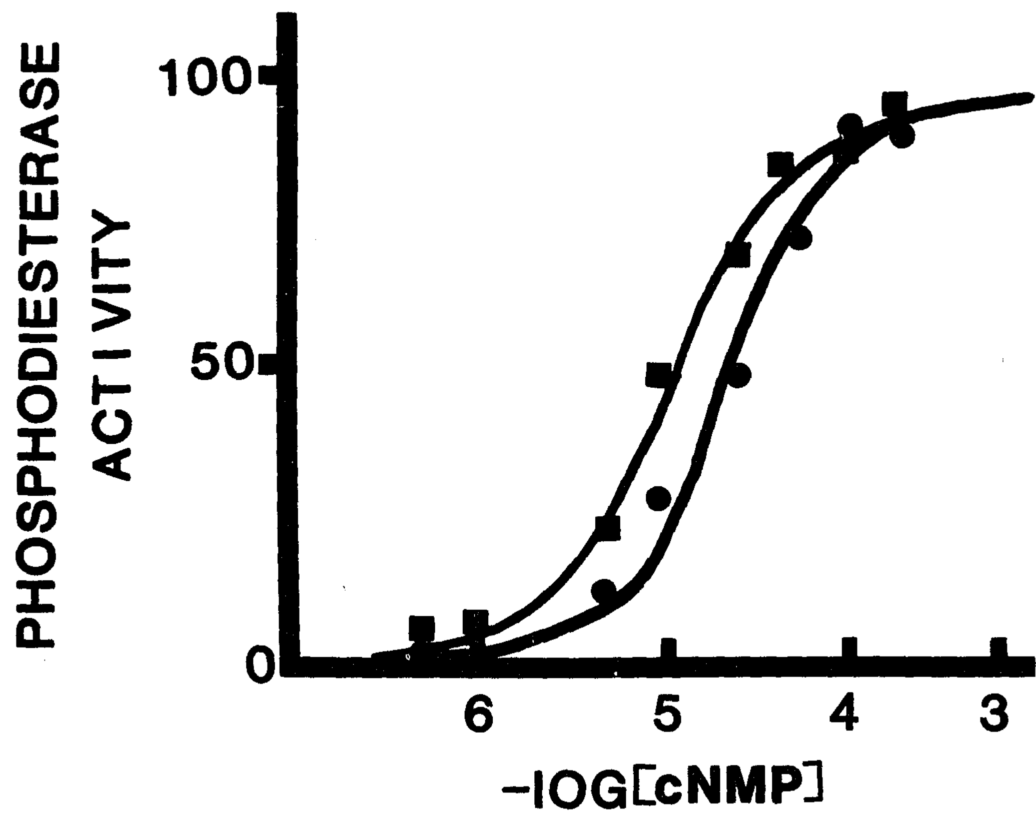


Figure 37.

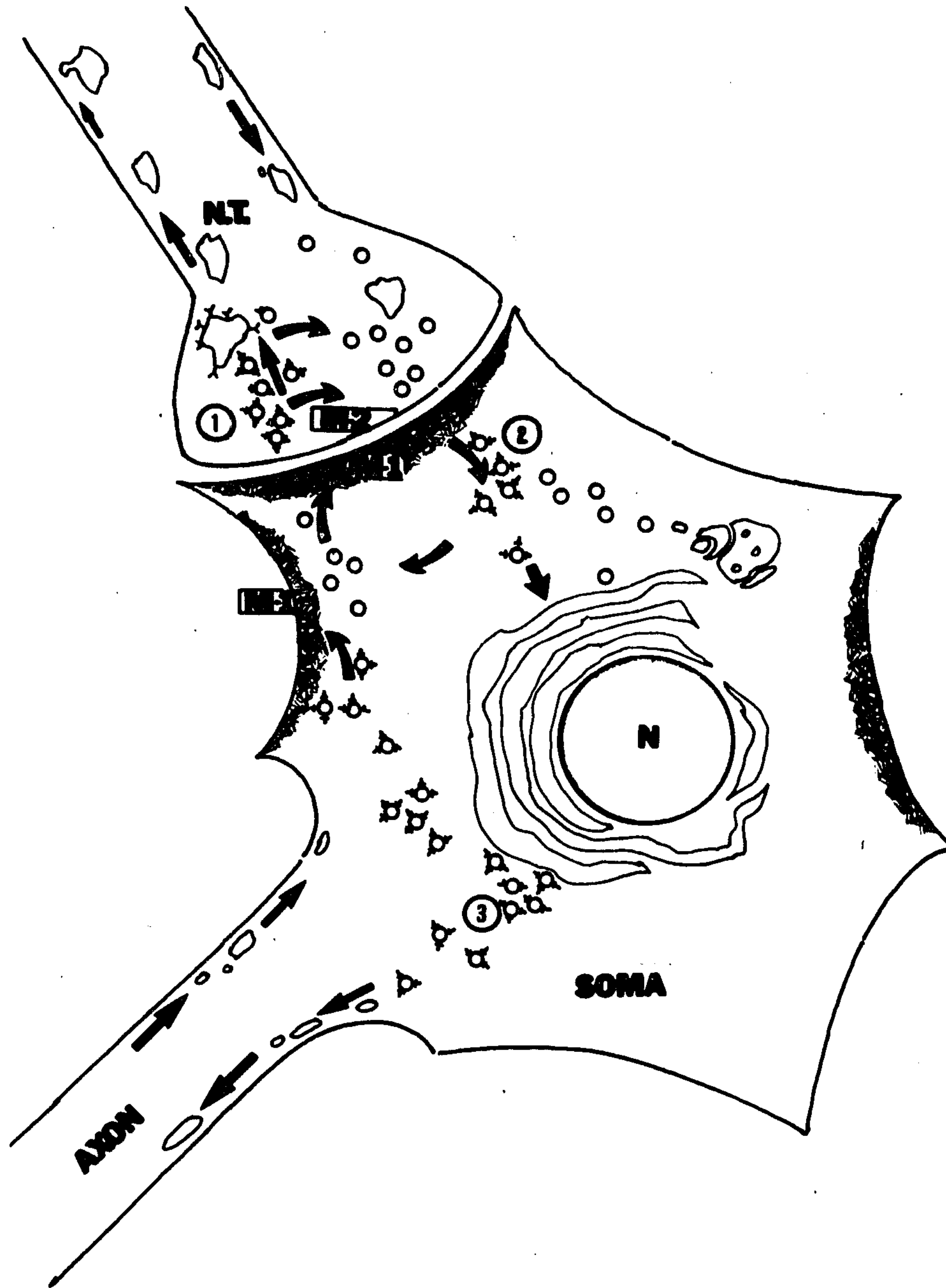


Figure 38.

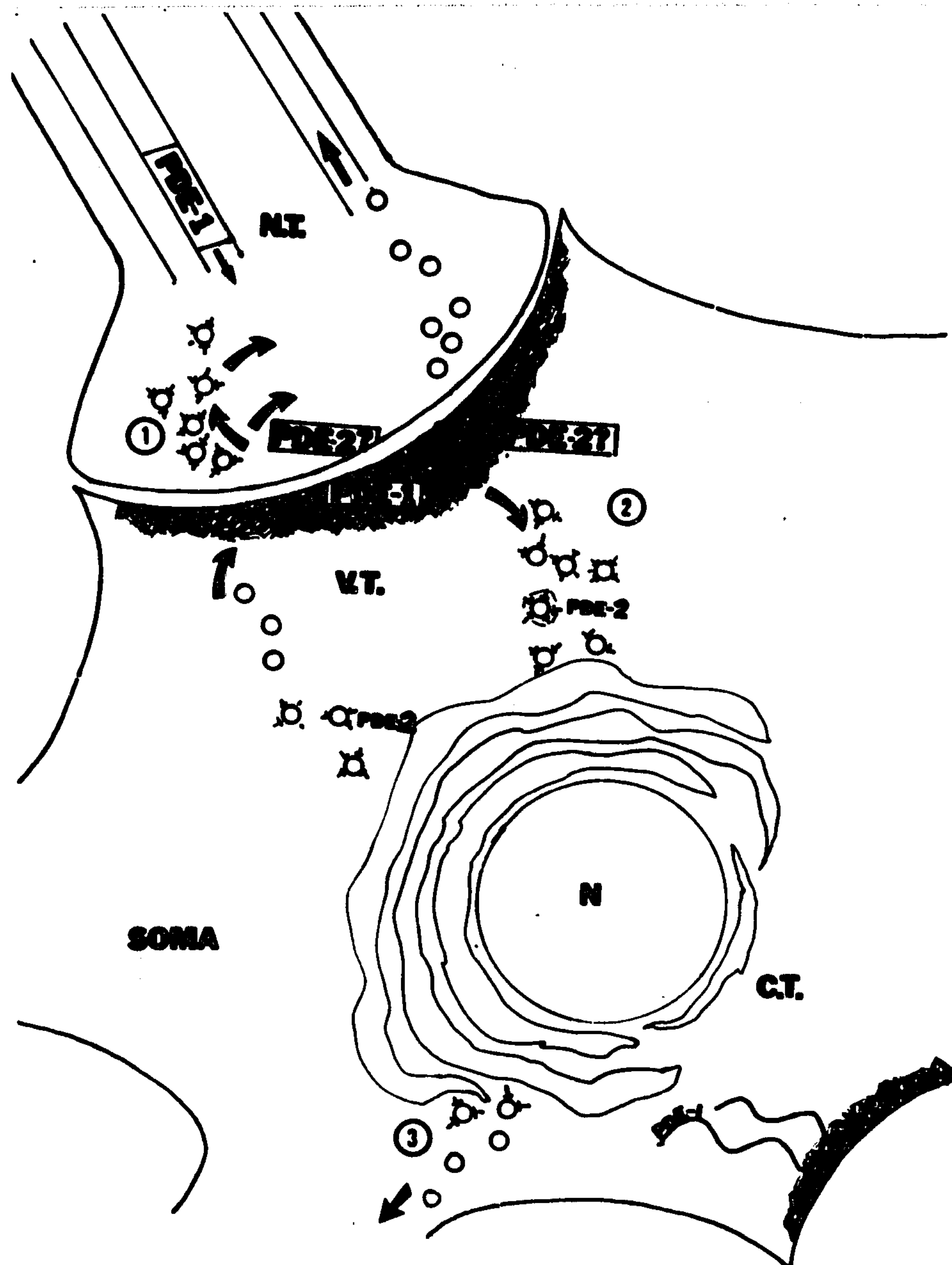
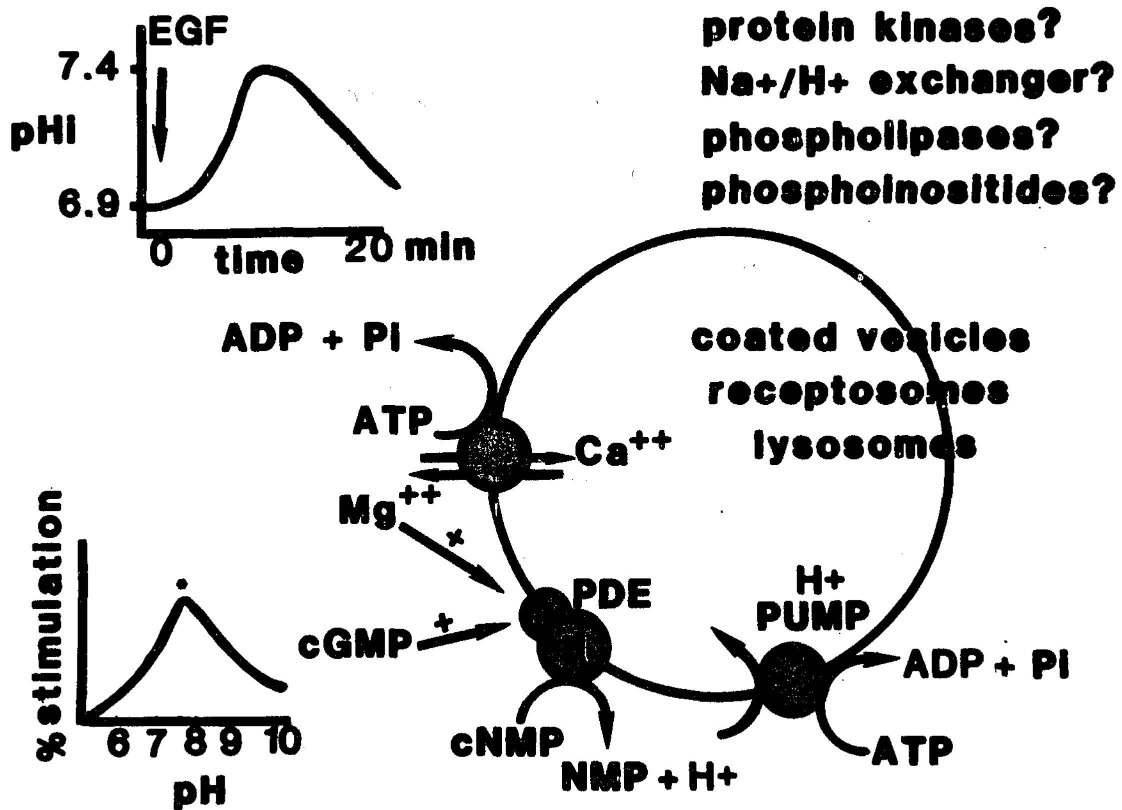


Figure 39.



BIBLIOGRAPHY

Aguilar, J.S., Salas P.J.I. and E. DeRobertis (1982). Cholinergic muscarinic receptor in synaptosomal membranes: Heterogeneity of binding sites for L-^{3}H-quinuclidinyl benzilate. Mol. Pharmacol. 22:304-309.

Aitchinson, R., West, D.W., and R.A. Clegg (1984). Insulin-stimulated high affinity cyclic AMP phosphdiesterase in rat mammary acini, FEBS Letts. 167: 25-28.

Alberts, P., and T. Bartfai (1976). Muscarinic acetylcholine receptor from rat brain. J. Biol. Chem. 251:1543-1547.

Ariano, M.A., and Adinolfi, A.M. (1977) Subcellular localization of cyclic nucleotide phosphodiesterase in the caudate nucleus. Exp. Neurol. 55, 84-94.

Ariano, M.J. and Appleman, M.M. (1979). Biochemical characterization of post-synaptically localized cyclic nucleotide phosphdiesterase. Brain Res. 177, 201-309.

Aronstram, R.S., L.G. Abood and W. Hoss (1978). Influence of sulfhydryl reagents and heavy metals on the functional state of the muscarinic acetylcholine receptor in rat brain. Molec. Pharamc. 14:575-586.

Aronstram, R.S. and M.E. Eldefrawi (1979). Reversible conversion between affinity states for agonists of the muscarinic acetylcholine receptor from rat brain. Biochem. Pharmacol. 28:701-703.

Baehr, W., M.J. Devlin and M.L. Applebury (1979). Isolation and characterization of cGMP phosphodiesterase from bovine rod outer segments. J. Biol. Chem. 254:11669-11677.

Barber, R., and R.W. Butcher (1980). the turnover of cyclic AMP in cultured fibroblasts. J. Cycl. Nucl. Res. 6:3-14.

Bastiani, M.J. and C.S. Goodman (1984). Neuronal growth cones: specific interactions mediated by filopodial insertion and induction of coated vesicles. Proc. Natl. Acad. Sci. 81: 1849-1853..

Beavo, J.A., Hardman, J.G. and Sutherland, E.W. (1970). Hydrolysis of cyclic guanosine and adenosine 3'5'-monophosphates by rat and bovine tissues. J. Biol. Chem. 245, 5649-5655.

Beavo, J.A., Hardman, J.G., and Sutherland, E.W. (1971). Stimulation of adenosine 3'5'monophosphate hydrolysis by guanosine 3',5'-monophosphate. J. Biol. Chem. 246:3841-3846.

Birdsall, N.J.M., Burgen, A.S.V., and E.C. Hulme (1978). The binding of agonists to brain muscarinic receptors. Mol. Pharmacol. 14: 723-736.

Blitz, L.A., Fine, E.G. and A.P. Toselli (1977). Evident that coated vesicles isolated from brain are calcium-sequestering organelles resembling sarcoplasmic reticulum. J. Cell Biol. 75: 135-147.

Blitz, A.L., and R.E. Fine (1980). Coated vesicles in neurons. In Coated Vesicles, edited by Ocklefored and Whyte, Cambridge Univ. Press, 1980, p. 104-134.

Bloom, W.W., Fields, K.L., Yen, S.R., Haver, K., School, W. and S. Puszkin (1980). Brain clathrin: immunofluorescent patterns in cultured cells and tissues. Proc. Natl. Acad. Sci. USA 77, 5520-5524.

Braell, A.W., Schlossman, M.D., Schmid, L.S., and E.J. Rothman. The dissociation of clathrin coats coupled to the hydrolysis of ATP: role of an uncoating ATPase. J. Cell Biol., in press.

Brodsky, F.M., N.J. Homes and P. Parham (1983). Tropomyosin-like properties of clathrin light chains allow a rapid, high-yield purification. J. Cell Bio. 96:911-914.

Brown, E., D.A. Kendall and S.R. Nahorski (1984). Inositol phospholipid hydrolysis in rat cerebral cortical slices: I. Receptor characterization. J. Neurochem. 42:1379-1387.

Brown, S.M., Anderson, G.W.R., and L.J. Goldstein (1983). Recycling receptors: The round-trip itinerary of migrant membrane proteins. Cell 32: 663-667.

Burgisser, E., DeLean, A., and R.J. Lefkowitz (1982). Reciprocal modulation of agonist and antagonist binding to muscarinic cholinergic receptor by guanine nucleotide. PNAS 79: 1732-1739.

Bursztajn, S., and G.D. Fischbach (1984). Evidence that vesicles transport acetycholine receptors to the surface membrane of chick myotubles. J. Cell Bio. 98: 498-506.

Butcher, R.W. (1984). Phosphodiesterase after twenty years. Adv. Cycl. Nucl. and Prot. Phosphoryl. 16:4-12.

Campbell, C., J. Squicciarini, M. Shia, P.F. Pilch and R.E. Fine (1984). Identification of a protein kinase as an intrinsic component of rat liver coated vesicles. Biochem. 23:4420-4426.

Carson, S. (1982). Cholate salt solubilizatin of bovine brain muscarinic receptors. Biochem. Pharmacol. 31: 1806-1809.

Champion, S. and J. Mauchamp, J. (1981). Muscarinic cholinergic receptors on cultured thyroid cells. I. Biological effect of carbachol and characterization of the receptor. Molec. Pharmac. 21:66-72.

Chasin, M. and D.N. Harris (1976). Inhibitors and activators of cyclic nucleotide phosphodiesterases. Adv. Cycl. Nucl. Res. 7:225-264.

Cheung, W.Y. (1970). Cyclic 3',5'-nucleotide phosphdiesterase. Demonstration of an activator. Biochem. Biophys. Res. Commun. 38:533-538.

Clayberger, C.A., Goodman, D.B.P., and H. Rasmussen (1981). Regulation of cyclic AMP metabolism in the rat erythrocyte during chronic B-adrenergic stimualtion. Evidence for calmodulin-mediated alteration of membrane bound phosphodiesterase activity. J. Membr. Biol. 58: 191-201.

Cochett, C., G.N. Gill, J. Meisenhelder, J.A. Cooper and T. Hunter (1984). C-kinase phosphorylates the epidermal growth factor receptor and reduces its epidermal growth factor - stimulated tyrosine protein kinase activity. J. Biol. Chem. 259:2553-2558.

Delhaye, M., J.M. DeSmet, P. DeNeef, J.C. Camus, J. Fontaine, M. Waelbroeck, P. Robberecht and J. Christophe (1984). A comparison between muscarinic receptor occupancy, adenylate cyclase inhibition and inotropic response in human heart. Naunyn-Schmiedberg's Arch. Pharmacol. 325:170-175.

DePeusner, I.C.W., C.J. Perec, and O.M. Tiscornia (1984). ^{3}H-QNB binding in submaxillary glands subsensitized by chronic alcohol feeding. Mount Sinai J. Med. 51:675-679.

Dumont, J.E., Miot, F., Erneux, C., Couchie,D., Cochaus, P., Gervy-Decoster, C., VanSande, J., and J.N. Wells (1984). Negative regulation of cyclic AMP levels by activation of cyclic nucleotide phosphodiesterases. The example of the dog thyroid. Adv. Cycl. Nucl. and Prot. Phosphoryl. Res. 16: 325-336.

Egrie, J.C., and Siegel, F.L. (1977) Adrenal medullary cyclic nucleotide phosphodiesterase. Subcellular distribution, partial purification and regulationof enzyme activity. Bioch. Bioph. Acta 483:348-366.

Ehlert, F.J., N. Kokka and A.S. Fairhurst (1980a). Altered (^{3}H) Quinuclidinyl Benzilate binding in the striatum of rats following chronic cholinesterase inhibiton with diisopropylfluorophophate. Molec. Pharmacol. 17: 24-30.

Ehlert, F.J., W.R. Roeske, L.B. Rosenberger, and H.I. Yamamura (1980b). The influence of guanyl-5'-imidodiphosphate and sodium on muscarinic receptor binding in the rat brain and longitudinal muscle of the rat ileum. Life Sci. 26:245-252.

Ehlert, J.F., Roeske, R.W., and H.I. Yamamura (1981). Muscarinic receptor: regulation by guanine nucleotides, ions and N-ethyl-maleimide. Fed. Proc. 40: 153-159.

Ehlert, F.J., Roeske, W.R., and H.I. Yamamura (1982). Muscarinic cholinergic receptor heterogeneity. Trend in Neurochem. Sc. 2: 230-339.

El-Fakahany, E., and E. Richelson (1980). Temperature dependence of muscarinic acetylcholine receptor activation, desensitization, and resensitization. J. Neurochem. 34:1288-1295.

El-Fakahany, E., and E. Richelson (1981). Phenoxybenzamine and dibenamine interactins with calcium channel effectors of the muscarinic receptor. Molec. Pharmacol. 20:519-525.

Engelhorn, R. (1981). Evaluation of selectivity of two different antimuscarinic substances. Scand. J. Gastr. 16: 49-57.

Erneux, C., D. Couchie, J.E. Dumont, J. Baraniak, W.J. Stec, E. Garcia Abad, G. Petridis and B. Jastorff (1982). Specificity of cyclic GMP activation of a mutli-substrate cyclic nucleotide phosphodiesterase from rat liver. Eur. J. Biochem. 115:503-510.

Erneux, C., Couchje, D., Dumont, J.E., and B. Jastorff (1984). Cyclic nucleotide derivatives as probes of phoshodiesterases catalytic and regulatory sites. Adv. Cycl. Nucl. and Prot. Phosphoryl. Res. 16: 107-118.

Erneux, C., F. Miot, J. Boeynams and J.E. Dumont (1981). Paradoxical stimulation by 1-methyl-3-isobutylxanthine of rat liver cyclic AMP phosphodiesterase activity. FEBS Letts 142:251-254.

Farquhar, G.M, and G.E. Palade (1981). The Golgi apparatus (complex)-(1954-1981)-from artifact to center stage. J. Cell Biol. 91: 77-103.

Fell, D.A. (1980). Theoretical analysis of the functioning of the high and low-Km cyclic nucleotide phosphodiesterase in the regulation of the concentration of adenosine 3':5'-cyclic monophosphate in animal cells. J. Theor. Biol. 84:361-385.

Fisher, S.K., P.D. Klinger and B.W. Agranoff (1983). Muscarinic agonist binding and phospholipid turnover. J. Biol. Chem. 258: 7358-7363.

Florendo, N.T., Barnett, R.J., and Greengard, P. (1971). Cyclic 3'5'-nucleotide phosphodiesterase: Cytochemical localization in cerebral cortex. Science 173, 745-747.

Forgac, M., Cantlay, L., Wiedenmann, B., Altstiel, L., and D. Branton (1983). Clathrin-coated vesicles contain an ATP-dependent proton pump. PNAS 80: 1300-1303.

Franks, D.J., and Macmanus, J.P. (1971) Cyclic GMP stimulation and inhibition of cyclic AMP phosphodiesterase from thymic lymphocytes. Biochem. Biophys. Res. Comm. 42, 844-849.

Fuder, H. (1981). The affinity of pirenzepine and other antimuscarinic compounds for pre and post-synaptic muscarinic receptors of the isolated rabbit and rat heart. Scand. J. Gastr. 16:79-85.

Fung, B.K.-K., J.B. Hurley and L. Stryer (1981). Flow of information in the light-triggered cyclic nucleotide cascade of vision. Proc. Natl. Acad. Sci. 78:152-156.

Furchgott, R.F and P. Bursztyn (1967). Comparison of dissociation constants and of relative efficacies of selected agonists acting on parasympathetic receptors. Ann. Acad. Sci. NY 144:882-889.

Furchgott, R.F. (1984). The role of endothelium in the responses of vascular smooth muscle to drugs. Ann. Rev. Pharmacol. Toxicol. 24:175-197.

Galper, J.B., W. Klein and W.A. Catterall (1977). Muscarinic acetylcholine receptors in developing chick heart. J. Biol. Chem. 252:8692-8699.

Galper, J.B., and T.W. Smith (1980). Agonist and guanine nucleotide modulation of muscarinic cholinergic receptors in cultured heart cells. J. Biol. Chem. 255:9571-9579.

Galper, J.B., L.C. Dziekan, D.S. O'Hara and T.W. Smith (1982). The biphasic response of muscarinic cholinergic receptors in cultured heart cells to agonists. J.

Biol. Chem. 257:10344-10356.

Gardier, R.W., E.J. Tsevdos, and D.B. Jackson (1978). The effect of pancuronium and gallamine on muscarinic transmission in the superior cervical ganglion. J. Pharamcol. and Exptl. Therap. 204: 46-50.

Geuze, H.J., J.W. Slot, G.J.A.M. Strous, H.F. Lodish and A.L. Schwartz (1983). Intracellular site of asialoglycoprotein receptor-ligand uncoupling: double-label immunoelectron microscopy during receptor-mediated endocytosis. Cell 42:277-287.

Giedroc, D.P., N. Ling and D. Puett (1983). Identification of B-endorphin residues 14-25 as a region involved in the inhibition of calmodulin-stimulated phosphodiesterase activity. Biochem. 22:5584-5591.

Giedroc, D.P., T.M. Keravis, J.V. Staros, N. Ling, J.N. Wells and D. Puett (1985). Functional properties of covalent B-endorphin peptide/calmodulin complexes. Chlorpromazine binding and phosphodiesterase activation. Biochem. 24:1203-1211.

Gilman, A.G. (1984). G proteins and dual control of adenylate cyclase. Cell 36: 577-579.

Goldstein, L.J., Anderson, W.G.R., and S.M. Brown (1979). Coated pits, coated vesicles and receptor mediated endocytosis. Nature 279: 679-685.

Gottlieb, M.H., Steer, C.J., Steven, A.C., and Chrambach, A. (1985) Applicability of agarose gel electrophoresis to the physical characterization of clathrin-coated vesicles. Anal. Biochem. 147:353-363.

Grab, D.J., Carlin, R.K., and Siekevitz, P. (1981). Function of calmodulin in postsynaptic densities. I. Presence of a calmodulin-activatable cyclic nucleotide phosphodiesterase activity. J. Cell Biol. 89, 433-439.

Grahame-Smith, D.G. (1985). Pharmacological adaptative responses occurring during drug therapy and in disease. Trends in Pharmacological Sciences 6:38-41.

Gray, E.G. (1961). The granule cells, mossy synapses and Purkinje spine synapses of the cerebellum: light and electron microscope observations. J. Anat. 95: 345-356.

Gross, R.A. and R.B. Clark (1976). Regulation of adenosine 3':5-monophosphate content in human astrocytoma cells by isoproterenol and carbachol. Molec. Pharmac. 13:242-250.

Hadjiconstantinou, M., P.E. Potter and N.H. Neff (1982). Trans-synaptic modulation via muscarinic receptors of serotonin-containing small intensely fourescent cells of superior cervical ganglion. J. Neurosci. 2: 1836-1839.

Haigler, H.T., F.R. Maxfield, M.C. Willingham and I. Pastan (1980). Dansylcadaverine inhibits internalization of ^{125}I-epidermal growth factor in BALB 3T3 cells. J. Biol. Chem. 255: 1239-1241.

Halim, S., H. Kilbinger and I. Wessler (1981). Pirenzepine does not discriminate

between pre- and post-synaptic muscarine receptors in the guinea-pig small intestine. Scand. J. Gastr. Suppl. 72: 87-94.

Halvorsen, S.W., and N.M. Nathason (1981). In vivo regulation of muscarinic acetylcholine receptor number and function in embryonic chick heart. J. Biol. Chem. 256: 7944-7948.

Hammer, R. (1981). Subclasses of muscarinic receptor and pirenzepine. Further experimental evidence. Scand. J. Gastr. 16: 59-65.

Hammer, R., and A. Giachetti (1982) Muscarinic receptor subtypes: M-1 and M-2 biochemical and functinal characterization. Life Sci. 31: 2991-2998.

Hammer, R., and A. Giachetti (1984). Selective muscarinic receptor antagonists. Trends in Pharmacol. Sci. 5: 18-20.

Hanley, M.R. and L.L. Iversen (1977). Muscarinic cholinergic receptors in rat corpus striatum and regulation of guanosine cyclic 3':5'-monophosphate. Mol. Pharmacol. 14:246-255.

Harden, T.K., A.G. Scheer and M.M. Smith (1982). Differential modification of the interaction of cardiac muscarinic cholinergic and Beta-adrenergic receptors with a guanine nucleotide binding components. Molec. Pharmac. 21: 570-580.

Hedlund, B., and T. Bartfai (1978). The importance of thiol- and disulfide groups in agonist and antagonist binding to the muscarinic receptor. Molec. Pharmacol. 15: 531-544.

Hedlund, B. and T. Bartfai (1981). Binding of ^{3}H pilocarpine to membrances from rat cerebral cortex. Naunyn-Schmiedeberg's Arch. Pharmacol. 317: 126-130.

Heller Brown, Jr., and S. Brown Masters (1984). Does phosphoinositide hydrolysis mediate "inhibitory" as well as "excitatory" muscarinic responses? Trends in Pharmacol. Sci. 5: 417-425.

Helenius, A., Marsh, M., and J. White (1980). The entry of viruses into animal cells. TIBS 5: 104-106

Heuser, E.J. and S.T. Reese (1973). Evidence for recycling of synaptic vesicle membrane during tansmitter release at the frog neuromuscular junctin. J. Cell Biol. 57: 315-344.

Heuser, J., and L. Evans (1980). Three dimensional visualization of coated vesicle formation in fibroblasts. J. Cell Biol. 84: 560-583.

Heyworth, C.M., S. Rawal and M.D. Houslay (1983). Guanine nucleotides activate the insulin-stimulated phosphodiesterase in liver plasma membranes. FEBS Letts 154: 87-91.

Higuchi, H., Matsumoto, K., Uchida, S., and H. Yoshida (1983). Binding properties of muscarinic cholinergic receptors in long term desensitization of smooth muscle. Japan J. Pharmacol. 33: 702-705.

Hootman, S.R. (1982). Regulation by isoproterenol of muscarinic acetylcholine

receptor numbers and sensitivity in rat submandibular, but not lacrimal, glands. Bioch. et Bioph. Acta 716: 341-347.

Houslay, M.D., Wallace, A.V., Marchmont, R.J., Marin, B.R., and C.M. Heyworth (1984). Insulin controls intracellular cyclic AMP concentrations in hepatocytes by activating specific cyclic AMP phosphodiesterases: phosphorylation of the peripheral plasma membrane enzyme. Adv. Cycl. Nucl. and Prot. Phosphoryl. Res. 16: 159-176.

Huang, C.Y., W. Chau, P.B. Chock, J.H. Wang and R.K. Sharma (1981). Mechanism of activation of cyclic nucleotide phosphodiesterase: requirement of the binding of four Ca^{+2} to calmodulin for activation. Proc. Natl. Acad. Sci. 78: 871-874.

Hurwitz, R.L., R.S. Hansen, S.A. Harrison, T.J. Martins, M.C. Mumby and J.A. Beavo (1984). Immunologic approaches to the study of cyclic nucleotide phosphodiesterases. Adv. Cycl. Nucl. Res. and Prot. Phosph. 16:89-106.

Jarv, J., B. Hedlund, and T. Bartfai (1979). Isomerization of the muscarinic receptor antagonist complex. J. Biol. Chem. 254: 5595-5598.

Jones, M.L., Kirk, J., and R.H. Mitchell (1982). Molecular events following activation of muscarinic receptors: the role of inositol phospholipids. Scand. J. Gastr. 72: 33-41.

Kadota, K., Usani, M., and A. Takahashi (1982). A protein kinase and its substrate associated with the outer coat and the inner core of coated vesicles from bovine brain. Briomed. Res. 5: 573-578

Kakiuchi, S. and R. Yamazaki (1970). Calcium dependent phosphodiesterase activity and its activating factor (PAF) from brain. Biochem. Biophys. Res. Commun. 41: 1104-110.

Kanaseki, T., and K. Kadota (1969). The vesicle in a basket. A morphological study of coated vesicles isolated from nerve endings in the guinea pig brain with special reference to membrane movements. J. Cell Biol. 42: 202-220.

Kauvar, L.M. (1982). Defective cyclic 3':5'monophosphate phosphodiesterase in the Drosophila memtory mutant dunce. J. Neurosci. 2: 1347-1358.

Keen, H.J., Willingham, C.M., and I. Pastan (1979). Clathrin-coated vesicles. Isolation, dissociation and factor-dependent reassociation of clathrin baskets. Cell 16: 303-312.

Keen, H.J., Willingham, C.M., and I. Pastan (1984). Clathrin and coated vesicle proteins. J. Biol. Chem. 256: 2538-2544.

Kilbinger, H. (1984). Presynaptic muscarinic receptors modulating acetylcholine release. Trends in Pharmacol. Sci. 5: 103-105.

Kilbinger, H., and I. Wessler (1983). The variation of acetylcholine release from myenteric neurones with stimulation frequency and train length. Role of presynaptic muscarine receptors. Naunyn-Schmiedberg's Arch. Pharmacol. 324: 130-133.

Klausner, R.D., J. van Renswoude, C. Kempf, K. Rao, J.L. Bateman and A.R. Robbins (1984). Failure to release iron from transferrin in a Chinese hamster ovary cell mutant pleiotropically defective in endocytosis. J. Cell Biol. 98: 1098-1101.

Klee, C.B., T.H. Crouch and M.H. Krinks (1979). Subunit structure and catalytic properties of bovine brain Ca^{++}-dependent cyclic nucleotide phosphodiesterase. Biochem. 18: 722-729.

Kloog, Y., Y. Egozi and M. Sokolovsky (1978). Characterization of muscarinic acetylcholine receptors from mouse brain: evidence for regional heterogeneity and isomerization. Molec. Pharmacol. 15: 545-558.

Konturek, S.J. (1983). Pharmacologic control of gastric acid secretion in peptic ulcer. Mt. Sinai J. Med. 50: 457-467.

Krinks, M.H., J. Haiech, A. Rhoads and C.B. Klee (1984). Reversible and irreversible activation of cyclic nucleotide phosphodiesterase: separation of the regulatory and catalytic domains by limited proteolysis. Adv. Cycl. Nucl. Res. and Prot. Phosph. 16: 31-48.

Krnjevic, K. (1974). Chemical nature of synaptic transmission in vertebrates. Physiol. Rev. 54: 418-540.

Krnjevic, K., R. Pumain and L. Renaud (1971). The mechanism of excitation by acetylcholine in the cerebral cortex. J. Physiol. 215: 247-268.

Kayoso, H. and M. Ui (1983). Functional uncoupling of muscarinic receptors from adenylate cyclase in rat cardiace membranes by the active component of islet-activating protein, pertussis toxin. J. Cycl. Nucl. and Prot. Phosphoryl. 9: 305-318.

Laduron, P. (1980). Axonal transport of muscarinice receptors. Nature 186: 287-288.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of bacteriophage T4. Nature 227: 680-685.

Lee, J., and E.E. El-Fakahany (1985). Heterogeneity of binding of muscarinic receptor antagonists in rat brain homogenates. J. Pharmacol. and Exptl. Therap. 233: 707-714.

Levin, R.M. and B. Weiss (1975). Mechanism by which psychotropic drugs inhibit adenosine cyclic 3':5'-monophosphate phosphodiesterase of brain. Mol. Pharmacol. 12: 581-589.

Liebman, P.A., and E.N. Pugh, Jr. (1981). ATP mediates rapid reversal of cyclic GMP phosphodiesterase activation in visual receptor membranes. Nature 287: 734-736.

Lisanti, M.P., M. Flanagan and S. Puszkin (1984). Clathrin lattice reorganization: theoretical considerations. J. Theor. Biol. 108: 143-157.

Lisanti, M.P., Shapiro, L.S., Moskowitz, N., Hua, E.L., Puszkin, S. and W. Schook (1982). Isolation and preliminary characterization of clathrin-associated proteins. Eur. J. Biochem. 125:463-

Lolley, R.N., D.B. Farber, M.E. Rayborn and J.G. Holleyfield (1977). Cyclic GMP accumulation causes degeneration of photoreceptor cells: simulation of an inherited disease. Science 196: 664-666.

Lowry, O.H., H.J. Rosenbrough, A.L. Farr and R.J. Randall (1951). Protein measurements with the Folin phenol reagent. J. Biol. Chem. 193: 265-275.

Luthin, G.R., and B.B. Wolfe (1984). Comparison of ^{3}H-Pirenzepine and 3-H-quinuclidinylbenzilate binding to muscarinic cholinergic receptors in rat brain. J. Pharmacol. and Exp. Therap. 228: 648-655.

Makino, H., and T. Kono (1980). Characterization of insulin sensitive phosphodiesterase in fat cells. II. Comparison of enzyme activities stimulated by insulin gnad isoproterenol. J. Biol. Chem. 255: 7850-7854.

Manganiello, V.C., B.C. Reed, J. Lieberman, M.D. Moss, M.D. Lane and M. Vaughn (1983). Alterations in cyclic AMP phosphodiesterase activities during differentiationof 3T3-L1 cells. J. Cycl. Nucl. Prot. Phosph. Res. 9, 143-154.

Manganiello, V.C., T. Yamamoto, M. Elks, M.C. Lin and M. Vaughan (1984). Regulation of specific forms.

Martins, T.J., Mumby, M.C., and J.A. Beavo (1982). Purification and characterization of a cyclic GMP-stimulated cyclic nucleotide phosphodiesterase from bovine tissues. J. Biol. Chem. 257: 1973-1979.

Mash, D.C., D.D. Flynn and L.T. Potter (1985). Loss of M2 muscarine receptors in the cerebral cortex in Alzheimer's disease and experimental cholinergic denervation. Science 228: 1115-1117.

Massini, E., R. Fantozzi, P. Blandina, S. Brunelleschi and P.R. Mannavoni (1983). Muscarinic cholinergic receptor binding in rat mast cells. Agents and Actions 13: 327-332.

Matsuzawa, H. and M. Nirenberg (1975). Receptor-mediated shifts in cGMP and cAMP levels in neuroblastoma cells. Proc. Natl. Acad. Sci. 72: 3472-3475.

Mattera, R., B.J.R. Pitts, M.L. Entman and L. Birnbaumer (1985). Guanine nucleotide regulation of a mammalian myocardial muscarinic receptor system. J. Biol. Chem. 260: 7410-7421.

Mazancourt, P., and Y. Giudecelli (1984). Guanine nucleotide- and GTP-dependent N-6-phenylisopropyladenosine stimulation of the membrane bound cyclic AMP high affinity phoshodiesterase in rat brain. FEBS letts. 167: 142-146.

McKinney, M., and J.T. Coyle (1982). Regulation of neocortical muscarinic receptors: effects of drug treatment and lesions. J. Neurosci. 2: 97-105.

McKinney, M., and E. Richelson (1984). The coupling of the neuronal muscarinic receptor to responses. Ann. Rev. Pharmacol. Toxicol. 24: 121-146.

Meeker, R.B., and T. Kendall Harden (1982). Muscarinic cholinergic receptor mediated activation of phosphodiestrase. Mol. Pharmacol. 22: 310-319.

Merion, M., Schlessinger, P., Brooks, R.M., Moering, J.M., Moehring, T., and W.S. Sly (1983). Defective acidification of endosomes in chinese hamster ovary cell mutant "cross-resistant" to toxins and viruses. PNAS 80: 5315-5319.

Merisko, E.M., M.G. Farguhar and G.E. Palade (1982). Coated vesicle isolation byuimmunoadsorption on Staphylococcus aureus cells. J. Cell Biol. 92: 846-857.

Meshul, C.K. and G.D. Pappas (1984). The relatonship of pinocytosis and synaptic vesicles at the frog neuromuscular junction. Brain Res. 290: 1-18.

Meyer, M.R., Gainer, M.W., and N.M. Nathaon (1982). In vivo regulation of muscarinic cholinergic receptors in embryonic chick brain. Mol. Pharmacol. 21: 280-286.

Michell, R.H. (1975). Inositol phospholipids and cell surface receptor function. Biochem. et Biophys. Acta 415: 81-147.

Miki, N., J.J. Keirns, F.R. Marcus, J. Freeman, and M.W. Bitensky (1973). Regulation of cyclic nucleotide concentrations in photoreceptors: an ATP-dependent stimulation of cyclic nucleotide phosphodiesterase by light. Proc. Natl. Acad. Sci. 70: 3820-3824.

Miller, M.T. and Heuser, E.J. (1984). Endocytosis of synaptic vesicle membrane at the frog neuromuscular junction. J. Cell Biol. 98, 685-698.

Miot, F., Erneux, C., Wells, J.N. and J.E. Dumont (1984). The effects of aklylated xanthines on cyclic AMP accumulation in dog thyroid slices exposed to carbamylcholine. Mol. Pharmacol. 25: 261-266.

Moore, J.B., Jr. and D.E. Schroedter (1982). Purification and characterization of a human lung calmodulin-independent cyclic AMP phosphodiesterase. Arch. Biochem. Biophys. 213: 276-287.

Moss, J., V.C. Manganiello and M. Vaughn (1977). Substrate and effector specificity of a guanosine 3'5'-monophosphate phosphodiesterase from rat liver. J. Biol. Chem. 252, 5:5211-5215.

Nandi, P.K., G. Irace, P/P. van Jaarsueld, R.E. Lippoldt and H. Edelhodi (1982). Instability of coated vesicles in concentrated sucrose solutions. Proc. Natl. Acad. Sci. 79: 5881-5885.

Nevorotin, A.J. (1980). Coated vesicles in different cell types: some functional implications. In, Coated Vesicles, edited by Ockleford and Whyte, Cambridge Univ. Press, 1980, p. 25-54.

Olianas, M.C., P. Onali, J.P. Schwartz, N.H. Neff and E. Costa (1984). The muscarinic receptor adenylate cyclase complex of rat striatum: desensitization following chronic inhibition of acetylcholinesterase activity. J. Neurochem. 42: 1439-1443.

Overstreet, D.H., R.W. Russell, A.D. Crocker and G.D. Schiller (1984). Selective breeding for differences in cholinergic functions: pre- and postsynaptic mechanisms involved in sensitivity to the anticholinesterase, DFP. Brain Res. 294: 327-332.

Pauloin, A., and P. Jolles (1984). Internal control of the coated vesicles pp-50-specific kinase complex. Nature 311: 250-252.

Pearse, B. (1976). Clathrin: a unique protein associated with intracellular transfer of membrane by coated vesicles. PNAS 73: 1255-1259.

Pearse, B. (1980). Coated vesicles. TIBS 5: 131-134.

Pearse, B.M.F. and M.S. Robinsin (1985). Purification and properties of 100 KD proteins from coated vesicles and their reconstitution with clathrin. Eur. J. Molec. Biol., in press.

Pfeffer, S.R. and R.B. Kelly (1985). The subpopulation of brain coated vesicles that carries synaptic vesicle proteins contains two unique polypeptides. Cell 40: 949-957.

Polson, J.B., Krazanowski, J.J., and A. Szentivanyi (1982). Inhibition of a high affinity cyclic AMP phosphodiesterase and relaxation of canine tracheal smooth muscle. Biochem. Pharmacol. 31: 3403-3406.

Purvis, K. and V. Hansson (1980). Calmodulin regulation of testicular cyclic nucleotide phosphodiesterase. Int. J. Anndrol. 3: 713-718.

Raffa, R.B. (1985). Coated pits and coated vesicles: histological findings and implications. Trends in Pharmacol. Sci. 6: 133-136.

Rhoads, A.R., A.K. Olowofoyeku, W.L. West and H.P. Morris (1976). Inhibition of a high molecular weight cyclic 3':5'-nucleotide phosphodiesterase isolated from rat liver. Biochem. Pharmacol. 25: 97-99.

Richelson, E. and E. El-Fakahany (1981). The molecular basis of neurotransmission at the muscarinic receptor. Biochem. Pharmacol. 30: 2887-2891.

Roth, T., and K. Porter (1964). Yolk protein uptake in the oocyte of the mosquito Aedes aegyptil. J. Cell Biol. 20: 313-332.

Rothman, J.E., H. Bursztyn-Pettegrew and R.E. Fine (1980). Transport of the membrane glycoprotein of vesicular stomatitis virus to the cell surface in two stages by clathrin-coated vesicles. J. Cell Biol. 86: 162-171.

Runge, M.S., P.B. Hewley, D. Puett and R.L. Williams (1979). Cyclic nucleotide phosphodiesterase activity in 10 nm-filaments and microtubule preparations from bovine brain. Proc. Natl. Acad. Sci. 76: 2561-2565.

Russell, T.R., W.L. Terasaki, and M.M. Appleman (1973). Separate phosphodiesterases for the hydrolysis of cyclic adenosine 3':5' monophosphate and cylic guanosine 3',5'-monophosphate in rat liver. J. Biol. Chem. 248:1334-1340.

Saitoh, Y., J.G. Hardman and J.N. Wells (1985). Differences in the association of calmodulin with cyclic nucleotide phosphodiesterase in relaxed and contracted arterial strips. Biochem. 24: 1613-1618.

Sakai, T., W.J. Thompason, V.R. Lavis and R.H. Williams (1974). Cyclic nucleotide phosphodiesterase activities from isolated fat cells. Correlation of subcellular distribution with effects of nucleotides and insulin. Arch. Biochem. Biophys. 162: 331-339.

Salisbury, J., Condeelis, J., and P. Satir (1980). Role of coated vesicles microfilaments and calmodulin in receptor-mediated endocytosis by cultured B lymphoblastoid cells. J. Cell Biol. 87: 312-141.

Salisbury, J., Coneelis, J.S., Maihle, N., and P. Satir (1981). Calmodulin localization during capping and receptor-mediated endocytosis. Nature 294: 164-166.

Scatchard, G. (1949). The attraction of proteins for small molecules and ions. Ann. Acad. Sci. NY 51: 6660-672.

Schlossman, D.M., S.L. Schmid, W.A. Braell, and J.E. Rothman (1984). An enzyme that removes clathrin coats: purification of an uncoating ATPase. J. Cell Biol. 99:723-733.

Sclessinger, J. (1980) The mechanisms and role of hormone-induced clustering of membrane receptors. TIBS 5:210-214.

Schmid, S.L., W.A. Braell, D.M. Schlossman and J.E. Rothman (1984). A role for clathrin light chains in the recognition of clathrin cages by 'uncoating ATPase". Nature 311: 228-231.

Schwartz, A.L. (1984). The hepatic asialoglycoprotein receptor. CRC critical Rev. in Biochem. 16: 207-233.

Segel, I.H. (1975). Enzyme Kinetics. Wiley-Interscience, New York.

Sellinger-Barnette, M., and L. Weiss (1984). Interaction of various peptides with calmodulin. Adv. Cycl. Nucl. Rev. and Prot. Phosph. 16: 261-276.

Shenolikar, S., W.J. Thompson and S.J. Strada (1985). Characterization of a Ca^{++} calmodulin-stimulated cyclic GMP phosphodiesterase from bovine brain. Biochem. 24: 672-678.

Shia, M.A., J.B. Rubin and P.F. Pilch (1983). The insulin receptor protein kinase. J. Biol. Chem. 258: 14450-14455.

Siegel, H., and J. Triggle (1983). Inhibition by concanavalin A, dansylcadaverine and bacitracin of muscarinic receptor-mediated desensitization in guinea pig ileal smooth muscle. J. Pharmacol. Exptl. Therap. 225: 534-538.

Simpson, L.L. (1981). The origin, structure, and pharmacological activity of Botulinum toxin. Pharmac. Rev. 33: 155-188.

Simpson, L.L. (1983). Ammonium chloride and methylamine hydrochloride antagonize clostridial neurotoxins. J. Pharm. Exp. Ther. 225: 546-552.

Sitaramaya, A., and P.A. Liebman (1983). Phosphorylation of rhodopsin and quenching of cyclic GMP phosphodiesterase activity by ATP at weak bleaches. J. Biol. Chem. 258: 12106-12109.

Smoake, J.A., L.S. Johnson, and G.T. Peake (1981). Calmodulin-dependent high affinity cylic-AMP phophodiesterase in liver membranes. Arch. Biochem. 206: 331-335.

Sokolovsky, M. (1984). Muscarinic receptors in the central nervous system. Int. Rev. Neurobiol. 25: 139-183.

Sokolovsky, M., and T. Bartfai (1981). Biochemical studies on muscarinic receptors. TIBS 6: 303-305.

Steinman, R.M., Mellman, I.S., Muller, W.A., and Z.A. Cohn (1983). Endocytosis and the recycling of plasma membrane. J. Cell Biol. 96: 1-27.

Stephenson, R.P. (1956). A modification of receptor theory. Br. J. Pharmacol. 11: 379-393.

Stone, G.C., Hammerschlag, R., and J.A. Bobinsky (1984). Involvement of coated vesicles in the initiation of fast axonal transport. Brain Res. 291: 219-228.

Strada, S.J., Martin, M.W., and W.J. Thompson (1984). General properties of multiple molecular forms of cyclic nucleotide phosphodiesterase in the nervous system. Adv. Cycl. Nucl. and Prot. Phosphoryl. Res. 16: 13-29.

Sudhof, T.C., J.-L. Goldstein, M.S. Brown and D.W. Russell (1985). The LDL receptor gene: a mosaic of exons shared with different proteins. Science 228: 815-824.

Sugiyama, H., M.P. Daniels and N. Nirenberg (1977). Muscarinic acetylcholine receptors of the developing retina. Proc. Natl. Acad. Sci. 74: 5524-5528.

Szelenyi, I. (1982). Does pirenzepine distinguish between subtypes of muscarinic receptors? J. Pharmac. 77: 567-569.

Takemoto, D.J., J. Hansen, L.J. Takemoto, M.D., Houslay and R.J. Marchmont (1984). Peptide mapping of cyclic nucleotide phosphodiesterases. Adv. Cycl. Nucl. Res. and Prot. Phosph. 16: 55-64.

Therien, H.M., and W.E. Mushynski (1979). Characterization of the cyclic 3'5'-nucleotide phosphodiesterase activity associated with synaptosomal plasma membranes and synaptic junctions. Biochim. Biophys. Acta. 585: 201-209.

Thompson, W.J., Terasaki, W.L., Epstein, P.M., and S.J. Strada (1979). Assay of cyclic nucleotide phosphodiesterase and resolution of multiple molecular forms of the enzyme. Adv. Cycl. Nucl. Res. 10: 69-92.

Thompson, W.J., Pratt, M.L., and S.J. Strada (1984). Biochemical properties of high-affinity cyclic AMP phosphodiesterase. Adv. Cycl. Nucl. and Prot. Phosphoryl. Res. 16: 137-148.

Tucker, M.M., J.B. Robinson and E. Stellwagen (1981). The effect of proteolysis on the calmodulin activation of cyclic nucleotide phosphodiesterase. J. Biol. Chem. 256: 9051-9058.

Unanue, E.R., Ungewichell, E., and D. Branton (1981). The binding of clathrin triskelions to membrane coated vesicles. Cell 26: 439-446.

Ungewickell, E. and D. Branton (1981). Assembly units of clathrin coats. Nature 289: 420-422.

Vale, M.R., R.V. Baker and D.B Hope (1984). Phosphodiesterase activities for cyclic nucleotides in nerve endings from the bovine posterior pituitary gland. J. Neurochem. 42: 377-383.

van Haarstetrt, P.J.M., and M.M. van Lookeren Campagne (1984). Transient kinetics of a cGMP-dependent cGMP-specific phosphodiesterase from dictyostelium discoideum. J. Cell Biol. 98: 709-716.

Venter, J.C. (1983). Muscarinic cholinergic receptor structure. J. Biol. Chem. 258: 4842-4848.

Venter, J.C., B. Eddy, L.M. Hall and C.M. Fraser (1984). Monoclonal antibodies detect the conservation of muscarinic cholinergic receptor structure from Drosophila to human brain and detect possible structural homology with alpha (one)-adrenergic receptors. Proc. Natl. Acad. Sci. 81: 271-276.

Waelbroeck, M., P. Robberecht, P. Chatelain and J. Christophe (1982). Rat cardiac muscarinic receptors. I. Effects of guanine nucleotides on high and low-affinity binding sites. Molec. Pharmacol. 21: 581-588.

Wallace, R.W., T.J. Lynch, A. Tallant and W.Y. Cheung (1978). Purification and characterization of an inhibitor protein of brain adenylate cyclcase and cyclic nucleotide phosphodiesterase. J. Biol. Chem. 254: 377-382.

Wamsely, J.K., Zarbin, M.A., and M.J. Kuhar (1981). Muscarinic receptors flow in the sciatic nerve. Brain Res. 217: 155-161.

Wamsely, J.K. (1983). Muscarinic cholinergic receptors undergo axonal transport in the brain. Eur. J. Pharmacol. 86: 309-310.

Weidenmann, B., K. Lawley, C. Grund and D. Branton (1985). Solubilizaiton of proteins from bovine brain coated vesicles by protein perturbants and triton-X-100. J. Cell Biol. 104: 12-18.

Weiler, M.H., V. Misgeld and D.K. Cheong (1984). Presynaptic muscarinic modulation of nicotinic excitation in the rat neostriatum. Brain Res. 296: 111-120.

Weinstein, H. (1975). Some new quantum chemical procedures for the analysis of drug-receptor interactions. Int. J. Quantum Chem: Quantum Biology Symp. No. 2:59-69.

Wells, J.N. and Hardman, J.G. (1977). Cyclic nucleotide phosphodiesterases. Adv. Cycl. Nucl. Res. 8:119-143.

Whyte, A. and C.D. Ockleford (1980). Structural aspects of coated vesicles function. In: Coated Vesicles, edited by C.D. Ockleford and A. Whyte, Cambridge University Press, pp. 283-302.

Willingham, M.C., and I. Pastan (1980). The receptosome: an intermediate organell or receptor-mediated endocytosis in cultured fibroblasts. Cell 21: 67-77.

Willingham, M.C., and I. Pastan (1983). Formation of receptosomes from plasma membrane coated pits during endocytosis: analysis by serial sections withimproved membrane labeling an dpreservation techniques. PNAS 80: 5617-5621.

Wojcikiewicz, R.J.H., P.R.M. Dobson and B.L. Brown (1984). Muscarinic acetylcholine receptor activation causes inhibition of cyclic AMP accumulation, prolactin and growth hormone secretion in GH_3 rat anterior pituitary tumour cells. Biochm. et Biophys. Acta 805: 25-29.

Woodward, M.P. and T.F. Roth (1978). Coated vesicles: characterization, selective dissociation and reassembly. Proc. Natl. Acad. Sci. 75:4384-4398.

Xie, X.S., Stone, D.K., and E. Racker (1983). Determinants of clathrin coated vesicles acidification. J. Biol. Chem. 258: 14834-14838.

Yamamoto, T., V.C. Manganiello and M. Vaughan (1983a). Purification and characterization of cyclic-GMP-stimulated cyclic nucleotide phosphodiesterase from calf liver. J. Biol. Chem. 258:12526-12533.

Yamamoto, T., S. Yamamoto, C.J. Osborne, Jr., C.V. Manganiello, and M. Vaughan (1983b). Complex effects of inhibitors on cyclic GMP-stimulated cyclic nucleotide phosphodiesterase. J. Biol. Chem. 258: 14173-14177.

Yamamoto, T., F. Lieberman, J.C. Osborne, V.C. Manganiello, M. Vaughan and H. Hidaka (1984). Selective inhibition of two soluble adenosine cyclic 3':5'-phosphate phosphodiesterases partially purified from calf liver. Biochem. 23: 670-675.

Yamamura, H.I., and S.H. Snyder (1974). Muscarinic cholinergic binding in rat brain. PNAS 71: 1725-1729.

Yamazaki, A., F. Bartucca, A. Ting and M.W. Bitensky (1982). Reciprocal effects of an inhibitory factor on catalytic activity and noncatalytic cGMP binding sites of rod phosphodiesterase. Proc. Natl. Acad. Sci. 79: 3702-3706.

Yamazaki, A., S. Uchida, P.J. Stein, G.L. Wheeler, and M.W. Bitensky (1984). Enzyme regulation and GTP binding protein: An algorithm of control which includes physical displacement of an inhibitory protein. Adv. Cycl. Nucl. Res and Prot. Phosphy. 16: 381-392.

Zarbin, M.A., Wamsley, J.K., and M.J. Kuhar (1982). Axonal transport of muscarinic cholinergic receptors in rat vagus nerve: high and low affinity agonists receptors move in opposite directions and differ in nucleotide sensitivity. J. Neurosci. 2: 934-943.

Zaremba, S. and J.H. Keen (1985). Limited proteolytic digestion of coated vesicle assembly polypeptides abolishes reassembly activity. In press.